THE ERYTHROID CELLS AND HAEMOGLOBINS OF THE CHICK EMBRYO

By GAIL A. P. BRUNS† and V. M. INGRAM, F.R.S.

Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts, U.S.A.

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† Present address: Division of Clinical Genetics, The Children's Hospital Medical Center, Harvard Medical School, Boston, Massachusetts, U.S.A.

The changes in the types of erythroid cells produced during embryogenesis of the chick have been correlated with the changes in the types of haemoglobins found in the embryo. Primitive erythroid cells constitute the only red blood cells of 2- to 5-day embryos. The first recognizable immature definitive erythroid cells appear in the embryonic circulation at 5 to 6 days and progressively replace the primitive cells, such that by 14 to 16 days the primitive cells constitute less than 1 % of the circulating erythroid cells.

Primitive erythropoiesis is strikingly different from definitive erythropoiesis. At any one time point between 2 and 16 days, all of the isolated primitive cells appear, by morphological criteria, to be at the same stage of maturation, and, although variation in cell size is observed, for an individual maturation stage, the small cells are not more mature than the medium-size cells, nor are the large cells less mature than the medium or small cells. Maturing primitive erythroid cells undergo the progressive changes in cell structure characteristic of erythroid maturation in mammalian erythropoietic systems, but do so as a uniform cell population.

Haemoglobin, isolated from primitive erythroid cells of 2- to 5-day embryos, shows two components on polyacrylamide gel electrophoresis, haemoglobin E and haemoglobin P. The haemoglobin E/P ratio is constant in lysates from 2- to 5-day embryos. At 6 to 7 days when the first haemoglobinized immature definitive erythroid cells appear in the embryonic circulation, two new haemoglobin components are observed in lysates of erythroid cells. These two new haemoglobin components are electrophoretically and immunologically identical to the two haemoglobin components of adult chickens, haemoglobins A and D. As the definitive erythroid cells replace the primitive erythrocytes in the embryonic circulation, the haemoglobins A and D increase in amount and replace haemoglobin P. Haemoglobin P cannot be detected immunologically in erythroid cell lysates from 16-day embryos which contain less than 1 % primitive cells.

In erythroid cell lysates from late embryos, which contained few, if any, primitive erythrocytes, a minor haemoglobin, electrophoretically similar to haemoglobin E on pH 10.3 polyacrylamide gels, is consistently observed. This component differs from haemoglobin E on pH 8.9 polyacrylmide gels, on Sephadex G-100 columns, on polyacrylamide gels of different porosities, and shows a reaction of only partial identity with haemoglobin E by two-dimensional immunodiffusion. This haemoglobin component, haemoglobin H, is detectable electrophoretically in lysates from 12-day embryos and immunologically in lysates from 8-day embryos. Haemoglobin H has not been observed in adult chickens. The switch from the production of primitive to definitive erythroid cells during development of the chick embryo is associated with the initiation of synthesis of three new haemoglobins, the two adult haemoglobins and haemoglobin H.

The haemoglobin D/A ratio of adult chicken haemoglobin, determined from the ratio of gel scan peak masses, is 0.30. When haemoglobins D and A first appear in erythroid cell lysates from 6- to 7-day embryos, the haemoglobin D/A ratio is about 0.9. The D/A ratio of lysates falls to 0.5 by 16 to 18 days, a time when 99% of the erythroid cells of the embryo are mature definitive erythrocytes. However, the haemoglobin D/A ratio of lysates from late embryos and young chicks of 0.5 to 20 days of age is consistently greater than that of adult chicken haemoglobin. Definitive erythrocytes of chick embryos and young chicks appear to differ from definitive cells of adult chickens in at least two ways: the presence of haemoglobin H and the higher haemoglobin D/A ratio.

Introduction and review of the literature

The maturation of erythroid cells in vertebrates has often been proposed as a model system for the study of regulation of differentiation in eukaryotic organisms. The maturing erythroid cell passes through a series of recognizable morphological stages and undergoes a progressive restriction of biosynthetic capacities which can be correlated with the changes in cell ultrastructure. Erythroid systems in a number of developing organisms including the human foetus and neonate, the mouse embryo, and the metamorphosing tadpole are characterized by changing erythroid cell populations, changing haemoglobins, and changing sites of erythropoiesis. Such systems afford an opportunity to investigate certain fundamental problems in developmental biology such as: the nature of the commitment of cells to a particular cell line; the regulation of cell populations in a developing organism; the factors which influence the 'switch' from the production of one type of cell to another; as well as the nature of activation and inactivation of structural genes for different proteins.

(*a*) *Erythropoiesis in the developing human embryo, foetus and neonate*

The first erythroid cells of the human embryo are found in the yolk sac blood sinuses at about 18 days of gestation (Bloom & Bartelmez 1940; Gilmour 1941), and are of the primitive cell series (a non-sustained erythroid cell line of young embryos, morphologically distinct from the sustained erythroid cell population). With establishment of the embryonic circulation at 25 days numerous early, mid, and late polychromatophilic primitive erythroblasts are observed in the circulation, and mitotic figures are common. Gilmour (1941) proposed that the expansion of the primitive erythrocyte pool after establishment of the circulation resulted from division of the circulating primitive cells. At 7 to 8 weeks most of the circulating primitive cells have been reported to be orthochromatic erythroblasts and reticulocytes, and mitotic figures are infrequently observed (Bloom & Bartelmez 1940). The primitive erythrocytes are not totally replaced by the definitive cell series until 12 weeks.

The first recognizable immature definitive erythrocytes (the sustained erythroid cell line of the human) were observed in the liver and yolk sac in a 10 mm embryo (crown rump (c.r.) length) by Gilmour (1941) and by Bloom & Bartelmez (1940) in a 12 mm embryo (6 weeks of age) and a few circulating definitive cells were observed in a 15 mm embryo. Although some definitive erythropoiesis has been reported to occur in the yolk sac until at least 9 weeks (Bloom & Bartelmez 1941), the liver is the major erythropoietic site for definitive erythroid cells between 6 and 12 weeks. Erythropoiesis commences in the bone marrow at 12 weeks. Significant erythropoiesis continues in the liver until about 6 months of gestation and then progressively declines and ceases by 5 days after birth. Although scattered foci of definitive erythropoiesis are observed in the spleen at 3 months, the spleen is not a significant erythropoietic site in the human embryo and foetus (Gilmour 1941).

During development of the human, two embryonic haemoglobins are recognized, Gower 1 with a chain composition of ε_4, and Gower 2 with a chain composition of $\alpha_2\varepsilon_2$ (Huehns, Flynn Butler & Beaven 1961; Huehns *et al.* 1964*a*; Huehns *et al.* 1964*b*; Hecht, Motulsky, Lemire & Shepard 1966). Haemoglobin Gower 1 and Gower 2 constitute the predominant haemoglobins of embryos of less than 8 weeks of gestation (25 mm c.r.) (Huehns *et al.* 1964*b*; Hecht *et al.* 1966). These haemoglobins were not found in embryos of greater than 3 months of age (10 cm c.r.) (Huehns *et al.* 1964*a*, *b*), although small amounts of Gower 2 haemoglobin have been detected in full term and young infants with D_1 trisomy (an aneuploid state for one of the D group chromosomes) (Huehns, Hecht, Keil & Motulsky 1964*c*). An additional embryonic haemoglobin, comparable to haemoglobin Portland 1 (Capp, Rigas & Jones 1967, 1970) which has a chain composition of $\gamma_2\chi_2$, was detected in embryos of 15 to 80 mm by Kaltsoya, Fessas & Stavropoulos (1966). Haemoglobin Portland 1 migrates very close to haemoglobin A on starch gel electrophoresis at pH 8.6 (Kaltsoya *et al.* 1966; Capp *et al.* 1967). Haemoglobin Portland 1 was not detected in embryos older than 11 weeks (Pataryas & Stamatoyannopoulos 1972), although traces of this haemoglobin have been found in normal term infants, in infants with D_1 trisomy (Hecht, Jones & Koler 1967), and in stillborn infants with homozygous α chain thalassaemia (Todd, Lai, Beaven & Huehns 1970; Weatherall, Clegg & Boon 1970).

The observations of Kaltsoya *et al.* (1966) and those of Hecht *et al.* (1966) that haemoglobin F was present in blood of the youngest embryos studied (16.3 mm c.r.) would indicate that γ chain synthesis is initiated by about 7 weeks of gestation. If the embryonic circulation at 7 to 9 weeks consists of mature primitive erythrocytes with only a few definitive cells, as reported by Bloom & Bartelmez (1940), γ chain synthesis would be initiated in primitive erythrocytes. The observations of Kleihauer, Tang & Betke (1967) suggest that haemoglobin Gower 1 and Gower 2 and haemoglobin F coexist within primitive erythrocytes from embryos of 8 to 12 week, of age.

The time of initiation of β chain synthesis during development of the human embryo is less clear. Huehns *et al.* (1964*a*, *b*) and Hecht *et al.* (1966) reported the presence of a haemoglobin migrating like haemoglobin A on starch gel electrophoresis at pH 8.4 in the youngest embryos studied (16.3 mm c.r., about 7 weeks of gestation). However, Walker & Turnbull (1955) first detected about 1 % haemoglobin A in embryonic blood at 13 weeks of gestation (about 56 mm c.r.) by alkaline denaturation kinetics. Using a pH 6.2 electrophoresis system capable of detecting 1 % haemoglobin A, Kaltsoya *et al.* (1966) found no haemoglobin A in embryos of less than 100 mm c.r. length. These authors, as well as Kleihauer & Stöffler (1968), suggest that the haemoglobin A observed by Huehns *et al.* (1964*a*, *b*) and by Hecht *et al.* (1966) was haemoglobin Portland 1.

Thomas, Lochte, Greenough & Wales (1960) studied the biosynthesis of haemoglobins A and F in the liver, spleen, and bone marrow of a 17-week foetus (13 cm c.r.) and in the liver of a 9-week foetus (2.1 cm c.r.). Although the ratio of haemoglobin A synthesized to that of haemoglobin F was higher in bone marrow than in liver or spleen of the 17-week foetus, both haemoglobins were synthesized in the liver and spleen and haemoglobin A in the bone marrow of the foetus. Both haemoglobins A and F appeared to be synthesized by the liver of the 9-week foetus. Hollenberg, Kaback & Kazazian (1971) and Kan *et al.* (1972) have reported the synthesis of haemoglobin A by circulating erythroid cells from 9- and 11-week foetuses respectively.

Foetal haemoglobin comprises the major haemoglobin of the human foetus from 8 weeks of gestation until birth. Two fractions of haemoglobin F have been described: F_1 (about 20 %)

which has two α chains, one γ chain with a free *N*-terminal glycine, and one γ chain with the *N*-terminal residue blocked as *N*-acetyl glycine; and F_2 (about 80 %) with a chain composition of $\alpha_2\gamma_2$ (Allen, Schroeder & Balog 1958; Schroeder & Matsuda 1958; Schroeder, Johnson, Matsuda & Fenninger 1962). The amount of haemoglobin A in foetal blood samples is about 10 % until the 35th week of gestation, when the amounts of haemoglobin A and F undergo a rapid reciprocal change. The amount of haemoglobin A in cord blood of full-term infants has been reported to vary from 11 to 39 % (Walker & Turnbull 1955; Cook, Brodie & Allen 1957; Beaven, Ellis & White 1960). The relative rate of synthesis of haemoglobin A and F by cord blood erythroid cells of new-born infants (25 to 43 weeks of gestation) is correlated with the gestational age and maturity of the infant (Bard, Markowski, Meschia & Battaglia 1970). Although some haemoglobin F is synthesized in normal postnatal infants (Jonxis 1949), the switch from the production of foetal to adult haemoglobin is essentially complete by 6 months following birth. Less than 1 % haemoglobin F can be found in normal adults (Gitlin, Sasaki & Vuopio 1968).

Haemoglobin A_2 ($\alpha_2\delta_2$) has been detected at about 8 months of gestation and increases in amount in parallel with haemoglobin A. Haemoglobin A_2 which constitutes about 0.25 to 0.58 % of the haemoglobin in normal term infants reaches normal adult values by about 80 days after birth (Wilson, Schroeder, Graves & Kach 1967).

One of the most interesting aspects of the switch from the production of haemoglobin F to haemoglobin A is that although both haemoglobins are found in the same cell (Tomoda 1964; Dan & Hagiwara 1967; Betke & Kleihauer 1958; Matioli & Thorell 1963; Gitlin *et al.* 1968), the relative quantities of the two haemoglobins are non-uniformly distributed in the erythrocytes. The non-uniformity of distribution of haemoglobin A and F is also observed in interacting thalassemia syndromes (Gitlin *et al.* 1968) and in haemolytic anaemia, leukemias, and pernicious anaemia associated with the reappearance of haemoglobin F (Bertles 1970). A non-uniform distribution of haemoglobin S and F has also been observed in sickle cell anaemia (Gitlin *et al.* 1968; Bertles & Milner 1968).

The mechanism of the switch from the production of haemoglobin F to haemoglobin A which occurs in the late foetus and young infant is as yet unknown, although several interesting hypotheses have been proposed by Ingram (1963, 1964), Baglioni (1963, 1966) and Kabat (1972). No information on the relationship of the stem cells for the primitive and definitive human erythroid cell lines is available.

(*b*) *Erythropoiesis in the developing C57BL/6J mouse embryo*

The first erythroid cells of the mouse embryo are of the primitive erythroid series formed in yolk sac blood islands at 7.5 to 8 days of gestation (Craig & Russell 1964; Kovach, Marks, Russell & Epler 1967; Barker 1968). Immature primitive erythrocytes are found in the embryonic circulation at 9 days (Barker 1968) where they mature as a cohort of cells (Fantoni, de la Chapelle & Marks 1969; Bank, Rifkind & Marks 1970). The yolk sac ceases to produce additional primitive cells after day 10 (Fantoni *et al.* 1969; de la Chapelle, Fantoni & Marks 1969; Bank *et al.* 1970). As the primitive erythroid cells mature in the embryonic circulation, the mitotic index falls to zero by 14 days; DNA synthesis is nil after 13 days; RNA synthesis ceases at 13 to 14 days; non-haem protein synthesis ceases at 12 days; and haemoglobin synthesis ceases by 14 days (Fantoni, de la Chapelle, Rifkind & Marks 1968; de la Chapelle *et al.* 1969). The loss of biosynthetic capacities is correlated with progressive nuclear pycnosis, although the

nucleus is not extruded, loss of cellular RNA, and the loss of polysomes (Kovack *et al.* 1967; Fantoni *et al.* 1968; Bank *et al.* 1970; Selander & de la Chapelle 1972). The primitive cells appear to be mature by 14 days and are subsequently lost from the embryonic circulation (Russell & Bernstein 1966).

Primitive erythrocytes synthesize three haemoglobins (Craig & Russell 1964; Barker 1968): E_1 with a chain composition of x_2y_2; E_2 with a chain composition of a_2y_2; and E_3 with a chain composition of a_2z_2 (Fantoni, Bank & Marks 1967; Fantoni *et al.* 1968; Gilman & Smithies 1968). The α chain is common to two of the embryonic haemoglobins and the adult haemoglobin of the C57BL/6J mouse (Fantoni *et al.* 1967, 1968). The amino acid sequence of both the x and z chains have been reported (Steinheider, Melderis & Osterlog 1972).

One of the most interesting aspects of the maturation of the mouse primitive erythrocytes is the observation that although non-haem protein synthesis is inhibited by actinomycin D, haemoglobin synthesis is not inhibited, even at the earliest time point studied, 9 days (Fantoni *et al.* 1968, 1969). The total embryonic haemoglobin per embryo and per primitive erythrocyte increases between day 9 and day 13. The relative amounts and the relative synthetic rates of the three embryonic haemoglobins change during this time period, although haemoglobin synthesis is not actinomycin D sensitive (Fantoni *et al.* 1969). The maturing primitive cells, in addition to synthesizing haemoglobin on an apparently stable messenger RNA, continue to synthesize DNA and undergo mitosis (de la Chapelle *et al.* 1969).

The second, or definitive, erythroid cells of the developing C57BL/6J mouse embryo arise in the liver at about 10 days of gestation (Russell & Bernstein 1966; Kovach *et al.* 1967; Rifkind, Chui & Epler 1969*b*). The definitive cell series demonstrate the well-known maturation stages: proerythroblast, basophilic erythroblast, polychromatophilic erythroblast, orthochromatic erythroblast, reticulocyte, and mature erythrocyte (Russell & Bernstein 1966; Rifkind *et al.* 1969*b*; Djaldetti, Chui, Marks & Rifkind 1970). The mouse definitive erythrocytes mature and extrude the pycnotic nucleus in the liver and are released into the embryonic circulation as reticulocytes, beginning on day 12 (Craig & Russell 1964; Kovach *et al.* 1967; Rifkind *et al.* 1969*b*). The definitive erythrocytes progressively replace the primitive cells in the embryonic circulation, such that by about 16 days no primitive cells are observed (Russell & Bernstein 1966). The definitive erythroid cells of the foetal liver or embryonic circulation appear to synthesize only adult mouse haemoglobin, which has a chain composition of a_2b_2 (Kovach *et al.* 1967; Barker 1968; Fantoni *et al.* 1968, 1969; Patton, Kirk & Moscona 1969; Bank *et al.* 1970).

Actinomycin D partially inhibits haemoglobin synthesis in immature definitive erythroid cells of 12-day foetal liver, but does not inhibit haemoglobin synthesis in immature definitive cells of foetal liver on subsequent days, athough non-haem protein synthesis is partially actinomycin D sensitive at all time points studied (Fantoni *et al.* 1968). It would appear that haemoglobin synthesis in proerythroblasts, basophilic erythroblasts, polychromatophilic erythroblasts and orthochromatic erythroblasts of 12-day foetal liver is actinomycin D sensitive, although haemoglobin synthesis in similar maturation stages of the definitive erythroid cells is actinomycin D resistant at 15 days (Djaldetti *et al.* 1970). No difference in morphology, RNA, DNA, or protein synthesis could be found between comparable maturation stages of the definitive erythroid cells of 12- and 15-day liver which could account for this difference in actinomycin D sensitivity (Djaldetti *et al.* 1970).

The regulatory mechanisms which program the cohort-like maturation of the primitive

erythroid cells (de la Chapelle *et al.* 1969), the shift of erythropoiesis from the yolk sac to the liver and subsequently to the spleen and bone marrow (Russell & Bernstein 1966), and the maturation of the mouse definitive erythroid cells (Djaldetti *et al.* 1970) remain unknown.

The role of erythropoietin in the regulation of embryonic, foetal and neonatal erythropoiesis requires clarification. Erythropoietin appears necessary for the production of recognizable erythroid cells in the adult mouse (Bleiberg, Liron & Feldman 1967; Curry, Trentin & Wolf 1967; Schooley 1967). However, the haematologic response of neonatal rodents, such as mice and rats, to anaemia, starvation, hypoxia, or nephrectomy (treatments which influence erythropoietin production in adult rodents) differs from that of adult animals subjected to similar treatment (Lucarelli *et al.* 1968; Lucarelli & Butturini 1967; Carmena, Howard & Stohlman 1968; Stohlman 1970). During early neonatal life the response of the young rodents becomes progressively more similar to that of adult animals (Carmena *et al.* 1968, Stohlman 1970). Similarly, the supression of erythroid colony formation in the spleens of lethally irradiated, plethoric mice injected with liver cell suspensions prepared from 12- to 19-day foetal mice was only partial (Bleiberg & Feldman 1969).

However, erythropoietin has been reported to stimulate haematopoiesis in cultures of foetal mouse and rat liver (Cole & Paul 1966; Paul & Hunter 1968, 1969; Hunter & Paul 1969; Gallien-Lartigue 1966, 1967), although no effect was observed on mouse or rat yolk sac erythroid cells. Preservation of immature mouse definitive erythroid precursors in cultures of foetal liver appears to be dependent on erythropoietin (Rifkind, Chui, Djaldetti & Marks 1969*a*; Chui, Djaldetti, Marks & Rifkind 1971; Marks 1972; Cantor, Morris, Marks & Rifkind 1972; Djaldetti, Preisler, Marks & Rifkind 1972; Marks & Rifkind 1972).

The question arises whether the stem cells for the definitive erythrocytes produced initially in the foetal mouse liver and subsequently in the spleen and bone marrow arise *in situ* or whether they are derived from migrating stem cells initially formed in the yolk sac. Moore & Metcalf (1970) have presented evidence suggesting that the development of liver erythropoiesis in the foetal mouse is dependent on the migration of yolk sac derived stem cells to the liver anlage. Cells capable of forming erythroid, granulocytic, and megakaryocytic colonies in the spleens of irradiated mice, assayed by the method of Till & McCulloch (1961), were first detected in the yolk sac, but not in the embryo, at 8 days. The number of yolk sac colony forming units (c.f.u.) reached a peak at 11 days and fell to zero by 13 days. *In vivo* c.f.u. were first detected in foetal liver at 10 days, and increased from a calculated total of 20 per liver on day 10 to 11000 per liver on day 13. *In vitro* c.f.u. were first detected in the embryonic circulation at 9 days and in the liver at 10 days. When 7-day mouse embryos with intact yolk sacs were cultured *in vitro* for 2 days, embryonic circulation developed and haematopoiesis was initiated in the liver. In these cultured embryos, *in vitro* c.f.u. were found in the yolk sac and also in the body of the embryo. When 7-day embryos were cultured *in vitro* for 2 days without the yolk sac, an embryonic circulation developed, as did the liver. However, it was reported that the embryonic circulation and liver of such embryos contained no erythroid cells and no *in vitro* c.f.u. When the yolk sac was cultured alone, *in vitro* c.f.u. were formed and persisted until 16 days, whereas in embryos developing *in vivo*, c.f.u. were not detected after 11 days. From these observations, and particularly from the absence of erythroid cells in the circulation and liver of embryos cultured without the yolk sac, it was considered that the *in vivo* and *in vitro* c.f.u. of the embryonic circulation and liver were derived from migration of yolk sac stem cells. *In vivo* c.f.u. were not assayed, however, in the embryos cultured *in vitro* with or without the yolk sac. It was pointed

out by the authors that the number of *in vitro* c.f.u. in the yolk sac, blood, and liver was much greater than the number of *in vivo* c.f.u. Furthermore, the relationship of *in vivo* c.f.u. which are capable of forming erythroid, granulocytic, and megakaryocytic colonies in spleens of irradiated mice and *in vitro* c.f.u. which form only granulocytic elements has not been clarified (Wu, Siminovitch, Till & McCulloch 1968; Bennett, Cudkowicz, Foster & Metcalf 1968; Bennett & Cudkowicz 1968; Chen & Schooley 1970; Haskill, McNeil & Moore 1970; Moore, McNeil & Haskill 1970; Van Bekkum, Noord, Maat & Dicke 1971).

Cells capable of forming colonies in the spleens of irradiated mice have been detected in the circulation of developing mouse embryos of 12 to 21 days (Barker 1970). The decrease in the total number of *in vivo* c.f.u. in mouse liver from 12 days until birth and the increase in *in vivo* c.f.u. in the spleens of late embryo and young neonatal mice (Barker, Keenan & Raphals 1969) would be compatible with migration of stem cells for the definitive erythroid series from the liver to the spleen. Alternative explanations for these observations, however, cannot be excluded.

(*c*) *Erythropoiesis during metamorphosis of the bullfrog tadpole*, Rana catesbeiana

The haemoglobins of the tadpole and adult frog of the species *Rana catesbeiana* differ (Herner & Frieden 1961; Baglioni & Sparks 1963; Hamada, Sakai, Shukuya & Kaziro 1964; Hamada & Shukuya 1966; Manwell 1966; Moss & Ingram 1965, 1968*a*, *b*; Stratton & Frieden 1967; Aggarwal & Riggs 1969; Wise 1970; Maniatis & Ingram 1971*b*, *c*). Similar differences in the tadpole and frog haemoglobins have been reported for *R. gryloi* and *R. heckscheri* (Herner & Frieden 1961), *R. clamitans* (Dessauer, Fox & Ramirez 1957) and *R. pipiens* (Maniatis 1969).

Baglioni & Sparks (1963), using starch electrophoresis, demonstrated four haemoglobins in the tadpole and four in frogs. Metamorphosing tadpoles had both tadpole and frog haemoglobins. Moss & Ingram (1965, 1968*a*, *b*), using polyacrylamide gel electrophoresis, demonstrated four to five haemoglobin components in tadpoles and five components in frog haemoglobin. Tadpoles undergoing natural or thyroxine induced metamorphosis showed a progressive replacement of the tadpole haemoglobin components by the components characteristic of frog haemoglobin (Moss & Ingram 1964, 1968*a*, *b*; De Witt 1968).

The chain composition of tadpole and frog haemoglobins of *R. catesbeiana* has been extensively investigated. Baglioni & Sparks (1963), on the basis of fingerprints of isolated frog and tadpole components, proposed that the tadpole haemoglobins were composed of at least three different peptide chains, while at least three additional peptide chains appeared necessary to generate the observed number of frog components. At most only one of these chains could be common to the tadpole and frog haemoglobins. Moss & Ingram (1968*a*), on the basis of low pH urea acrylamide gel electrophoresis of the isolated globin of the major frog and tadpole components, concluded that these components did not share a common chain. Similar results were obtained by Stratton & Freiden (1967) and Aggarwal & Riggs (1969) using chemical methods, and by Wise (1970) and Maniatis & Ingram (1971*b*) using immunological techniques. However, Hamada & Shukuya (1966) have reported that the major frog and tadpole haemoglobins shared one common chain.

Since the metamorphosis of the *R. catesbeiana* tadpole is associated with a switch in the type of haemoglobin found in circulating erythrocytes, the question arose whether this switch was associated with the appearance of a new erythrocyte population or occurred within erythrocytes of the same cell line. Moss & Ingram (1965, 1968*a*) and De Witt (1968) showed that following

administration of thyroxine to tadpoles the ability of circulating erythroid cells to incorporate radioactive amino acids into protein showed a sharp initial drop, followed by the appearance, in the circulation of the metamorphosing tadpole, of immature cells which were actively synthesizing frog haemoglobin. It was postulated that thyroxine inhibited the production of new immature tadpole cells and initiated the synthesis of frog haemoglobin either by triggering a new cell line or by turning on the synthesis of frog haemoglobin in erythroid cells previously committed to the synthesis of tadpole haemoglobin.

From early morphological studies (Jordan & Speidel 1923*a*, *b*) the kidney had been considered the major site of erythropoiesis in the tadpole, while, during metamorphosis, erythropoiesis was reported to shift to the spleen. However, studies of Maniatis & Ingram (1971*a*, *c*) indicated that the liver was the major site of erythropoiesis in both the tadpole and metamorphosing tadpole. Using specific antisera against the major tadpole or frog haemoglobin, conjugated with two different fluorescent dyes, Maniatis & Ingram (1971*c*) determined that the immature erythroid cells arising in the liver during thyroxine induced metamorphosis contained only frog haemoglobin. No erythroid cells, in the liver, spleen, kidney, or circulating blood, contained both tadpole and frog haemoglobin. These results were confirmed by Maniatis & Ingram (1971*c*) using the Gitlin technique (1968) for detection of antigens in single cells.

The decrease in protein and RNA synthetic capacity and the loss of ribosomal RNA in circulating tadpole erythrocytes following administration of thyroxine (Moss & Ingram 1965; McMahon & De Witt 1968), the absence of immature erythrocytes containing tadpole haemoglobin in the liver of metamorphosing tadpoles (Maniatis & Ingram 1971*a*, *c*), and the morphological characteristics of the immature, synthetically active erythrocytes found in the circulation of the metamorphosing tadpole (De Witt 1968; Vankin, Brandt & De Witt 1970) are compatible with the hypothesis that a second erythroid cell line, producing only frog haemoglobin, arises during natural or thyroxine induced metamorphosis. The observations of Hollyfield (1966) on the appearance, during thyroxine induced metamorphosis, of morphologically different, circulating erythrocytes with a smaller mean area and area distribution than erythroid cells of premetamorphoric tadpoles have been similarly interpreted. The recent observations of De Witt, Price, Vankin & Brandt (1972) and of Maniatis & Ingram (1972) on the appearance of very immature erythroid cells producing only tadpole haemoglobin in premetamorphoric tadpoles treated with phenylhydrazine suggest that the population of erythroid cells producing tadpole haemoglobin and the population of cells producing frog haemoglobin are independently regulated and arise from different progenitor cells.

(*d*) *Heterogeneity of haemoglobins in other vertebrates*

Changes in the types of haemoglobins produced in developing organisms have also been reported for a number of other species (Gratzer & Allison 1960; Muller 1961); for example, the cyclostome (Adinolfi, Chieffi & Siniscalco 1959), the rat (Hunter & Paul 1969), the monkey, *Macaca speciosa* (Kitchen, Eaton & Stenger 1968), cattle, pigs and sheep (Kleihauer & Stöffler 1968; Kleihauer, Brauchle & Brandt 1966; Grimes & Duncan 1959), and goats (Adams, Wrightstone, Miller & Huisman 1969). In cattle, pigs and sheep, two embryonic haemoglobins, similar in chain composition to Gower 1 and Gower 2 of the human embryo, are progressively replaced by foetal haemoglobin, and subsequently by the adult type haemoglobin. The presence of the embryonic haemoglobins is temporally correlated with the presence of circulating primitive erythrocytes (Kleihauer & Stöffler 1968).

Similarly, the presence of two populations of erythroid cells, a primitive and definitive cell line, during development of vertebrates is not unusual (Bloom & Bartelmez 1940). Whether the primitive and definitive cells develop at the same site appears to vary depending on the species. For example, in the rabbit, guinea-pig and cat, large numbers of definitive cells develop in the yolk sac (Maximow 1909); in man, few definitive cells arise in the yolk sac (Bloom & Bartelmez 1940); and in the mouse and rat definitive erythrocytes do not develop in the yolk sac (Bank *et al.* 1970: Hunter & Paul 1969).

(*e*) *Erythropoiesis during development of the chick embryo*

(i) *Sites of erythropoiesis*

The earliest erythroid elements of the developing chick embryo are formed in blood islands lying in the horseshoe-shaped area surrounding the posterior and posteriolateral parts of the area pellucida (Romanoff 1960; Murray 1932). Localized thickenings of the extra-embryonic mesoderm are noted in this area at the head fold stage, and clumps of cells are apparent in the deep layers of the mesoderm at the 2- to 4-somite stage (Sabin 1920). At the 5-somite stage, the peripheral cells of these aggregates form the vascular epithelium of the blood islands, and enclose the cells which form the first, or primitive, erythrocytes of the embryo (Dantschakoff 1908; Sabin 1920; Murray 1932). The formation of blood islands occurs initially at the peripheral zone of the area vasculosa and spread centrally, the blood islands becoming a network of capillaries. The embryonic circulation becomes established at 16 to 17 somites (45 to 49 h). Several additional waves of blood island formation occurring after the establishment of the circulation have been noted by Sabin (1920), but were not observed by Small (1969). With increasing age of the embryo the vascular network of the yolk sac increases in size by a process of proliferation of solid masses of endothelial cells which secondarily form lumina continuous with the pre-existing vessels (Sabin 1920). Whether this expansion of the extra-embryonic blood vessel network is accompanied by additional formation of erythropoietic cells has been disputed (Dantschakoff 1909*a*, *b*; Jolly 1939). With the exception of foci of haemocytoblasts (the multipotential haematopoietic stem cell of Dantschakoff (1908) and Maximow (1924)) in the caudal dorsal aorta in the 8- to 9-somite embryo and on the ventral wall of the aorta in the 3- to 4-day embryo (Dantschakoff 1909*a*; Sabin 1920) formation of intra-embryonic blood vessels does not appear to be associated with formation of erythropoietic foci (Jolly 1939).

The yolk sac remains the major erythropoietic site of the chick embryo until 18 to 20 days (Dantschakoff 1908), and thus is also the site of initial formation of the second, or definitive, erythroid cell line of the chick embryo. Dantschakoff (1908) reported an increased number of haemocytoblasts in the yolk sac of 4- to 5-day embryos, a stage when the yolk sac membranes grow into the yolk sac. Small (1969) confirmed these observations and, in a 7-day embryo, observed folds of yolk sac entoderm containing immature definitive erythrocytes extending into the yolk mass.

Neither the liver nor the spleen of the developing chick embryo produce significant numbers of erythrocytes (Stricht 1891; Haff 1914; Danchakoff 1916). Bone marrow erythropoiesis becomes significant on days 10 to 12 of incubation (Dantschakoff 1909*b*: Dawson 1936) and becomes the major site of erythropoiesis at the time of hatching. It has been reported that, in contrast to the maturation of erythrocytes in the yolk sac, marrow derived erythrocytes are released into the embryonic circulation as mature cells (Dantschakoff 1908).

White blood cells are formed in the extra-vascular tissue of the yolk sac at 3 to 4 days (Dantschakoff 1908; Sugiyama 1926). Thromboblasts and thrombocytes are found in the yolk sac vasculature at 3 to 4 days (Sugiyama 1926). Granulopoiesis begins in the spleen at 9 days (Danchakoff 1916; Sandreuter 1951), the liver at 11 days (Haff 1914; Sandreuter 1951), the thymus at 9 to 10 days (Terni 1924), the bone marrow at 10 to 12 days (Dantschakoff 1909*a*, *b*), and in the bursa of Fabricus near hatching (Romanoff 1960). The thymus has become a lymphoid organ by 10 days (Terni 1924).

(ii) *Erythroid cell populations*

Primitive erythrocytes. The first erythroid cells formed in the blood islads of 5- to 7-somite embryos (Dantschakoff 1908; Sugiyama 1926; Murray 1932; Sabin 1920) constitute the only recognizable erythroid cells until about 5 days of age (Dantschakoff 1908; Sugiyama 1926; Dawson 1936; Fennel 1947; Lemez & Rychter 1956; Lucas & Jamroz 1961; Edmonds 1966; Ceresa-Castellani & Leone 1969; Small 1969; Weintraub, Cambell & Holtzer 1971; Cambell, Weintraub, Mayall & Holtzer 1971). Beginning at 5 days, the primitive cells are progressively replaced by the definitive cells (Dantschakoff 1908; Sugiyama 1926; Dawson 1936; Fennel 1947; Lemez & Rychter 1956; Lucas & Jamroz 1961), and only an occasional primitive cell is found in the circulation of late embryos and young chicks (Dawson 1936; Lemez & Rychter 1956; Lucas & Jamroz 1961).

On the basis of morphological studies with the light microscope, several sequential stages of the maturing primitive cells have been described: the proerythroblast, basophilic erythroblast, early, mid, and late polychromatophilic erythroblasts, reticulocyte and mature primitive erythrocyte (Sugiyama 1926; Dawson 1936; Fennel 1947; O'Connor 1952; Romanoff 1960; Lucas & Jamroz 1961; Small 1969).

The morphological characteristics associated with maturation of the primitive erythroid cells, as observed in the electron microscope, substantiate the changes observed by light microscopy (Edmonds 1966; Ceresa-Castellani & Leone 1969; Small 1969). The proerythroblast found in the embryonic vessels at 1.5 to 2 days (Small 1969) is characterized by a large central nucleus with homogeneously arranged chromatin and one to two prominent nucleoli, numerous mitochondria, and numerous free ribosomes and polysomes. Cytoplasmic yolk inclusion bodies are frequently observed. The basophilic erythroblast, observed at 1.5 to 2.5 days (Small 1969), has a large nucleus with beginning peripheral chromatin clumping, prominent nucleoli, and a very high concentration of cytoplasmic ribosomes. Small groups of cytoplasmic microtubules, precursors of the marginal band, are observed near the cell periphery. The early, mid, and late polychromatophilic erythroblasts, progressively observed in the embryonic vessels from 2.5 to 6 days of incubation (Small 1969), show a decreasing nuclear-cytoplasm ratio, progressive clumping of nuclear chromatin, less prominent nucleoli, a decreasing concentration of cytoplasmic ribosomes and a progressive increase in the density of the cytoplasmic matrix, presumably due to the increasing haemoglobin content of the cells. The early polychromatophilic erythroblast, observed at 2.5 to 3.5 days (Small 1969), is the first cell of the primitive erythroid series to have a complete marginal band of microtubules. The appearance of the marginal band is correlated with the change from a spherical to an ovaloid shape of the primitive cells. The primitive reticulocyte, observed from 6 to 8 days, has marked condensation of the nuclear chromatin, a low concentration of cytoplasmic ribosomes, some mitochondria, and a fragmented Golgi apparatus. Mature primitive erythrocytes, observed after 8 days, have

few, if any ribosomes, occasional mitochondria, very pronounced clumping of the nuclear chromatin, and a dense haemoglobin filled cytoplasm. The biosynthetic capacities of maturing primitive erythroid cells correlate with the cell ultrastructure (Hagopian & Ingram 1971; Grinnell & Lee 1972).

Definitive erythrocytes. The first immature definitive erythrocytes are found in the embryonic circulation at 4 to 5 days of incubation (Dantschakoff 1908; Sugiyama 1926; Fennel 1947; Lemez & Rychter 1956; Lucas & Jamroz 1961; Edmonds 1966; Small 1969) when erythropoiesis is confined to the yolk sac. Both Dantschakoff (1908) and Edmonds (1966) reported that the first definitive cells arise from haemocytoblasts or mulipotential stem cells similar to and perhaps derived from the blood island cells of the 5- to 7-somite embryo. The several sequential maturation stages of the definitive erythroid cells include: the proerythroblast; basophilic erythroblast; early, mid, and late polychromatophilic erythroblasts; reticulocyte; and mature definitive cells (Dawson 1936; Fennel 1947; Lucas & Jamroz 1961; Cameron & Prescott 1963; Cameron & Kastberg 1969; Small 1969). The maturation stages of the definitive cells are similar to those described for foetal rabbit liver erythroid cells (Grasso, Woodward & Swift 1963), rabbit bone marrow cells (Borsook 1966), foetal mouse liver definitive erythroid cells (Rifkind *et al.* 1968*b*), adult mouse spleen and bone marrow erythroid precursors (Russell & Bernstein 1966), and human bone marrow cells (Wintrobe 1956).

Studies of definitive cell maturation with the electron microscope (Edmonds 1966; Small 1969) substantiate the maturation changes observed by light microscopy. The ultrastructure changes in maturing definitive erythroid cells are quite similar to those of comparable maturation stages of the primitive erythroid cells (Edmonds 1966; Ceresa-Castellani & Leone 1969; Small 1969); foetal rabbit liver erythroid cells (Grasso, Swift & Ackerman 1962; Sorenson 1960); primitive and definitive mouse erythroid precursors (Kovach *et al.* 1967; Rifkind *et al.* 1968*b*; Djaldetti *et al.* 1970); adult mouse spleen erythroid cells (Orlic, Gordon & Rhodin 1965); and human foetal liver erythroid cells (Grasso *et al.* 1962; Zamboni 1965). Several maturation stages of definitive cells are simultaneously observed in the circulation of embryos from 5 to 6 days until 18 to 20 days (Dawson 1936; Fennel 1947; Sandreuter 1951; Lucas & Jamroz 1961; Small 1969). The first definitive cells seen in the embryonic circulation at about 5 days include a few proerythroblasts, basophilic erythroblasts and early polychromatophilic erythroblasts. On days 6 to 8, early, mid, and late polychromatophilic erythroblasts are the predominant definitive cells. At 9 to 10 days round definitive cells with round nuclei, comparable to reticulocytes, and the first mature, definitive erythrocytes are observed (Dawson 1936; Lemez 1964). With increasing age of the embryo, very immature definitive cells (basophilic erythroblasts and early polychromatophilic erythroblasts) are infrequently observed, and mid and late polychromatophilic erythroblasts constitute only a small fraction of the definitive cells. This increasing maturity of the circulating definitive cells occurs while the blood volume and total number of definitive cells per embryo is greatly increasing (Rychter, Kopecky & Lemez 1955; Schønheyder 1938; Sendju 1927; Ramsay 1950; O'Connor 1952). Mitoses among circulating definitive cells are rarely observed after 6 to 7 days (Dawson 1936; Lucas & Jamroz 1961). Therefore, with increasing age of the embryo, immature definitive cells appear to remain outside the circulation until the late polychromatophilic erythroblast and reticulocyte stages (Dawson 1936; Lucas & Jamroz 1961).

The first mature definitive cells are round cells with round nuclei, whereas mature definitive cells of 18- to 21-day embryos and of adult chickens are oval with oval nuclei (Dantschakoff

1908; Fennel 1947; Lemez 1953; Lucas & Jamroz 1961). It was postulated that the round definitive cells with round nuclei were reticulocyte precursors to the mature oval definitive cells with oval nuclei (Keller 1933). However, many of the round, highly haemoglobinized cells with round nuclei are not reticulocytes (Dawson 1936; Lemez 1957). Lemez (1957, 1964) proposed that the mature round definitive cells with round nuclei represented the first generation of definitive erythrocytes (E_1) and the mature oval definitive cells with oval nuclei represented a second series of embryonic definitive cells (E_2). Dantschakoff (1908, 1909*a*) had also proposed that several (2 to 4) successive generations of definitive cells occurred during embryogenesis of the chick. The evidence for the multiple generations of definitive cells was based on the cytoplasmic coloration, nuclear shape, and degree of nuclear pycnosis (Lucas & Jamroz 1961).

(iii) *Haemoglobins of chick embryos and adult chickens*

Haemoglobins of the adult chicken. Haemoglobin of adult White Leghorn chickens can be resolved by electrophoresis or column chromatography into several components. Van der Helm & Huisman (1958) observed two components on paper electrophoresis and IRC-50 column chromatography of adult chicken haemoglobin. The major component constituted about 85 % and the minor component, about 15 % of the total haemoglobin. The amino acid composition of the two components were quite different. Two haemoglobins of widely different amino acid composition were also observed by Saha (1964), Saha & Ghosh (1965), Schnek *et al.* (1966), and Fraser (1961) using ion exchange chromatography. The latter two authors reported that the major haemoglobin constituted about 70 % and the minor component, about 30 % of the total haemoglobin.

One major component, one minor component, and a trace component constituting about 73, 23 and 4 % respectively of the total haemoglobin were observed by Matsuda & Takei (1963) using CM-cellulose chromatography. Hashimoto & Wilt (1966) also isolated a major component, a minor component, and a trace component from cyanmethaemoglobin by CM-cellulose chromatography of freshly prepared or stored haemoglobin. The major component constituted 65 to 70 % of the total haemoglobin, the minor component, 30 to 35 %, and the trace component, less than 1 %. Starch gel electrophoresis of the isolated components, however, revealed additional heterogeneity. The major component migrated as a single component, while the minor component split into two fractions, and the trace component migrated as a diffuse band. Re-electrophoresis of the components eluted from the starch gels indicated that each component retained its electrophoretic position.

Bargellesi, Callegarini & Conconi (1969) and Callegarini, Bargellesi & Conconi (1969) using IRC-50 chromatography observed one major, one minor, and a trace component which constituted 70, 20 and 9 % respectively of the haemoglobin. Starch gel electrophoresis of similarly prepared cyanmethaemoglobin showed two components, constituting 70 to 75 % and 25 to 30 % of the total haemoglobin.

Three components, constituting 73, 26 and 0.9 % of the total haemoglobin, were isolated by Moss & Thompson (1969*a*) and Moss (1970) using Bio-Rex 70 chromatography. Carbonmonoxyhaemoglobin of adult chickens showed one major and one minor component on both starch gel and polyacrylamide gel electrophoresis. In contrast to the observations of Hashimoto & Wilt (1966), both the major and minor components isolated by column chromatography showed only one band on both starch gel and polyacrylamide gel electrophoresis. The major and minor components isolated from the columns migrated similarly to the major

and minor haemoglobin components of unfractionated adult chicken haemoglobin. These observations are similar to those of Müller (1961).

Using agar gel electrophoresis, D'Amelio (1966) observed one major, one minor, and a trace component in adult chicken haemoglobin samples. One major component (80 %) and one minor component (20 %) were observed on starch gel electrophoresis by Manwell, Baker, Rosklansky & Foght (1963) and by Manwell, Baker & Betz (1966). Haemoglobin isolated from White Leghorn, New Hampshire, and Columbian chickens showed similar electrophoretic patterns. Kabat (1968) observed one major and one minor component (3:1 ratio) with both IRC-50 chromatography and acrylamide gel electrophoresis of adult chicken haemoglobin.

Washburn (1968*a*, *b*), studying the haemoglobins of Athens–Canadian chickens, found three different types of electrophoretic patterns using cellulose acetate electrophoresis. One adult chicken was observed to have, in addition to the major haemoglobin component, two minor components, one migrating at the normal minor band position, and the second migrating faster than the usual minor component. Genetic studies indicated that the chicken with two minor haemoglobin components was heterozygous for a mutation involving the gene for one of the chains of the minor haemoglobin. Callegarini *et al.* (1969) have reported similar homozygous and heterozygous mutants in a White Leghorn line of chickens.

It would appear, therefore, that adult chicken haemoglobin can be resolved into two components on starch or acrylamide gel electrophoresis, and two components plus a trace component on ion exchange chromatography. The major component constitutes about 70 % of the haemoglobin while the minor component constitutes about 30 % of the total. The trace component has been reported to constitute from 1 to 9 % of the total haemoglobin.

There are, however, several reports of additional haemoglobin components. Marchis-Mouren & Lipmann (1965) observed five components on IRC-50 chromatography of the ribosome free supernatant of lysed adult chicken reticulocytes. In Rhode Island Red chickens, Simons (1966) observed four haemoglobin components using CM-cellulose chromatography. Schurch, Godet, Nigon & Blanchet (1968) observed one major and one minor component in adult haemoglobin of White Leghorn chickens on polyacrylamide gels at pH 8.9 (25 °C). As noted by Manwell *et al.* (1963), Hashimoto & Wilt (1966), Denmark & Washburn (1969*b*) and Moss & Thompson (1969*a*), freezing and thawing of adult chicken haemoglobin, storage of samples, and changes in the valance state of the haem iron may generate artefactual components.

An area of controversy in the literature concerns the chain composition of the major and minor adult chicken haemoglobins (table 1). Saha (1964) concluded from the amino acid composition of the isolated globin chains of the two haemoglobins that all four chains were unique. Using pH 1.9 starch gel electrophoresis of globins prepared from the major and minor adult components, D'Amelio (1966) and Manwell *et al.* (1966) proposed that the adult major and minor components shared a common β chain. Hashimoto & Wilt (1966), who resolved the minor component into two components on starch gel electrophoresis, concluded that the adult major component and the two minor components shared a common β chain although the α chains of the three components appeared unique as judged by pH 1.9 starch gel electrophoresis. Bargellesi *et al.* (1969) concluded from pH 3.5 urea starch gel electrophoresis that the adult major and minor components shared a common α chain.

Moss & Thompson (1969*a*) and Moss (1970) concluded from the identical electrophoretic mobility of the isolated β chains of the major and minor components and the 'fingerprints' of these globin chains that the β chains of the two haemoglobin components were very similar.

However, the amino acid composition of the two isolated β chains was not identical. The isolated α chain globins of the major and minor haemoglobins were electrophoretically different and had significantly different 'fingerprints' and amino acid compositions. These authors concluded that all four chains of the adult chicken haemoglobins were unique although the β chains of the two components were quite similar. The amino acid sequence of the α chain of the major adult chicken haemoglobin has been reported by Matsuda, Takei, Wu & Shiozawa (1971).

TABLE 1. SUMMARY OF THE LITERATURE ON THE CHAIN COMPOSITION OF THE MAJOR AND MINOR HAEMOGLOBINS OF ADULT CHICKENS

author	method	conclusions
Saha (1964)	amino acid composition of isolated globins	major and minor components: no common chains
Manwell *et al.* (1966); D'Amelio (1966)	pH 1.9 starch gel electrophoresis of globins	major and minor components: common β chain
Hashimoto & Wilt (1966)	pH 1.9 starch gel electrophoresis of globins	major and split minor components: common β chain
Bargellesi *et al.* (1969)	pH 3.5 starch gel electrophoesis of globins	major and minor components: common α chain
Moss & Thompson (1969*a*); Moss (1970)	pH 1.9 starch gel electrophoresis of globins, fingerprints of separated chains, amino acid composition of separated chains	major and minor component: β chains of identical electrophoretic mobility, similar fingerprints but amino acid composition not identical

An additional area of uncertainty has been whether any of the chains of adult chicken haemoglobin have acetylated *N*-terminal amino acids. Satake, Sasakawa & Maruyama (1963) described the isolation of *N*-acetyl-Val-Thr-Leu from unfractionated adult chicken globin. Matsuda, Maita & Nakajima (1964) and Matsuda, Maekawa & Otsubo (1965) found approximately 4 mol DNP-valine per mol major haemoglobin component and 2 mol DNP-valine per mol minor component. No additional DNP amino acids could be isolated from the separated minor component. When the *N*-terminal residues of the separated α and β chains of the major and minor adult chicken globins were examined, the α chains of the major component had *N*-terminal Val-Leu residues and the β chains, *N*-terminal Val-His residues. The β chains of the minor component had *N*-terminal Val-His residues, but no *N*-terminal residues could be detected for the α chains.

Schnek *et al.* (1966), however, found 4 mol *N*-terminal valine per mol minor component and 2 mol *N*-terminal valine per mol major component plus 1.9 mol acetate per mol major component by hydrazinolysis. Marchis-Mouren & Lipmann (1965) observed that ^{14}C acetate from acetyl-CoA could be incorporated into four of the five haemoglobin components from phenylhydrazine treated adult chickens, and at least some of the incorporated acetate was in *N*-terminal residues.

Moss & Thompson (1969*a*) and Moss (1970), using White Leghorn chickens, found that the *N*-terminal residues of the isolated α chains of the major component were Val-Leu-Ser-, and of the isolated β chains of the major component, Val-His-Gly-. The *N*-terminal residues of the isolated α chains of the minor component were Met-Leu-Thr-, and of the isolated β chains of the minor component, Val-His-Gly-. However, 2 mol acetate per mol major component and about 0.5 mol acetate per mol minor component were found by gas chromatography after

hydrazinolysis of the haemoglobins (Moss & Thompson 1969*b*). The acetate was localized to the α chains of both haemoglobin components. The acetate did not represent *O*-acetylation, and was subsequently localized in tryptic peptides 13 to 14 and 13 to 15 of the α chain of the major adult haemoglobin. This sequence contains two lysine residues, one of which must be *N*-acetylated. The α chain acetate of the minor component was not further characterized (Moss 1970). The observations are summarized in table 2. The difference in the results of Matsuda *et al.* (1964, 1965), of Schnek *et al.* (1966), and of Moss & Thompson (1969*a*, *b*) and Moss (1970) remain unexplained, but could perhaps reflect strain differences in the White Leghorn chickens used by these authors.

TABLE 2. SUMMARY OF THE LITERATURE ON THE *N*-TERMINAL RESIDUES OF THE MAJOR AND MINOR ADULT CHICKEN HAEMOGLOBINS

author	component	conclusions
Satake *et al.* (1963)	total globin	*N*-acetyl-Val-Thr-Leu
Matsuda *et al.* (1964, 1965)	major component	4 mole DNP-valine/mol globin
	minor component	2 mol DNP-valine/mol globin
	major component α chain	*N*-terminal Val-Leu
	major component β chain	*N*-terminal Val-His
	minor component α chain	blocked *N*-terminal
	minor component β chain	*N*-terminal Val-His
Schnek *et al.* (1966)	major component	2 mol *N*-terminal valine and 1.9 mol of acetate/mol globin
	minor component	4 mol of *N*-terminal valine/mol globin
Moss & Thompson (1969*a*, *b*); Moss (1970)	major component α chain	*N*-terminal Val-Leu-Ser-
	major component β chain	*N*-terminal Val-His-Gly-
	minor component α chain	*N*-terminal Met-Leu-Thr-
	minor component β chain	*N*-terminal Val-His-Gly

Haemoglobins of the chick embryo. The haemoglobins of the developing chick embryo have been compared to adult chicken haemoglobin at several time points during embryogenesis by a number of investigators. Fraser (1961), using paper electrophoresis at pH 5.9 and CM-cellulose chromatography, reported that haemoglobin of 5-, 7-, 11- and 16-day embryos had the same two components observed in adult chicken haemoglobin, athough the proportions of the two components differed from adult haemoglobin. In a subsequent publication, Fraser (1963) noted the presence of a third haemoglobin component in embryos of 7 to 21 days of age. When haemoglobins of 4- to 19-day embryos were chromatographed on phosphonic acid cellulose (Cellex-P) as many as 13 components were observed during development of the embryos (Fraser, Horton, Dupourque & Chernoff 1972).

Manwell *et al.* (1963), using starch gel electrophoresis at pH 7.2 and 8.2, observed two haemoglobin components in 4- to 5-day embryos which were not found in adult chicken haemoglobin. The major component constituted 80 % of the total haemoglobin and the minor component, 20 % of the total. Haemoglobin of 7-day embryos could be resolved into four components, the two embryonic haemoglobins plus the two adult components. The two embryonic haemoglobins were not detected in embryos of 12 days of age, although a trace haemoglobin component, migrating more slowly than the minor embryonic haemoglobin, was observed in late embryos (Manwell *et al.* 1966).

Three components were observed in haemoglobin of 5-day embryos by Hashimoto & Wilt

(1966) using starch gel electrophoresis. Additional heterogenetiy was not observed on re-electrophoresis of the isolated haemoglobins. Two of the components appeared to correspond to the major embryonic component of Manwell *et al.* (1963) and constituted 47 and 43 % of the total haemoglobin. The minor haemoglobin component, constituting 10 % of the total haemoglobin, was electrophoretically similar to the minor component of Manwell *et al.* (1963).

Huisman & Schillhorn Van Veen (1964) observed three haemoglobin components in lysates prepared from 14- and 17-day embryos and young chicks. The major component was electrophoretically comparable to the major adult haemoglobin component. The minor component of lysates from 14- and 17-day embryos migrated more slowly than the minor adult component. The minor component of haemoglobins from young chicks migrated faster than that of late embryo haemoglobins, but more slowly than the adult minor component. However, following dialysis of the haemoglobin of adult chickens or young chicks against 0.2 M phosphate buffer, pH 7.4, the electrophoretic mobility of the minor component was comparable to that of the minor component of late embryo haemoglobins. The third, or trace, haemoglobin component of late embryos, which constituted about 8 % of the total haemoglobin of 14-day embryos, was no longer detectable in young chicks 32 days following hatching.

Godet (1967) compared the haemoglobins of 5- to 18-day chick embryos with the haemoglobin of 4-day-old chicks and adult chickens. Adult chicken haemoglobin was resolved into one major slow component and four minor faster components. Haemoglobin of 4-day-old chicks had the five adult components plus two trace components migrating more slowly than the major haemoglobin component. Haemoglobin of 5- and 6-day chick embryos was resolved into three components, electrophoretically similar to the components observed by Manwell *et al.* (1963) and Hashimoto & Wilt (1966) in haemoglobin of 5-day embryos. At 7 days, a component migrating similarly to one of the adult minor fast bands was observed in addition to the three embryonic components. Haemoglobins of 10- to 18-day embryos contained the major adult component, the two slowly migrating trace bands seen in haemoglobin of 4-day-old chicks, and the faster migrating minor band observed in 7-day embryo haemoglobin.

Denmark & Washburn (1969*a*, *b*), using cellulose acetate strip electrophoresis, observed three haemoglobin components in red cell lysates from 3- to 5-day chick embryos comparable to the components reported by Manwell *et al.* (1963) and Hashimoto & Wilt (1966). The major component constituted 75 to 80 %, and the minor component, 20 to 25 % of the total haemoglobin. Three components were observed in haemoglobins of 11- to 18-day chick embryos and young chicks less than 12 weeks of age: the adult major and minor components plus the slowly migrating minor embryonic component. The latter component constituted about 7 % of the total haemoglobin in late embryos. These authors also studied the haemoglobins of chick embryos homozygous and heterozygous for the mutant minor adult haemoglobin component (Washburn 1968*a*).

The chain composition of the haemoglobins of chick embryos has also been investigated. Manwell *et al.* (1966), using pH 2 starch gel electrophoresis, reported that the major and minor embryonic haemoglobins did not share a common chain. However, the minor embryonic haemoglobin appeared to have an α chain in common with the major adult component. The major embryonic haemoglobin component could be split into two bands under some electrophoretic conditions. Chain composition studies suggested that the split major embryonic components had a common α chain. The β chain of one of these components appeared to be similar to the β chains of both the major and minor adult haemoglobins.

Hashimoto & Wilt (1966), on the basis of pH 1.9 starch gel electrophoresis, reported that the three embryonic haemoglobnis shared a common β chain, which was distinct from the β chain of the adult haemoglobins. The minor embryonic haemoglobin component showed an α chain similar to the α chain of the major adult component. The α chains of the two major embryonic components were heterogeneous, but no α chains common to these two embryonic components were observed. Only one of the four α chains of these two major embryonic haemoglobins appeared similar to the α chain of the adult minor component.

TABLE 3. SUMMARY OF THE LITERATURE ON THE CHAIN COMPOSITION OF THE HAEMOGLOBINS OF CHICK EMBRYOS

author	method	chain composition of globins of chick embryos	comparison with the chain composition of adult chicken globins
Manwell *et al.* (1966)	pH 2 electrophoresis of globins	major and minor components: no common chain	minor embryonic and adult major component: common α chain
		split major components: common α chain	one of the split major embryonic components and adult major and minor components: common β chain
Hashimoto & Wilt (1966)	pH 1.9 starch electrophoresis of globins	major and minor components: common β chain	minor embryonic and adult major component: common α chain
			one of the split major embryonic components and adult minor component: common α chain

Although the observations of Hashimoto & Wilt (1966) on the number of haemoglobin components in red cell lysates from 5-day chick embryos and on the electrophoretic mobilities of the components were similar to the observations of Manwell *et al.* (1963, 1966), the chain composition formulation of the two groups were significantly different (table 3).

(iv) *Regulation of erythropoiesis*

Little is known about the nature of the regulatory events by which certain cells of the chick blastoderm become committed first to primitive erythropoiesis and subsequently to definitive erythropoiesis, or when such events occur during embryogenesis of the chick. Settle (1954) mapped the areas of the chick blastoderm capable of forming blood islands *in vitro* from pre-streak blastoderms to blastoderms of four to six somites. The position changes of the areas of potential erythropoiesis were similar to those of the epiblast areas forming the posterior and posteriolateral extra-embryonic mesoderm described by Spratt (1942, 1946, 1947) and Rudnick (1944, 1948). Settle proposed, therefore, that certain areas of the chick blastoderm became committed to erythropoiesis before the pre-streak stage (less than 6 to 7 h incubation).

Miura & Wilt (1969) observed that the *in vitro* formation of blood islands in ectomesodermal tissue of the presumptive area vasculosa of definitive primitive streak embryos (20 h incubation), if not totally dependent upon interaction with endodermal tissue, was significantly enhanced by interaction with endodermal tissue. The endodermal layer is formed in the developing blastoderm prior to the pe-streak stage (Lillie 1952). Miura & Wilt (1971) also observed that the formation of erythropoietic foci in cultures of re-aggregated presumptive area vasculosa of definitive primitive streak embryos (20 h incubation) was inhibited if 5-bromodeoxyuridine

(BUdR) was included in the culture medium for the first 6 h following explantation. After the first 6 h, the formation of erythropoietic foci was not inhibited by BUdR, although the analogue was incorporated into cellular DNA during both the sensitive and insensitive periods. These authors proposed that during the first 6 h of culture of presumptive erythropoietic tissue from definitive primitive streak embryos, a regulatory event, critical for subsequent differentiation of erythropoietic cells, occurs.

Additional regulatory events, occurring prior to the appearance of haemoglobin in blood islands of the 6- to 9-somite embryo have been described by several investigators (O'Brien 1961; Hell 1964). O'Brien (1961) observed an inhibition of haemoglobin formation in explanted blastoderms when 8-azaguanine was added to the culture medium prior to the 4-somite stage. The analogue had no effect on haemoglobin formation of explanted blastoderms after the 4-somite stage. Wilt (1962, 1965) observed that actinomycin D, 5-fluorouracil, and 5-bromodeoxyuridine inhibited haemoglobin formation in explanted blastoderms if added prior to the head fold stage (19 to 22 h), but had no effect if added to the cultures after this stage. Inhibition of haemoglobin formation was observed when 8-azaguanine was added to the cultures prior to the 7-somite stage, whereas no inhibition was observed when this analogue was added after the 7-somite stage. It was proposed, therefore, that the messenger RNA for haemoglobin synthesis by the primitive cells was formed and stabilized a number of hours before the onset of haemoglobin synthesis. Levere & Granick (1967) proposed that ALA synthetase (the first enzyme in the haem biosynthetic pathway) was rate limiting for haem synthesis and haemoglobin formation in chick blastoderms. The inhibition of haemoglobin synthesis observed when explanted blastoderms of less than six somites were treated with actinomycin D could be overcome by addition of δ-aminolevulinic acid (ALA), suggesting that the messenger RNA for ALA synthetase is transcribed prior to the head process stage (23 to 25 h). These observations would place the time of synthesis of at least some of the messenger RNA for globin synthesis earlier than 23 to 25 h. Similar observations have been reported by Wainwright & Wainwright (1966, 1967*a*, *b*, *c*).

Hagopian & Ingram (1971) have shown that blastoderms, de-embryonated and placed in organ culture at 1.5 days of incubation, are capable of producing primitive and definitive erythrocytes, as well as the embryonic and adult haemoglobins associated with two erythrocyte populations. The number of primitive cells increase in number between day 1.5 and day 7.5 at a rate comparable to that observed *in ovo* when expressed as cells/cm^2 of tissue. The mitotic indices and the cell cycle times of the primitive cells are also comparable to primitive cells formed *in ovo* (Dawson 1936; O'Connor 1951; Weintraub *et al.* 1971). The information necessary for the formation of additional primitive cells, as well as the progenitor cells for the definitive erythroid series, and the 'biological clock' mechanism for the initiation of the production of the second erythroid cell series is contained in the area vasculosa of 1.5 day blastoderms and does not require the continued presence of the embryo.

Cell suspensions of 24 h chick blastoderms which contain no haemoglobinized erythroid precursors are also capable of producing both primitive and definitive erythrocytes and the associated embryonic and adult haemoglobins when cultured *in vitro* (Hagopian, Lipke & Ingram 1972).

These observations, while indicating that the progenitor cells of the definitive erythroid series are present in the area vasculosa of 24 to 36 h blastoderms, do not provide information on whether such progenitor cells are in cell cycle (Becker, McCulloch, Siminovitch & Till 1965;

Dutton & Mishell 1967) or in G_0 (Gelfant 1964; DeCosse & Gelfant 1968; Burns & Tannock 1970), in the intact embryo developing *in ovo*.

The studies of Moore & Owen (1965, 1967*a*) indicated that cell suspensions prepared from the yolk sac of 7-day chick embryos, 14-day embryo spleens, and 16- to 18-day embryo bone marrow were capable of repopulating the erythroid, myeloid, and lymphoid tissues of irradiated chick embryos. Circulating cells capable of establishing thymus and bursa of Fabricus chimeras were also detected in chick embryos (Moore & Owen 1966, 1967*b*), although the precise time of development of such cells was not determined.

TABLE 4. COMPARISON OF THE NEW NOMENCLATURE OF HAEMOGLOBIN COMPONENTS OF YOUNG EMBRYOS WITH THE NOMENCLATURE OF OTHER AUTHORS

investigators	major haemoglobin	minor haemoglobin	other haemoglobin components
Bruns (1971); Hagopian & Ingram (1971)	P	E	—
Hashimoto & Wilt (1966)	E_2+E_1	E_3	—
Manwell *et al* (1963)	embryo major haemoglobin	embryo minor haemoglobin	—
Denmark & Washburn (1968*a*)	M	E_1	E_2 type I T type II E_2, T type III

TABLE 5. COMPARISON OF THE NEW NOMENCLATURE OF HAEMOGLOBIN COMPONENTS OF LATE EMBRYOS, YOUNG CHICKS, AND ADULT CHICKENS WITH THE NOMENCLATURE OF OTHER AUTHORS

investigators	major haemoglobin	minor haemoglobin	trace haemoglobin	other haemoglobin components
Bruns (1971); Hagopian & Ingram (1971)	A	D	H	—
Callegarini *et al.* (1969)	Hb_2	Hb_{1a}	—	Hb_1
D'Amelio (1966)	C_1	C_2	—	C_3
Hashimoto & Wilt (1966)	A-3	A-2 $[A_{2f}+A_{2s}]$	—	A-1
Huisman *et al.* (1964)	major	minor	slow	—
Manwell *et al.* (1963)	adult major Hb	adult minor Hb	trace Hb of foetuses and chicks	—
Matsuda & Takei (1963)	A_{II}	A_I	—	A_{III}
Moss & Thompson (1969*a*)	Hb_I	Hb_{II}	—	Hb_{III}
Saha (1964)	Hb_1	Hb_2	—	—
Schnek *et al.* (1966)	P_2	P_1	—	—
Washburn (1968*b*)	M	type I M_1, type II M_2	E	type II T

These studies indicate that multipotential stem cells are present in the yolk sac of 7-day chick embryos. However, whether progenitor cells for the definitive erythrocyte series exist in embryos of less than 5 days of age as cells committed to definitive erythropoiesis, capable of forming foci of definitive erythroid cells, *in vivo* or *in vitro*, is unknown. It is also not known whether definitive stem cells arise from cells previously committed to primitive erythropoiesis, or whether two separate groups of progenitor cells are formed early in embryogenesis, one group of cells giving rise to primitive erythrocytes, and the second, to definitive erythrocytes (Ingram 1972).

(*f*) *Nomenclature of haemoglobin components*

A simple nomenclature for the haemoglobin components of chick embryos, young chicks, and the adult chicken has been adopted to facilitate the description of the changes in the haemoglobins of the developing embryo. The major electrophoretic component observed in embryos of 2 to 5 days is denoted haemoglobin P, while the minor component of these early embryos has been called haemoglobin E. The major and minor components of adult chicken haemoglobin are called haemoglobins A and D, respectively. The minor slow component observed in late embryos and young chicks is denoted haemoglobin H.

A comparison of the nomenclature used by previous authors for the haemoglobin components of 3- to 5-day embryos with the new nomenclature is shown in table 4. Similarly, a comparison of the nomenclature of previous authors for the haemoglobin components of late embryos, young chicks, and the adult chicken with the new nomenclature is shown in table 5. Haemoglobin components consistently observed by previous authors, but not detected in this study, are also presented in tables 4 and 5.

(*g*) *Outline of experiments*

The present investigation was undertaken to correlate the changes in the types of erythroid cells produced during development of the chick embryo with the changes in the haemoglobins of the embryo. To this end the time of appearance, the morphological characteristics of the several maturation stages of the primitive cells, the relationships of the several size classes of primitive cells, and the number of primitive cells per embryo were determined and related to the stage of embryogenesis. Similarly, the time of appearance of the definitive cells, the morphological characteristics of the several maturation stages of the definitive cells, the total number of definitive cells per embryo and the distribution of the definitive cells of the embryos among the several maturation stages were determined and correlated with the age of the embryo.

To resolve the discrepancies in the literature, the number and types of haemoglobin components, the time of appearance and disappearance of the haemoglobins and the relative quantities of the several haemoglobin components were determined during development of the chick embryo and correlated with the numbers and types of erythroid cells of the embryo.

This study forms the framework for experiments designed to elucidate the biochemical events associated with the switch from primitive to definitive erythropoiesis in the chick embryo (Brown 1972; Brown & Ingram 1972; Chan & Ingram 1972; Hagopian & Ingram 1972; Lipke & Ingram 1972; Moss, Joyce & Ingram 1972).

2. Materials and methods

Eggs and chickens

Fresh fetilized eggs of the Hy-Line strain of White Leghorn chickens were obtained from the Fairweather Farm, Andover, Massachusetts. Fertilized eggs of the Mt Hope Line of White Leghorn chickens were obtained from Spafas, Inc., R.F.D. no. 3, Norwich, Connecticut. These two strains were demonstrated to be haematologically equivalent (Bruns 1971). Eggs, stored no longer than 7 days at 15 °C, were incubated blunt end up without rotation in a Sears Roebuck & Co. egg incubator (model no. 228–735) at a dry-bulb temperature of 37.5 °C and a wet-bulb temperature of 29.5 °C (Abbott 1967). Timing of incubation was begun when the eggs

were placed in the incubator. Hatched chicks were obtained from incubated eggs as detailed by Abbott (1967). Adult hens of the Hy-Line strain of White Leghorn chickens were obtained from the Fairweather Farm, Andover, Massachusetts.

Preparation of erythroid cell suspensions

Circulating erythroid cells were obtained from embryos of 2.5 to 21 days of age by nicking the major embryonic vessels *in ovo* and collecting the cells streaming from the cut vessels. No anticoagulant was necessary for embryos less than 16 days of age. For older embryos the collecting pipettes were prerinsed in a solution of sodium heparin, 2 mg/ml in Howard Ringer solution (DeHaan 1967). Alternatively, erythroid cells were obtained from the embryonic circulation and vascular cords of the area vasculosa of 1.5- to 10-day embryos. The embryos and surrounding blastodisks were removed from the eggs, the major vessels cut, the vascular cords gently teased with dissecting needles, and the erythroid cells collected in Howard Ringer solution containing 1 % bovine serum albumin (H.R. b.s.a. solution). The erythroid cell suspension was filtered through one layer of gauze.

In one set of experiments it was desirable to obtain the circulating erythroid cells of the embryos at least in part separately from those erythroid cells which might be sequestered in the yolk sac sinusoids. The embryos and surrounding area vasculosa were removed from the eggs and placed in a Petri dish containing H.R. b.s.a. solution. The major vessels were cut and the blood allowed to flow out into the collecting fluid. The embryos and vascular tissue was transferred to a second Petri dish and any easily obtained erythroid cells collected. The remaining vascular tissue was transferred to a third Petri dish and the vascular cords minced with scissors and teased gently with dissecting forceps to obtain any sequestered erythroid cells. The contents of each Petri dish were kept separate and designated fraction 1, 2, and 3 respectively.

Young chicks were bled from the heart and adult hens from the wing vein with syringes prerinsed with a solution of sodium heparin (2 mg/ml in H.R. solution).

The erythroid cells were centrifuged at 3300 rev/min for 5 min in an International clinical centrifuge at 2 °C and washed three times in H.R. b.s.a. solution. Cell recovery was approximately 97.5 %. Cell counts were performed with phase optics. Duplicate cell counts differed by no more than 7 %.

Fixed preparations of erythroid cells

For the preparation of slides, aliquots (0.2 ml) of the erythroid cell suspensions (about 5×10^5 cells/ml) in H.R. b.s.a. solution were centrifuged at 700 rev/min for 5 min in a Shandon cytocentrifuge. Slides were fixed in methanol and stained with May Greenwald, giemsa; Benzidine, giemsa (Bruns 1971), or Wright, giemsa (Hagopian & Ingram 1971).

Reticulocyte counts

One volume of whole blood or one volume of an erythroid cell suspension was incubated for 30 to 45 min with one volume of 1 % new methylene blue N and slides prepared with the cytocentrifuge. Preparations were counterstained with Wright giemsa.

Histological examination of erythroid cell preparations

The percentages of primitive and definitive cells were determined by scoring 1000 cells per slide. Mitotic indices of primitive cells were determined on 1000 to 4000 cells per slide. Mitoses among definitive cells were rarely observed except in occasional preparations from 6-day

embryos, and the mitotic index was expressed as a fraction of the total number of definitive cells on the slide. Differential counts of definitive cells were determined on 1000 definitive cells per slide or on the total number of definitive cells on the slide. Definitive cells were assigned to the several maturation stages according to the criteria of Lucas & Jamroz (1961).

Measurement of cell and nuclear diameters

Fixed preparations. Cell and nuclear diameters of primitive cells and of the several maturation stages of definitive cells were measured on May Greenwald, giemsa-stained preparations of erythroid cells of 2- to 19-day embryos at × 1250 magnification with an eyepiece reticle having an etched 1 cm scale divided into 100 0.1 mm increments. Since primitive cells of 2- to 7-day embryos can be classified as large, medium, and small when observed in fixed preparations, the cell and nuclear diameters of at least 20 primitive cells of each size class were measured at each time point. After 7 days, only medium and small primitive cells were observed in fixed preparations and at least 20 cells of each size class were measured at each time point between 7 and 16 days. For rapid assignment of primitive erythroid cells to the large, medium, and small size classes, fixed preparations of erythroid cells from embryos of a particular age were scanned at × 1250 magnification and the largest cells classified as 'large', intermediate sized cells classified as 'medium', and the smallest cells observed classified as 'small'. The cell diameter limits set for large cells of 2-day embryos were not, therefore, necessarily the same as the cell diameter limits for large primitive erythroid cells of 3.5-day embryos. To assess the accuracy of assigning primitive cells to size classes by visual inspection, cells from 2- and 3-day embryos were classified as large, medium or small and, following classification, the diameter of each cell was measured. Four of 67 cells were incorrectly assigned (6%) and these errors occurred at the limit diameters of the size classes.

For the definitive cell series, where possible, cell and nuclear diameters of at least 20 definitive cells of each maturation stage were measured at each time point between 5 and 19 days. If 20 cells of a particular maturation stage were not present in the preparation, every cell of that stage on the slide was measured. Both the major and minor axes of the ellipse were measured for oval definitive cells and for definitive cells with oval nuclei. The cytoplasmic and nuclear diameters of a comparable number of definitive erythrocytes of adult chickens were also measured.

Wet mount preparations. To determine the size distribution histograms of primitive cells, the diameters of 200 randomly selected primitive cells from 2.3-, 3-, 3.5- and 4-day embryos were measured on wet mount preparations at × 600 magnification with a Vickers A.E.I. image splitting measuring eyepiece (Vickers Instruments, Inc., Malden, Mass). To prevent size distortions due to drying, fresh wet mounts were prepared after two to three cells had been measured.

Microscope calibration. The microscope eye reticle and the Vickers instrument were calibrated for each objective with a Bausch & Lomb stage micrometer (0.1 and 0.01 mm divisions) for objectives of × 40 or less. For the × 100 objectives, the calibration was done indirectly using a primitive cell which had been measured with the × 40 objective as a reference standard.

Preparation of haemoglobin

An aliquot of the washed cell suspension used for morphological studies was centrifuged for 3 min at 3300 rev/min in an International clinical centrifuge, the supernatant removed, and the cells suspended in 1 volume of lysis buffer (0.024 M tris, 0.0013 M H_3PO_4, 0.02% saponin,

0.05 % DNase I, pH 7.4). The lysate was incubated at 37 °C for 15 min, 1/5 volume of CCl_4 added, and the mixture centrifuged for 15 min at 3300 rev/min. The supernatant was removed, and phosphate buffer added to a final concentration of 0.05 M (pH 7.4). The CCl_4 interface material was re-extracted once with 0.9 % NaCl or until the interface material was essentially colourless, and the extract centrifuged for 15 min at 3300 rev/min. Potassium phosphate buffer (pH 7.4) was added to the NaCl extract to a final concentration of 0.05 M. Both the initial lysate and the NaCl extract were centrifuged at 15000 *g* for 15 min in a Lourdes centrifuge. In certain experiments the initial lysate and the NaCl extract were combined, while in other experiments the two extracts were kept separate. In experiments where the quantity of haemoglobin recovered from a known number of erythroid cells was determined, the volume of the initial aliquot of the cell suspension, as well as the volumes of the initial lysate and the NaCl extract were measured. Except where otherwise indicated, all manipulations were performed at 2 °C. Haemoglobin samples were stored in aliquots at −196 °C in a MVE nitrogen cryoflask.

Determination of haemoglobin concentration

Spectra of oxyhaemoglobin or cyanmethaemoglobin solutions were obtained with a Cary model 14 recording spectrophotometer or with a Zeiss spectrophotometer. In the latter case readings were taken every 5 nm. Haemoglobin was converted to cyanmethaemoglobin by addition of $\frac{1}{10}$ volume of Drabkin's solution (1 g $NaHCO_3$, 50 mg KCN, and 200 mg $K_3Fe(CN)_6$ per litre of water (Drabkin & Austin 1935)).

A sample of adult chicken haemoglobin which showed oxyhaemoglobin absorption maxima at 415, 540 and 575 nm and cyanmethaemoglobin absorption maxima at 420 and 540 nm was used as a reference standard. The ratios of the oxyhaemoglobin absorption at 575/560 and 540/560 nm, and the ratios of the cyanmethaemoglobin absorption at 551/545 and 540/545 nm of this standard were comparable to those of human haemoglobin (Drabkin & Austin 1932, 1935; Horecker 1943). The concentration of the adult chicken haemoglobin standard was determined as cyanmethaemoglobin using a millimolar extinction coefficient/haemoglobin chain of 11 (Eilers 1967). From the concentration and the observed 420/540 nm spectral ratio, a millimolar extinction coefficient at 420 nm of 107.8 was calculated for adult chicken cyanmethaemoglobin. Similarly, the concentration of the adult chicken haemoglobin standard as oxyhaemoglobin was determined using the millimolar extinction coefficient/haemoglobin chain of 15.0 (Horecker 1943). A millimolar extinction coefficient of 130.5 at 415 nm was calculated for adult chicken oxyhaemoglobin.

The cyanmethaemoglobin spectra of haemoglobin D and haemoglobin A of 17-day embryos and of haemoglobin P and haemoglobin E of 5-day embryos were comparable to the cyanmethaemoglobin spectrum of adult chicken haemoglobin (Bruns 1971). The cyanmethaemoglobin spectrum of haemoglobin H, isolated by preparative polyacrylamide gel electrophoresis from erythroid cell lysates of 5-day chicks, was also comparable to that of similarly isolated haemoglobins E and P of 5-day embryos and haemoglobins D and A of 5-day chicks (Bruns 1971).

Determination of the total haemoglobin of chick embryos

Embryos of 3.5 to 9 days. Eggs incubated 3.5, 4 or 5 days were cracked into a bowl containing H.R. solution and the entire blastoderm was removed without tearing the blood vessels. Eggs incubated 6 to 9 days were cracked near the pointed end of the egg, a cut made across the yolk sac and the yolk emptied from the shell leaving the embryo and blastoderm attached to the

blunt end of the egg. The entire embryo and membranes were removed from the egg without tearing the vessels and stored at −20 °C or −70 °C. For the isolation of haemoglobin the samples were thawed and homogenized with a Dual glass homogenizer in lysis buffer (0.024 M tris, 0.0013 M H_3PO_4, pH 7.4). One-tenth volume of 0.2 mg/100 ml saponin and $\frac{1}{100}$ volume of DNase 1 (10 mg/ml) was added and the lysate incubated at 37 °C for 30 min. The solution was centrifuged at 3300 rev/min in an International clinical centrifuge for 10 min. The supernatant was removed, and the pellet was extracted once with lysis buffer and 1 to 2 times with 0.9 mg/100 ml NaCl with centrifugation between each extraction. The pellet remaining after the final extraction was essentially colourless. The extracts were combined, $\frac{1}{5}$ volume of CCl_4 added, and the solution centrifuged at 12000 *g* for 15 min. The supernatant was removed from the colourless pellet and potassium phosphate buffer (pH 7.4) added to a final concentration of 0.05 M. The haemoglobin solution was centrifuged at 15000 *g* for 2 h. All manipulations were performed at 2 °C.

Embryos of 10 *to* 18 *days.* Embryos of 10 days or older cannot be removed from the shell membrane without tearing the blood vessels. Hence, whole eggs of 10, 12, 14, 16 and 18 days of age were homogenized in a Waring blender in calcium-free H.R. solution containing 0.02 M PO_4, pH 7.4 for 60 s in 15 s intervals. The homogenate was filtered through one layer of gauze and stored at −20 °C or −70 °C. For the preparation of haemoglobin, an aliquot of the homogenate was thawed and centrifuged at 3300 rev/min in an International clinical centrifuge. The pellet was extracted once with calcium free H.R. solution containing 0.02 M phosphate, pH 7.4 and the extract recentrifuged. The supernatants were combined, $\frac{1}{5}$ volume of CCl_4 added, and the solution centrifuged at 12000 *g* for 30 min. The supernatant was separated from the colourless pellet, made 0.05 M in phosphate, pH 7.4, and centrifuged at 15 000 *g* for 2 h. All manipulations were performed at 2 °C.

Oxyhaemoglobin and cyanmethaemoglobin spectra were obtained on all haemoglobin solutions with a Cary 14 recording spectrophotometer. The 540/415 nm absorption ratio of oxyhaemoglobin solutions prepared from whole embryos did not differ from the value observed with adult chicken haemoglobin by more than 10 %.

Determination of the average haemoglobin content of chick erythroid cells

Alkaline haematin method. Erythroid cells, obtained from the embryonic circulation of 3.5- to 18-day embryos or from adult chickens, were suspended in H.R. solution. Duplicate cell counts were performed on two dilutions of each erythroid cell suspension. Aliquot of the erythroid cell suspensions (25 to 100 µl, 1–4 × 10^8 cells/ml) were treated with NaOH to convert the haemoglobin to alkaline haematin as described by King, Gilchrist & Delory (1944) and by Horecker (1948). The alkaline haematin spectrum was scanned from 650 to 350 nm with a Cary 14 recording spectrophotometer. All assays were performed in triplicate and both a human haemoglobin and an adult chicken haemoglobin reference standard were included with each group of samples.

A sample of adult human haemoglobin of known concentration having spectral absorption ratios in agreement with literature values (Drabkin & Austin 1932, 1935; Horecker 1943) was used to characterize the alkaline haematin method. The alkaline haematin spectrum of this haemoglobin was comparable to that published by Horecker (1948). The 395/570 nm spectral ratio was 10.5 (s.d. ± 0.29, $n = 5$). The coloration was stable for at least 60 min and was linear with haemoglobin concentration at least over the range of 0.25 to 2.0 mg/ml of assay

solution. The degree of coloration was independent of the time of heating over the range of 2 to 10 min. The assay procedure did not require alteration when adult chicken haemoglobin or adult chicken erythrocytes were substituted for human haemoglobin.

Calculations. The concentration of the previously described adult chicken haemoglobin reference standard was determined both as oxyhaemoglobin and as cyanmethaemoglobin. Aliquots of the haemoglobin were converted to alklaine haematin and the absorption at 395 and 570 nm determined. The 395/570 nm absorption ratio of this standard was 9.86 (s.d. ± 0.17, $n = 7$). From the absorption at two wavelengths and the concentration of the haemoglobin standard, the optical density at 395 and 570 nm to be expected for conversion of 1 mg of adult chicken haemoglobin to alkaline haematin was calculated. The assumption was made that these conversion factors would be applicable to the conversion of the haemoglobins of embryonic and adult chicken erythroid cells to alkaline haematin. Since the spectral absorption ratios of the haemoglobins isolated from erythroid cells of chick embryos were comparable to those of adult chicken haemoglobin (Bruns 1971), and the presence of erythrocytes from chick embryos or from the adult chicken did not alter the alkaline haematin spectra of the human or adult chicken haemoglobin reference standard, this assumption appeared warranted. The 395/570 nm absorption ratios of alkaline haematin spectra of adult chicken erythrocytes and erythroid cells of chick embryos differed from that of the adult chicken haemoglobin reference standard by no more than 5 %.

Analytical polyacrylamide gel electrophoresis

pH 10.3 *electrophoresis.* Cylindrical disk gel electrophoresis was performed as described by Moss & Ingram (1968*a*), at pH 10.3, 2 °C. The resolving gel of 7.5 % acrylamide was 6 cm in length and 0.5 cm diameter. The stacking gel was 1 cm in length and 0.5 cm diameter. Haemoglobin samples were prepared for electrophoresis by mixing 10 µl of haemoglobin solution in 0.05 M phosphate, pH 7.2, 20 µl of a solution containing 12.8 ml of 1 M H_3PO_4, 2.85 g tris, and 20 g sucrose/100 ml, pH 7.2, and 10 µl of a solution containing 0.5 g KCN, 2.0 g $K_3Fe(CN)_6$, and 0.1 g $NaHCO_3$/100 ml. Of this mixture 10 µl was loaded onto the gels. When gels were to be stained with benzidine, 4 to 6 µg of haemoglobin was placed on each gel, and when gels were to be stained with amido black, 10 to 12 µg of haemoglobin were loaded on each gel. Electrophoresis was performed in a Canalco model 66 unit at 2 °C. Gels were run at 1 mA/tube for 20 min to permit stacking, followed by a 2.5 h electrophoresis at 2 mA/tube. Alternatively, samples were electrophoresed at 3 mA/tube for 2 h.

pH 8.9 *electrophoresis.* Gel solutions were prepared as previously except that the pH 7.9 tris, HCl, *N,N,N′,N′*-tetramethylethylenediamine (TEMED) solution of Hedrick & Smith (1968) was substituted for the pH 8.9 tris, HCl, TEMED solution of Moss & Ingram (1968*a*). When gels were to be stained with benzidine 4 to 6 µg of haemoglobin were placed on each gel, and when gels were to be stained with amido black 10 to 12 µg of haemoglobin was loaded on each gel. Gels were run at 1 mA/tube for 20 min, followed by a 2.75 h electrophoresis at 2 mA/tube.

Staining of acrylamide gels

For haemoglobin staining, gels were fixed in 1 M acetic acid for 15 min and then placed for 15 min in a solution of 0.2 mg/100 ml benzidine HCl in 1 M acetic acid to which $\frac{1}{500}$ volume of 30 g/100 ml H_2O_2 had been added immediately prior to staining. Full blue-green coloration developed by 15 min, and the gels were transferred to tubes containing a solution of 0.5 M

acetic acid, 0.5 M sodium acetate. The tubes were placed at 2° C in the dark to preserve the colour. Benzidine stained gels are very light sensitive and the original blue-green colour becomes grey, grey-brown, and finally brown. If benzidine gels are to be scanned, it is necessary to rigorously control the staining and storage conditions.

For protein staining, the gels were placed in 1 mg/100 ml amido black 10B in 7 g/100 ml acetic acid for 10 min, and destained with 7 g/100 ml acetic acid.

Benzidine stained gels were scanned at 500 nm with a Gilford 240 spectrophotometer with a linear transporter 24 h after staining. Amido black stained gels were scanned at 500 nm and unstained gels at 420 nm.

Quantification of the relative amounts of haemoglobin components

To quantify the changes in the relative quantities of the haemoglobins of the chick embryo the peak mass ratios of the components were determined from gel scans. Xerox copies of the scans were made, areas under the peaks assigned, and the peaks cut out and weighted. The ratios of the areas under the several haemoglobin peaks were then calculated. Figure 1 demonstrates the assignment of peak areas for haemoglobins electrophoresed on pH 10.3 polyacrylamide gels. Isolated peaks such as haemoglobins E and D were taken to be symmetrical. In the pH 10.3 system haemoglobins A and P were incompletely resolved. For area assignment the largest of the two peaks was drawn symmetrically, and the smaller of the two peaks was drawn as a shoulder of the larger peak. In haemoglobin samples from late embryos, where no primitive erythrocytes could be found among the circulating erythroid cells, and in samples from adult chickens, the anterior shoulder of the A band, which migrates in the position of haemoglobin P, was denoted A′. Since, in adult chicken haemoglobin samples, the ratio of haemoglobin A/A′ was constant over a 20-fold range of haemoglobin/gel, it was assumed that a similar fraction of the haemoglobin A of lysates from embryos migrated in a position coincident with that of haemoglobin P. When both haemoglobinized definitive erythroid cells and primitive erythrocytes were present in the embryonic circulation, the peak corresponding to haemoglobin P was denoted P plus A′. Similarly, because haemoglobin H migrates coincidently with haemoglobin E, the peak corresponding to haemoglobin E was denoted E plus H after definitive cells had entered the embryonic circulation. After all primitive erythrocytes had been lost from the circulation, this peak would be denoted haemoglobin H.

The peak area assignments for haemoglobins electrophoresed at pH 8.9 are shown in figure 2. Isolated peaks such as haemoglobins E and D were assumed to be symmetrical. Assignment of area to the diffuse band P′ migrating faster than haemoglobin P was difficult. In the absence of haemoglobin D, the assignment was as indicated in figure 2*a*, *b*. When haemoglobin D was present in the lysate, but where the P′ component was clearly observed on the gels, assignment was as indicated in figure 2*c*, *d*. In lysates from late embryos, where no primitive erythrocytes were present among the circulating erythroid cells, and in adult haemoglobin samples, the component migrating faster than haemoglobin A was denoted A′. Since the A/A′ ratio of haemoglobin samples from adult chickens was constant over a 20-fold range of haemoglobin/gel, it was assumed that a comparable fraction of haemoglobin A of lysates from chick embryos migrated in a similar position. Therefore, when both haemoglobinized definitive erythroid cells and primitive erythrocytes were present in the embryonic circulation, the haemoglobin P peak was denoted P plus A′. Since mixtures of haemoglobins E and H were not totally resolved with the pH 8.9 electrophoresis system, the peak corresponding to haemoglobin E

was denoted E plus H after appearance of definitive erythroid cells in the circulation. After all primitve cells had been lost from the embryonic circulation, this peak would be denoted haemoglobin H.

The reproducibility of the peak area assignments was 10 % for pH 10.3 polyacrylamide gels and 15 % for pH 8.9 gels. In order to use the peak mass ratios to estimate the quantities of the several haemoglobins in lysates from developing embryos, mixtures containing known quantities of haemoglobin from 5-day embryos and from an adult chicken were electrophoresed at

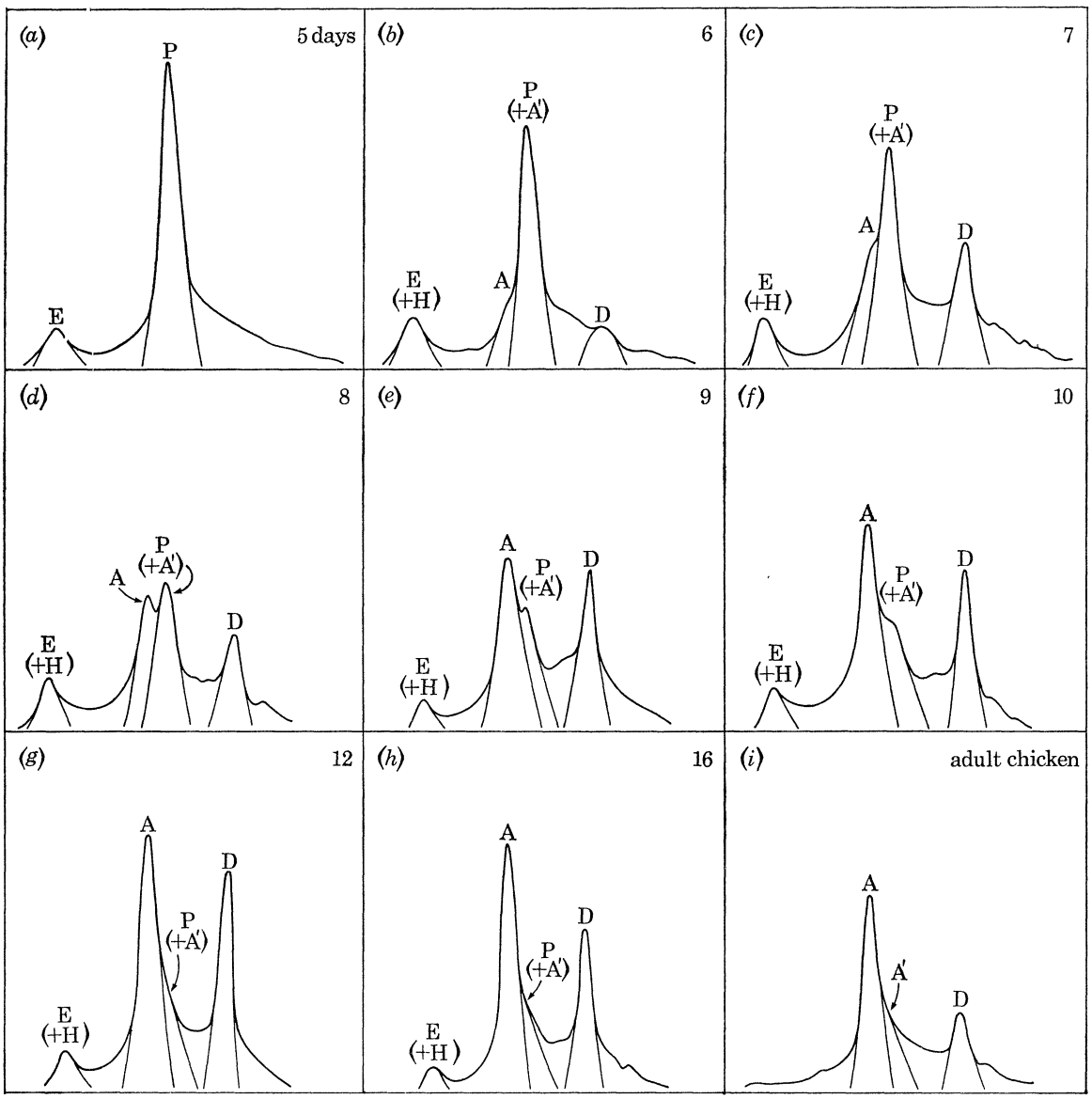


FIGURE 1. Assignment of peak areas on gel scans of embryonic and adult haemoglobins (pH 10.3 electrophoresis). Amido black stain. (*a*) Haemoglobin from 5-day embryos. The letters E and P denote haemoglobins E and P. (*b*) Haemoglobin from 6-day embryos. The letters A and D indicate haemoglobins A and D. The notation E(+H) indicates haemoglobin E plus haemoglobin H. The notation P(+A′) indicates haemoglobin P plus the component of haemoglobin A which migrates in the position of haemoglobin P. (*c*) Haemoglobin from 7-day embryos. (*d*) Haemoglobin from 8-day embryos. (*e*) Haemoglobin from 9-day embryos. (*f*) Haemoglobin from 10-day embryos. (*g*) Haemoglobin from 12-day embryos. (*h*) Haemoglobin from 16-day embryos. (*i*) Haemoglobin from an adult chicken.

pH 10.3 or pH 8.9, the peak masses determined, and the observed peak mass ratios compared with the ratios calculated from the percentage of the several haemoglobins in the mixtures. This method of calibration provided an estimate of the errors associated with peak mass assignment and permitted appropriate correction factors to be applied to data obtained on lysates from embryos of different ages (Bruns 1971).

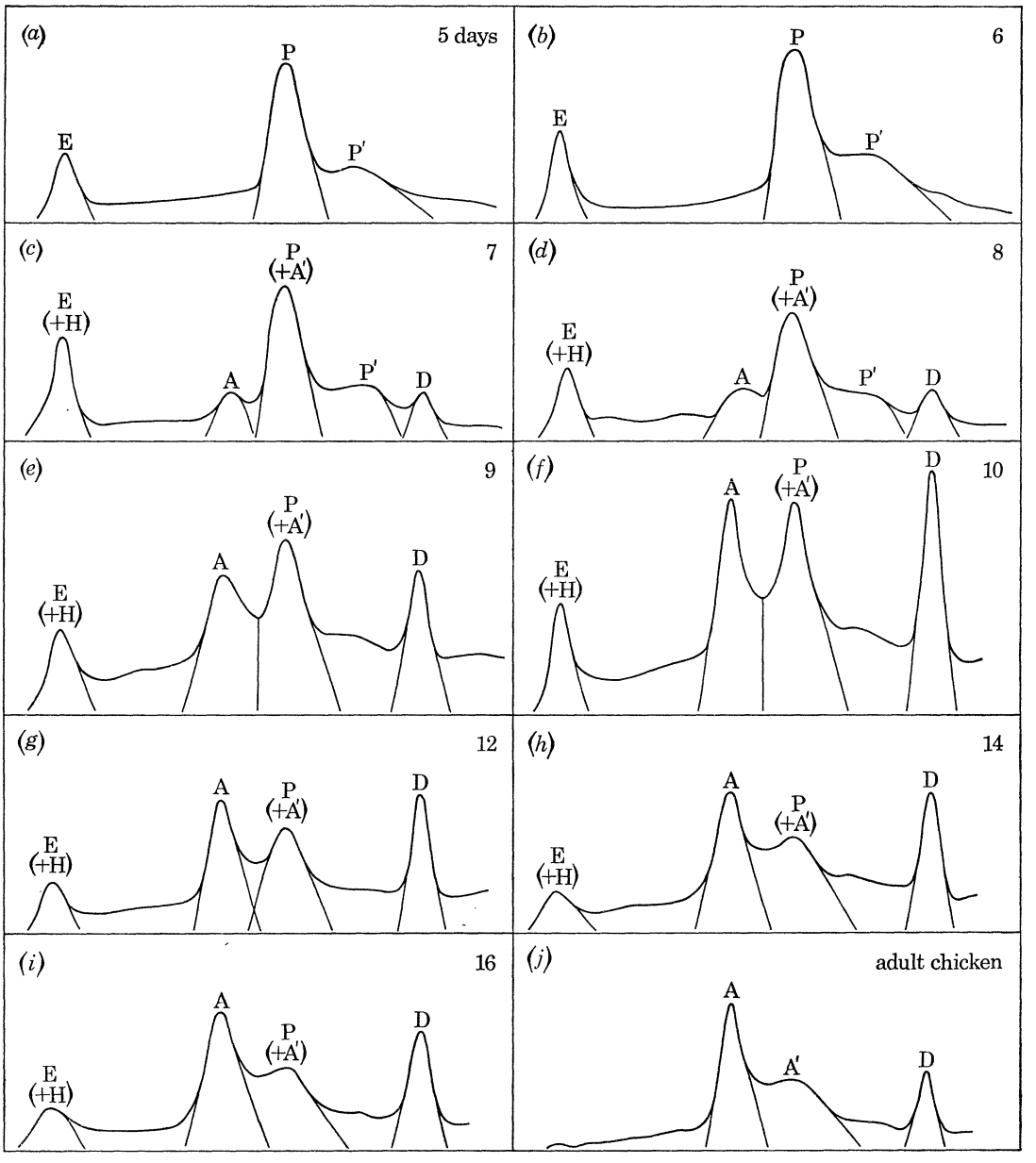


FIGURE 2. Assignment of peak areas on gel scans of embryonic and adult haemoglobins (pH 8.9 electrophoresis). Amido black stain. (*a*) Haemoglobin from 5-day embryos. The letters P and E denote haemoglobins P and E. The letter P′ denotes the diffuse component of haemoglobin P which migrates faster than haemoglobin P in this electrophoresis system. (*b*) Haemoglobin from 6-day embryos. (*c*) Haemoglobin from 7-day embryos. The letters A and D indicate haemoglobins A and D. The notations E(+H) and P(+A′) are explained in figure 1. (*d*) Haemoglobin from 8-day embryos. (*e*) Haemoglobin from 9-day embryos. (*f*) Haemoglobin from 10-day embryos. (*g*) Haemoglobin from 12-day embryos. (*h*) Haemoglobin from 14-day embryos. (*i*) Haemoglobin from 16-day embryos. (*j*) Haemoglobin from an adult chicken.

Preparative polyacrylamide gel electrophoresis

Preparative polyacrylamide gel electrophoresis was performed in 10 cm × 1.3 cm (i.d.) tubes with a 7 cm column of resolving gel and a 1 cm column of stacking gel (Maniatis & Ingram 1971*b*). Haemoglobin solutions (3.5 to 8 mg) were converted to cyanmethaemoglobin immediately prior to electrophoresis, $\frac{1}{4}$ volume of 40 % sucrose was added and the sample (0.3 to 0.4 ml) was loaded on the gel. Electrophoresis was performed at 7 mA/tube for 1.5 h followed by 2.5 to 3 h at 15 mA/tube.

Following electrophoresis the gels were removed from the tubes, the desired bands cut from the gel and the haemoglobin eluted from the gel with 0.9 mg/100 ml NaCl for 18 h at 2 °C.

Determination of the approximate molecular mass of proteins by gel electrophoresis

Hedrick & Smith (1968) described a method for determining whether proteins are charge and/or size isomers and for assessing the approximate molecular mass of proteins based on the migration rate of the protein relative to a tracking dye on polyacrylamide gels of different porosities. Polyacrylamide gels containing 4, 5, 6, 7.5, 8.5 and 9.5 % acrylamide were prepared as described for the pH 8.9 electrophoresis system. The acrylamide/bisacrylamide ratio remained constant. Haemoglobin samples from 5-day embryos and 1-day-old chicks were prepared as described above except that the loading buffer contained 8 mg/100 ml bromophenol blue.

Gels were electrophoresed at 1 mA/tube for 45 min and then at 2 mA/tube for 1.5 h. The electrophoresis was terminated when the tracking dye reached the bottom of the gel column in the tubes containing 4 % acrylamide. The gels were removed from the tubes, the dye front marked and the gels stained with amido black. Following destaining the migration distance of the tracking dye and of the several haemoglobin bands were measured. The logarithm of the migration distance of each haemoglobin band relative to the migration of the tracking dye was plotted versus the percentage of acrylamide in the gel.

Immunization of rabbits with individual haemoglobin components

The major and minor components of haemoglobin isolated from 4-day embryos were separated by preparative polyacrylamide gel electrophoresis. The D and A haemoglobin components were similarly prepared by electrophoresis of adult chicken haemoglobin. Representative separations of the four haemoglobin components are shown in figure 13, plate 28. The bands containing the separated haemoglobin components were removed from the gels and the haemoglobins eluted. Haemoglobin components used for immunization were prepared 18 h before use and were not frozen.

Male albino rabbits received two injections, 10 days apart, of the haemoglobin component (0.5 to 1.5 mg) emulsified in complete Freund's adjuvant. One-fifth of the total volume was injected into each foot pad and the remainder injected intramuscularly. Rabbits were bled 40 ml from the ear vein 15 days after the second injection. Rabbits immunized with the major or minor components of haemoglobin from 4-day embryos received three intravenous injections of 0.25 to 1.0 mg of the haemoglobin component at weekly intervals. Rabbits immunized with A or D haemoglobin received two intravenous injections of 0.5 to 2.0 mg of the haemoglobin component 7 days apart. One week following the last injection the rabbits were bled approximately 40 ml on each of 2 consecutive days, and the sera pooled. Sera were stored at −20 °C.

Characterization of rabbit antisera to individual haemoglobin components

Two-dimensional immunodiffusion was performed as described by Stollar & Levine (1963) in 50 × 12 mm falcon Petri dishes with tight lids. Ten μl of serum were placed in the centre well, and 10 μl of the haemoglobin solutions (0.1 to 1.0 mg/ml in 0.9 mg/100 ml NaCl) were placed in peripheral wells. The serum of the first and second bleeding of each rabbit was tested against each of the individual haemoglobin components.

Gel filtration of haemoglobin on Sephadex G-100

Sephadex G-100 (Pharmacia Fine Chemicals, Inc.) was equilibrated with 0.1 M tris, 0.1 M NaCl, pH 8.2. A 2.5 cm × 90 cm Sephadex K25 column was poured with a pressure head of 20 cm, and one inclusion volume of buffer was passed through the column. The void volume and inclusion volume were determined with dextran blue 2000 and a solution of $K_3Fe(CN)_6$. Haemoglobin which had been dialysed for 18 h at 2 °C against two changes of column buffer in Visking dialysis tubing (Union Carbide Corp.) was applied to the top of the column in 1 to 2 ml. Elution of the haemoglobin was obtained by continued passage of column buffer. The flow rate was 7 to 9 ml/h and fractions of 3.5 to 4.5 ml were collected. The column eluate was monitored at 260, 280, 420 and 540 nm on a Zeiss spectrophotometer. The column was calibrated with the following molecular mass markers: α-amylase (bacterial type 11-A); egg white lysozyme; and bovine serum albumin.

3. Erythrocytes of the chick embryo

The first erythroid cells of the developing chick embryo, the primitive cells, arise in the blood islands of the area vasculosa at about 36 h of incubation (6 to 9 somites). The primitive cells constitute the only recognizable erythroid cells of the embryo until about 5 days when immature cells of the second, or definitive, erythroid cell line enter the embryonic circulation. The definitive erythroid cells progressively replace the primitive cells, such that by 16 days less than 1 % of the erythroid cells of the embryo are of the primitive cell series. The percentage of primitive and definitive erythroid cells in the embryonic circulation of 2- to 19-day embryos in a typical experiment is shown in figure 3.

(*a*) *Maturation of primitive erythroid cells*

The progressive maturation stages of the primitive cells are shown in figure 11, plate 27, beginning with the very immature cells seen at 2 days and culminating in the mature primitive erythrocyte seen after 8 days. A few cells, morphologically quite similar to erythroid cells of 2-day embryos, but with more vacuolation and frequent pseudopodia, can be isolated from the area vasculosa of 1.5-day embryos. The morphological changes which characterize the maturation of primitive cells as illustrated in figure 11 include: a decrease in cytoplasmic basophilia; a decrease in cell and nuclear size and a decrease in the nuclear to cytoplasm ratio; the loss of prominent nucleoli; progressive chromatin clumping; and progressive nuclear pycnosis. Maturation of primitive cells is accompanied by increasing haemoglobinization as judged by the ability of the cells to stain with benzidine. The percentage of primitive cells which stain with benzidine increases from 47 % at 2 days to 100 % at 5 days (table 6). The reaction of 2-day cells with benzidine is very weak, but progressively increases in intensity as the cells mature.

Although the morphological changes associated with maturation of the primitive cells are similar to those of the definitive erythroid cells of the chicken and mammals (Wintrobe 1956; Lucas & Jamroz 1961; Russell & Bernstein 1966), the changes are more gradual and the discrete maturation stages characteristic of definitive erythroid cell lines are not seen. Primitive cell erythropoiesis differs from definitive erythropoiesis in another characteristic way. During definitive erythropoiesis, a number of different maturation stages of the erythroid cells are

TABLE 6. BENZIDINE POSITIVE PRIMITIVE CELLS†

age of embryos/days	benzidine positive cells/%	age of embryos days	benzidine positive cells/%
2.0	46.6	5.0	100
2.5	69.6	6.0	100
3.0	87.0	8.0	100
3.5	94.4	10.0	100
4.0	98.0		

† Fixed preparations of erythroid cells were stained with benzidine, giemsa. One thousand cells were counted at each time point. The numbers represent the average value for three experiments.

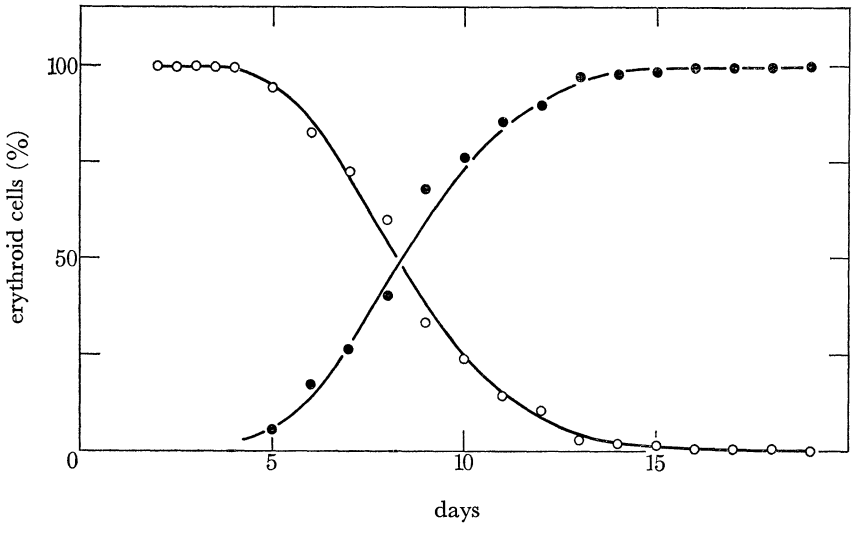


FIGURE 3. Percentage of primitive and definitive erythroid cells in red blood cell suspensions from embryos of 2 to 19 days of age. Eyrthroid cells from embryos of 2 to 4 days were obtained from mechanically dissociated blastoderms. Erythroid cells from embryos of 5 to 19 days of age were obtained from the embryonic circulation. Fixed preparations of erythroid cells were prepared with the Shandon cytocentrifuge and stained with May Greenwald, giemsa stain. At least 1000 cells were counted at each time point. The time schedule for the switch from the production of primitive to definitive erythroid cells will vary as much as 12 to 24 h depending upon the temperature and humidity of the incubator. In this study, eggs were incubated without rotation at a dry-bulb temperature of 37.5 °C and a wet-bulb temperature of 29.5 °C (Abbott 1967). ○, Primitive cells; ●, definitive cells.

simultaneously present in erythropoietic tissues or in peripheral blood and the size of the erythroid precursors is correlated with their maturation stage, the smaller cells being more mature than the large immature precursors (Wintrobe 1956; Lucas & Jamroz 1961; Russell & Berntein 1966; Borsook 1966). However, during primitive cell erythropoiesis in the chick embryo, at each time point studied several sizes of primitive cells are observed but cells of quite different size appear to be at the same stage of maturation as judged by morphological criteria (figure 11,

plate 27). Primitive cells isolated from mechanically dissociated vascular tissue of embryos are not more or less mature than cells isolated from the embryonic circulation.

Preparations of erythroid cells from embryos of 2 to 5 days of age contain approximately 98 % primitive cells. The other 2 % consists of a mixture of thrombocyte precursors (Sugiyama 1926; Lucas & Jamroz 1961) and cells of unknown function which do not show progressive maturation changes and do not stain positively with benzidine. In one experiment in which

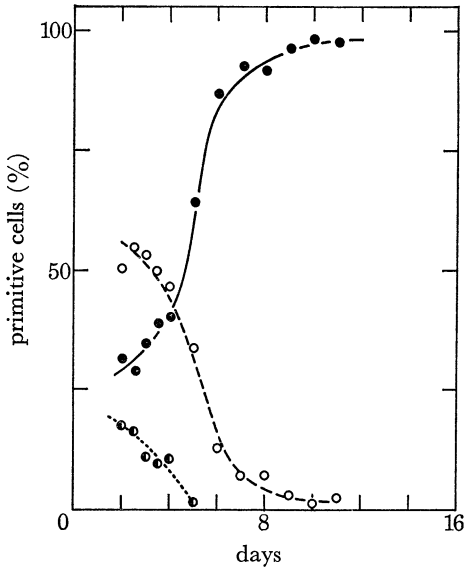


FIGURE 4

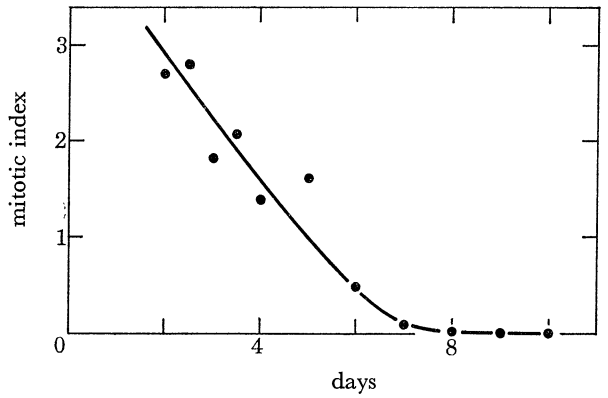


FIGURE 5

FIGURE 4. Percentage of large, medium, and small primitive cells observed in fixed preparations of erythroid cells of 2- to 11-day embryos. Erythroid cells from embryos of 2 to 4 days were obtained from mechanically dissociated blastoderms. Erythroid cells from embryos of 5 to 11 days of age were obtained from the embryonic circulation. Fixed preparations of erythroid cells were prepared with the Shandon cytocentrifuge and stained with May Greenwald, giemsa stain. At least 1000 cells were scored at each time point. ◐, Large primitive cells; ○, medium primitive cells; ●, small primitive cells.

FIGURE 5. Mitotic indices of primitive erythroid cells of 2- to 10-day embryos. Erythroid cells from 2- to 4-day embryos were obtained from mechanically dissociated blastoderms. Erythroid cells from 5- to 10-day embryos were obtained from the embryonic circulation. Preparations of erythroid cells were prepared with the Shandon cytocentrifuge and stained with benzidine, giemsa stain. The mitotic indices are average values from three experiments for embryos of 2 to 4 days and from two experiments for embryos of 5 to 10 days. Approximately 4000 to 6000 primitive cells were scored for each time point in each experiment.

erythroid cells were obtained from single eggs at time points between 2 and 5 days, no primitive cells corresponding morphologically to less mature maturation stages of primitive cells were seen. In a second experiment in which cells from 48 eggs were pooled at each time point, 4 to 5 % of the primitive cells of 2.6-, 3- and 3.5-day embryos and 1.4 % of the primitive cells of 4- and 5-day embryos appeared morphologically equivalent to less mature primitive cells. In this second experiment, although embryos with blastoderm diameters markedly larger or smaller than the modal diameter were discarded, some variation in the maturation stages of the embryos in the pool would not be unexpected.

Variation in cell size in morphologically equivalent primitive cells was observed in fixed preparations at all time points studied (figure 4), although the variation decreased with maturation of the primitive cells. The percentage of large primitive cells decreased between

day 2 and day 5 and these cells constituted only about 1 % of the primitive cells after day 5. Similarly, the percentage of medium size primitive cells decreased to less than 5 % of the primitive cells by day 9. The cytoplasmic and nuclear diameters and the nuclear/cytoplasm ratios of maturing primitive cells (measured on fixed preparations) gradually decrease between day 2 and days 6 to 8 and then remain essentially constant (table 7).

TABLE 7. CYTOPLASMIC AND NUCLEAR DIAMETERS OF PRIMITIVE CELLS IN FIXED PREPARATIONS†

age of embryos days	large primitive cells			medium primitive cells			small primitive cells		
	cytoplasm diameter	nuclear diameter	*n/c*	cytoplasm diameter	nuclear diameter	*n/c*	cytoplasm diameter	nuclear diameter	*n/c*
2.0	21.0 (20)	13.1	0.63	17.6 (20)	10.4	0.59	14.1 (20)	8.5	0.61
2.5	20.4 (20)	12.8	0.63	16.8 (20)	10.6	0.63	14.0 (20)	8.6	0.62
3.0	19.9 (20)	11.1	0.56	16.4 (20)	9.8	0.60	13.2 (20)	8.3	0.62
3.5	18.8 (14)	9.4	0.50	15.7 (20)	7.6	0.48	12.5 (20)	6.6	0.53
4.0	18.5 (15)	9.0	0.51	15.3 (20)	7.6	0.50	12.9 (20)	6.2	0.51
5.0	16.6 (15)	7.3	0.44	14.5 (15)	6.9	0.47	11.4 (20)	5.4	0.48
6.0	16.4 (12)	7.6	0.46	14.4 (10)	5.6	0.39	11.5 (20)	5.0	0.44
8.0	—	—	—	14.4 (10)	5.4	0.38	11.6 (20)	4.4	0.38
10.0	—	—	—	13.8 (5)	5.0	0.36	11.1 (20)	4.0	0.36

† Erythroid cells from 2- to 4-day embryos were obtained from mechanically dissociated blastoderms. Erythroid cells from embryos of 5 to 10 days were obtained from the embryonic circulation. Fixed preparations were prepared with the Shandon cytocentrifuge and stained with May Greenwald, giemsa stain. The cell and nuclear diameters were measued at × 1250 magnification with a calibrated microscope eyepiece reticle. The numbers in brackets indicate the number of cells of each type measured.

Mitotic figures were observed in primitive cells isolated either from the total area vasculosa or from the embryonic circulation of 2.5- to 7-day embryos. The mitotic index of the primitive cells of these two types of preparations did not differ. The mitotic index falls from about 2.8 % in primitive cells of 2-day embryos to about 0.1 % in primitive cells of 7-day embryos (figure 5). As about 75 % of the observed mitotic figures were in metaphase, the true mitotic index of the primitive cells is probably somewhat higher (O'Connor 1952; Odartchenko, Cottier, Feinendegen & Bond 1964; Thrasher 1966). Mitoses were not confined to a morphologically recognizable subpopulation of primitive cells, and mitotic figures were observed in highly haemoglobinized cells.

The mitotic index data as well as the changes in the cytoplasmic and nuclear diameters suggest that primitive cells are approaching maturity by 6 to 7 days. The transition from the reticulocyte to the mature erythrocyte is characterized by the loss of cytoplasmic staining with new methylene blue N or brilliant cresyl blue (Wintrobe 1956; Russell & Bernstein 1966; Borsook 1966). To determine when the primitive cells become mature erythrocytes, the percentage of primitive cells which were stained with the supra-vital dye, new methylene blue N, was determined. All primitive cells from embryos of 8 days or less showed cytoplasmic reticulum. The reticulum of primitive cells, which was faint and diffuse in cells from 3-day embryos, became more defined and clumped between day 4 and day 6. At 7 days the reticulum was clustered about the cell nucleus and, by 8 days, the primitive cells had only traces of reticulum about the nucleus. Only about 4 % of primitive cells from 10-day embryos contained even traces of reticulum and by 12 days none of the primitive cells contained reticulum.

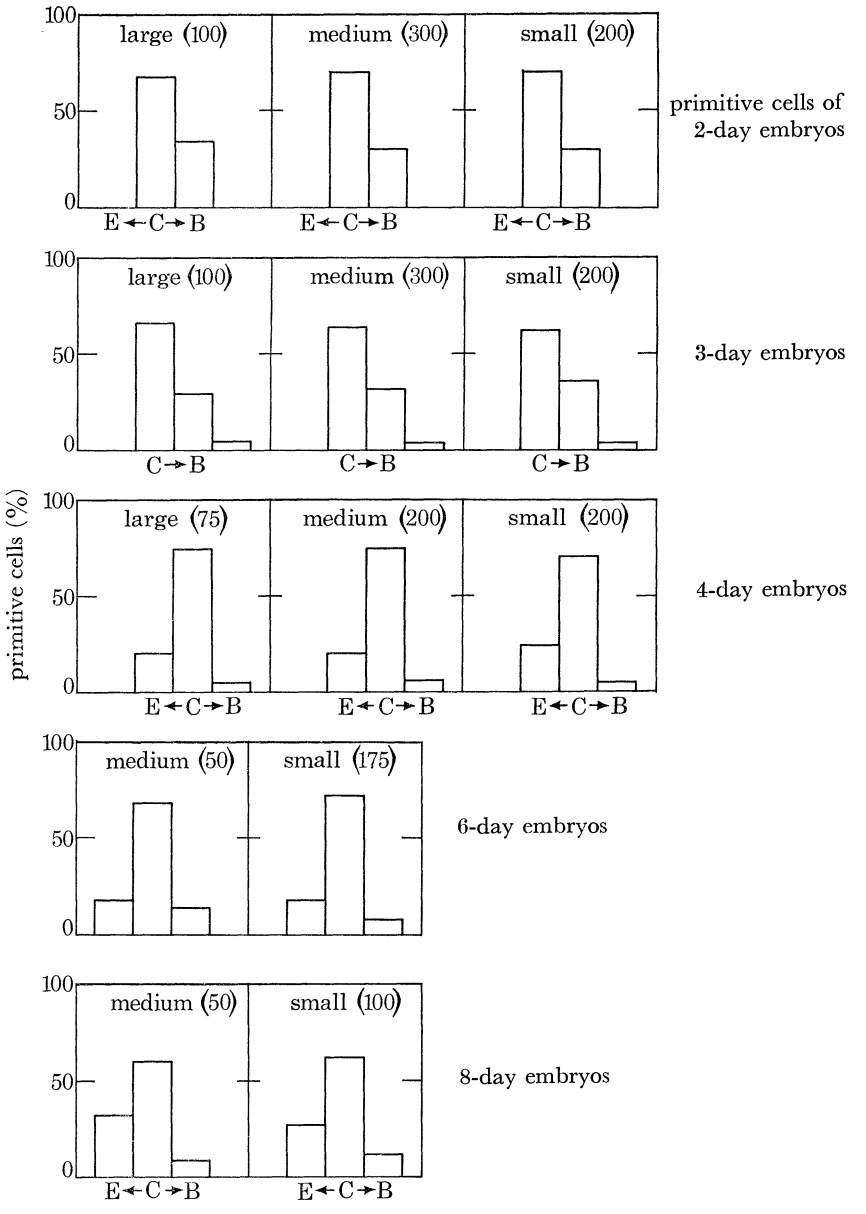


FIGURE 6. Correlation of cell size and cell maturity in preparations stained with May Greenwald, giemsa. The histograms show the percentages of large, medium, and small primitive cells of 2- to 8-day embryos which have more or less cytoplasmic eosinophilia than the commonest value judged on a three-point scale of intensity. In all histograms, the tallest column represents the fraction of cells with the most common degree of cytoplasmic eosinophilia (denoted C). The fraction of cells with greater cytoplasmic eosinophilia is plotted to the left of the tallest column (denoted E→) and the fraction of cells with the greater cytoplasmic basophilia is plotted to the right of the tallest column (denoted →B). The commonest degree of cytoplasmic eosinophilia is comparable for the large, medium, and small cells of the population of erythroid cells isolated from embryos of one particular age. However, the degree of cytoplasmic eosinophilia increases as the primitive cells mature. Therefore the commonest degree of cytoplasmic eosinophilia for primitive cells isolated from 2-day embryos is not the same as that of primitive cells isolated from 5-day embryos. The numbers in the figure captions indicate the number of cells examined for each histogram.

(b) Interrelations of the size classes of primitive cells

To study more carefully the possible correlation of cell size within a population of erythroid cells from embryos of a particular age with the maturation state of the cell, the percentage of primitive erythroid cells of each size class, which had more or less cytoplasmic eosinophilia than the majority of the cells, was determined for populations of erythroid cells from 2- to 8-day embryos (figure 6). The histograms of the small cells did not differ from those of medium and large cells at any time point studied. The percentage of primitive cells of each size class which showed a greater or lesser staining intensity with benzidine than the majority of the cells was determined for populations of erythroid cells from 2- to 6-day embryos (figure 7). The histograms of small cells were not very different from those of the medium size cells. Large cells of 2- and 2.5-day embryos showed fewer intensely stained cells and a greater number of benzidine negative cells than the medium or small cells. However, by 3 days the histograms of the large, medium, and small cells were quite comparable.

To determine whether the primitive cells fall into several different discrete size classes and to obtain the relative frequencies of cells of the several sizes, cell diameters were measured on wet mount preparations of 2.3-, 3-, 3.5- and 4-day primitive cells. The refractile characteristics and flow characteristics of the cells examined in wet mount preparations suggested that the cells were spherical under the experimental conditions. On the assumption that the cells were spheres, volume distributions were calculated from the measured diameters and are presented in figure 8. Statistical analysis (Bruns 1971) indicated that both the diameter and volume distributions were skewed normal distributions. The cell volumes range from 200 to about 1600 μm^3 and the range was quite similar at the four time points studied. The mean cell volume of primitive cells from 2.3-day embryos was 640 μm^3 and that of 4-day primitive cells was 560 μm^3. The mean diameter of primitive cells decreased only about 5 % between day 2.3 and day 4. Since primitive cells of embryos older than 4 days are ovaloid in wet mount preparations and were not converted to spheres under the experimental conditions, it was not possible to determine the cell diameter and volume distributions of more mature primitive cells.

Variation in the size of primitive cells has therefore been observed in both wet mount and fixed preparations. It is not unreasonable to propose that the large, medium and small cells observed in fixed preparations arise from the large, medium and small cells observed in the wet mount preparations. The larger cell diameters of primitive cells observed in fixed and stained preparations are probably due to deformation during preparation of the slides with the Shandon cytocentrifuge. The decrease in the mean cell diameter of primitive cells, measured on fixed preparations, stands in contrast to the constancy of the mean cell diameter of primitive cells in wet mount preparations. These observations suggest that a change in cell deformability occurs during maturation of primitive erythroid cells, perhaps secondary to a change in the red cell membrane.

The cell volume distributions provide additional information on the interrelations of the several sizes of primitive cells. It might be proposed that the size distribution of the cells reflects their position in the cell cycle, i.e. the large cells representing G_2 cells, the small cells representing G_1 cells and the medium size cells distributed throughout S phase. However, the observed distributions do not show the theoretical relative frequencies of large, medium and small cells expected for a simple parent–progeny relationship of the larger and smaller cells (Walker 1954; Stanners & Till 1960; Killander & Zetterberg 1965; Alpen & Johnston 1967).

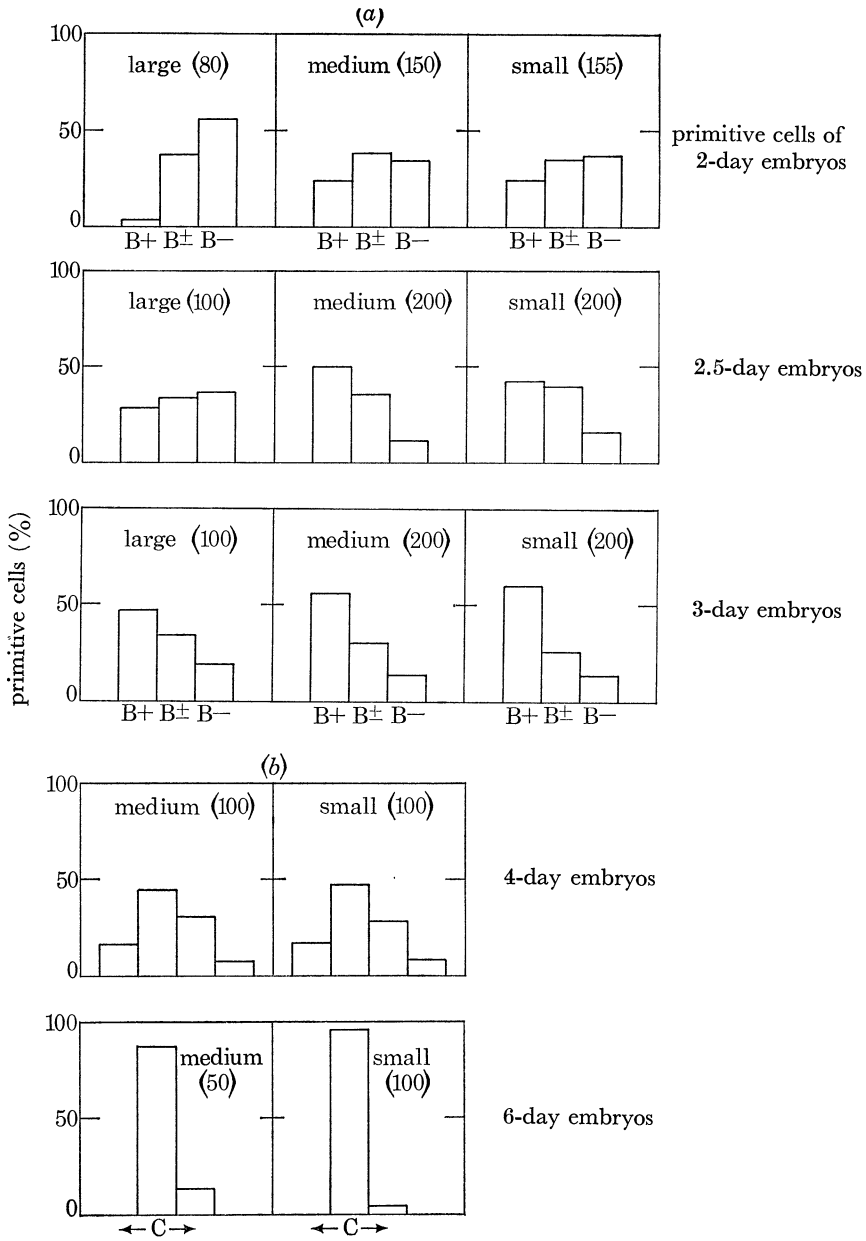


FIGURE 7. Correlation of cell size and cell maturity in preparations stained with benzidine, giemsa. (*a*) The histograms show the percentage of large, medium and small primitive cells of 2- to 3-day embryos which are benzidine positive (B+), weakly benzidine positive (B±), and benzidine negative (B−). The numbers in the figure captions indicate the number of cells examined for each histogram. (*b*) The histograms show the percentage of medium and small primitive cells of 4- to 6-day embryos which have a greater or lesser staining intensity with benzidine than the commonest value judged on a three point scale of intensity. In these histograms the tallest column represents the fraction of cells with the most common intensity of benzidine staining (denoted C). The fraction of cells staining more intensely with benzidine is plotted to the left of the tallest column (denoted ←) and the fraction of cells staining less intensely with benzidine is plotted to the right of the tallest column (denoted →). The most common intensity of benzidine staining is comparable for the medium and small cells of the population of erythroid cells isolated from embryos of one particular age. However, the intensity of benzidine staining increases as the primitive cells mature. Histograms for large primitive cells are not shown as only a few such cells were observed on the slides prepared from erythroid cells of 4- to 6-day embryos in this experiment. The numbers in the figure captions indicate the number of cells examined for each histogram.

If the large cells were G_2 cells, the small cells G_1 progeny of the large G_2 cells, and the medium-size cells distributed throughout S phase, it would be predicted that mitotic figures would be seen only in large cells and not in medium and small cells. To test this prediction, the percentage of the observed mitotic cells which were large, medium and small was determined on fixed preparations of primitive cells to 2- to 4-day embryos and compared with the percentage of large, medium and small cells in the erythroid cell populations (table 8). There is a relative excess of mitoses among large cells but at least 45 % of the mitoses were observed in medium size cells.

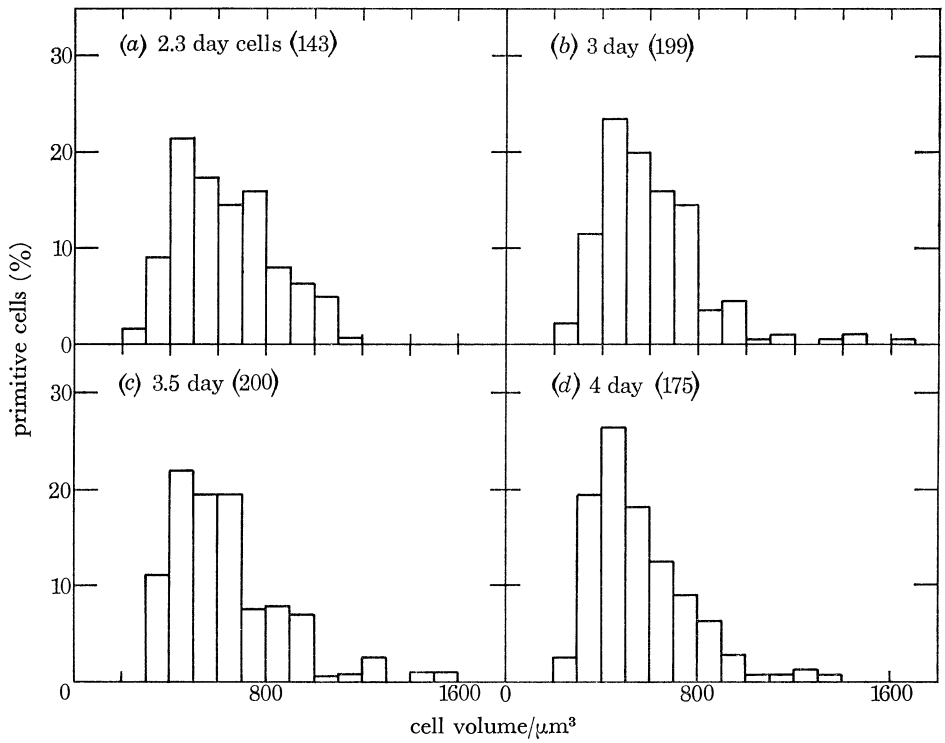


FIGURE 8. Cell volume histograms of primitive erythroid cells of 2.3- to 4-day embryos (wet mount preparations). Cell diameters of randomly selected primitive cells were measured in wet mount preparations and cell volumes were calculated assuming that the cells were spheres. The numbers in the figure captions indicate the number of cells measured for each histogram. (*a*) Primitive cells from 2.3-day embryos. (*b*) Primitive cells from 3-day embryos. (*c*) Primitive cells from 3.5-day embryos. (*d*) Primitive cells from 4-day embryos.

TABLE 8. FREQUENCY OF MITOSES AMONG LARGE, MEDIUM AND SMALL PRIMITIVE CELLS†

age of embryos/ days	mitotic index (total population)	percentage of mitoses in			number of mitoses counted	percentage of		
		large cells	medium cells	small cells		large cells	medium cells	small cells
2.0	2.7	44.7	55.2	—	52	17.4	50.9	31.8
2.5	2.8	51.0	44.0	4.9	41	16.7	52.4	30.8
3.0	1.8	21.5	70.7	4.9	41	10.8	52.9	34.8
3.5	2.1	22.5	63.6	13.6	22	8.3	49.5	39.2
4.0	1.4	55.0	45.0	—	20	11.1	46.8	40.5

† Erythroid cells were obtained from mechanically dissociated blastoderms. Fixed preparations were prepared with the Shandon cytocentrifuge and stained with May Greenwald, giemsa stain.

(c) *Maturation of definitive erythroid cells*

The morphological characteristics of the several maturation stages of definitive erythroid cells of the chick embryo are remarkably similar to the sequential maturation stages of the definitive cells of the foetal and adult mouse, the rabbit, and the human. The maturing definitive elements of the chick embryo can be classified as proerythroblasts, basophilic erythroblasts, early, mid, and late polychromatophilic erythroblasts, reticulocytes, and mature erythrocytes (Lucas & Jamroz 1961). Representative stages of maturing definitive erythroid cells are shown in figure 12, plate 27. The relative frequency of each of the several maturation stages of definitive

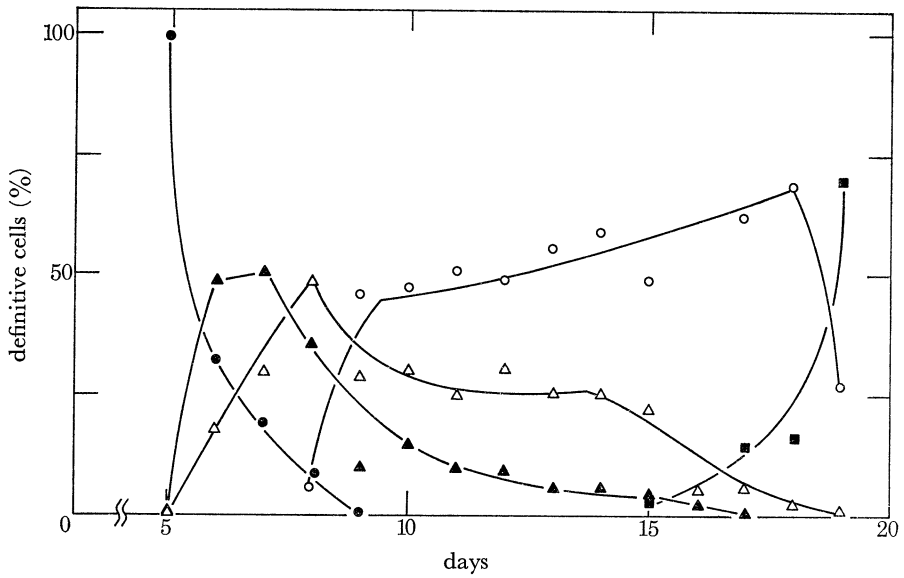


FIGURE 9. Time of appearance in the embryonic circulation of the several maturation stages of definitive erythroid cells. Erythroid cells were obtained from the embryonic circulation. Fixed preparations of erythroid cells were prepared with the Shandon cytocentrifuge and stained with May Greenwald, giemsa stain. Differential cell counts were determined on at least 1000 definitive cells at each time point. Assignment of definitive cells to the several maturation stages was done according to the criteria of Lucas & Jamroz (1961): ●, basophilic erythroblasts, large and small early polychromatophilic erythroblasts; ▲, large and small mid-polychromatophilic erythroblasts; △, large and small late polychromatophilic erythroblasts; ○, mature cells with round nuclei; ■, mature cells with oval nuclei.

TABLE 9. RELATIVE FREQUENCIES (%) OF DEFINITIVE CELL TYPES AMONG THE CIRCULATING ERYTHROID CELLS OF YOUNG CHICKS AND ADULT CHICKENS

age of donor/ days	immature definitive cells†	mature round definitive cells with round nuclei	mature oval definitive cells with round nuclei	mature oval definitive cells with oval nuclei	definitive cells in the sample
			young chicks		
0.5	1.1	8.0	1.9	88.6	100
5	9.6	9.4	7.2	73.9	100
10	0.9	8.1	1.1	90.2	100
15	2.2	8.2	0.5	89.6	100
20	3.2	8.4	—	88.3	100
			adult chickens		
	0.2	9.0	0.2	90.4	100

† Immature definitive cells: basophilic erythroblasts, early, mid and late polychromatophilic erythroblasts, and grey and pale oval cells.

cells, expressed as a percentage of the definitive cells in erythroid cell suspensions obtained from the vascular network of 5- to 20-day embryos, is shown in figure 9. Comparable data for young chicks and adult chickens is presented in table 9.

The first recognizable definitive elements, basophilic erythroblasts and early polychromatophilic erythroblasts, appear in the embryonic circulation at about 5 days. Basophilic erythroblasts are rarely seen after 7 days, and early polychromatophilic erythroblasts are only oaccasionally observed after day 8. Mid and late polychromatophilic erythroblasts appear at 6 to 7 days. These early definitive elements appear as round cells in fixed preparations and as round or slightly oval cells in wet mount preparations. Mid polychromatophilic erythroblasts persist in the circulation, although with decreasing frequency, until about day 15 and are occasionally found among the circulating erythroid cells of late embryos and young chicks. Similarly, late polychromatophilic erythroblasts, which can constitute 50 % of the definitive cells of 8-day embryos, are found in the circulation with decreasing frequency until hatching and can constitute several % of the circulating definitive elements in young chicks.

The first definitive erythrocytes which appear to be fully haemoglobinized in May Greenwald, giemsa-stained fixed preparations are round cells, similar in size to late polychromatophilic erythroblasts, but with more condensed nuclei. These cells appear in the embryonic circulation at 8 to 9 days and constitute the largest fraction of definitive erythroid cells from day 10 to about day 18. Oval cells with round, condensed nuclei and a comparable degree of haemoglobinization appear in the embryonic circulation at about day 9 and constitute about 10 to 20 % of definitive cells until about day 18. It is not unlikely that the round and oval cells with round nuclei represent one class of cells, the change of shape occurring during preparation of the slides. Wet mount preparations suggest that the majority of the definitive cells are oval or slightly oval by 8 to 10 days. At 8 to 9 days incompletely haemoglobinized oval cells, comparable to mid and late polychromatophilic erythroblasts, also appear. It should be recalled that the mid and late polychromatophilic erythroblasts which appear in the embryonic circulation at 6 to 7 days are round in fixed preparations.

An unusual characteristic of the nuclei of late polychromatophilic erythroblasts, fully haemoglobinized round and oval cells, and incompletely haemoglobinized oval cells has been consistently observed beginning at about 11 days. The nuclei of approximately 10 to 30 % of each of these cell types is enlarged and shows a characteristic loosening or 'fraying' of the chromatin. This phenomenon may represent nuclear reorganization prior to the final condensation of the nucleus.

Definitive cells with oval nuclei first appear in the embryonic circulation at 15 to 16 days (figure 9). The rapid decrease in the percentage of mature erythrocytes with round nuclei and the concomitant increase in the percentage of mature cells with oval nuclei was consistently observed to occur near the time of hatching. If the highly haemoglobinized round and oval cells with round nuclei are not precursors of the erythroid cells with oval nuclei, it becomes necessary to postulate that most of the erythroid cell mass of the embryo is turned over during a 1- to 2-day period immediately before hatching.

The nuclear and cytoplasmic diameters and the nuclear/cytoplasm ratio of the several maturation stages of definitive cells were determined on fixed preparations of erythroid cells from embryos of 5 to 19 days of age. For a particular maturation stage of the definitive cell series, the nuclear and cytoplasmic diameters were independent of the age of the embryos. The average values of these parameters are shown in table 10.

The morphological studies on May Greenwald, giemsa-stained preparations suggested that the first mature definitive erythrocytes appear in the embryonic circulation at 8 to 9 days. To further study this point the fraction of definitive cells which contained cytoplasmic reticulum, as determined by supravital staining with new methylene blue N, was correlated with the

TABLE 10. NUCLEAR AND CYTOPLASMIC DIAMETERS OF DEFINITIVE ERYTHROCYTES OF CHICK EMBRYOS AND ADULT CHICKENS DETERMINED ON FIXED PREPARATIONS*

	cytoplasm		nuclear		
cell type†	$\bar{D}$‡ ± s.d.	n	$\bar{D}$‡ ± s.d.	n	$\frac{\text{nuclear}}{\text{cytoplasm}}$
chick embryo					
BE	12.9 ± 0.55	10	8.3 ± 0.48	10	0.64
LEP	10.5 ± 0.60	35	6.2 ± 0.84	35	0.59
SEP	9.0 ± 0.71	36	5.9 ± 0.54	36	0.66
P	8.8 ± 0.37	35	4.6 ± 0.55	35	0.52
LP	10.5 ± 0.61	34	5.4 ± 0.55	34	0.51
O	9.0 ± 0.64	37	4.5 ± 0.55	37	0.50
O(N*)	9.0 ± 0.88	30	5.2 ± 0.53	30	0.58
LO	10.6 ± 0.69	19	4.9 ± 0.81	19	0.46
PO	11.3 ± 0.84 × 7.2 ± 0.51	31	4.2 ± 0.59	31	—
PO(N*)	11.0 ± 0.69 × 7.2 ± 0.66	22	5.2 ± 0.42	22	—
MR(RN)	9.8 ± 0.78	35	4.1 ± 0.49	35	0.42
MR(N*)	9.5 ± 0.60	36	5.0 ± 0.22	36	0.53
MO(RN)	11.3 ± 0.69 × 7.3 ± 0.60	36	4.0 ± 0.33	36	—
MO(N*)	11.1 ± 0.62 × 7.0 ± 0.70	35	5.2 ± 0.49	35	—
MR(ON)	10.2 ± 0.48	30	4.7‡ ± 0.46	30	—
MO(ON)	11.7 ± 0.65 × 7.1 ± 0.54	36	4.4 ± 0.49 × 3.0 ± 0.19	36	—
adult chicken					
MR(ON)	9.6 ± 0.60	20	4.6‡ ± 0.49	20	—
MO(ON)	11.5 ± 0.51 × 6.7 ± 0.35	20	5.0 ± 0.40 × 2.7 ± 0.47	20	—

† Cell type code: BE, basophilic erythroblast; LEP, large early polychromatophilic erythroblast; SEP, small early polychromatophilic erythroblast; P, mid-polychromatophilic erythroblast; LP, large mid-polychromatophilic erythroblast; O, late polychromatophilic erythroblast; LO, large late polychromatophilic erythroblast; PO, pale oval cell with a round nucleus (similar to the late polychromatophilic erythroblast); MR(RN), mature round erythrocyte with a round nucleus; MO(RN), mature oval erythrocyte with a round nucleus; MR(ON) mature round erythrocyte with an oval nucleus; MO(ON), mature oval erythrocyte with an oval nucleus; O(N*), late polychromatophilic erythroblast with 'frayed' nuclear chromatin pattern; PO(N*), pale oval cell with 'frayed' nuclear chromatin pattern; MR(N*), mature round erythrocyte with 'frayed' nuclear chromatin pattern; MO(N*), mature oval erythroctye with 'frayed' nuclear chromatin pattern.

Abbreviations: $\bar{D}$, average diamter; s.d., standard deviation of the average diameter; n, number of cells measured; ‡, for oval cells with round or oval nuclei, the long and short axes of the cytoplasmic ellipse were measured, and the average lengths and standard deviations calculated. For round cells with oval nuclei, only the long axis of the nucleus was measured. For oval cells with oval nuclei, both the long and short axes of the nuclear ellipse were measured, and the average lengths and standard deviations calculated; *, the nuclear and cytoplasmic diameter of a particular maturation stage of the definitive cell series does not change significantly with increasing age of the embryo. Thus, for example, the average diameters given for mid-polychromatophilic erythroblasts are valid for this maturation stage when seen in the 7-day embryo or in the 15-day embryo.

frequencies of the several maturation stages of definitive cells (table 11). The reticulum of basophilic erythroblasts and early and mid polychromatophilic erythroblasts is extensive and diffusely spread over the cytoplasm. Clumping of reticulum is observed in the more haemoglobinized late polychromatophilic erythroblasts. Although about 15 % of definitive cells from 8-day embryos could be classified as mature erythrocytes on May Greenwald, giemsa-stained

preparations, all the definitive cells of the 8-day embryos contained some reticulum. However, by 10 days, about 40% of the definitive cells no longer contained reticulum and therefore represent mature definitive erythrocytes.

TABLE 11. TIME OF APPEARANCE OF MATURE DEFINITIVE ERYTHROCYTES

Determined by morphological criteria and by the presence or absence of cytoplasmic reticulum†‡

age of donor/days	BE+LEP+ SEP	P+LP	O+LO+PO	mature cells with RN or ON	cells with cytoplasmic reticulum	cells without cytoplasmic reticulum
5	100	—	—	—	100	—
6	17.6	67.4	15.0	—	100	—
8	1.0	21.4	62.6	14.6	100	—
10	0.4	20.4	38.4	41.2	57.5	42.5
12	—	10.6	32.4	57.0	54.0	46.0
14	0.6	16.8	23.8	59.0	47.7	52.3
16	—	4.8	18.4	77.0	31.9	69.1
19	—	2.4	4.0	93.6	8.7	91.3
21	—	—	3.6	96.8	6.6	93.4
adult 1	—	—	0.6	99.4	2.5	97.5
adult 2	—	—	0.4	99.6	1.2	98.8

† Determined by supravital staining of the erythroid cells with new methylene blue, N.

‡ Cell type designations: BE+LEP+SEP, basopholic erythroblasts plus large and small early polychromatophilic erythroblasts; P+LP, large and small mid-polychromatophilic erythroblasts; O+LO+PO, large and small late polychromatophilic erythroblasts plus pale oval cells; mature cells, mature cells (round or oval) with round or oval nuclei.

The time course of appearance and disappearance of the several maturation stages of the definitive cells in the embryonic circulation indicates that, although very immature definitive precursors are released into the circulation at the initiation of definitive erythropoiesis at 5 days, with increasing age of the embryo progressively more mature definitive erythroid cells are released from the erythropoietic sites. Mitoses among definitive cells were rarely observed except at 6 days when the mitotic index was about 1% (the number of definitive cells in erythroid cell preparations from 5-day embryos is so low that the mitoses would not be observed unless the mitotic index was quite high). At 6 days mitotic figures were observed among basophilic erythroblasts as well as in early, mid and late polychromatophilic erythroblasts. Although the latter cell types are present among the circulating erythroid cells of older embryos, mitoses were only rarely seen. The definitive erythroid cell series of the chick embryo is a sustained cell population in contrast to the primitive cells. This fact, plus the observation that the definitive elements found in the embryonic circulation become progressively more mature during the period of extensive expansion of the embryonic circulation, requires that a sequestered compartment of immature definitive precursors and progenitor cells exists while erythropoiesis is confined to the yolk sac.

(*d*) *Changes in the total number of erythrocytes of the embryo*

To relate the changes in the types of erythroid cells of the developing embryos to the concomitant expansion of the embryonic circulation, it was necessary to obtain an estimate of the number of erythroid cells and the total amount of haemoglobin of embryos of different ages. The total haemoglobin of embryos of 3.5 to 18 days of age was estimated from oxyhaemoglobin spectra of haemoglobin solutions prepared from total embryos and blastoderms. The results

are presented in table 12. No corrections for myoglobin or cytochromes were made as the contribution of these proteins to the total haem protein pool of the embryos was calculated to be insignificant (Kagen & Linder 1968; Kagen, Linder & Gurevich 1969; Akeson, von Ehrenstein, Hevesy & Theorell 1960; Romanoff 1967).

TABLE 12. CHANGES IN THE AVERAGE AMOUNT OF HAEMOGLOBIN PER CIRCULATING ERYTHROID CELL AND IN THE TOTAL HAEMOGLOBIN PER EMBRYO DURING DEVELOPMENT OF THE CHICK EMBRYO†

age of embryos	haemoglobin per 10^6 erythroid cells/μg	haemoglobin per embryo
days		mg
3.5	38.0	0.36
4	51.3	0.78
5	56.3	2.82
6	59.9	5.01
7	47.9	6.91
8	43.7	13.70
9	38.5	18.10
10	35.0	—
12	36.1	51.10
14	35.0	100.60
16	34.6	139.90
18	29.8	139.60
adult chicken	30.6	—

† The haemoglobin per cell values were determined by the alkaline haematin method of King *et al.* (1944) and of Horecker (1948). The haemoglobin per embryo values were calculated from the spectra of oxyhaemoglobin solutions. The ratio of the absorption at 415 and 540 nm of the haemoglobin solutions prepared from whole embryos was within 5% of the value observed with adult chicken haemoglobin with the exception of the values for 3.5, 4- and 5-day embryos where the observed ratio of the absorption at 415 and 540 nm was within 10% of the value observed with adult chicken haemoglobin.

The average haemoglobin content of circulating erythroid cells was determined by the alkaline haematin method of King *et al.* (1944) and Horecker (1948) on circulating erythroid cells from embryos of 3.5 to 18 days of age and on adult chicken erythrocytes (table 12). It would have beeen desirable to determine the average haemoglobin content of erythroid cells obtained from the entire erythroid cell compartment of the embryo. This was not technically possible as the turbidity introduced by yolk material present in erythroid cell suspensions prepared from mechanically dissociated embryonic vascular tissue altered the 395/570 nm absorption ratio of alkaline haematin.

If the values for the total haemoglobin per embryo are divided by the values for the average haemoglobin content per circulating erythroid cell, the resulting numbers are an estimate of the number of erythroid cells of the embryos. These numbers will be artificially low if a compartment of erythroid cells with a lower haemoglobin content per cell exists outside the embryonic circulation. The presence of such a compartment with a different haemoglobin per cell value than that determined for circulating erythroid cells might be predicted to exist in embryos of 6 to 9 days, the time when partially haemoglobinized definitive cells are first seen in the embryonic circulation. In embryos of greater than 9 to 10 days of age, when the definitive cells of the circulation are becoming progressively more mature, the existence of a compartment of less mature definitive cells outside the circulation must, of necessity, be postulated.

To obtain some information on the size and composition of such a compartment, erythroid cells were collected from the embryonic circulation of one group of embryos and from the total

vascular tissue of a second group of comparable embryos by a method which permitted collection of erythroid cells from the major vessels (fraction 1), from partially dissociated vascular tissue of the embryos and membranes (fraction 2), and from mechanically dissociated vascular sinusoids of the membranes (fraction 3). It would be expected that if a sequestered compartment of definitive cells, less mature than those of the embryonic circulation, was of significant size and was accessible by mechanical dissociation of the vascular tissue, the values for the haemoglobin/erythroid cell and the differential counts of the cells of fractions 2 and 3 would differ from the values of these parameters measured on erythroid cells isolated from the embryonic circulation and on fraction 1.

When the differential counts of the erythroid cells of fractions 1, 2 and 3 of 6- to 9-day embryos were compared to those of erythroid cells isolated from the embryonic circulation, there were no significant differences in the types of definitive cells found. Only in 6-day embryos did the percentage of primitive cells found among circulating erythroid cells differ from the values of fractions 1 to 3. The percentage of primitive and definitive cells of the three fractions were similar. To compare the values of the average haemoglobin content of erythroid cells of fractions 1 to 3, haemoglobin was isolated from a known number of erythroid cells of each of the fractions from embryos of 6 to 8 days and from fractions 1 and 2 of 9-day embryos. The total amount of isolated haemoglobin was then divided by the number of cells used for the isolation of haemoglobin, and a value for the average haemoglobin content per erythroid cell was obtained. Although this method is less accurate than the alkaline haematin method because of the number of manipulative steps, the values for the average haemoglobin content per erythroid cell of the three fractions from 6-, 7- and 8-day embryos and for the two fractions from 9-day embryos were comparable. For the 6- to 9-day embryos it was possible to compare the average haemoglobin content of erythroid cells isolated from the embryonic circulation with the values for erythroid cells of fraction 1 using the alkaline haematin method. The results were similar. Hence it was not possible to locate a compartment of erythroid cells in embryos of 6 to 9 days which had a different distribution of primitive and definitive cells, a different differential count of definitive cells, or a different average haemoglobin content per erythroid cell than erythroid cells isolated from the embryonic circulation.

To obtain an estimate of the number of primitive and definitive erythroid cells per embryo, the number of erythroid cells per embryo was multiplied by the percentage of primitive and definitive cells in samples of circulating erythroid cells. The results are shown in figure 10. The primitive erythroid cells appear to have a lifespan shorter than the gestational age of the embryo.

The increase in the number of definitive cells per embryo is striking. As shown in figure 10 there was a 500 % increase between day 6 and day 18. The number of cells per embryo in each of the several maturation stages of the definitive cells (table 13) was estimated from the number of definitive cells per embryo and the differential counts of the definitive cells.

Because the numbers of erythroid cells per embryo presented in figure 10 and table 13 were calculated values obtained indirectly from the haemoglobin content of embryos and the average haemoglobin content of erythroid cells, the number of erythroid cells which could be easily isolated from mechanically dissociated vascular tissue of embryos of 2 to 9 days of age was determined in order to verify the rate of increase in the number of erythroid cells per embryo. These numbers are presented in table 14. The numbers of primitive and definitive cells were obtained by multiplying the number of isolated cells by the percentage of primitive and

definitive cells in the cell suspensions. The number of erythroid cells which could be easily isolated from embryos was about half of the calculated number of erythroid cells per embryo. The 24 h increment in the number of isolated erythroid cells per embryo for embryos of 3.5 to 9 days of age was quite similar to the 24 h increment in the number of erythroid cells per embryo calculated from the total haemoglobin of the embryos and the average haemoglobin content of erythroid cells.

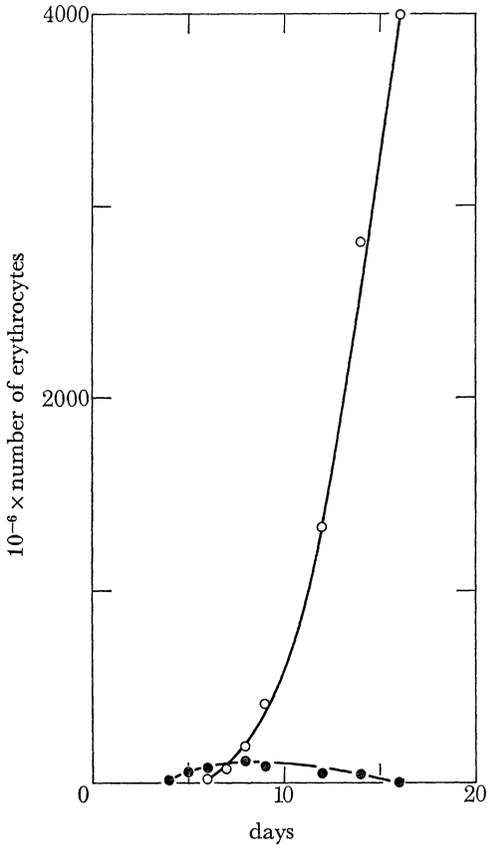


FIGURE 10. Estimated number of primitive and definitive erythroid cells in embryos of different ages. The number of erythroid cells per embryo was calculated by dividing the amount of haemoglobin per embryo by the average haemoglobin content per erythroid cell. The total number of erythroid cells per embryo was then multiplied by the percentage of primitive and definitive cells in the red cell suspensions to obtain an estimate of the number of primitive and definitive cells per embryo. ○, Definitive cells; ●, primitive cells.

Primitive erythroid cell populations isolated from mechanically dissociated vascular tissue of embryos were morphologically identical to primitive cell populations isolated from the embryonic circulation of comparable embryos. In neither type of preparation were primitive cells significantly less mature or less haemoglobinized than the predominant erythroid cell type observed. Isolation of primitive cells from mechanically dissociated vascular tissues of the embryo should give a random sample of the primitive cells from all accessible compartments, whereas isolation of primitive cells from the embryonic circulation should give a random sample of circulating erythroid cells only. It would therefore appear that any compartment of primitive cells containing less mature cells must be either small or not accessible by mechanical dissociation of the vascular areas of the embryos.

The data of table 15 on the increase of the number of cells in each of the several maturation

TABLE 13. ESTIMATED NUMBER OF CELLS IN EACH OF THE SEVERAL MATURATION STAGES OF DEFINITIVE ERYTHROID CELLS IN EMBRYOS OF VARIOUS AGES†

age of embryos days	BE ($\times 10^6$)	LEP+SEP ($\times 10^6$)	P+LP ($\times 10^6$)	O+LO ($\times 10^6$)	M(RN) ($\times 10^6$)	PO ($\times 10^6$)	M(ON) ($\times 10^6$)
5	0.18	0.2	—	—	—	—	—
6	0.19	2.7	4.3	1.7	—	—	—
7	0.64	12.0	32.0	19.0	—	—	—
8	0.76	16.0	50.0	92.0	12.0	19.0	—
9	0.41	12.0	41.0	120.0	190.0	52.0	—
12	—	17.0	130.0	430.0	660.0	88.0	—
14	—	17.0	180.0	740.0	1680.0	190.0	—
18	—	—	46.0	83.0	1260.0	18.0	3280.0

† The number of cells in each of the several maturation stages of the definitive cells was calculated from the number of definitive cells per embryo (figure 10) and the differential counts of definitive cells in the erythroid cell preparations.

Letter code: BE, basophilic erythroblasts; LEP+SEP, large and small early polychromatophilic erythroblasts; O+LO, large and small late polychromatophilic erythroblasts; M(RN), mature cells (round or oval) with round nuclei; PO, pale oval cells similar to late polychromatophilic erythroblasts; M(ON), mature cells (round or oval) with oval nuclei.

TABLE 14. NUMBER OF ERYTHROID CELLS PER EMBRYO OBTAINED FROM MECHANICALLY DISSOCIATED VASCULAR TISSUE†

age of embryos days	erythroid cells ($\times 10^6$)	primitive cells ($\times 10^6$)	definitive cells ($\times 10^6$)	primitive cells/%	definitive cells/%
2	0.26	0.26	—	100	0
2.5	0.53	0.53	—	100	0
3	1.7	1.7	—	100	0
3.5	3.0	3.0	—	100	0
4	5.8	5.8	—	100	0
5	25.0	25.0	—	100	0
6	49.0	39.0	10.0	79.8	20.2
7	60.0	33.0	27.0	54.3	45.7
8	179.0	63.0	116.0	35.1	64.9
9	346.0	29.0	317.0	8.5	91.5

† The number of erythroid cells per embryo was determined by multiplying the number of erythroid cells per ml of cell suspension obtained from mechanically dissociated vascular tissue by the volume of the cell suspension. The numbers of primitive and definitive cells per embryo were calculated by multiplying the number of erythroid cells per embryo by the percentage of primitive and definitive cells in the suspension.

TABLE 15. NUMBER OF CELLS PER EMBRYO IN EACH OF THE SEVERAL MATURATION STAGES OF DEFINITIVE CELLS AMONG DEFINITIVE ERYTHROID CELLS ISOLATED FROM MECHANICALLY DISSOCIATED VASCULAR TISSUE†

age of embryos days	BE ($\times 10^6$)	LEP+SEP ($\times 10^6$)	P+LP ($\times 10^6$)	O+LO ($\times 10^6$)	M(RN) ($\times 10^6$)	PO ($\times 10^6$)	M(ON) ($\times 10^6$)
6	0.78	3.7	4.5	0.77	—	—	—
7	0.60	5.9	7.5	13.0	—	—	—
8	0.92	4.3	12.0	98.0	—	—	—
9	1.90	7.6	32.0	275.0	—	—	—

† The number of cells per embryo in each of the several maturation stages of definitive cells was calculated from the number of definitive cells per embryo (table 14) and the differential counts of the definitive cells. The letter code is the same as in table 13.

stages of the definitive cells in the accessible erythroid cell compartment of 6- to 9-day embryos, suggests that there is a continued input of new definitive cells into this compartment. For example, in view of the virtual absence of mitotic figures in isolated definitive cells, the number of definitive cells in the accessible compartment on day 8 is insufficient to account for the number of accessible definitive cells on day 9.

4. Changes in the types of haemoglobins

(*a*) *Haemoglobins of 2- to 5-day embryos*

(i) *Normal components*

The haemoglobin components of freshly prepared red cell lysates of 2- to 5-day embryos are resolved on pH 10.3 polyacrylamide gels into a major fast component (haemoglobin P) and a minor slow component (haemoglobin E) (figure 14*a*, plate 28). In addition, two trace haemoglobin components were occasionally observed: a minor band migrating faster than the major component in a position similar to that of the minor adult haemoglobin; and a trace band migrating between haemoglobins E and P. Haemoglobins E and P were also resolved on pH 8.9 polyacrylamide gels (figure 14*b*).

Table 16. Comparison of the E/P peak mass ratios of haemoglobins prepared from circulating erythroid cells and haemoglobins prepared from erythroid cells isolated from mechanically dissociated blastoderms

age of embryos / days	E/P (amido black stained gels): haemoglobin prepared from circulating erythroid cells	E/P (amido black stained gels): haemoglobin prepared from erythroid cells of dissociated blastoderms
2.5	—	0.18
3.0	—	0.23
3.5	0.24	0.20
4.0	0.22	0.24
5.0	0.26	0.24

The relative quantity of these two components, determined from gel scan peak masses and expressed as the E/P ratio, was constant between day 2 and day 5 (table 16). The E/P ratios of unstained gels, benzidine stained gels, and amido black stained gels were comparable. The E/P ratios of haemoglobins prepared from circulating erythroid cells of embryos and from erythroid cells of mechanically dissociated whole blastoderms were comparable (table 16).

When erythroid cells were collected in H.R. b.s.a. solution the CCl_4 interface residue of the cell lysate contained additional haemoglobin. To determine whether the residual haemoglobin differed from the bulk of the haemoglobin, the interface material was re-extracted with isotonic NaCl and the two extracts electrophoresed separately. No additional haemoglobin components were observed in the NaCl extract of the CCl_4 interface residue. However, the amount of haemoglobin E relative to haemoglobin P (determined from gel scan peak masses) was 1.5 to 2 times that of the first extract. The fraction of the total haemoglobin of the erythroid cells found in the NaCl extract was 38 % in 2.5-day embryos, 25 % in 3-day embryos, and about 15 % in 3.5-, 4-, and 5-day embryos.

The number of erythroid cells which can be obtained from very young embryos is small, and the resulting haemoglobin solutions are dilute. Such haemoglobin solutions are unstable when stored on ice or when frozen at −20 °C. However, the electrophoretic patterns of haemoglobins stored in liquid nitrogen were identical to the patterns of freshly prepared haemoglobins from 2- to 5-day embryos on both benzidine and amido black stained gels. The E/P ratios of haemoglobins stored in liquid nitrogen were entirely comparable to those of freshly prepared haemoglobins. Aliquots of haemoglobin solutions could be stored in liquid nitrogen for at least two months without generation of additional electrophoretic components.

(ii) *Minor artefactual haemoglobins*

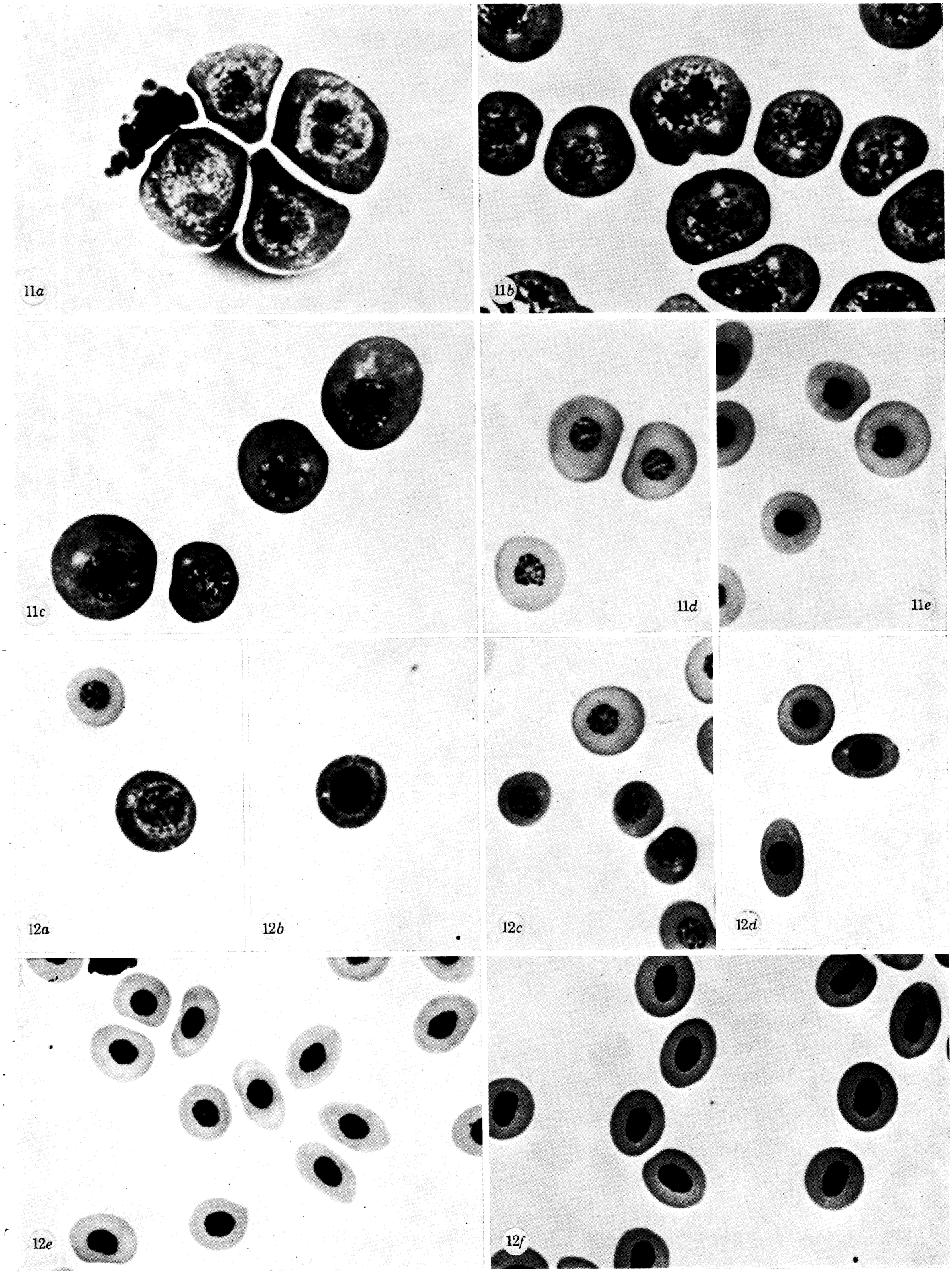
When haemoglobin solutions from 2- to 5-day embryos were frozen at −20 °C or stored for more than 18 h on ice, several additional components were observed on polyacrylamide gels. A component migrating faster than haemoglobin P was observed in haemoglobins frozen at −20 °C (band P′ in figure 14*c*). The minor band (P″) which migrates near the position of the minor adult haemoglobin D was also observed in this sample. An additional component which migrated between haemoglobins E and P was observed in samples stored at 0 to 4 °C for more than 18 h (figure 14*d*). When haemoglobins of 2- to 5-day embryos were stored at 0 to 4 °C for several days, the E band could be totally lost. As the E band decreased in amount the band 1′ (figure 14*d*) increased in intensity. The band P′ also increased in intensity as the time the haemoglobins were held at 0 to 4 °C increased. The complex pattern of haemoglobin components observed when haemoglobin of 4-day embryos was eluted from Sephadex G-100 and concentrated by vacuum dialysis is shown in figure 14*e*.

To determine the origin of these minor bands, haemoglobins E and P were isolated from 4.5-day embryos by preparative polyacrylamide gel electrophoresis. The isolated components were re-electrophoresed and the electrophoretic patterns compared with those of the adult A and D components which had been isolated from adult chicken haemoglobin by preparative polyacrylamide gel electrophoresis. As shown in figure 14*f*, *g* the two bands (P′, P″) which migrate faster than haemoglobin P can arise from haemoglobin P while the band (1′) which migrates between haemoglobins E and P arises from the haemoglobin E. Two other bands (2′ and 3′) which arise from haemoglobin E migrate similarly to two of the bands arising from haemoglobin A (figure 14*f*).

Description of plate 27

Figure 11. Maturation stages of primitive cells of 2- to 14-day embryos. Fixed preparations of erythroid cells were prepared with the Shandon cytocentrifuge and stained with Wright, giemsa stain. (*a*) Erythroid cells from 2-day embryos. (*b*) Erythroid cells from 3-day embryos. (*c*) Primitive erythroid cells from 5-day embryos. (*d*) Primitive erythroid cells from 7-day embryos. (*e*) Erythroid cells from 14-day embryos. The large cell is a mature primitive erythrocyte. Two well haemoglobinized round definitive cells with round nuclei are also observed.

Figure 12. Maturation stages of definitive cells of 6- to 16-day embryos. Fixed preparations of erythroid cells were prepared with the Shandon cytocentrifuge and stained with May Greenwald, giemsa. (*a*) Definitive erythroid cells of 6-day embryos. The large, immature cell is a basophilic erythroblast and the small cell is a late polychromatophilic erythroblast. (*b*) Early polychromatophilic erythroblast of 6-day embryos. (*c*) Erythroid cells of 7-day embryos. The large cell is a primitive erythrocyte. The small cells represent mid and late polychromatophilic erythroblasts. (*d*) Definitive erythroid cells of 12-day embryos. Well haemoglobinized round and oval erythroid cells with round nuclei are observed. (*e*) Erythroid cells of 16-day embryos. (*f*) Erythroid cells of an adult chicken.



FIGURES 11 AND 12. For legends see facing page.

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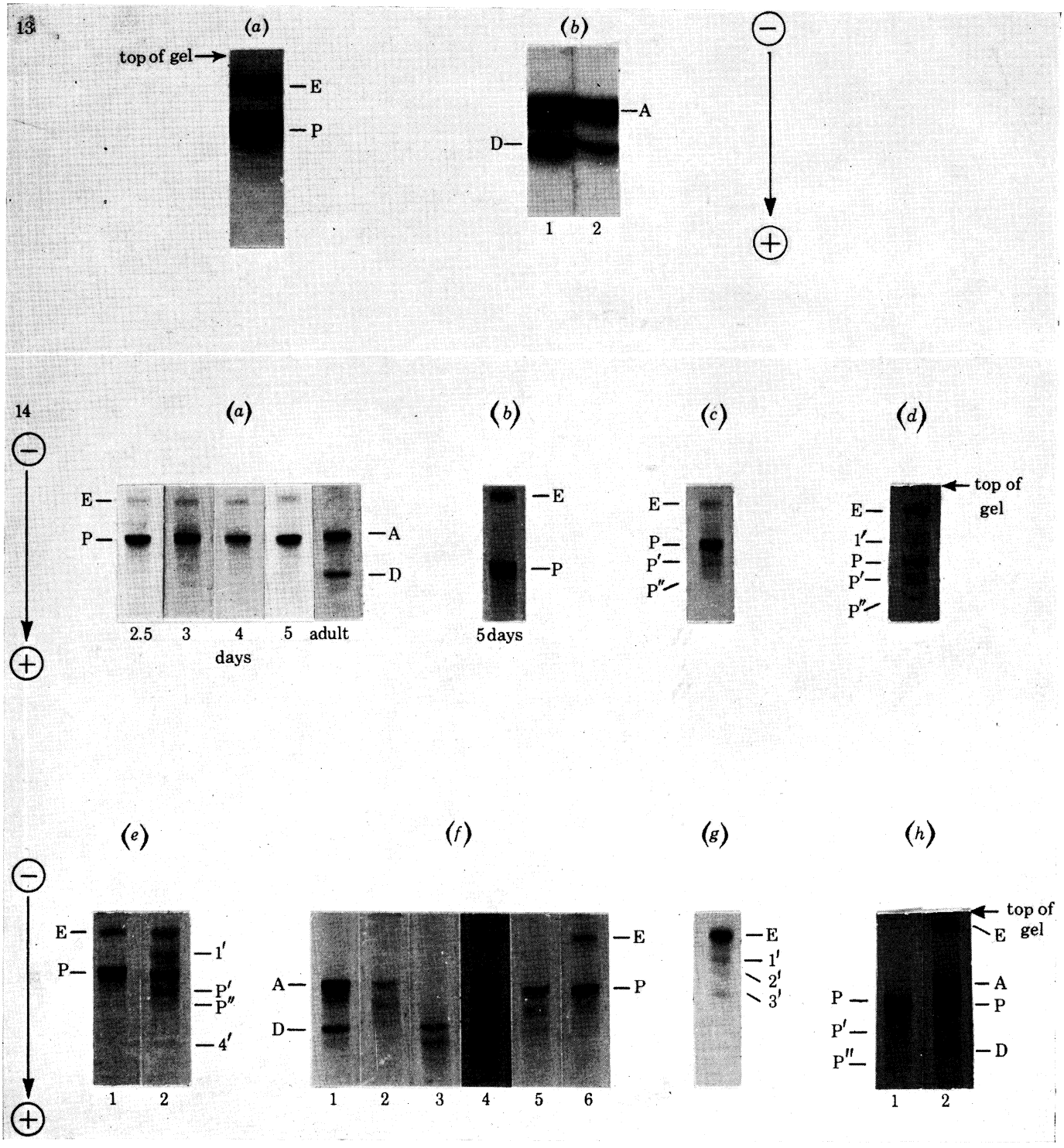

FIGURES 13 AND 14. For legends see facing page.

Electrophoresis of isolated haemoglobin P at pH 10.3 suggested that the P″ component migrated slightly faster than haemoglobin D. Electrophoresis of isolated haemoglobin P on polyacrylamide gels at pH 8.9 indicated that this component differs in electrophoretic mobility from haemoglobin D (figure 14*h*).

(*b*) *Haemoglobins of* 6- *to* 21-*day embryos and of young chicks*

(i) pH 10.3 *electrophoresis*

At 6 to 7 days when the first haemoglobinized immature definitive erythroid cells appear in the embryonic vascular system, two new haemoglobin components became detectable in freshly prepared erythroid cell lysates (figure 15*a*, plate 29). These two new components were observed on benzidine or amido black stained gels. In the 6-day lystate a component is observed which migrates similarly to the minor adult haemoglobin (haemoglobin D). A very faint band, migrating slightly more slowly than haemoglobin P, is also observed. This component migrates similarly to the major adult haemoglobin (haemoglobin A). Although the electrophoretic mobilities of the two new components are similar to the mobilities of the two adult components, the relative quantities of the two new components differ from those of adult chicken haemoglobin. Indeed, in some lysates from 6-day embryos, only the component similar to haemoglobin D was observed. The variation in the quantities of the two new components in lysates from 6- to 7-day embryos correlated with the percentage of definitive erythroid cells in the cell suspension used for the preparation of haemoglobin.

As the embryos increase in age, the two new haemoglobin components increase in amount

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Description of plate 28

Figure 13. Preparative polyacrylamide gel electrophoresis of haemoglobin of 4-day embryos and of an adult chicken, pH 10.3, 2 °C. Unstained gels. (*a*) Haemoglobin of 4-day embryos. The bands labelled E and P were eluted from the gels and used as the immunogens. (*b*) Haemoglobin of an adult chicken. The band labelled D (1) was eluted and used as the immunogen for preparation of antisera to haemoglobin D. The band labelled A (2) was similarly treated and used as the immunogen for preparation of antisera to haemoglobin A.

Figure 14. Analytical polyacrylamide gel electrophoresis of haemoglobin of young embryos. (*a*) Haemoglobin of 2- to 5-day embryos compared to adult chicken haemoglobin (pH 10.3 electrophoresis, amido black stain). The letters E and P indicate the two components of early embryos. A and D indicate the major and minor adult components respectively. (*b*) Haemoglobin of 5-day embryos electrophoresed at pH 8.9 (amido black stain). (*c*) Five-day embryo haemoglobin frozen at −20 °C. In addition to the E and P components, two additional bands, P′ and P″, are seen (pH 10.3 electrophoresis, amido black stain). (*d*) Five-day embryo haemoglobin stored at 0 °C for more than 18 h (pH 10.3 electrophoresis, amido black stain). In addition to the E and P components, a band (1′) running between haemoglobins E and P, as well as the two bands migrating faster than haemoglobin P (P′ and P″) are observed. (*e*) Comparison of the electrophoretic pattern (pH 10.3) of haemoglobin from 4-day embryos prior to Sephadex G-100 column chromatography (1) and a pooled fraction of the eluate (2) (amido black stain). (*f*) Comparison of the electrophoretic patterns of haemoglobins A, D and P (2, 3, and 5 respectively) which had been isolated by preparative polyacrylamide gel electrophoresis with the patterns of adult chicken haemoglobin (1), 5-day embryo haemoglobin (6), and a sample containing haemoglobins E, A, P and D (4) (pH 10.3 electrophoresis, amido black stain). (*g*) Electrophoretic pattern of haemoglobin E isolated by preparative polyacrylamide gel electrophoresis. In addition to the E band, three additional components were seen. The migration of the 1′ band is similar to the band migrating between haemoglobins E and P. The bands labelled 2′ and 3′ migrate similarly to two of the bands associated with isolated haemoglobin A (pH 10.3 electrophoresis, benzidine stain). (*h*) Comparison of the electrophoretogram of isolated haemoglobin P (1) with that of a haemoglobin sample containing the E, A, P and D components (2). The letters P′ and P″ denote the two minor bands observed in samples of haemoglobin P isolated by preparative polyacrylamide gel electrophoresis (pH 8.9 electrophoresis, amido black stain).

and the quantity of haemoglobin P decreases (figure 15*b*). It cannot be determined from the electrophoretograms when haemoglobin P is no longer present in the lysates since this component migrates as an anterior shoulder of the major component by 12 to 14 days, a time when primitive erythrocytes constitute less than 5 % of the circulating erythroid cells. A trace haemoglobin component which migrated on pH 10.3 polyacrylamide gels similarly to haemoglobin E was consistently observed in lysates from late embryos (16 to 21 days), although primitive erythrocytes constituted less than 0.1 % of the circulating erythroid cells of these embryos (figure 15*b*). This component was not observed in adult chicken haemoglobin.

In lysates from young chicks (0.5 to 20 days) the two haemoglobins corresponding in mobility to haemoglobins A and D of the adult chicken were observed as well as the minor component which migrated similarly to haemoglobin E (figure 15*c*). This component constituted about 5 to 7 % of the haemoglobin of late embryos and about 2.5 % of the haemoglobin of young chicks. This component was present in lysates prepared from 21 individually bled chicks.

The haemoglobin electrophoretograms of figure 15*a*, *b* are of samples stored on ice no longer than 18 h before electrophoresis. Haemoglobins of older embryos and adult chickens are also unstable at 0 to 4 °C or when frozen at −20 °C. Two additional bands which migrate between haemoglobins A and D have been observed on pH 10.3 polyacrylamide gels of haemoglobins from late embryos, young chicks, and adult chickens which had been frozen at −20 °C or stored on ice for longer than 18 h. The qantities of these two components increased as the storage time increased. When haemoglobins of 6- to 21-day embryos, young chicks, and adult chickens were stored in liquid nitrogen for up to two months, the electrophoretograms were identical to those of freshly prepared haemoglobins.

About 10 % of the haemoglobin of erythroid cells from embryos of 6 days of age or older was retained in the CCl_4 interface material following the first centrifugation of the lysate. No additional haemoglobin components were observed when the NaCl extract of the interface material was electrophoresed on pH 10.3 polyacrylamide gels. However, in the NaCl extracts prepared from erythroid cells of 6- to 9-day embryos the percentage of haemoglobin E was 1.5 to 2 times that observed in the initial lysate. The percentages of the several haemoglobin components were comparable in the initial lysates and in the NaCl extracts prepared from embryos of greater than 9 days of age.

(ii) pH 8.9 *electrophoresis*

To confirm the changes in the types of haemoglobins produced by the developing embryo, a second polyacrylamide gel system at pH 8.9 was employed. Two major components (haemoglobins E and P) were observed in lysates from 4- to 5-day embryos (figure 15*d*). A diffuse band which migrated more rapidly than haemoglobin P was also observed. This component, denoted P′, was observed when haemoglobin P, isolated by preparative polyacrylamide gel electrophoresis from haemoglobin of 4.5-day embryos, was re-electrophoresed at pH 8.9 (figure 14*h*). In the pH 8.9 system adult chicken haemoglobin had the two components (haemoglobin A and D) observed in the pH 10.3 system, but also showed a diffuse band, denoted A′, migrating between haemoglobins A and D (figure 15*d*). When haemoglobin A, isolated by preparative polyacrylamide gel electrophoresis of adult chicken haemoglobin, was re-electrophoresed at pH 8.9, this more rapidly migrating, diffuse component was also observed.

When lysates from 6- to 7-day embryos were examined on polyacrylamide gels at pH 8.9, the E, P and P′ components were observed. In addition, a component with an electrophoretic

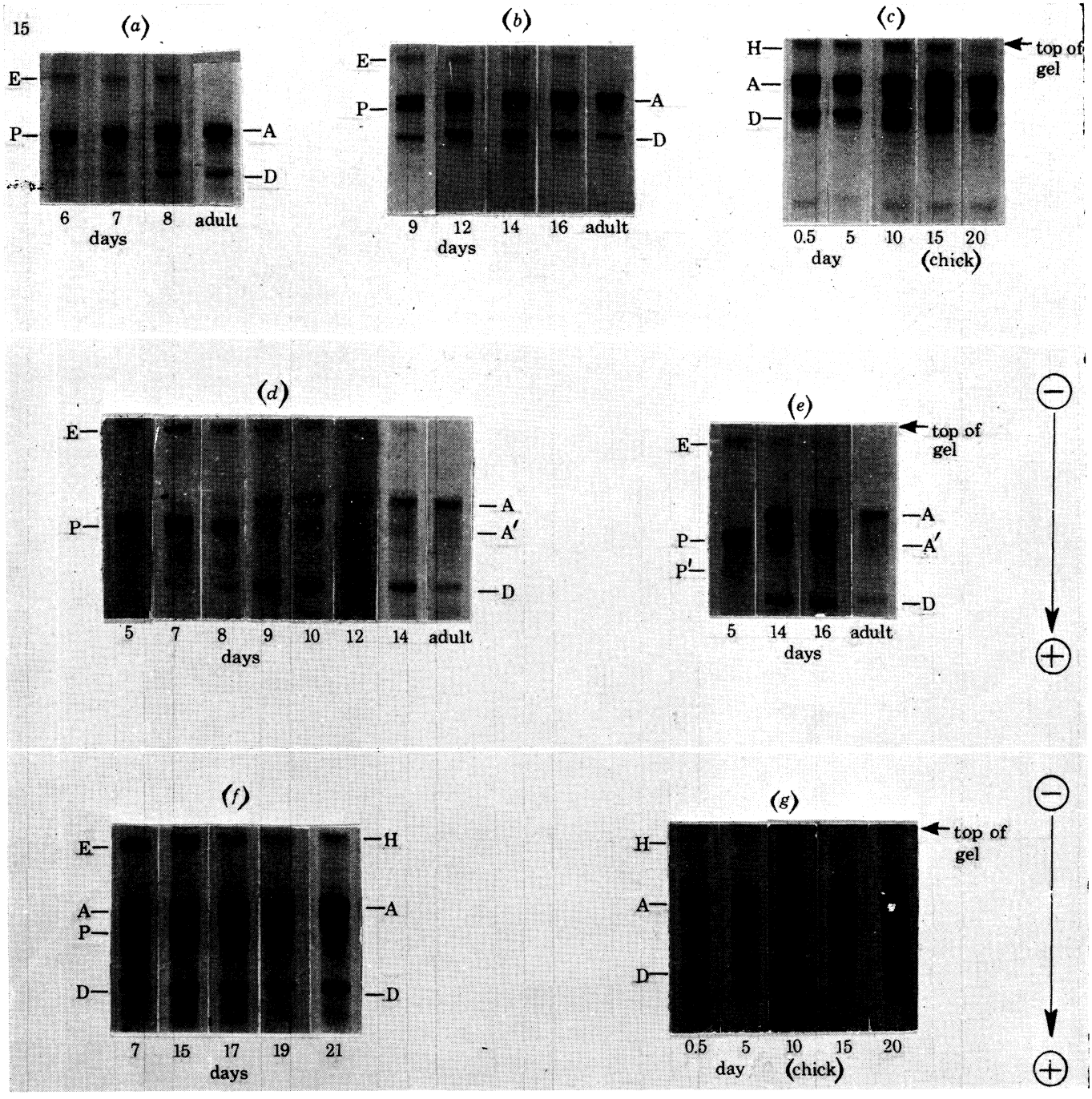

FIGURE 15. Analytical polyacrylamide gel electrophoresis of haemoglobins of embryos of 6 to 21 days and of 0.5- to 20-day chicks (amido black stain). (*a*) Comparison of haemoglobin of 6-, 7- and 8-day embryos with haemoglobin of an adult chicken (pH 10.3 electrophoresis). The letters E and P denote the two embryonic haemoglobin components. The letters A and D denote the major and minor adult haemoglobins. (*b*) Haemoglobins of 12- to 16-day embryos compared to haemoglobins of 9-day embryos and of an adult chicken (pH 10.3 electrophoresis). (*c*) Haemoglobins of 0.5- to 20-day-old chicks. The letter H indicates the minor component of haemoglobin of late embryos which migrates in the pH 10.3 system similarly to haemoglobin E (pH 10.3 electrophoresis). (*d*) Comparison of haemoglobins of 6- to 14-day embryos with haemoglobins of 5-day embryos and of an adult chicken on pH 8.9 polyacrylamide gels. The letters E, P, A and D indicate the respective haemoglobin components. The letter A′ refers to the diffuse component associated with haemoglobin A which migrates between haemoglobins A and D in the pH 8.9 electrophoresis system. (*e*) Comparison of the migration of haemoglobin P of 5-day embryos with the adult haemoglobin component A′ and the similarly migrating component of lysates from 14- and 16-day embryos (pH 8.9 electrophoresis). (*f*) Comparison of the haemoglobins of late embryos with the haemoglobins of 7-day embryos. The letter H indicates the minor component of haemoglobins of late embryos and young chicks which migrates on pH 8.9 polyacrylamide gels slightly more slowly than haemoglobin E (pH 8.9 electrophoresis). (*g*) Haemoglobins of 0.5 to 20-day-old chicks (pH 8.9 electrophoresis).

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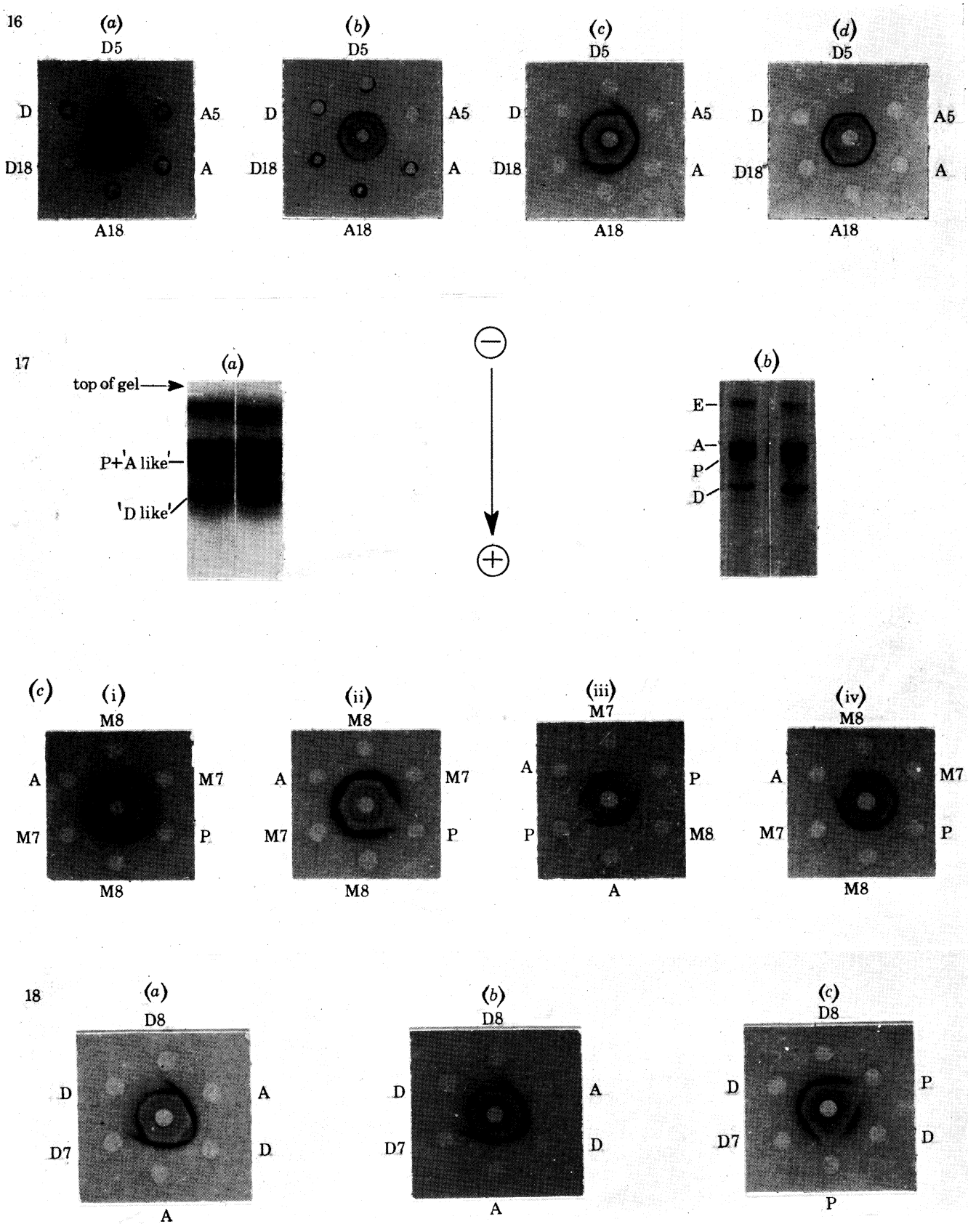


FIGURES 16 TO 18. For legends see facing page.

mobility comparable to that of the adult haemoglobin D and a faint band migrating similarly to the adult haemoglobin A were detected (figure 15*d*). The relative quantities of these two new haemoglobins differed from the relative amounts of the A and D components of adult chicken haemoglobin. As the embryos increased in age, these new components progressively replaced haemoglobins E and P. However, a prominent band, which appeared to migrate near the position of haemoglobhn P was observed in lysates from 14- and 16-day embryos although less than 5 % of the erythroid cells of these embryos were primitive erythrocytes. Upon careful examination, this component was observed to migrate slightly faster than P′ and corresponded to the component A′ (figure 15*e*). The minor haemoglobin component of lysates from late embryos appeared to migrate slightly more slowly than haemoglobin E of 5-day embryos on pH 8.9 polyacrylamide gels (figure 15*f*). This component was denoted haemoglobin H.

When lysates from young chicks (0.5 to 20 days) were electrophoresed on pH 8.9 polyacrylamide gels, the two new components corresponding to the adult haemoglobins D and A, the A′ band, and the minor component which migrated slightly more slowly than haemoglobin E were observed (figure 15*g*). The percentages of haemoglobin H in lysates electrophoresed at pH 8.9 were comparable to the values obtained when the same lysates were run on pH 10.3 polyacrylamide gels. On these heavily loaded gels, a minor component, migrating faster than haemoglobin D was observed. This component was observed when haemoglobin D of adult chickens, isolated by preparative polyacrylamide gel electrophoresis, was rebanded on analytical gels (figure 14*f*).

Description of plate 30

Figure 16. Comparison of the 'D like' and 'A like' haemoglobins of 18-day embryos with haemoglobins D and A of 5-day chicks and of an adult chicken by two-dimensional immunodiffusion. Two micrograms of haemoglobin were placed in the peripheral wells. The plates were stained with amido black. (*a*) Reaction with rabbit antiserum to haemoglobin E. The letter D indicates adult chicken haemoglobin D; D5, haemoglobin D of 5-day chicks; and D18, 'D like' haemoglobin of 18-day embryos. The letter A notations are similar. (*b*) Reaction with rabbit antiserum to haemoglobin P. (*c*) Reaction with rabbit antiserum to haemoglobin A. (*d*) Reaction with rabbit antiserum to haemoglobin D.

Figure 17. Comparison of a mixture of haemoglobin P and 'A like' haemoglobin from 7- and 8-day embryos with haemoglobin A of an adult chicken and haemoglobin P of 5-day embryos by two-dimensional immunodiffusion. (*a*) Preparative polyacrylamide gel electrophoresis of lysates from 7- and 8-day embryos, pH 10.3, 2 °C. Unstained gels. The bands euted from the gels are indicated by the lettered lines. (*b*) Analytical polyacrylamide gel electrophoresis of the lysates from 7- and 8-day embryos used for the preparation of the mixture of haemoglobin P and 'A like' haemoglobin and 'D like' haemoglobin (pH 10.3 electrophoresis). (*c*) Comparison of the mixture of haemoglobin P and 'A-like 'haemoglobin from 7- and 8-day embryos with haemoglobin A of an adult chicken and haemoglobin P from 5-day embryos. The letters M7 and M8 refer to the mixture of haemoglobin P and 'A like' haemoglobin isolated from 7- and 8-day embryos, respectively. Two micrograms of haemoglobin were placed in the peripheral wells. Plates were stained with amido black. (i) Reaction with rabbit antiserum to haemoglobin E. (ii) Reaction with rabbit antiserum to haemoglobin A. (iii) Reaction with a second rabbit antiserum to haemoglobin A. (iv) Reaction with rabbit antiserum to haemoglobin P.

Figure 18. Comparison of 'D like' haemoglobin of 7- and 8-day embryos with haemoglobin D of an adult chicken and haemoglobin P of 5-day embryos by two-dimensional immunodiffusion. (*a*) Reaction of haemoglobin D of an adult chicken and 'D like' haemoglobin of 7- and 8-day embryos with rabbit antiserum to haemoglobin E. The letters D7 and D8 denote the 'D-like' haemoglobin isolated from 7- and 8-day embryos by preparative polyacrylamide gel electrophoresis. (*b*) Reaction of haemoglobin D of adult chickens and 'D like' haemoglobin of 7- and 8-day embryos with rabbit antiserum to haemoglobin A. (*c*) Reaction of haemoglobin P of 5-day chick embryos and 'D like' haemoglobin of 7- and 8-day embryos with rabbit antiserum to haemoglobin A.

(iii) *Comparison of the 'D like' and 'A like' haemoglobin of chick embryos with haemoglobin D and A of adult chickens by two-dimensional immunodiffusion*

To further substantiate the identity of the 'D like' and 'A like' haemoglobins of chick embryos with haemoglobin D and haemoglobin A of adult chickens, these components were isolated by preparative polyacrylamide gel electrophoresis from 18-day embryos and the immunological reactivity of the isolated components compared to haemoglobins D and A isolated from 5-day chicks and from an adult chicken. The 'D like' and 'A like' haemoglobins of 18-day embryos showed reactions of identity with haemoglobin D and haemoglobin A respectively with all antisera tested (figure 16, plate 30).

'D like' haemoglobin and a mixture of haemoglobin P and 'A like' haemoglobin were isolated from erythroid cell lysates of 7- and 8-day embryos by preparative polyacrylamide gel electrophoresis (figure 17*a*, plate 30). Haemoglobin P and 'A like' haemoglobin of 7- and 8-day embryos were not resolved on the preparative gels. However, electrophoresis of these lysates on analytical polyacrylamide gels indicated that both the P and 'A like' components were present (figure 17*b*).

With an antiserum to haemoglogin E, m7 and m8 (the mixtures of haemoglobin P and the 'A like' haemoglobin of 7- and 8-day embryos) showed precipitin lines which were continuous with that of haemoglobin A from an adult chicken (figure 17*c* (i)). The precipitin lines of m7 and m8 showed spurs with haemoglobin P of 4.5-day embryos (figure 17*c* (i), (ii)). M7 and m8 showed reactions of identity with haemoglobin A with an antiserum which did not react with haemoglobin P (figure 17*c* (iii)). The presence of both haemoglobin P and haemoglobin A in m8 was demonstrated with an antiserum to haemoglobin P. With this antiserum m8 showed two precipitin lines: one continuous with that of haemoglobin P, and the second continuous with that of haemoglobin A of an adult chicken (figure 17*c* (iv)).

The 'D like' haemoglobin of 7- and 8-day embryos showed reactions of identity with haemoglobin D of adult chickens with all antisera tested (figure 18, plate 30). Since an occasionally observed minor haemoglobin component of lysates from embryos of less than 6 days of age migrated in the pH 10.3 electrophoresis system close to the position of haemoglobin D, the isolated 'D like' haemoglobin of 7- and 8-day embryos was compared by two-dimensional immunodiffusion with haemoglobin P of 4.5-day embryos. Haemoglobin P did not cross-react with an antiserum to haemoglobin A, although haemoglobin D of adult chickens reacted strongly. 'D like' haemoglobin of 7- and 8-day embryos showed reactions of identity with haemoglobin D with this antiserum (figure 18*c*).

(iv) *Quantification of the relative amounts of the haemoglobin components*

The change in the haemoglobin D/A ratio of lysates prepared from circulating erythroid cells of 7- to 21-day embryos and of 0.5- to 20-day chicks is shown in figure 19. The D/A ratio of adult chicken haemoglobin, electrophoresed simultaneously with the embryo and young chick samples, is shown for comparison. The ratio change was similar for haemoglobins electrophoresed at pH 10.3 or pH 8.9, for benzidine or amido black stained gels and for haemoglobins electrophoresed within 18 h of preparation or stored in liquid nitrogen prior to electrophoresis. In each of five separate experiments the haemoglobin D/A ratio of lysates from late embryos was higher than the ratio observed for adult chicken haemoglobin electrophoresed simultaneously with the embryonic haemoglobins. The D/A ratio of haemoglobins from 20

individually bled chicks of 0.5 to 20 days of age was also higher than that of adult chicken haemoglobin (table 17). The haemoglobin D/A ratios of lysates from young chicks did not overlap the range of values of this ratio observed with adult chicken haemoglobin.

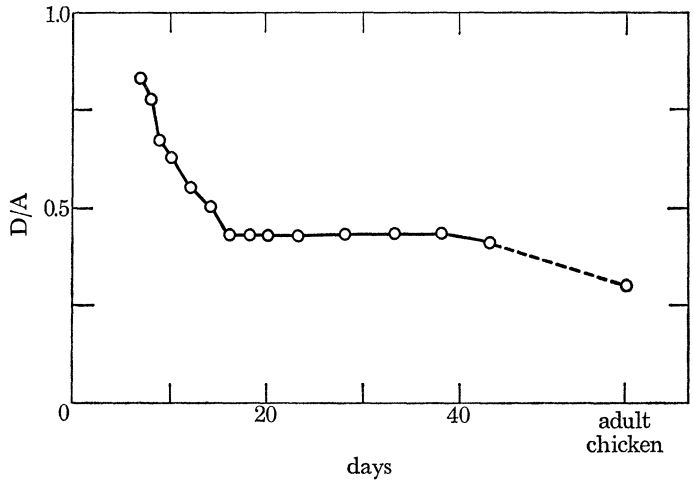


FIGURE 19. Changes in the haemoglobin D/A ratio during development of the chick embryo and young chick. The haemoglobin D/A ratios were determined from gel scan peak masses of haemoglobins separated on pH 8.9 polyacrylamide gels. The ratios were corrected for errors resulting from the method of peak mass assignment by use of mixtures of adult chicken haemoglobin and haemoglobin of 5-day embryos (Bruns 1971).

TABLE 17. HAEMOGLOBIN D/A PEAK MASS RATIOS OF LYSATES FROM CIRCULATING ERYTHROCYTES OF INDIVIDUAL YOUNG CHICKS (pH 10.3 ELECTROPHORESIS)†

age of chick/days	haemoglobin D/A peak mass ratio
0.5	0.43, 0.42, 0.40, 0.43
5	0.40, 0.41, 0.44, 0.48
10	0.46, 0.42, 0.41, 0.46
15	0.43, 0.44, 0.46
20	0.39, 0.39, 0.40

† The average haemoglobin D/A peak mass ratio of the adult chicken standard was 0.31 (0.28–0.34); $n = 5$.

TABLE 18. HAEMOGLOBIN D/A PEAK MASS RATIOS OF HAEMOGLOBINS ISOLATED FROM INDIVIDUAL ADULT CHICKENS

	D/A pH 10.3 system			D/A pH 8.9 system		
chicken	ratio	standard deviation	number of assays	ratio	standard deviation	number of assays
1	0.31	±0.05	5	—	—	—
2	0.34	±0.05	5	—	—	—
3	0.30	±0.01	3	—	—	—
4	0.30	±0.04	3	0.31	±0.03	3
5	0.33	±0.01	3	0.36	—	2
6	0.31	±0.03	3	0.33	±0.04	3
average	0.33	±0.01	22	0.33	±0.02	8

The difference in the D/A ratio of haemoglobin from late embryos or young chicks and that of adult chicken haemoglobin cannot be ascribed to an anomally of one adult chicken. Haemoglobin was prepared from six different adult chickens and each sample was electrophoresed several times at pH 10.3. The D/A ratios of these six haemoglobin samples were essentially

identical (table 18). Haemoglobin from three of the six chickens was also electrophoresed several times at pH 8.9. The D/A ratios were essentially identical and were comparable to those obtained with the pH 10.3 electrophoresis system (table 18).

To determine whether the change with time in the haemoglobin D/A ratios was a systematic methodological artefact, mixtures of 5-day embryo haemoglobin and adult chicken haemoglobin were electrophoresed at pH 10.3 and 8.9. The total quantity of haemoglobin of each mixture loaded on the gels was comparable to the amount of haemoglobin per gel used for analysis of the chick embryo haemoglobins. The quantities of 5-day embryo haemoglobin and adult chicken haemoglobin in the mixtures were arranged such that the gel scans of the mixtures would mimic as closely as possible the scans of lysates of embryonic erythroid cells. The electrophoretic patterns of these mixtures are shown in figure 20*a*, *b*, plate 31 for the pH 10.3 and 8.9 systems respectively. Comparison of the electrophoretograms of mixtures 2 and 3 of figure 20*a* with the 6- and 7-day samples of figure 15*a*, plate 29, clearly indicates that the D/A haemoglobin ratios of the early embryo haemoglobins differ from those of mixtures of 5-day embryo and adult chicken haemoglobins. A similar conclusion can be drawn from comparison of the electrophoretograms of mixtures 2 to 4 of figure 20*b* with the 7- and 8-day samples of figure 15*d*.

TABLE 19. COMPARISON OF THE HAEMOGLOBIN D/A AND THE HAEMOGLOBIN A/(P+A′) PEAK MASS RATIOS OF HAEMOGLOBIN ISOLATED FROM EMBRYOS WITH THE RATIOS OF HAEMOGLOBIN MIXTURES (pH 8.9 ELECTROPHORESIS)†

age of embryos (days)	ratio		mixture	ratio	
	D/A	A/(P+A′)		D/A	A/(P+A′)
7	1.0	0.23	87.5% 5DE; 12.5% AC	0.52	0.26
8	0.83	0.30	75.5% 5DE: 24.5% AC	0.46	0.40
9	0.77	0.71	64.5% 5DE; 35.5% AC	0.44	0.57
10	0.73	0.90	52.6% 5DE; 47.4% AC	0.47	0.69
12	0.69	1.00	43.5% 5DE; 56.5% AC	0.43	0.81
14	0.62	1.20	33.9% 5DE; 66.1% AC	0.42	0.94
16	0.54	1.20	24.6% 5DE; 75.4% AC	0.38	1.00
adult chicken	0.35	1.00	16.1% 5DE; 83.9% AC	0.38	1.10
			7.9% 5DE; 92.1% AC	0.39	1.20
			100% AC	0.35	1.30

† Abbreviations: 5DE, haemoglobin of 5-day embryos; AC, haemoglobin of an adult chicken.

For each of these mixtures electrophoresed at pH 10.3 and 8.9 peak areas were assigned and the observed peak mass ratios calculated. The observed ratios were compared with the peak mass ratios calculated from the percentages of the several haemoglobins in each mixture. A comparison of the observed A/(P+A′) and D/A ratios of the mixtures electrophoresed at pH 8.9 and of the haemoglobins from 7- to 16-day embryos (figure 15*d*) is presented in table 19. Since the mixtures were designed to mimic the gel scans of the A and P+A′ peaks of experimental samples the errors in the estimation of the A and P+A′ peaks should be comparable in the mixtures and in the embryonic haemoglobin samples. The A/(P+A′) ratio observed for haemoglobin from 7-day embryos can be compared to that of the mixtures, and, at matching A/(P+A′) ratio values, the D/A ratio to be expected if the amount of D haemoglobin were comparable to that observed in adult chicken haemoglobin compared to the observed D/A ratio of the 7-day haemoglobin sample. When this was done, it was noted that for all the embryonic haemoglobins examined the observed D/A ratio exceeded the expected ratio. Similar results were

obtained when the A/(P+A′) and D/A ratios of embryonic haemoglobin samples electrophoresed at pH 10.3 were compared with the A/(P+A′) and D/A ratios of the mixtures electrophoresed at pH 10.3.

Several additional pieces of pertinent information can be obtained from figure 20. These electrophoretograms indicate that the lower limit of detection for haemoglobin A with the pH 10.3 electrophoresis system is about 5 % (mixture 2) and for haemoglobin D, about 2.8 % (mixture 3). Haemoglobin P would appear not to migrate as a separate band in the presence of haemoglobin A when haemoglobin P constitutes less than about 25 % of the mixture (mixture 8). The lower limit of detectability for haemoglobin E appears to be about 2.5 % (mixture 10). The lower limits of detectability for haemoglobin E and D are only applicable to gels loaded with about 12 μg of haemoglobin, which was the usual amount of haemoglobin placed on the pH 10.3 gels. With the pH 8.9 system (about 20 μg of haemoglobin per gel), the limits of detectability of haemoglobins A, D, and E were similar to those of the pH 10.3 electrophoresis system.

(v) *Absence of haemoglobin P in erythroid cell lysates from late embryos*

It was of interest to determine whether the loss of primitive erythrocytes from the embryonic circulation was correlated with the loss of the haemoglobins associated with primitive erythroid cells, haemoglobins E and P. The persistence in lysates from late embryos and young chicks of a minor haemoglobin component which migrated at or near the position of haemoglobin E raised the question whether haemoglobins E and P were also synthesized in definitive erythroid cells. Since it was not possible to determine the time of disappearance of haemoglobin P with either the pH 10.3 or pH 8.9 electrophoresis system, an antiserum to haemoglobin P which showed two precipitin lines with mixtures of haemoglobins A and P was utilized to ascertain the presence or absence of haemoglobin P in erythroid cell lysates from late embryos and young chicks.

The major electrophoretic component of erythroid cell lysates of 10-, 12-, 16-, and 18-day embryos and of 5-day chicks was isolated by preparative polyacrylamide gel electrophoresis and tested for the presence of haemoglobin P. Haemoglobin P was present in the 10- and 12-day eluate but was not detected in the isolated major haemoglobin component of 16- or 18-day embryos or of 5-day chicks. When the approximate values for the percentages of haemoglobin P in the major haemoglobin component of 10- and 12-day lysates were converted to the percentages of haemoglobin P in the lysates, the 10-day lysate contained at least 14 % haemoglobin P and the 12-day lysate contained 4 to 10 % haemoglobin P. When the amount of haemoglobin P which would have been undetected by the immunological assay was estimated for the late embryo and young chick lysates, the 16-day lysate contained less than 7 % haemoglobin P and the lysates from 18-day embryos and from 5-day chicks contained less than 2 % haemoglobin P.

(*c*) *Nature of haemoglobin H of late embryos and young chicks*

(i) *Electrophoretic and chromatographic characteristics*

The minor haemoglobin component of late embryos and young chicks (haemoglobin H), which migrated similarly to haemoglobin E in the pH 10.3 electrophoresis system, migrated slightly more slowly than haemoglobin E in the pH 8.9 electrophoresis system (figure 15*e*, *f*, plate 29).

To substantiate this observation, a mixture of 5-day embryo haemoglobin and haemoglobin from a 0.5-day chick was electrophoresed at pH 8.9 and the electrophoretic pattern was compared to the patterns of the haemoglobins of 5-day embryos, 7-day embryos, and 0.5-day chicks. As shown in figure 21, plate 31, the minor haemoglobin component of 0.5-day chicks migrated more slowly than haemoglobin E and the electrophoretogram of the mixture suggested a double band in the area of haemoglobin E and H. When the same haemoglobins were electrophoresed at pH 10.3, the minor haemoglobin component of 0.5-day chicks migrated similarly to haemoglobin E, and no double band was observed in the mixture. When mixtures of 5-day embryo haemoglobin and adult chicken haemoglobin were electrophoresed at pH 8.9, the electrophoretic mobility of haemoglobin E did not differ from that observed in 5-day embryo haemoglobin. Thus the presence of haemoglobins A and D did not alter the electrophoretic characteristics of haemoglobin E.

Haemoglobin E of 5-day embryos and the haemoglobin H of 18-day embryos and 0.5-day chicks were isolated by preparative polyacrylamide gel electrophoresis and the electrophoretic mobilities of the isolated components compared on pH 8.9 gels. The isolated haemoglobin H migrated more slowly than haemoglobin E.

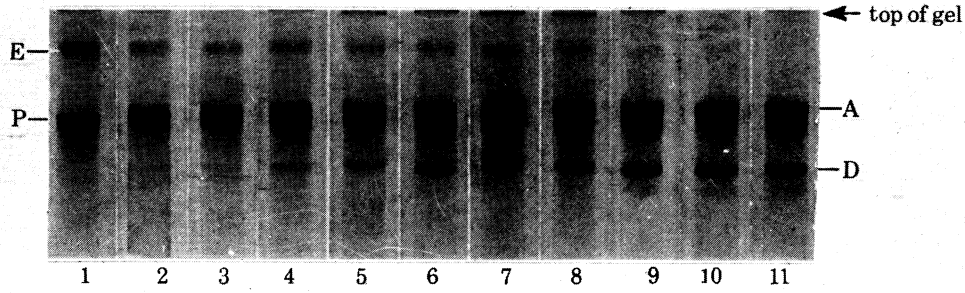
The slow mobility of haemoglobin E of 5-day embryos and of haemoglobin H suggested that these components might be higher molecular mass haemoglobin aggregates. When the haemoglobin of 5-day chicks was chromatographed on Sephadex G-100 the haemoglobin was eluted as one symmetrical peak and no haem-containing protein was eluted in the void volume. However, when fractions of the column eluate containing the eluted haemoglobin were electrophoresed and the percentage of haemoglobin H determined, fractions from the leading edge of the peak had a considerably higher percentage of haemoglobin H than fractions from the trailing edge of the peak (figure 25). Some fractions from the trailing edge of the peak contained no detectable haemoglobin H. The haemoglobin D/A peak mass ratio was essentially constant throughout the peak.

Description of plate 31

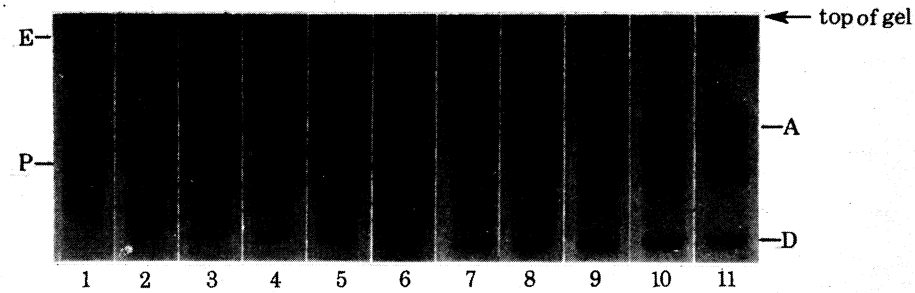
Figure 20. Haemoglobin electrophoretograms of mixtures of haemoglobin from 5-day embryos and an adult chicken (amido black stain). (*a*) Electrophoresis of the mixtures at pH 10.3. The number below each gel refers to the mixture number: (1) 100 % 5-day embryo haemoglobin; (2) 91.7 % 5-day embryo haemoglobin plus 8.3 % adult haemoglobin; (3) 83 % 5-day embryo haemoglobin plus 17 % adult haemoglobin; (4) 74.1 % 5-day embryo haemoglobin plus 25.9 % adult haemoglobin; (5) 64.6 % 5-day embryo haemoglobin plus 35.4 % adult haemoglobin; (6) 55 % 5-day embryo haemoglobin plus 45 % adult haemoglobin; (7) 44.9 % 5-day embryo haemoglobin plus 55.1 % adult haemoglobin; (8) 34.4 % 5-day embryo haemoglobin plus 65.6 % adult haemoglobin; (9) 23.2 % 5-day embryo haemoglobin plus 76.8 % adult haemoglobin; (10) 11.8 % 5-day embryo haemoglobin plus 88.2 % adult haemoglobin; (11) 100 % adult chicken haemoglobin. (*b*) Electrophoresis of the mixtures at pH 8.9. The number below each gel refers to the mixture number; (1) 100 % 5-day embryo haemoglobin; (2) 87.5 % 5-day embryo haemoglobin plus 12.5 % adult haemoglobin; (3) 75.5 % 5-day embryo haemoglobin plus 24.5 % adult haemoglobin; (4) 64.5 % 5-day embryo haemoglobin plus 35.5 % adult haemoglobin; (5) 52.6 % 5-day embryo haemoglobin plus 47.4 % adult haemoglobin; (6) 43.5 % 5-day embryo haemoglobin plus 56.5 % adult haemoglobin; (7) 33.9 % 5-day embryo haemoglobin plus 66.1 % adult haemoglobin; (8) 24.6 % 5-day embryo haemoglobin plus 75.4 % adult haemoglobin; (9) 16.1 % 5-day embryo haemoglobin plus 83.9 % adult haemoglobin; (10) 7.9 % 5-day embryo haemoglobin plus 92.1 % adult haemoglobin; (11) 100 % adult chicken haemoglobin.

Figure 21. Demonstration of the electrophoretic difference between haemoglobin E of 5-day embryos and haemoglobin H of late embryos and young chicks. Haemoglobins were electrophoresed on pH 8.9 polyacrylamide gels. Gels were stained with amido black: (1) Haemoglobin of 5-day embryos; (2) mixture of haemoglobin of 5-day embryos and a 0.5-day chick; (3) haemoglobin of a 0.5-day chick.

20 (*a*)



(*b*)



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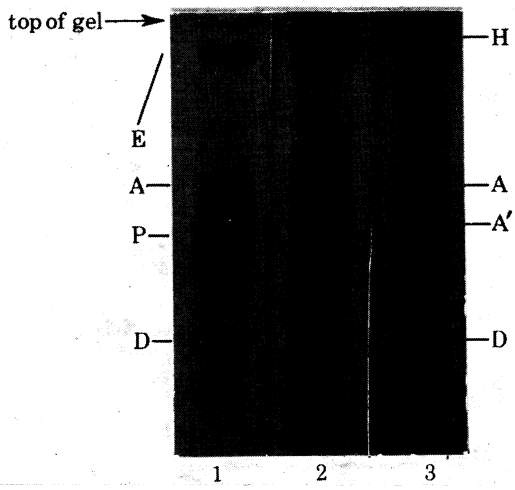

FIGURES 20 AND 21. For legends see facing page.

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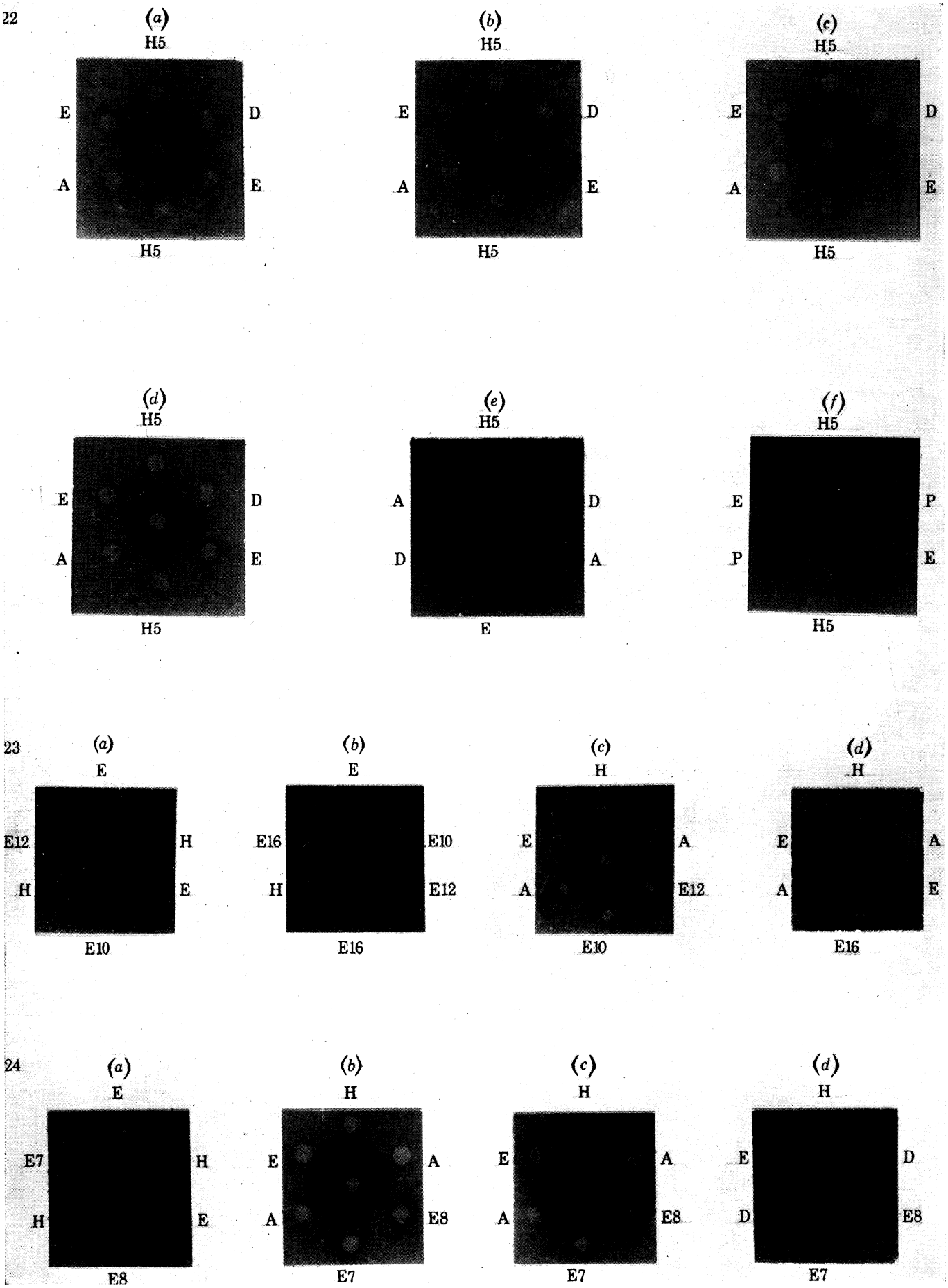

FIGURES 22 TO 24. For legends see facing page.

When haemoglobin of 5-day embryos was chromatographed on Sephadex G-100 the haemoglobin peak was not symmetrical, but had a significant trailing edge. Electorphoresis of fractions of the column eluate indicated that little or no haemoglobin E was observed in fractions from the leading edge of the haemoglobin peak. Haemoglobin E was found in fractions of the trailing edge of the peak (figure 26). The latter fractions also contained the artifactual electrophoretic components observed when haemoglobins of 5-day embryos were frozen at -20 °C or stored in ice in dilute solution. Addition of adult haemoglobin to the 5-day embryo haemoglobin prior to chromatography on Sephadex G-100 did not alter the elution characteristics of haemoglobin E of 5-day embryos. The behaviour of haemoglobin H suggests that this component is a higher molecular mass aggregate of haemoglobin molecules with a high dissociation constant under the conditions of Sephadex G-100 chromatography (Guidotti & Craig 1963; Andrews 1964; Nichol, Bethune, Kegeles & Hess 1964).

Hedrick & Smith (1968) described a method for determining whether proteins are charge and/or size isomers based on the electrophoretic mobilities of the proteins in polyacrylamide gels of different porosities. Haemoglobin of 5-day embryos and of 0.5-day chicks was electrophoresed at pH 8.9 on gels containing 4 to 9.5 % acrylamide (the acrylamide-bisacrylamide ratio remained constant) and the mobilities of the E and P components of 5-day embryo

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Description of plate 32

Figure 22. Comparison by two-dimensional immunodiffusion of the reactviity of haemoglobin H of a 5-day chick with the reactivities of haemoglobins E, P, A and D with rabbit antiserum to isolated haemoglobin components. Plates were stained with amido black. (*a*) Reactivity with antiserum to haemoglobin E of 5-day embryos. The letters E, A and D denote the respective haemoglobins. H5 indicates haemoglobin H of a 5-day chick. 2 μg haemoglobin/well. (*b*) Reactivity with antiserum to haemoglobin A of an adult chicken. 2 μg haemoglobin/well. (*c*) Reactivity with antiserum (D_1) to haemoglobin D of an adult chicken. 2 μg haemoglobin/well. (*d*) Reactivity with a second antiserum (D_2) to haemoglobin D of an adult chicken. 2 μg haemoglobin/well. (*e*) Reactivty with the same antiserum as in (*d*). The haemoglobins were placed in the wells such that the reactivity of the antiserum with haemoglobins A and D and with haemoglobins H, A and D could be observed on the same plate. 1 μg haemoglobin/well. (*f*) Reactivity with an antiserum to haemoglobin A of an adult chicken which does not cross react with haemoglobin P. 2 μg haemoglobin/well.

Figure 23. Immunological assay for haemoglobin H in lysates of circulating erythroid cells from 10- to 16-day embryos by two-dimensional immunodiffusion. Two micrograms of haemoglobin were placed in the peripheral wells. Plates were stained with amido black. (*a*) Comparison of the reactivity, with an antiserum (D_2) to haemoglobin D, of the minor haemoglobin component of 10-day (E10) and 12-day embryos (E12) with the reactivity of isolated haemoglobins E and H. (*b*) Comparison of the reactivity, with an antiserum (D_2) to haemoglobin D of the minor haemoglobin component of 10-day (E10), 12-day (E12), and 16-day embryos (E16) with the reactivity of isolated haemoglobins E and H. (*c*) Comparison of the reactivity, with a second antiserum (D_1) to haemoglobin D, of the minor haemoglobin component of 10-day (E10) and 12-day embryos (E12) with the reactivities of haemoglobins E, H and A. (*d*) Comparison of the reactivity, with a second antiserum (D_1) to haemoglobin D, of the minor haemoglobin component of 16-day embryos (E 16) with the reactivities of haemoglobins E, H and A.

Figure 24. Immunological assay for the presence of haemoglobin H in lysates of circulating erythroid cells from 7- and 8-day embryos by two-dimensional immunodiffusion. Two micrograms of haemoglobin were placed in peripheral wells. Plates were stained with amido black. (*a*) Comparison of the reactivity, with an antiserum (D_1) to haemoglobin D, of the isolated E component of 7-day (E7) and 8-day embryos (E8) with the reactivities of haemoglobin E from 5-day embryos and haemoglobin H. (*b*) Comparison of the reactivity, with an antiserum (D_1) to haemoglobin D, of the isolated E component of 7-day (E7) and 8-day embryos (E8) with the reactivities of isolated haemoglobins E, H and A. (*c*) Comparison of the reactivity, with an antiserum to haemoglobin A, of the isolated E component of 7-day (E7) and 8-day embryos (E8) with the reactivities of isolated haemoglobins E, H and A. (*d*) Comparison of the reactivity, with a second antiserum (D_2) to haemoglobin D, of the isolated E component of 7-day (E7) and 8-day embryos (E8) with the reactivities of isolated haemoglobins E, H and D.

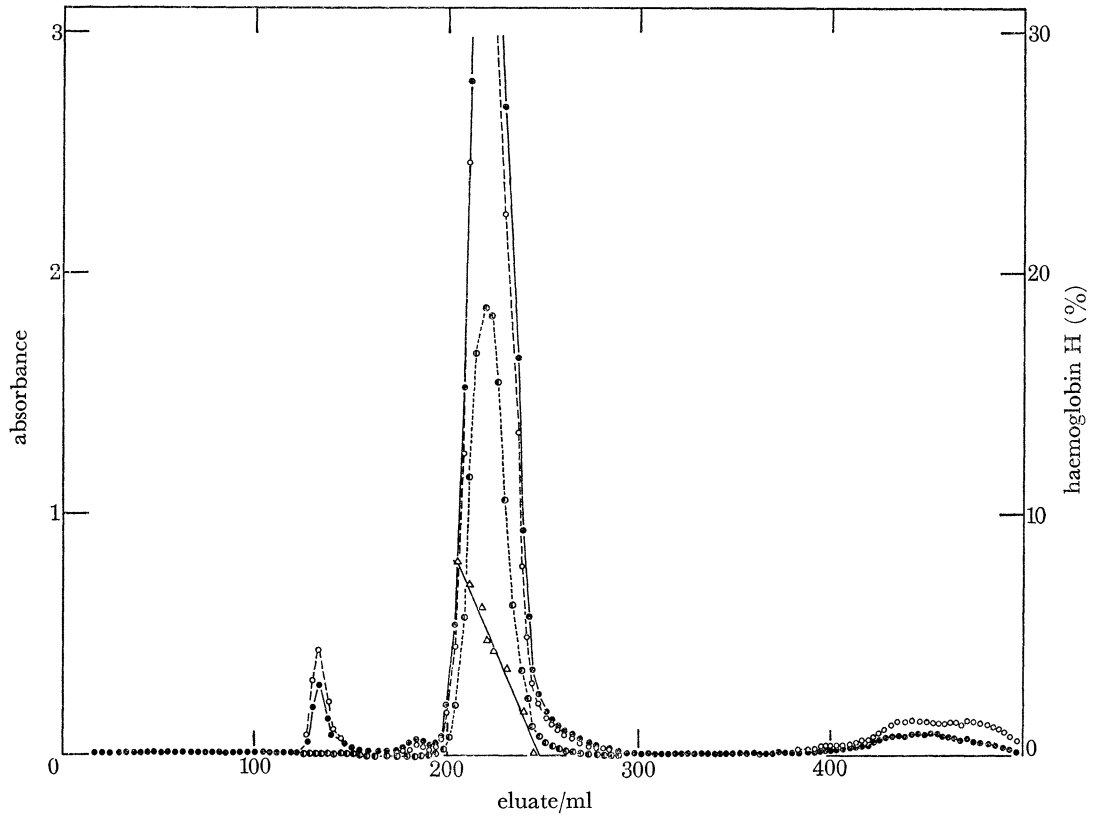

FIGURE 25. Gel filtration of haemoglobin of a 5-day old chick on Sephadex G-100. Buffer: 0.1 M tris, pH 8.3, 0.1 M NaCl. Column: 2.5 cm × 95 cm. V_0: 135 ml. V_I: 399 ml. Haemoglobin recovery: 98.5 %. ◐, Absorbance, 540 nm; ●, absorbance, 280 nm; ○, absorbance, 260 nm; △, haemoglobin H (%).

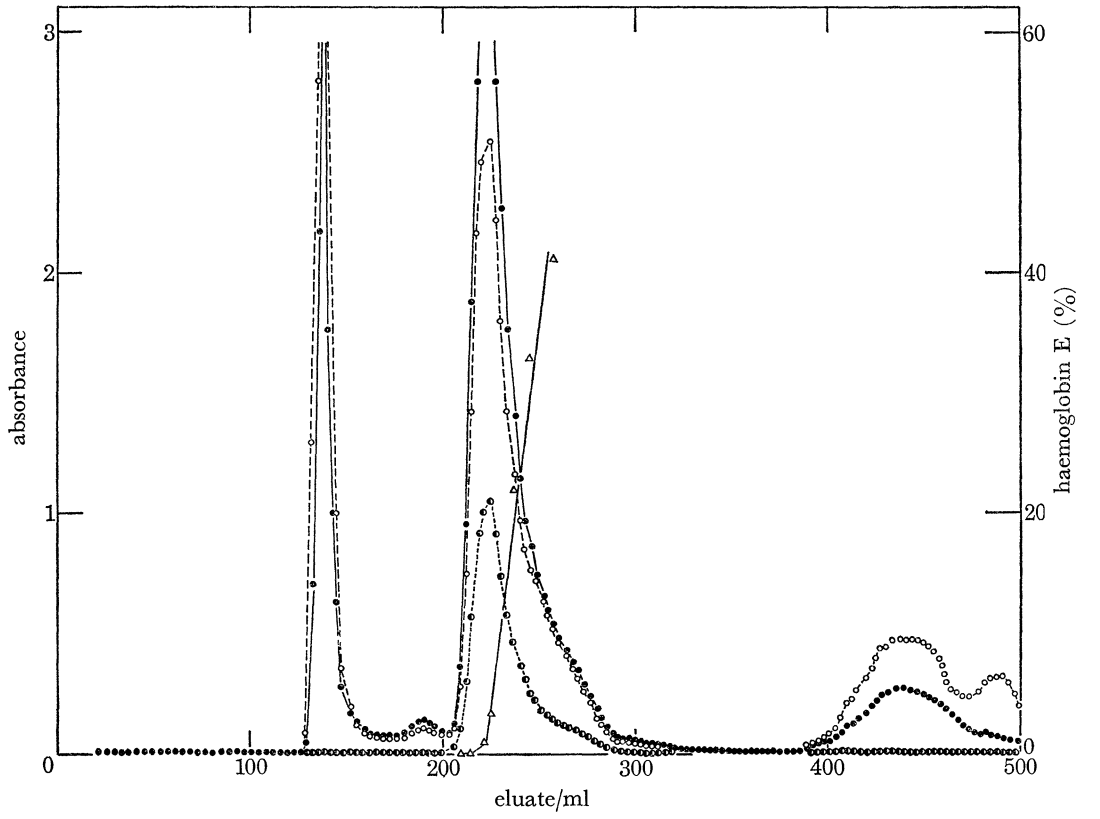

FIGURE 26. Gel filtration of haemoglobin of 5-day embryos on Sephadex G-100. Buffer: 0.1 M tris, pH 8.3, 0.1 M NaCl. Column: 2.5 cm × 95 cm. V_0: 135 ml. V_I: 399 ml. Haemoglobin recovery: 93.8 %. ◐, Absorbance, 540 nm; ●, absorbance, 280 nm; ○, absorbance, 260 nm; △, haemoglobin E (%).

haemoglobin and of the A, D and H components of 0.5-day chick haemoglobin were compared to the mobility of the tracking dye, bromphenol blue. The logarithm of the relative mobility of each haemoglobin component was plotted versus the percentage of acrylamide in the gel (figure 27). Proteins which are charge, but not size, isomers will show parallel lines on such plots; whereas proteins which are size, but not charge, isomers will show non-parallel lines which extrapolate to a common point at 1 to 2 % acrylamide. Proteins which are both charge and size isomers will show non-parallel lines which intersect at acrylamide concentrations greater than 1 to 2 % (Hedrick & Smith 1968). The results shown in figure 27 indicate that the haemoglobins A, D, P and E are charge isomers, while haemoglobin H would appear to be both a charge and size isomer of the other haemoglobin components.

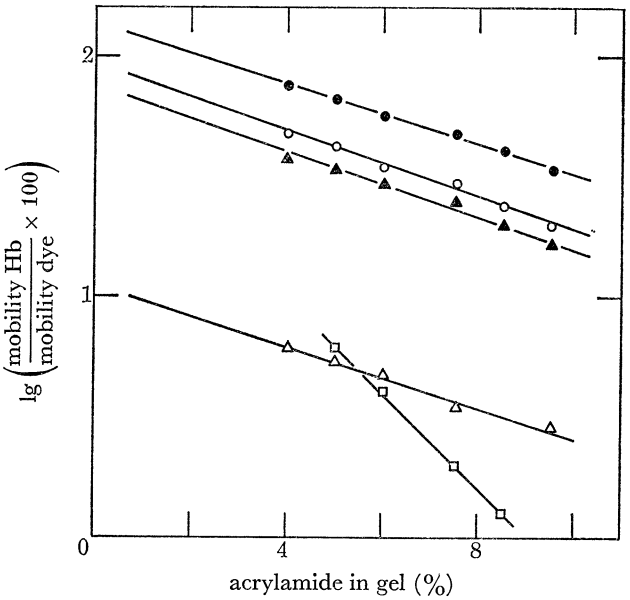

FIGURE 27. Plot of the logarithm of the mobility of haemoglobins A, P, D, E and H relative to the tracking dye against the percentage of acrylamide in the gels (pH 8.9 electrophoresis, 2 °C). The acrylamide:bisacrylamide ratio was 30:0.8. ●, Hb A; ○, Hb P; △, Hb D; ▲, Hb E; □, Hb H.

(ii) *Immunological characteristics of haemoglobin H*

To obtain further information on the similarities and differences of haemoglobins E and H, the immunological reactivity of haemoglobin H, isolated by preparative polyacrylamide gel electrophoresis from 5-day chicks, was compared, by use of antisera to haemoglobin E, P, A or D, to the reactivity of haemoglobin E of 5-day embryos. The immunological reactivities of haemoglobin H were also compared to those of isolated haemoglobins A, D and P.

Haemoglobin H of 5-day chicks showed a reaction of identity with haemoglobin E of 5-day embryos with an antiserum to haemoglobin E (figure 22*a*, plate 32). However, with this antiserum, haemoglobin A also shows a reaction of identity with haemoglobin E of 5-day embryos and with haemoglobin H of 5-day chicks (figure 22*a*). With one antiserum to haemoglobin A, haemoglobin E, haemoglobin H and haemoglobin A showed reactions of identity (figure 22*b*). However, with an antiserum (D_1) to haemoglobin D, haemoglobin E of 5-day embryos and haemoglobin H of 5-day chicks showed a reaction of only partial identity (figure 22*c*). With a second antiserum (D_2) to haemoglobin D, haemoglobin E of 5-day embryos reacted weakly whereas haemoglobin H reacted strongly (figure 22*d*).

With all four antisera haemoglobin H reacted differently from haemoglobin D. Haemoglobin H also reacted differently from haemoglobin P (figure 22*f*). When the haemoglobin samples were placed in the wells of the Ouchterlony plates such that the reactivities of haemoglobins A, D and H with antiserum D_2 could be compared on the same plate, haemoglobin A and haemoglobin H showed a reaction of identity, while haemoglobin D and haemoglobin H showed a reaction of only partial identity (figure 22*e*).

Identical results were obtained when the reactivity of haemoglobin H from 18-day embryos was compared to the reactivities of haemoglobins E, P, A and D.

Although haemoglobin H shares some antigenic determinants with haemoglobin E, these two haemoglobins are immunologically not identical. Haemoglobin H is not identical to either haemoglobin P or haemoglobin D. Although haemoglobin H showed reactions of apparent identity with haemoglobin A with all antisera tested, the reactivity of haemoglobin H with antiserum D_2 would suggest that haemoglobin A and haemoglobin H are not identical.

Because of the similarities of the immunological reactivities of haemoglobin H and haemoglobin A, it was considered possible that haeomoglobin H was contaminated with haeomoglobin A. However, when haemoglobin H was electrophoresed on pH 10.3 polyacrylamide gels, haemoglobin A was not detected.

In summary, haemoglobin H and haemoglobin E differ in electrophoretic mobility on 7.5 % polyacrylamide gels at pH 8.9 and on polyacrylamide gels of different porosities, in elution behaviour on Sephadex G-100, and in immunological reactivity with a number of antisera.

(iii) *Time of appearance of haemoglobin H*

Since haemoglobin H is observed in lysates from 18-day embryos and young chicks, where the percentage of primitive cells among the circulating erythrocytes ranges from 0.4 to 0 %, it would appear that haemoglobin H is produced by definitive cells of the chick embryo. To determine when haemoglobin H first appeared during development of the chick embryo, lysates from 5- to 16-day embryos were electrophoresed at pH 8.9.

Haemoglobin H was detectable electrophoretically in lysates from 12-, 14-, and 16-day embryos, but not in lysates from embryos of less than 12 days of age.

To confirm the presence of haemoglobin H in lysates from 12- to 16-day embryos, haemoglobin of 10-, 12- and 16-day embryos was electrophoresed on preparative polyacrylamide gels and the component migrating in the area of haemoglobins E and H eluted from the gels and compared immunologically with haemoglobins E, H, P, D and A. Haemoglobin H could be detected in the eluate of this band from 10-day embryos, although haemoglobin H was not detectable electrophoretically (figure 23*b*, plate 32). Haemoglobin H was also demonstrated in the lysates from 12- and 16-day embryos.

Lysates from 7- and 8-day embryos were also electrophoresed on preparative polyacrylamide gels, the E band eluted from the gels and the eluate tested for the presence of haemoglobin H. Haemoglobin H was easily demonstrable in lysates from 8-day embryos (figure 24*b*, plate 32). With antiserum D_1 the spur between haemoglobin H and the E component of 7-day embryos was less prominent than the spur between haemoglobin E of 5-day embryos and haemoglobin H (figure 24*a*). Similarly, the spur between the haemoglobin E component of 7-day embryos and haemoglobin A was less prominent than the spur between haemoglobin E of 5-day embryos and haemoglobin A with an antiserum to haemoglobin A (figure 24*c*). These observations suggested that haemoglobin H was present in the lysate from 7-day embryos.

However, the spur between haemoglobin A and the haemoglobin E component of 7-day lysates was quite pronounced with antiserum D_1 (figure 24*b*). With this antiserum a prominent spur between haemoglobin E of 5-day embryos and haemoglobin A was consistently observed. A mixture of 80 % haemoglobin E and 20 % haemoglobin H showed a small spur with haemoglobin A with antiserum D_1, while a mixture of 40 % haemoglobin H and 60 % haemoglobin E showed no spur with haemoglobin A. The E band of 7-day lysates contained, therefore, less than 40 % haemoglobin H and probably less than 20 % haemoglobin H.

These experiments indicate that haemoglobin H is produced by definitive erythroid cells and appears in lysates of circulating erythroid cells of the embryo when haemoglobinized definitive cells enter the circulation.

5. Discussion

(*a*) *Erythrocytes of the chick embryo*

(i) *Maturation of primitive erythroid cells*

The changes in cell morphology associated with maturation of the primitive erythroid cells from the very immature cells observed at 1.5–2 days to the mature primitive cells observed at 8 days are comparable to the maturation changes seen in other erythroid systems; and include losss of cytoplasmic basophilia, progressive haemoglobinization, a decrease in the nuclear/cytoplasm ratio, loss of nucleoli, progressive condensation of nuclear chromatin and nuclear pycnosis. Similar changes during maturation of primitive cells have been reported by Dawson (1936), Fennel (1947), and Lucas & Jamroz (1961). The morphological characteristics of maturing primitive cells, observed by light microscopy in fixed and stained preparations, have been correlated with the changes in cell ultrastructure by Edmonds (1966), Ceresa-Castellani & Leone (1969) and Small (1969).

Primitive cell erythropoiesis in the chick embryo differs strikingly from definitive cell erythropoiesis of the chick embryo, the mouse, the rabbit, and the human. In contrast to the latter erythroid systems, where several different maturation stages of erythroid precursors are simultaneously observed in erythropoietic tissues or in peripheral blood, all of the primitive erythroid cells which can be isolated from the embryonic circulation or from mechanically dissociated vascular tissue of the chick embryo at any particular time point, appear to be at the same stage of maturation as judged by morphological criteria. The primitive cells appear to mature as a cohort of cells and, hence, form a relatively uniform population of erythroid cells which undergo the changes in cell structure characteristic of erythroid cell maturation. The primitive erythroid series would therefore appear to be an ideal system for further investigation of the biochemical processes occurring during erythroid cell maturation. The cohort-like nature of primitive cell erythropoiesis has also been recently noted by Small (1969), Weintraub *et al.* (1971) and Campbell *et al.* (1971) and appears to be similar to the maturation of foetal mouse yolk sac erythroid cells (Fantoni *et al.* 1969; Bank *et al.* 1970).

However, when primitive cells were viewed in fixed and stained preparations, size variation was observed at all time points until 8 to 10 days, although at any particular time point, the large cells were not less mature and the small cells more mature than the medium-size cells. Similar size variation among primitive cells was noted by Fennel (1947) and by Lucas & Jamroz (1961). The cell diameter measurements reported by Fennel (1947) on stained smears of primitive cells are quite similar to the cell diameters observed on slides prepared with the cytocentri-

fuge. Variation in cell size of primitive cells from embryos of 2.3 to 4 days of age was confirmed by determination of the distributions of cell diameters measured in wet mount preparations. The cell diameter and cell volume distributions could not be determined on primitive cells from embryos greater than 4 days of age as the cells maintained an ovaloid shape in the wet mounts. Primitive cells from 2.3 to 4 days behaved as spheres under the experimental conditions. The appearance of ovaloid primitive cells does not appear to correlate with the appearance of microtubules in the marginal band in the erythroid cells (Small 1969), but may be related to the change in structure of the marginal band in maturing primitive cells described by Small (1969). The mean diameter and diameter distributions of primitive cells measured in wet mount preparations does not change significantly between 2.3 and 4 days, although the cell diameters measured in fixed preparations do decrease during this period. These observations suggest that a change in cell deformability occurs as the cells mature. Because cell diameters could not be accurately measured on more mature primitive cells the contribution of a decrease in cell deformability to the decrease in cell diameter and in the variability of cell size in primitive cells from 4- to 8-day embryos cannot be assessed. The decrease in cell size variation of the primitive cells may reflect the cessation of division among these cells as they mature.

The variation in cell diameter and volume of a population of cells, which, by morphological criteria, appear otherwise comparable raises the question of the significance of the size variation. The most simple model would hold that the small cells are G_1 cells, the medium-size cells are distributed throughout S phase, and the large cells are G_2 cells. It might then be proposed that the large cells are G_2 parent cells of the small cells. A specific prediction of such a model would be that mitotic figures should only be observed in the large cells. It would also be predicted that, among erythroid cells isolated at short time intervals following administration of tritiated thymidine to embryos, the labelling indices and the average grain counts of labelled cells of both the large and small size classes would be less than that of the medium size cells.

However, the frequency distributions of the cell diameters determined on wet mount preparations and of the calculated cell volumes are not compatable with such a simple model (Stanners & Till 1960; Weintraub *et al.* 1971). In addition, assuming that the cell volume decreases by a factor of two at division, the volumes of the large cells are too great for these cells to be the parent cells of the small cells. The progeny of the large cells should be medium size cells on the basis of cell volume. The volumes of the medium-size cells are sufficient to produce daughter cells with the volumes observed for the small cells. Measurement of the diameters of late telophase daughter cells indicated that while many such cells had diameters comparable to the small cells, some had diameters comparable to medium size cells. Fennel (1947), studying mitoses of primitive cells in wet mount preparations, also observed that medium-size daughter cells arose from large cells while small daughter cells arose from medium-size cells.

Mitoses are uncommon among the small cells. However, about 50 % of the observed mitotic figures of primitive cells from 2- to 4-day embryos were in medium-size cells. Although the large cells constituted less than 20 % of the primitive cells, about 50 % of the observed mitoses were in large cells.

When embryos, 2 to 4 days of age, were treated *in ovo* with tritiated thymidine and erythroid cells isolated at 1 to 6 h after administration of the isotope, the labelling indices and the average grain counts of small primitive cells were less than the values of these parameters observed in medium size primitive cells (Bruns 1971). The frequency distribution histograms of grains per labelled cell and the average grain counts of labelled cells were comparable for the medium

and large primitive cells, although the labelling indices of large primitive cells were occasionally somewhat less than those of the medium-size cells (Bruns 1971).

It would appear, therefore, that the available data on the interrelationships of the several size classes of primitive erythroid cells do not support the simple model. A hypothesis compatable with the data would hold that the small cells are G_1 and early S phase progeny of medium-size G_2 cells. The medium-size cells would include G_1 and early S phase progeny of the large G_2 cells plus S phase and G_2 parent cells for the small cells. The large cells would include S phase and G_2 parent cells for the medium-size cells.

(ii) *Expansion of the primitive cell compartment*

Between day 1.5 and day 5, when primitive erythroid cells constitute the only detectable erythroid elements of the developing chick embryo, a significant expansion of the embryonic circulation occurs. When the number of primitive cells of the embryos was estimated from the total haemoglobin of the embryos and the average haemoglobin content of primitive erythroid cells, there appeared to be about a 300 % increase in the number of cells between day 4 and day 5, and an additional 50 % increase between day 5 and day 6. When the expansion of the primitive cell pool of the embryos was assessed, using the number of primitive cells obtained from mechanically dissociated blastoderms, there appeared to be about a 650 % increase in the number of primitive cells between day 2 and day 3, a 350 % increase between day 3 and day 4, a 300 % increase between day 4 and day 5 and an additional 60 % increase between day 5 and day 6. Primitive cells isolated from mechanically dissociated vascular tissue of embryos were not less mature than primitive cells obtained from the embryonic circulation of embryos of comparable age. The mitotic index of primitive cells isolated from mechanically dissociated vascular tissue of embryos was also comparable to that of circulating primitive erythroid cells. The rate of increase in the number of primitive cells of embryos observed in the present investigation was quite similar to that determined by Lemez (1964) and by Weintraub *et al.* (1971). A similar rate of increase in the number of primitive cells per blastoderm has been observed by Hagopian & Ingram (1971) in de-embryonated, cultured blastoderms.

The question has arisen whether the increase in the number of primitive cells per embryo between any two time points can be accounted for solely by division of the primitive cells existing at the first time point. Lemez (1964) concluded that the rate of increase in the number of circulating primitive cells per embryo could not be accounted for by division of circulating primitive cells and that formation of new primitive cells and migration of such cells into the embryonic circulation was required. Hagopian & Ingram (1971) have also proposed, from data obtained on cultured de-embryonated blastoderms, that additional mesenchymal elements are recruited into the primitive cell pool. The kinetics of labelling of primitive cells from embryos of 2–5 days of age with tritiated thymidine *in ovo* (Bruns 1971) are most easily explained by recruitment of additional mesenchymal elements into the primitive cell pool until at least 4.5 days.

Weintraub *et al.* (1971) and Campbell *et al.* (1971) have proposed a model for primitive erythropoiesis in which primitive cells undergo six divisions, beginning at 35 h of incubation, the first three of which have a cycle time of 9.6 h, the next two have a cycle time of 17 h, and the final division has a cycle time of 29 h. In this model primitive cells can arise from primitive stem cells and enter the compartment of maturing primitive cells until day 2.5. Therefore on day 3, for example, the primitive cell population would include cells in the fourth and fifth

division cycles as well as cells in the second and third division cycles. Those cells in the second and third division cycles on day 3 would be at the same maturation stage as those cells of 2.3- to 2.75-day embryos which had begun the maturation sequence at 35 h. This model was considered to be compatible with the observed rate of increase in the number of primitive cells per embryo (Weintraub *et al.* 1971). However, it is difficult to explain a fourfold increase in the number of primitive cells per embryo between day 3 and day 4 when the predominant cell cycle time at day 3 is 17 h and the mitotic index was reported to be 2.7 %. This model would predict that erythroid cell suspensions from 3-day embryos should contain cells equivalent to primitive cells of 2.3-day embryos and that erythroid cell suspensions from 2.5-day embryos should contain cells equivalent to primitive cells of 1.5-day embryos. It is unlikely that such a range of cell maturation stages would have been overlooked in the present study.

(iii) *Lifespan of primitive erythrocytes*

Primitive erythrocytes were only rarely found in the embryonic circulation after day 16. Among approximately 20000 erythroid cells examined from 18 young chicks less than 21 days of age, only one primitive erythrocyte was observed. These observations are similar to those of Sugiyama (1926), Fennel (1947), Lemez (1964), Lucas & Jamroz (1961) and Small (1969). Dawson (1936), however, reported that primitive cells persist as long as 2 weeks following hatching. To determine whether the loss of primitive cells reflected solely a dilution of these cells as the embryonic circulation expanded, an estimate of the number of primitive cells per embryo was obtained for embryos of 3.5 to 18 days of age. The estimated number of primitive cells per embryo increased from 9.4×10^6 at 3.5 days to 120×10^6 at 8 days and subsequently declined to 10×10^6 at day 16. These observations would indicate that primitive erythrocytes have a finite lifespan shorter than the gestational period of the embryo. Similar values for the number of primitive cells per embryo have been reported by Lemez & Rychter (1956) and Lemez & Josifko (1957). These authors estimated the number of primitive cells from the blood volumes of the embryo, the number of erythroid cells/ml of blood and the percentage of primitive cells among the circulating erythroid cells of the embryos. Weintraub *et al.* (1971) have reported comparable numbers for embryos of 2 to 5 days of age.

The maximum lifespan of primitive erythroid cells would encompass the time between the initial formation of immature primitive cells at 1.5 days until hatching, about 19.5 days, and the average lifespan about 8.5 days. If primitive cells are considered to be mature erythrocytes at 8 to 9 days, the maximum lifespan of mature primitive erythrocytes would be 12 to 13 days and the average lifespan of mature primitive cells, about 6 days. Similar estimates of the lifespan of primitive cells were reported by Lemez (1964).

(iv) *Comparison of definitive and primitive erythropoiesis*

The morphological characteristics of the several maturation stages of the definitive erythroid cells described in this study and by Dawson (1936), Fennel (1947), Lemez & Rychter (1956), Lucas & Jamroz (1961) and Small (1969) are quite similar to the morphological characteristics of comparable maturation stages of the definitive erythroid cells of the mouse (Orlic *et al.* 1965; Russell & Bernstein 1966; Rifkind *et al.* 1969*b*), the rabbit (Grasso *et al.* 1963; Borsook 1966), and the human (Wintrobe 1956; Pease 1956; Zamboni 1965).

Although the changes in cell morphology associated with the maturation of immature definitive cells are similar to those of the maturing primitive erythroid cells and include loss of

cytoplasmic basophilia, a decrease in the nuclear/cytoplasm ratio, progressive clumping of nuclear chromatin, and nuclear pycnosis, the maturation stages of the definitive cells are quite distinct, and the changes from one maturation stage to the next are marked. During the maturation of the primitive cells, although the same changes in cell structure are occurring, the several maturation stages of primitive cells are not clearly demarcated, i.e., the early polychromatophilic erythroblast is not distinctly different from the mid-polychromatophilic erythroblast. The nature of this difference between the maturation of primitive and definitive erythroid cells remains unexplained.

In contrast to primitive erythropoiesis, with the initiation of definitive erythropoiesis at 5 days, a number of maturation stages of the definitive erythroid cells are simultaneously present in the embryonic circulation. The changing peripheral blood picture of embryos between day 5 and days 16 to 18 when the definitive erythroid cells are replacing the primitive cells is striking. At 5 days, the first recognizable definitive erythroid cells found in the embryonic circulation are proerythroblasts, basophilic erythroblasts, and early polychromatophilic erythroblasts. These cells constitute 1 to 4 % of the circulating erythroid elements. At 6 days, where, in a typical experiment, the percentage of definitive cells in the circulation has increased about sevenfold, approximately 80 % of the definitive cells are early and mid-polychromatophilic erythroblasts. At 7 days when the definitive cells constitute about 50 % of the circulating cells, about 80 % of the definitive cells are mid and late polychromatophilic erythroblasts. At 8 to 10 days definitive cells of all maturation stages from basophilic erythroblasts to highly haemoglobinized erythrocytes are observed, although cells less mature than mid-polychromatophilic erythroblasts constitute less than 10 % of the definitive cells. On each successive day, the percentage of immature definitive cells in the embryonic circulation decreases such that by day 16, when definitive cells constitute essentially 100 % of the circulating erythroid cells, only about 10 % of the definitive cells are late polychromatophilic erythroblasts and more immature cells constitute less than one percent of the cells. These observations are in agreement with those of Fennel (1947), Sandreuter (1951), Lemez & Rychter (1956), and Lucas & Jamroz (1961).

The primitive cells of the embryos are therefore replaced by progressively more mature definitive cells. To appreciate the full significance of this observation, cognizance must be taken of the expansion of the embryonic circulation which occurs simultaneously with the replacement of the primitive erythrocytes by definitive erythroid cells. Between day 5 and day 16 the total haemoglobin of the embryos increases 50-fold (Schønheyder 1938; Ramsay 1950; Bruns 1971). When the expansion of the embryonic circulation is related to the estimated number of definitive cells per embryo, there would appear to be as much as a 2000- to 8000-fold increase in the number of definitive cells per embryo between day 5 and day 16, and as much as a 400-fold increase between day 6 and day 16. When the increase in the estimated number of definitive cells per embryo is calculated for a 24 h period, the increase is also striking. There can be as much as a 20-fold increase between day 5 and day 6, a sevenfold increase between day 6 and day 7, a threefold increase between day 7 and day 8, and a twofold increase over each subsequent 24 h period until day 16. A similar rate of increase in the number of definitive cells per embryo has been reported by Rychter *et al.* (1955) and by Lemez (1964).

Since the definitive erythroid cells of the developing chick embryo are a self-perpetuating cell line, definitive stem cells and immature definitive erythroid precursors capable of producing the large numbers of additional definitive cells needed as the embryonic circulation expands must be available beginning at the time of initiation of definitive erythropoiesis at 5 days of

incubation. Mitoses are infrequently observed among the immature definitive cells isolated from the embryonic circulation or from mechanically dissociated blastoderms except at 6 days (see also Dawson (1936), and Lemez (1964)). It would appear that as the embryo matures, definitive cells are retained in the haematopoietic foci until the late polychromatophilic erythroblast and reticulocyte stages of maturation (Dawson 1936; Lucas & Jamroz 1961). Although erythropoeisis begins in the bone marrow of the chick embryo at 10 to 12 days (Dantschakoff 1909*b*; Dawson 1936), the yolk sac remains the major erythropoietic site until 18 to 20 days (Dantschakoff 1908). Neither the liver nor the spleen of the chick embryo are significant erythropoietic tissues (Stricht 1891; Haff 1914; Danchakoff 1916).

Proliferation of haemocytoblasts and formation of erythropoietic foci in the ventral wall of the aorta of 4- to 7-day embryos has been described (Dantschakoff 1908, 1909*b*). If these foci produced definitive cells, the very immature definitive erythroid cells observed in the embryonic circulation between days 5 and 7 might arise from such non-sequestered foci. The foci of definitive erythropoiesis observed by Small (1969) in the capillaries of entodermal folds extending into the yolk sac in 7-day embryos might represent sequestered foci comparable to the bone marrow of older embryos and adult chickens.

(v) *Relationship of definitive erythrocytes with round nuclei to definitive erythrocytes with oval nuclei*

The first highly haemoglobinized definitive cells which appear to be mature definitive erythrocytes in May Greenwald, giemsa-stained preparations are found in the embryonic circulation at 8 to 9 days. However, such cells are late reticulocytes. The first mature definitive erythrocytes, which appear in the embryonic circulation on day 10, are round cells with round condensed nuclei. At approximately the same time, oval mid and late polychromatophilic erythroblasts and mature oval cells with round nuclei are first observed. Mature round or oval definitive erythrocytes with round condensed nuclei increase in frequency until day 18 to 19. The first mature definitive erythrocytes with partially condensed oval nuclei appear in the embryonic circulation at 15 to 16 days, and then rapidly replace the mature cells with round nuclei over a 1- or 2-day period between 19 and 21 days.

The appearance of mature definitive erythrocytes with round nuclei prior to the appearance of mature definitive erythrocytes with oval nuclei during embryogenesis of the chick was previously noted by Dantschakoff (1908), Fennel (1947), Lemez (1964), and Lucas & Jamroz (1961). Lemez (1964) demonstrated that the highly haemoglobinized cells with round nuclei were not reticulocytes, and proposed that such cells represented mature cells of the first generation of embryonic definitive cells, whereas the mature erythrocytes with oval nuclei represented a second generation of embryonic definitive cells. Dantschakoff (1908, 1909*b*) had also proposed that several generations of embryonic definitive cells occurred during development of the chick embryo.

It might be proposed that the mature definitive erythrocytes with round nuclei are yolk sac derived definitive cells while the mature erythrocytes with oval nuclei are of bone marrow origin. However, in this study, immediately before the rapid replacement of mature cells with round nuclei by mature cells with oval nuclei, no immature definitive cells with oval nuclei were observed. The less mature definitive cells (late polychromatophilic erythroblasts) of late embryos had round nuclei. Similarly, the small number of circulating immature definitive cells of young chicks had round nuclei. If the blood volume per gram of body mass is constant between hatching and 20 days following hatching, it can be estimated that the haemoglobin and the

number of erythroid cells per chick doubles during these 20 days. However, no immature definitive cells with oval nuclei are observed. Immature definitive erythroid cells with oval nuclei are observed in the circulation of adult chickens made anaemic with acetophenylhydrazine (Bruns 1971). In light of these observations, the concept that mature definitive erythrocytes with round nuclei and comparable cells with oval nuclei represent two different generations of embryonic definitive cells requires further evaluation.

(*b*) *Changes in the types of haemoglobins*

(i) *Major haemoglobin components*

Three distinct stages of erythropoiesis can be recognized during development of the chick embryo: the first, between 1.5 and 5 days when only primitive erythroid cells are found in the embryonic circulation; the second, between 6 and 16 days when both primitive and definitive erythroid cells exist; and the third, after 16 days when only definitive erythroid cells are found in the embryonic circulation. The number and types of haemoglobins produced during development of the embryo correlate with these three stages of erythropoiesis.

Between day 2 and day 5, the two electrophoretic components, haemoglobins E and P, are observed in lysates of embryonic erythroid cells. One minor component, which migrated near the position of haemoglobin D in the pH 10.3 electrophoresis system, was shown to have a mobility faster than that of haemoglobin D in the pH 8.9 electrophoresis system. If haemoglobin D is present in lysates of erythroid cells from embryos of less than 6 days of age, this component constitutes less than 2.5 % of the haemoglobin. Similarly, haemoglobin A, if present prior to 6 days, constitutes less than 5 % of the haemoglobin.

The two haemoglobins which first appear in lysates of embryonic erythroid cells at 6 to 7 days, when the first haemoglobinized definitive erythroid cells have entered the embryonic circulation, have been shown to be electrophoretically identical to the A and D components of adult chicken haemoglobin in two different electrophoresis systems, and to exhibit reactions of immunological identity with haemoglobins A and D of adult chickens. Peptide maps of haemoglobins A and D isolated from 7.5-day embryos have been shown to be identical to the peptide maps of the A and D components of adult chicken haemoglobin (Brown 1971; Brown & Ingram 1972).

The absence of haemoglobin P in red cell lysates from embryos of 16 days of age or older is correlated with the virtual absence of primitive erythrocytes in the cell suspensions used for the preparation of the lysates. If haemoglobin P is present in lysates prepared from erythroid cell suspensions which contain only definitive erythroid cells, this component constitutes less than 2 % of the total haemoglobin. The time of disappearance of haemoglobin E from lysates of embryonic erythroid cells is obscured by the presence of haemoglobin H in lysates from late embryos. It is not unlikely, however, that haemoglobins E and P are simultaneously lost.

These observations indicate that the presence of the P and probably E haemoglobin components in red cell lysates of developing chick embryos is correlated with the presence of primitive cells among the embryonic erythroid cells. Thus, the three distinct stages of erythropoiesis during development of the chick embryo can be defined, not only in terms of the types of erythroid cells found in the embryos, but also in terms of the types of haemoglobins found in lysates of embryonic erythroid cells.

It has been assumed that the haemoglobins E and P coexist within the same erythroid cell. This is not, however, an obligate assumption. Since the haemoglobin E/P ratio is constant

between day 2 and day 5, if the two components do not exist in the same cell, the ratios of the cells containing haemoglobin E and those containing the P component and the rate of increase of haemoglobinization of such cells must remain constant. Since haemoglobins A and D have been shown to exist in the same cell in adult chickens (Matioli & Thorell 1963), it is not unreasonable to assume that both haemoglobins A and D coexist in the embryonic definitive cells.

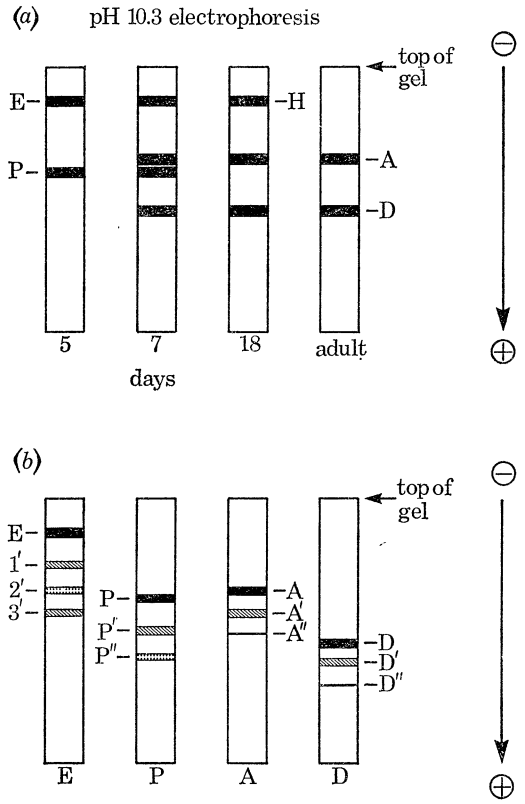

FIGURE 28. Schematic representation of the major and minor haemoglobins of chick embryos and of adult chickens on polyacrylamide gels at pH 10.3. (*a*) Haemoglobin components observed in freshly prepared lysates or in lysates stored in liquid nitrogen prior to electrophoresis on analytical polyacrylamide gels at pH 10.3. The letters, E, P, A, D and H indicate the appropriate haemoglobins. The positions of the bands are drawn to scale. However, the widths of the bands are not meant to indicate the relative proportions of the haemoglobins. (*b*) Haemoglobin components observed when haemoglobins E and P of 5-day embryos and haemoglobins A and D of an adult chicken, isolated by preparative polyacrylamide gel electrophoresis, are re-electrophoresed on analytical polyacrylamide gels at pH 10.3. The relative quantities of the components are indicated by the gradation of coloration of the bands. For example, when haemoglobin E is re-electrophoresed, the E band is the most prominent, the bands labelled 1′ and 3′ are of about equal prominence and the band labelled 2′ is the least prominent component. The positions of the bands are drawn to scale.

(ii) *Minor haemoglobin components*

The haemoglobins of chick embryos and adult chickens are unstable and components not observed in fresh lysates or in lysates stored in liquid nitrogen were observed when lysates were stored at −20 °C or for more than 18 h at 0 °C. The electrophoretic patterns of freshly prepared lysates of chick embryo and adult chicken erythroid cells are presented schematically in figures 28 and 29. The electrophoretic mobilities and relative quantities of the additional components observed when haemoglobins E, P, A and D, which had been isolated from preparative polyacrylamide gels, were re-electrophoresed are also shown (figures 28 and 29). It is therefore possible to tentatively determine the origin of the commonly seen artifactual haemoglobin

components. For example, the additional components most often observed in lysates from 2- to 5-day embryos include a band migrating between the E and P components, comparable to the 1′ band of haemoglobin E, and one or two bands migrating faster than the P component, comparable to bands P′ and P″. It is likely that these artifactual components arise from the haemoglobin E and P components, respectively.

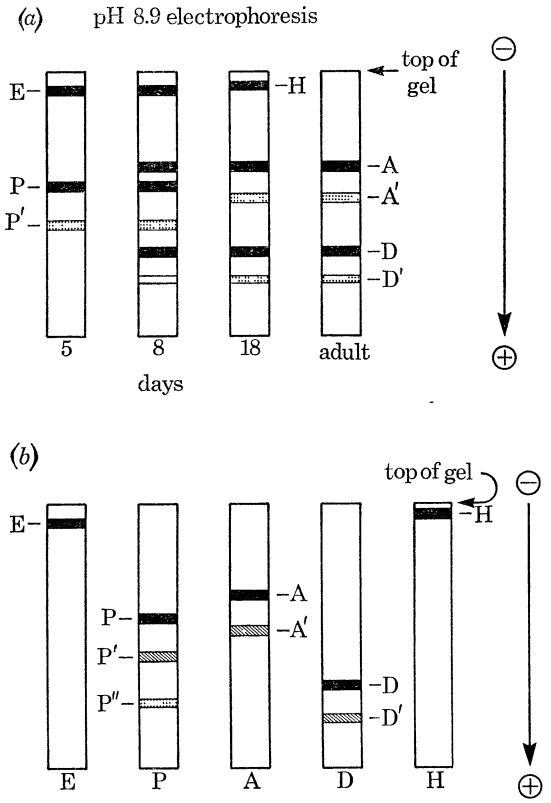

FIGURE 29. Schematic representation of the major and minor haemoglobins of chick embryos and adult chickens on polyacrylamide gels at pH 8.9 (*a*) Haemoglobin components observed in freshly prepared lysates or in lysates stored in liquid nitrogen prior to electrophoresis on analytical polyacrylamide gels at pH 8.9. The letters E, P, A, D and H indicate the appropriate haemoglobins. The letters P′, A′ and D′ indicate less prominent components associated with haemoglobins P, A and D, respectively, which are consistently observed on pH 8.9 polyacrylamide gels. The positions of the bands are drawn to scale. The widths of the black bands are not meant to indicate the relative proportions of the haemoglobins. (*b*) Haemoglobin components observed when haemoglobins E and P of 5-day embryos, haemoglobins A and D of an adult chicken, and haemoglobin H of 18-day embryos, isolated by preparative polyacrylamide gel electrophoresis, are re-electrophoresed at pH 8.9. The relative quantities of the components are indicated by the gradation of coloration of the bands. For example, when haemoglobin P is re-electrophoresed, the P band is the most prominent, the P′ band is less prominent than the P band, and the P″ band is the least prominent band. The positions of the bands are drawn to scale.

(iii) *Haemoglobin H*

The minor haemoglobin component of late embryos and young chicks which migrates similarly to haemoglobin E in the pH 10.3 electrophoresis system was shown to differ in electrophoretic mobility from haemoglobin E in the pH 8.9 electrophoresis system. With several different antisera, haemoglobin H showed reactions of only partial immunological identity with haemoglobin E. This component also differed immunologically from haemoglobins D, P and A. Haemoglobin H and haemoglobin E behaved differently on Sephadex G-100 columns. Although

haemoglobin H eluted from the column with the major haemoglobin peak, this component was concentrated at the leading edge of the peak, whereas haemoglobin E was concentrated at the trailing edge of the haemoglobin peak. When the mobility of haemoglobin H was compared to the mobilities of haemoglobins E, P, A and D on polyacrylamide gels of different porosities, haemoglobin H appeared to be a charge and size isomer of the other four haemoglobins. The slope of the plot of the logarithm of the relative mobility of a protein against the percentage acrylamide in the gel has been correlated with the molecular mass of the protein. Using the data of Hedrick & Smith (1968) for the relation of the slope of such plots to molecular masses, haemoglobins E, P, A and D had the expected molecular mass of about 65000, while haemoglobin H appeared to have a molecular mass of about 500000. However, on gels of 6 to 9.5% acrylamide, the mobility of haemoglobin H was small, and less than that of haemoglobin E. Hence, small errors in measuring the position of the band could introduce a significant error into the molecular mass estimation.

These two haemoglobins appear to be different proteins, although the nature of this difference and the relation of haemoglobins H and A remain unknown. Since haemoglobin H reacts with antiserum to haemoglobin D, whereas haemoglobin E reacts weakly or not at all with anti-D antisera, it might be postulated that haemoglobin H is more closely related to haemoglobin D than is haemoglobin E.

Since haemoglobin H can be detected electrophoretically in lysates of erythroid cells from 12- to 14-day embryos and by immunological reactivity in lysates from 8-day embryos, this component appears to be synthesized by definitive cells, perhaps beginning as early as 6 to 7 days.

Several previous investigators have reported the presence of a minor haemoglobin component in late embryos and young chicks which migrated at or near the position of haemoglobin E (Manwell *et al.* 1963; Huisman & Schillhorn Van Veen 1964; Washburn 1968*b*; Schurch *et al.* 1968; Denmark & Washburn 1969*a*), although no attempts to characterize this haemoglobin were described. Washburn (1968*b*) reported that this trace haemoglobin became undetectable in lysates from normal chickens at 90 days after hatching, although this component persisted in adult chickens heterozygous or homozygous for a mutant haemoglobin D. Fraser *et al.* (1972) noted the persistence in late embryos and young chicks of less than 50 days of age of a trace haemoglobin component which differed slightly from the minor haemoglobin of early embryos in its elution profile from DEAE-cellulose columns. Borgese & Bertles (1965) noted the presence in late duck embryos and young ducklings of a slow-moving trace haemoglobin component electrophoretically similar to the minor embryonic haemoglobin.

(iv) *Changes in the haemoglobin D/A ratio*

When the two adult components, haemoglobins D and A, were first detected in lysates of embryonic erythroid cells at 6 to 7 days of incubation, the ratio of the quantities of haemoglobin D and haemoglobin A was about 2 times greater than the haemoglobin D/A ratio of adult chicken haemoglobin. The D/A ratio decreased rapidly between day 6 and days 14 to 17 to the plateau values observed in late embryos and young chicks (0.5 to 20 days of age). The haemoglobin D/A ratio of late embryos and young chicks was consistently 40 to 50% greater than that of adult chicken haemoglobin. In no case did the D/A ratio of lysates from late embryos or from individual young chicks equal the ratio or overlap the range of values of the ratio observed for adult chicken haemoglobin electrophoresed simultaneously with the late embryo and young chick erythroid cell lysates.

Analysis of the rapid change in the haemoglobin D/A ratio between day 6 and days 14 to 17 is complex. The D/A ratio on any day n will be the average of the contribution of the circulating definitive erythroid cells already in the embryonic circulation at day $n-1$ and of new definitive cells entering the circulation between day $n-1$ and n. Additional data is required to analyse the multiple variables influencing the D/A ratio between day 6 and days 14 to 17. The phase of rapid change in the D/A ratio does correlate with the period during the development of the chick embryo when definitive cells are first entering the circulation in large numbers and rapidly replacing the primitive erythrocytes. This time period is also one during which the immature definitive cells, such as early and mid-polychromatophilic erythroblasts, are being replaced by late polychromatophilic erythroblasts, retiuclocytes, and mature definitive erythrocytes.

It might be proposed that although the D/A ratio at 7 days, for example, is about twice that of late embryos, the haemoglobinization of the immature definitive cells of the 7-day embryos (mid and late polychromatophilic erythroblasts) is sufficiently low that synthesis of more haemoglobin A than haemoglobin D in late polychromatophilic erythroblasts and reticulocytes would result in mature definitive cells with a haemoglobin D/A ratio similar to that of late embryos (Burka & Marks 1964; Rieder & Weatherall 1966; Winslow & Ingram 1966; Baglioni 1966). However, the amount of haemoglobin D/definitive erythroid cell of 7- to 8-day embryos is equal to or greater than the amount of haemoglobin D/definitive cell of late embryos although the immature definitive cells of 7- to 8-day embryos are only about 70 % haemoglobinized. At 9 to 12 days, when the average circulating definitive cell is almost fully haemoglobinized, the amount of haemoglobin D per definitive cell appears to be about 30 % greater than the haemoglobin D content of late embryo definitive cells. These observations suggest that the ratio of haemoglobin D and haemoglobin A synthesis and/or accumulation by definitive cells is reprogrammed during development of the chick embryo. At least in the acetophenylhydrazine treated adult chicken, the presence of 25 to 35 % early and mid-polychromatophilic erythroblasts and 30 to 40 % late polychromatophilic erythroblasts and reticulocytes among circulating erythroid cells was not associated with an alteration in the haemoglobin D/A ratio either during the period of acute anaemia or during the recover phase (Kabat & Attardi 1963; Bruns 1971).

The stabilization of the haemoglobin D/A ratio of late embryos at a value higher than that of adult chicken haemoglobin occurs at a time during development of the chick embryo when at least 90 % of the circulating definitive erythroid cells are mature erythrocytes. The stability of the D/A ratio is maintained in spite of a twofold increase in the size of the erythroid cell compartment of the embryo between day 14 and day 21 (Ramsay 1950, Rychter *et al.* 1955, Lemez 1964). If it is assumed that both haemoglobin D and haemoglobin A exist within the same cell (Matioli & Thorell 1963), these observations suggest that mature definitive erythrocytes of embryonic origin have been programmed to contain different quantities of haemoglobins D and A than mature erythrocytes of adult chickens. A similar hypothesis applicable to definitive erythrocytes of young chicks is suggested by the stability of the haemoglobin D/A ratio of 0.5- to 20-day chicks. If the blood volume per gram of body mass is constant between hatching and 15 days following hatching, it can be estimated that the haemoglobin and the number of erythroid cells per chick doubles during these 15 days without a discernible change in the haemoglobin D/A ratio.

A difference between the haemoglobin D/A ratio of late embryos, young chicks of less than 100 days of age, and adult chickens has been reported by Washburn (1968*b*) and Denmark &

Washburn (1969*b*). The data of Huisman & Schillhorn Van Veen (1964), however, do not show any significant differences between the quantities of the major and minor components of large embryos, young chicks, and adult chickens. Borgese & Bertles (1965) observed a higher ratio of the minor/major haemoglobin component in late duck embryos and young ducklings of less than 84 days of age than in adult ducks. The absolute amount per erythrocyte of the minor haemoglobin was about 3 times greater than the quantity of this component in erythrocytes of adult ducks.

In summary the definitive cells of the chick embryo appear to differ from those of the adult chicken in at least two ways: the production of haemoglobin H and the different haemoglobin D/A ratio of mature erythrocytes. The data of Moss *et al.* (1972) on the histones of definitive erythrocytes of late chick embryos and of mature erythrocytes of adult chickens are consistent with this hypothesis.

When profound anaemia was produced in adult chickens with acetophenylhydrazine, the immature definitive cells were morphologically different from cells of comparable maturation stages of the chick embryo. Mid and late polychromatophilic erythroblasts of anaemic chickens were slightly oval in fixed preparations and had oval nuclei with a looser chromatin pattern than the corresponding cells of embryos, which in fixed preparations are round cells with round nuclei. The nuclei of well haemoglobinized round and oval cells of anaemic chickens were larger and had a looser chromatin pattern than similar well haemoglobinized round or oval cells of the embryos. The nuclear diameters and the nuclear/cytoplasm ratios of the cells of each of the several maturation stages found in the blood of anaemic chickens were greater than those of comparable cells from chick embryos. The cytoplasmic diameters of cells from anaemic chickens and from chick embryos were comparable.

The morphological differences between immature definitive cells of anaemic chickens and comparable maturation stages of immature definitive cells of chick embryos would be compatible with a hypothesis that the definitive cells of embryos and adult chickens represent two different cell populations. The two types of definitive cells might, however, represent only one cell population, the differences between the embryonic definitive cells and the definitive cells of the adult chicken resulting from different environmental factors (Barker 1968; Wolf & Trentin 1968; Trentin 1970).

The relationship between the change in the haemoglobin D/A ratio of developing chick embryos and the concomitant changes in oxygen affinity of the haemoglobins (Hall 1935; Huisman, Schillhorn Van Veen, Dozy & Nechtman 1964; Huisman & Schillhorn Van Veen 1964; Benesch, Benesch & Yu 1968; Benesch & Besnech 1969) requires illucidation. The parallels between the changes in the types of haemoglobins and erythroid cells of the developing chick embryo and of the developing human foetus and neonate are striking.

Recently an additional haemoglobin, called haemoglobin M has been detected in this laboratory by column chromatography and apparently occurs in primitive cells; its electrophoretic behaviour is similar to haemoglobin P (Brown 1972).

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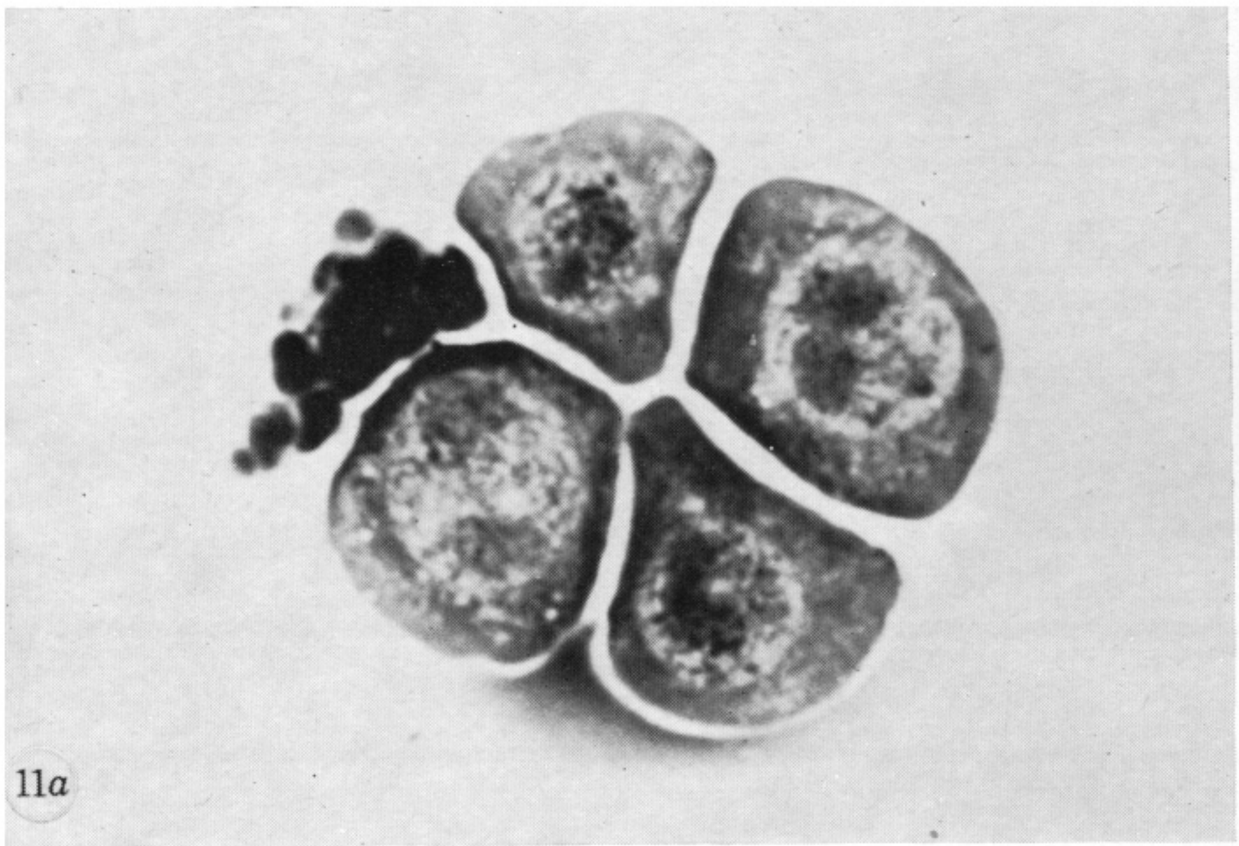

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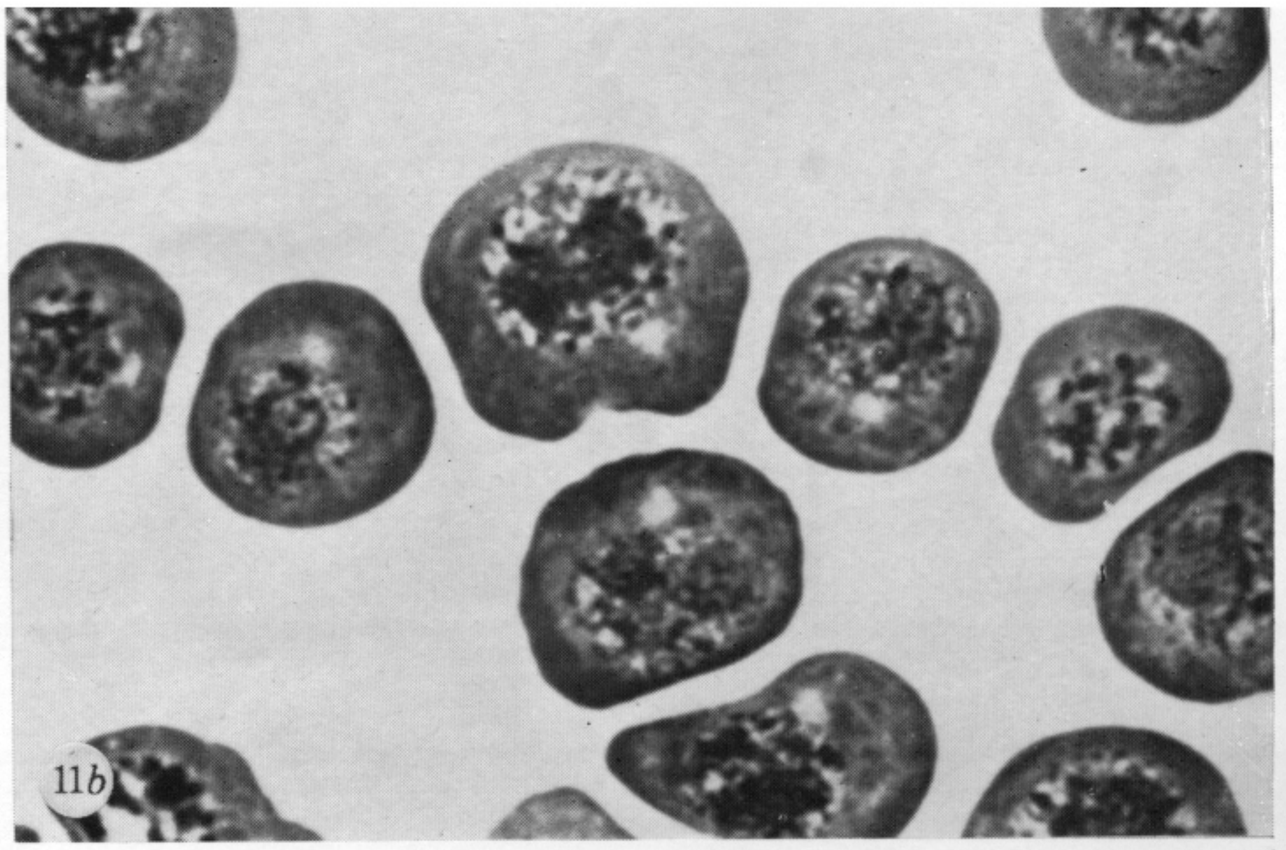

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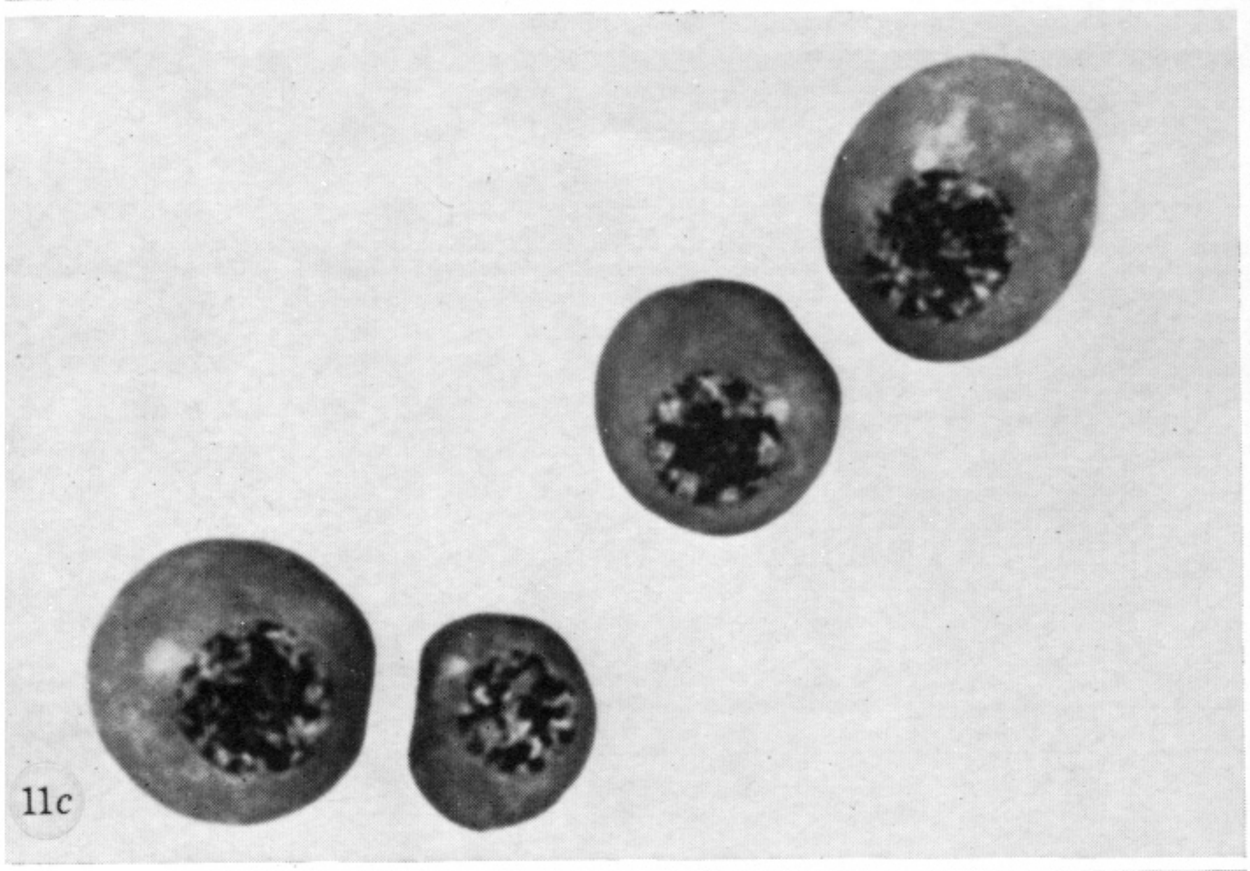

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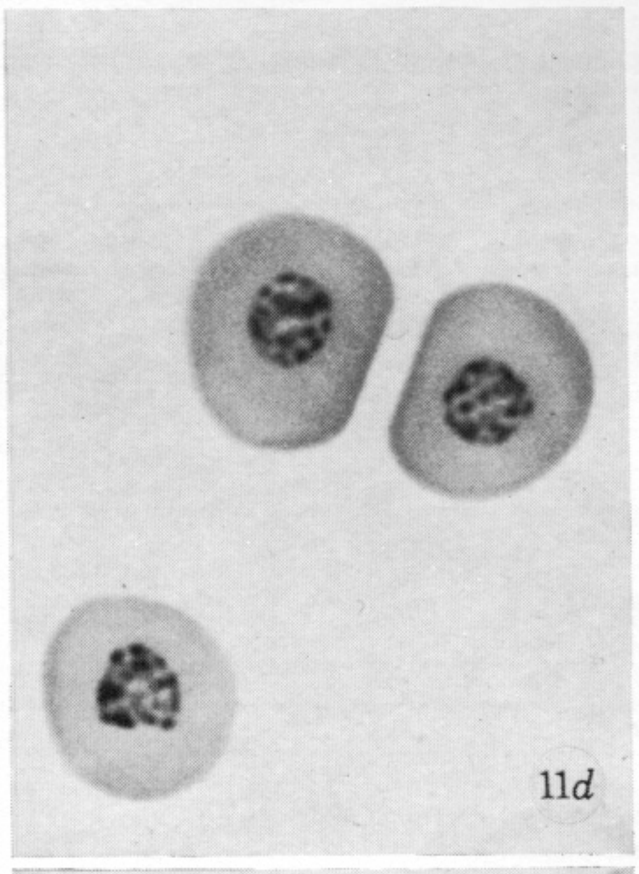

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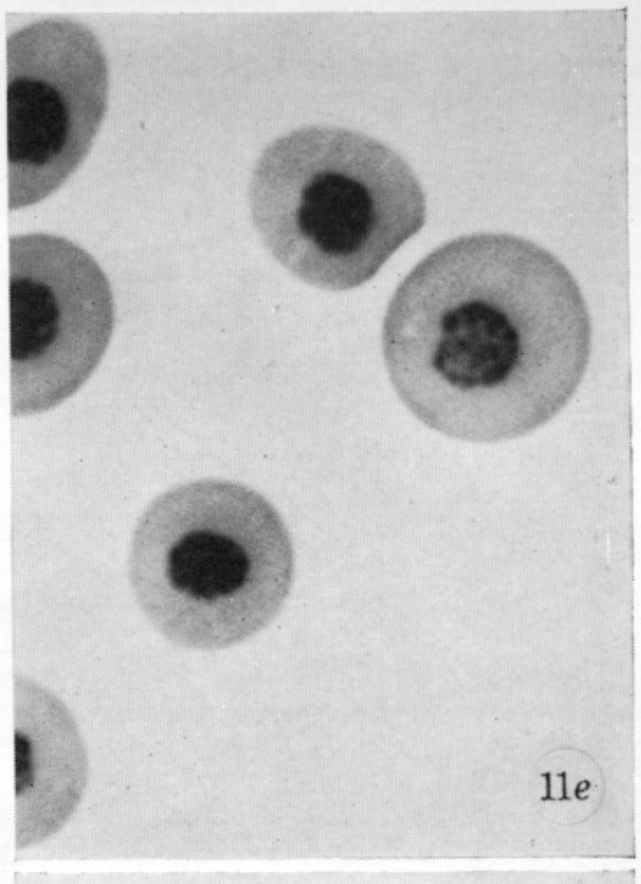

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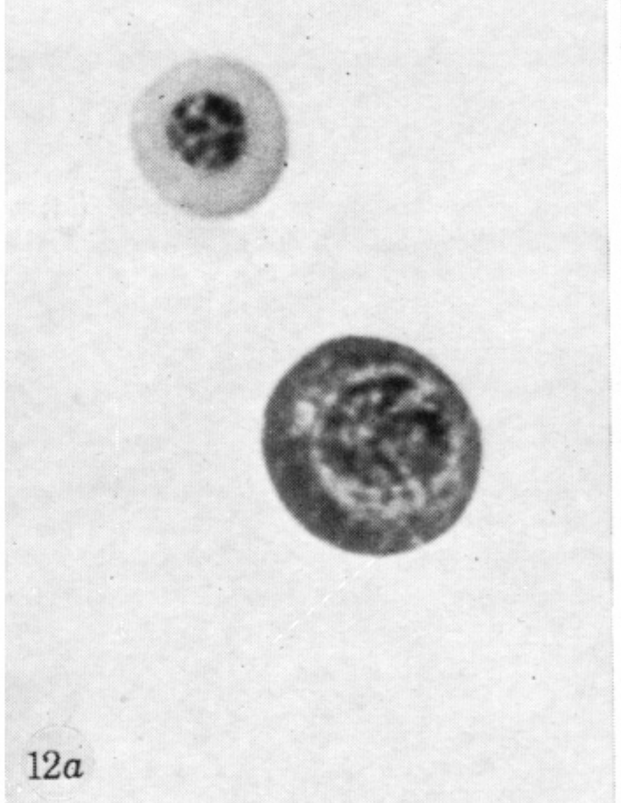

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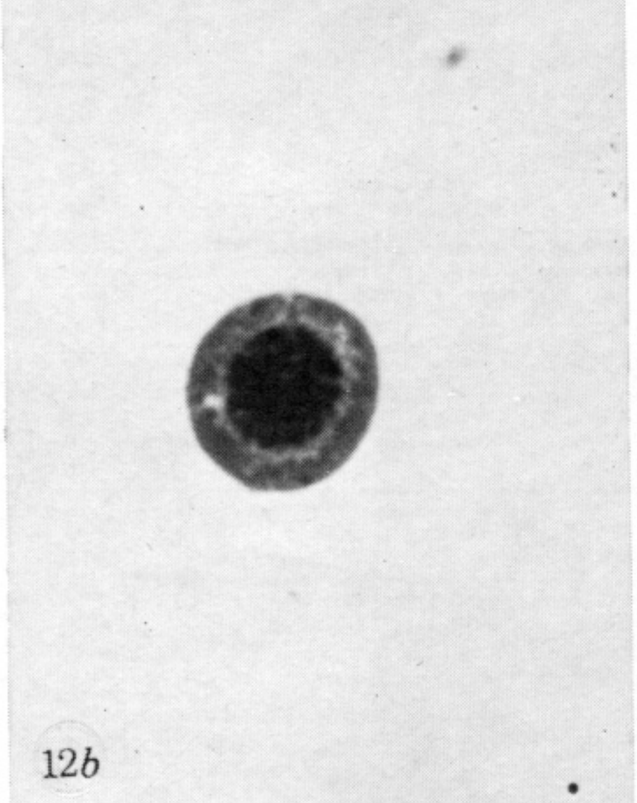

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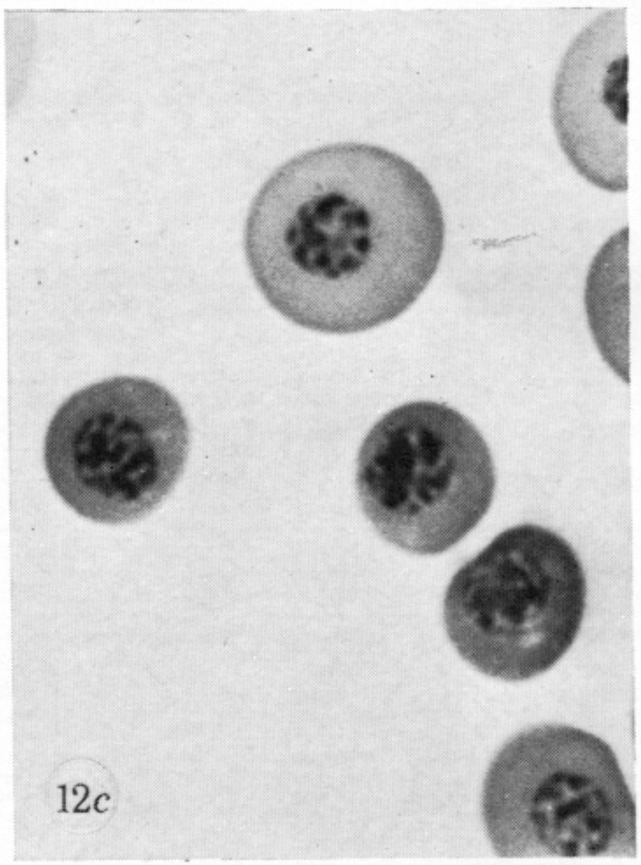

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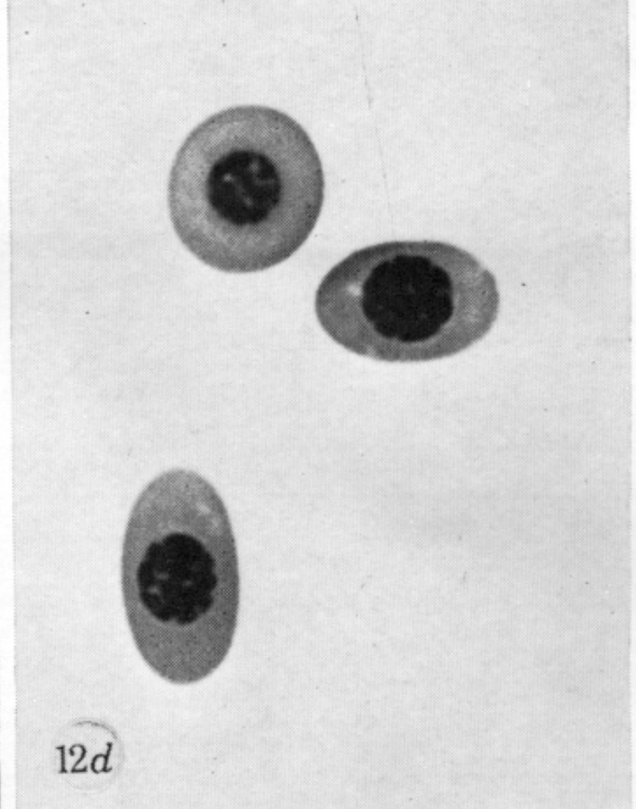

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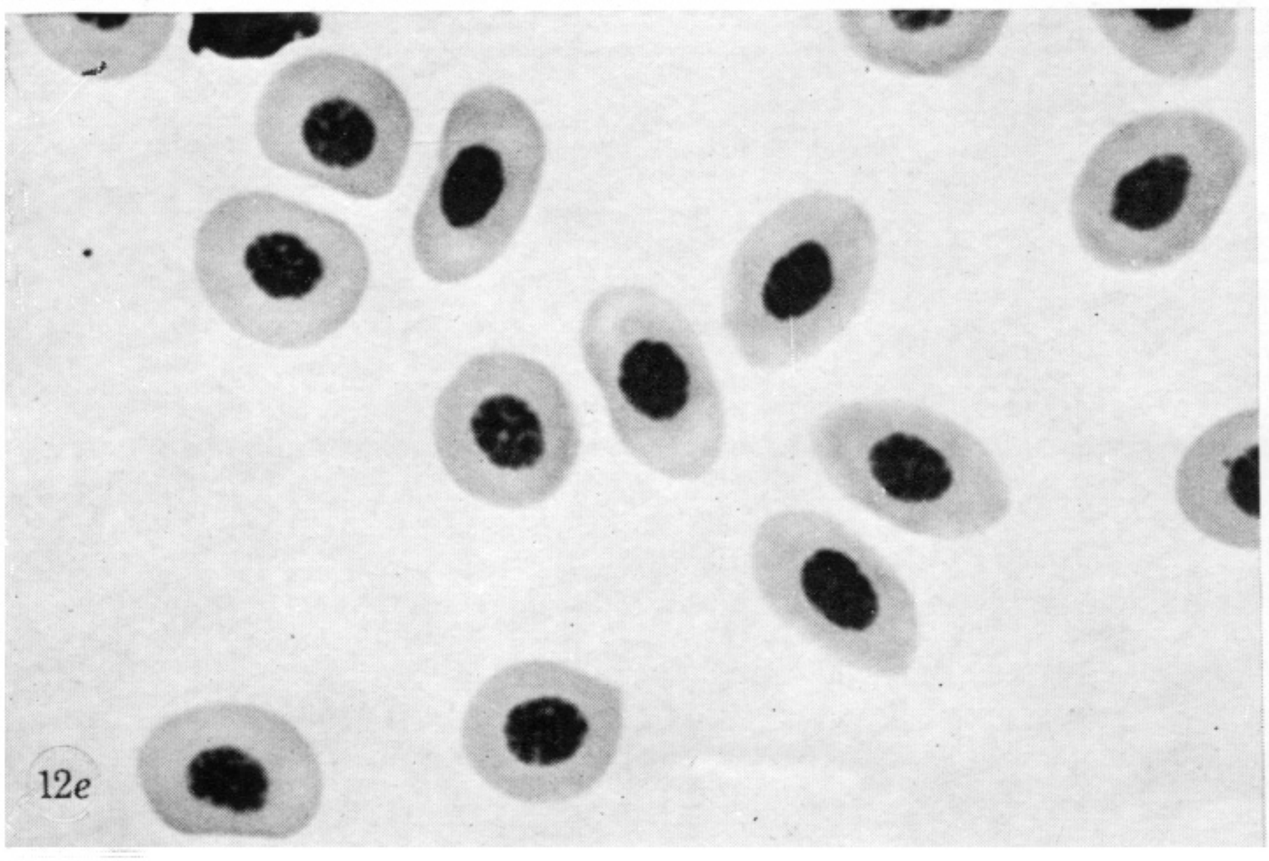

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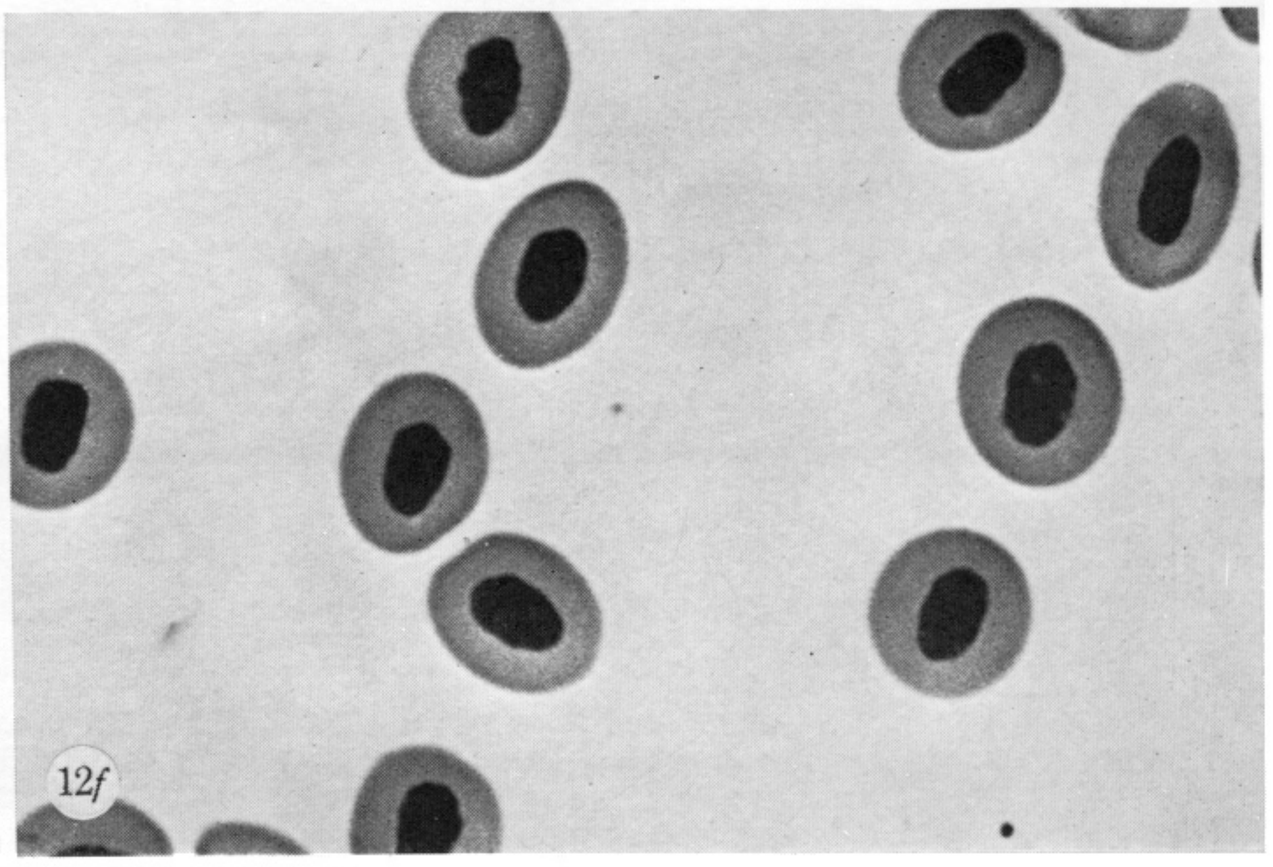

12*f*

FIGURES 11 AND 12. For legends see facing page.

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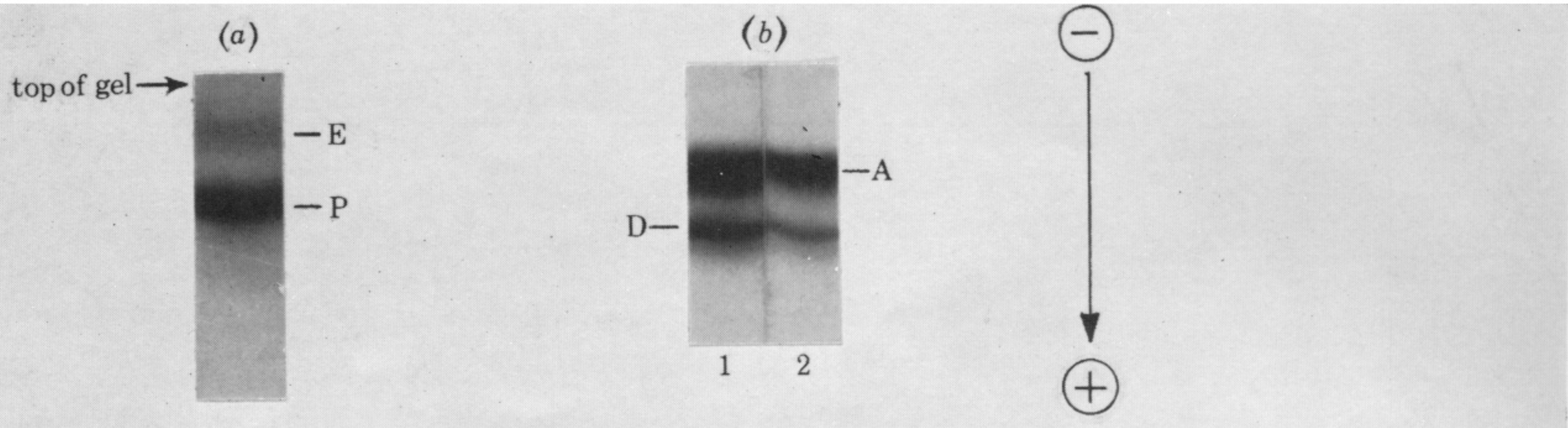

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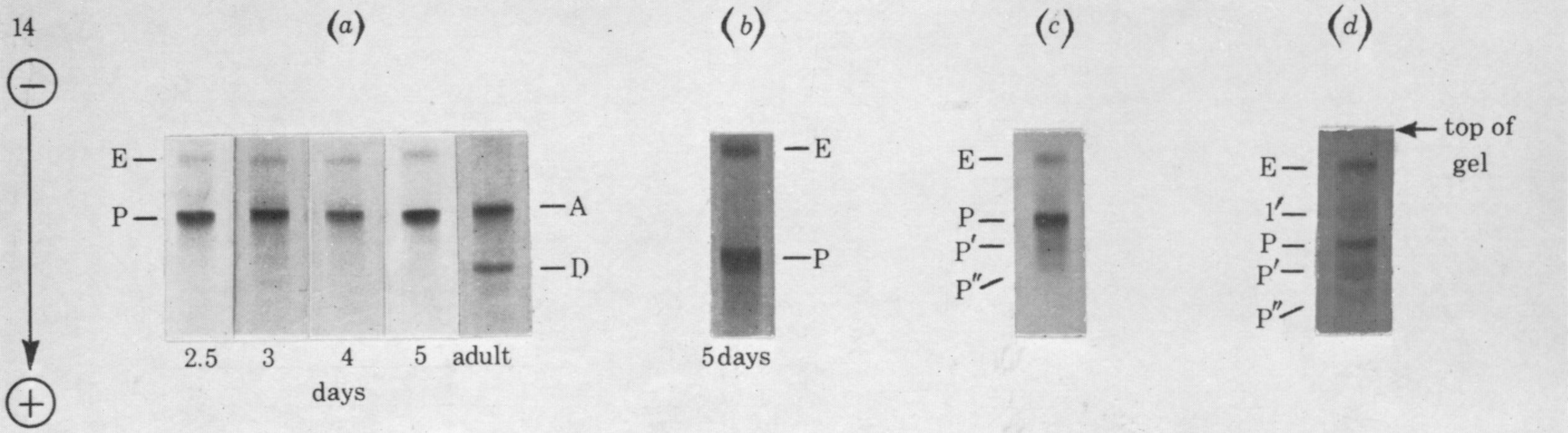
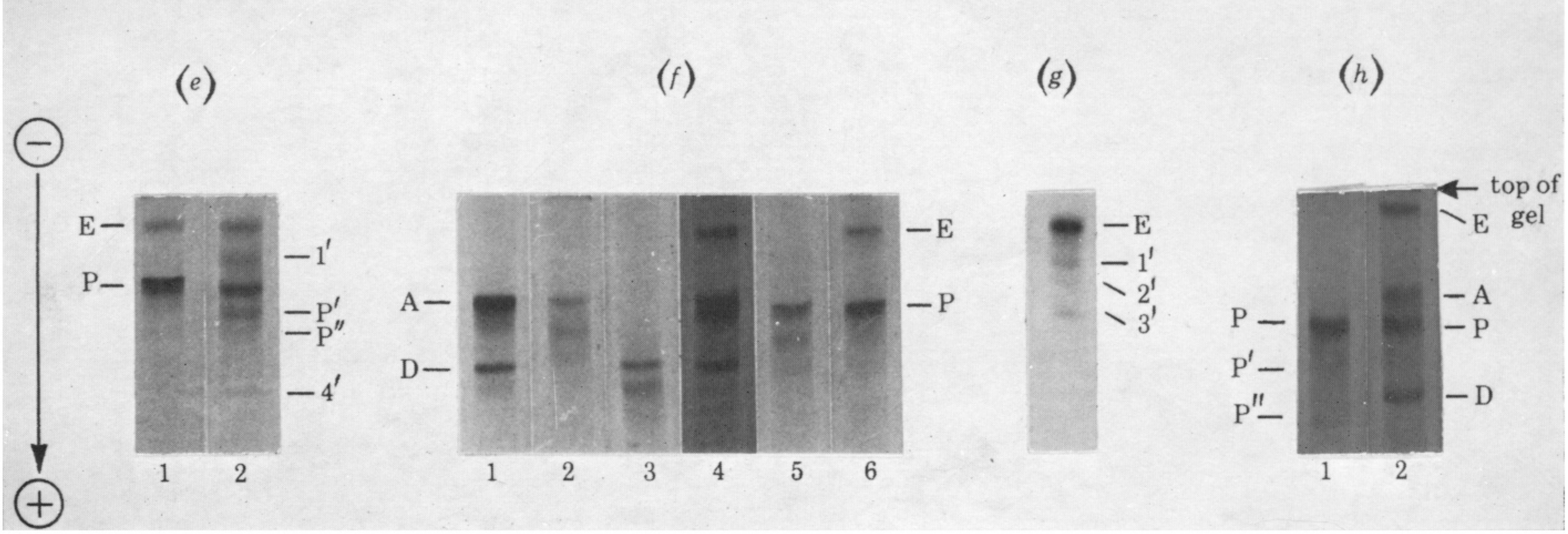




FIGURES 13 AND 14. For legends see facing page.

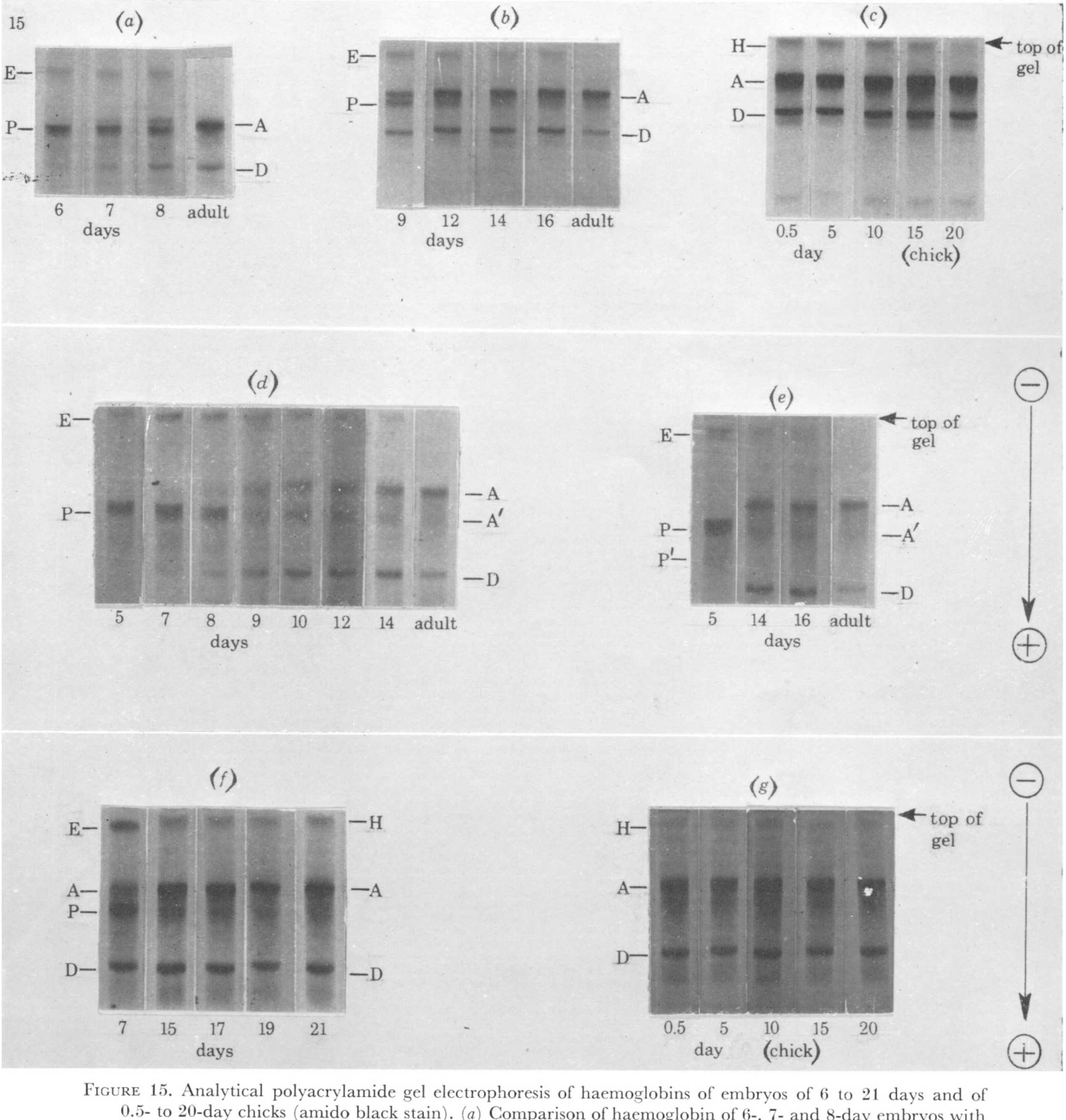
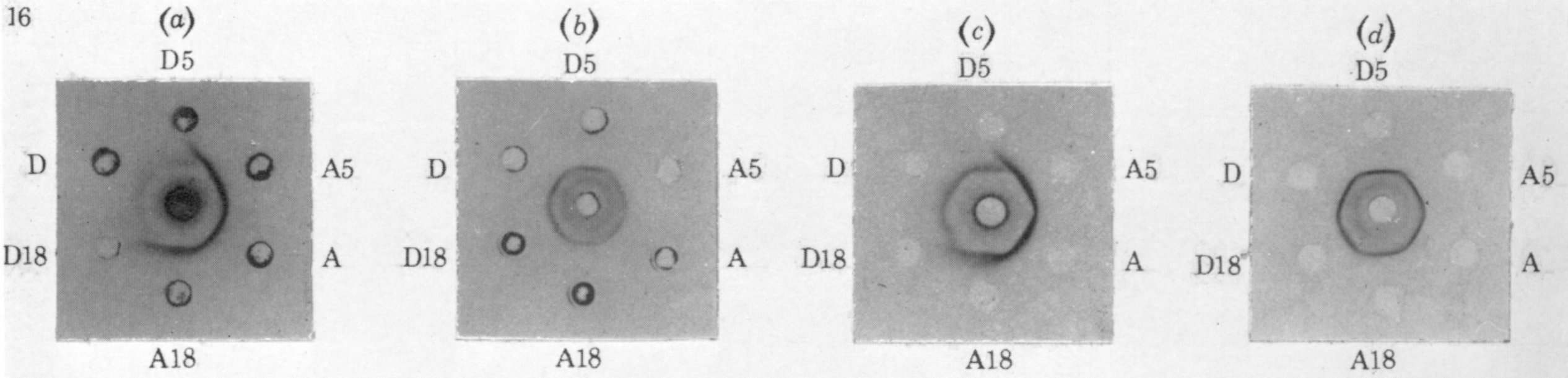


FIGURE 15. Analytical polyacrylamide gel electrophoresis of haemoglobins of embryos of 6 to 21 days and of 0.5- to 20-day chicks (amido black stain). (*a*) Comparison of haemoglobin of 6-, 7- and 8-day embryos with haemoglobin of an adult chicken (pH 10.3 electrophoresis). The letters E and P denote the two embryonic haemoglobin components. The letters A and D denote the major and minor adult haemoglobins. (*b*) Haemoglobins of 12- to 16-day embryos compared to haemoglobins of 9-day embryos and of an adult chicken (pH 10.3 electrophoresis). (*c*) Haemoglobins of 0.5- to 20-day-old chicks. The letter H indicates the minor component of haemoglobin of late embryos which migrates in the pH 10.3 system similarly to haemoglobin E (pH 10.3 electrophoresis). (*d*) Comparison of haemoglobins of 6- to 14-day embryos with haemoglobins of 5-day embryos and of an adult chicken on pH 8.9 polyacrylamide gels. The letters E, P, A and D indicate the respective haemoglobin components. The letter A′ refers to the diffuse component associated with haemoglobin A which migrates between haemoglobins A and D in the pH 8.9 electrophoresis system. (*e*) Comparison of the migration of haemoglobin P of 5-day embryos with the adult haemoglobin component A′ and the similarly migrating component of lysates from 14- and 16-day embryos (pH 8.9 electrophoresis). (*f*) Comparison of the haemoglobins of late embryos with the haemoglobins of 7-day embryos. The letter H indicates the minor component of haemoglobins of late embryos and young chicks which migrates on pH 8.9 polyacrylamide gels slightly more slowly than haemoglobin E (pH 8.9 electrophoresis). (*g*) Haemoglobins of 0.5 to 20-day-old chicks (pH 8.9 electrophoresis).

16



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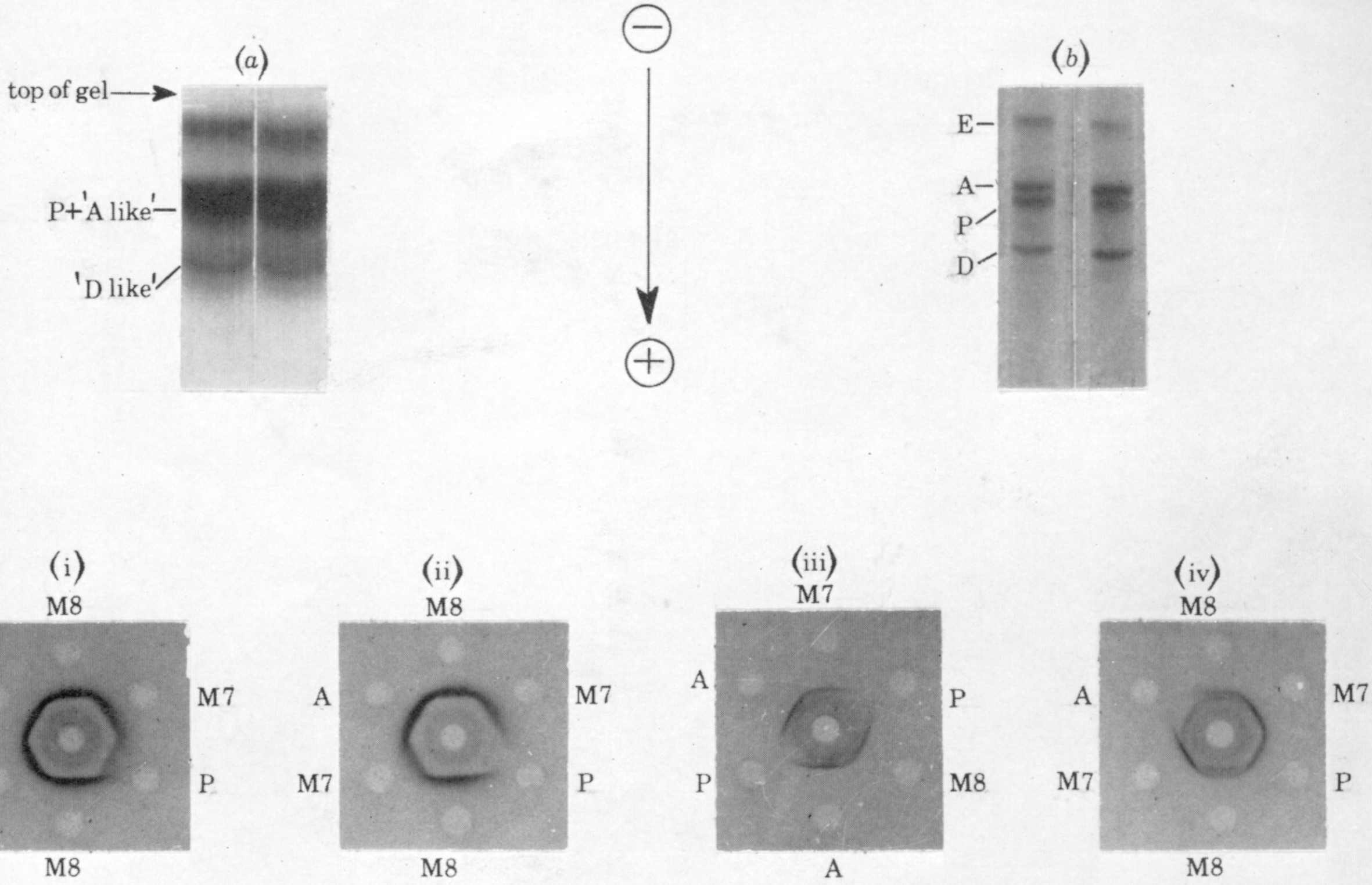

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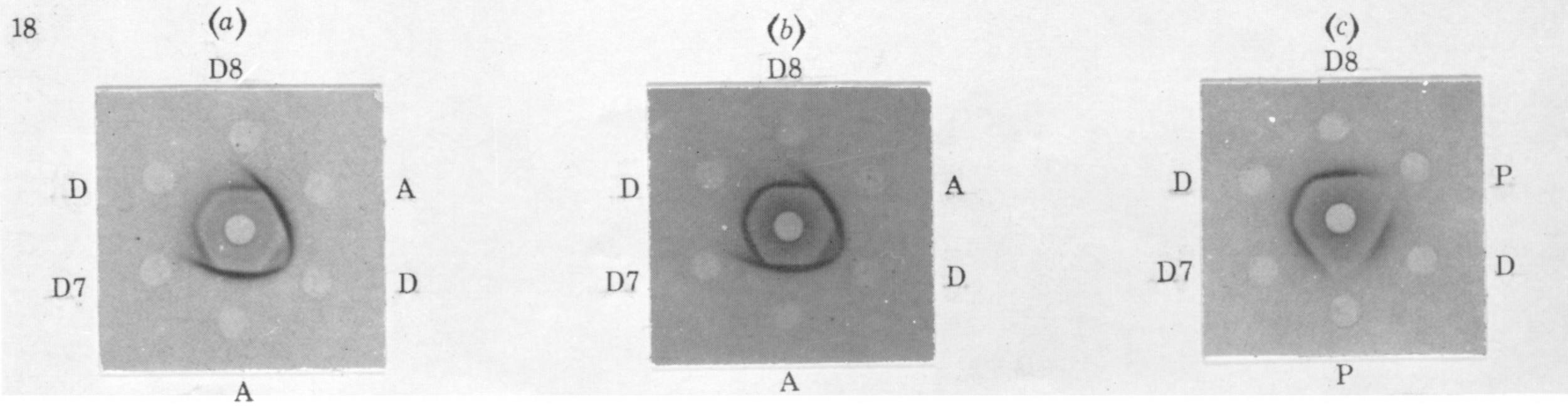

FIGURES 16 TO 18. For legends see facing page.

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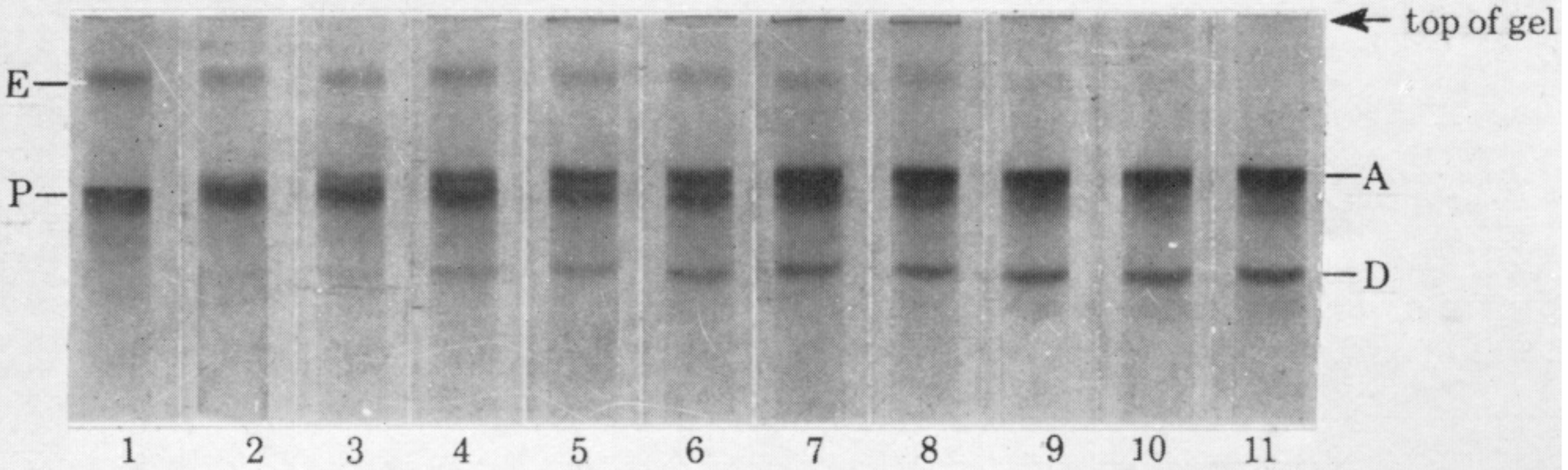

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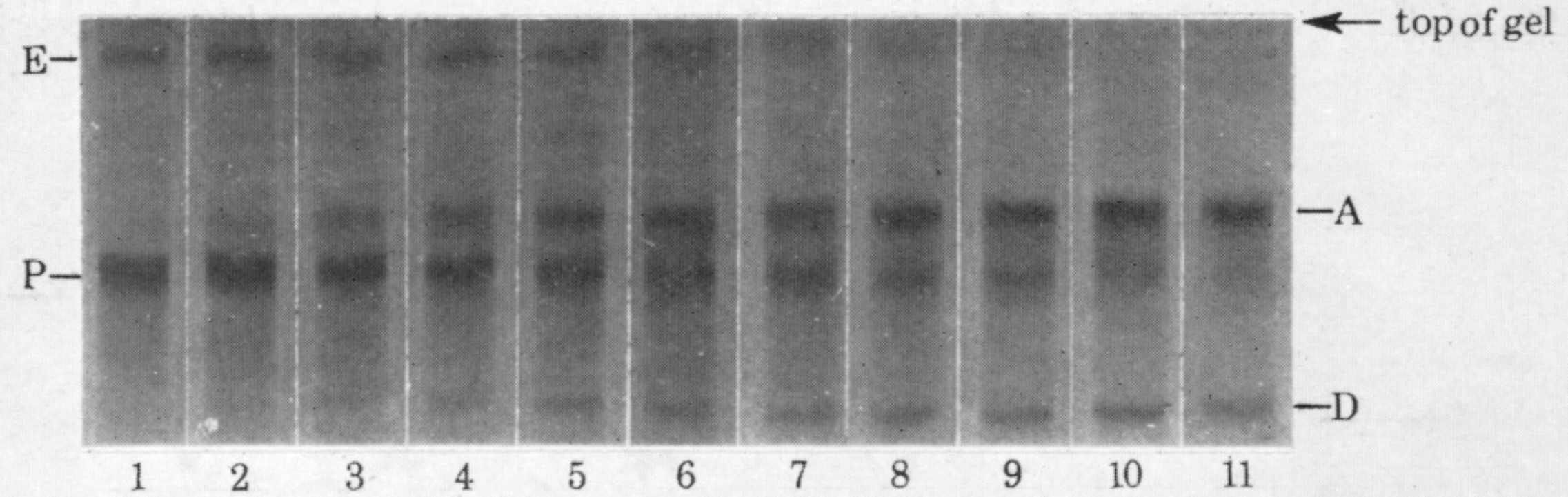

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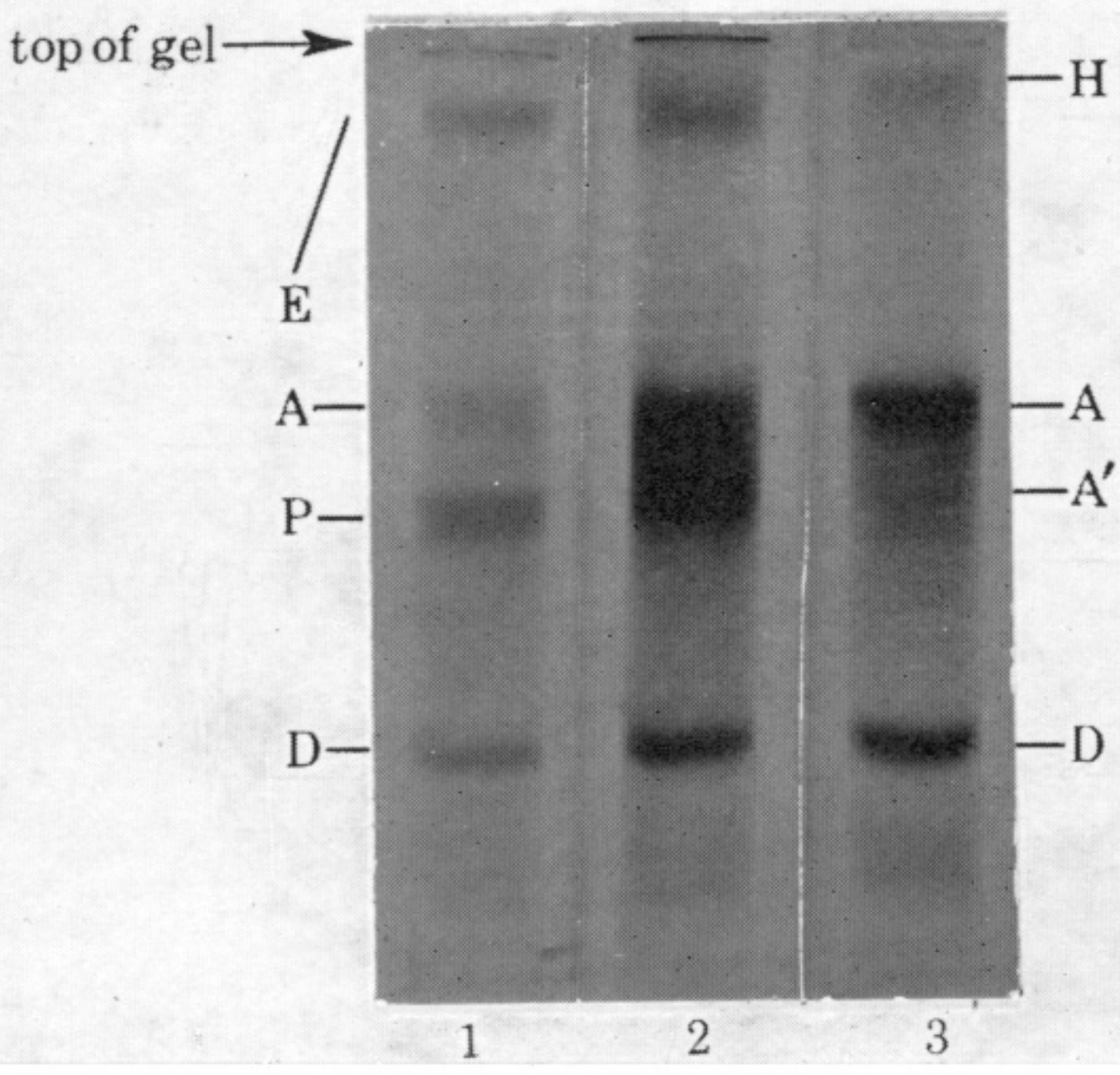

FIGURES 20 AND 21. For legends see facing page.

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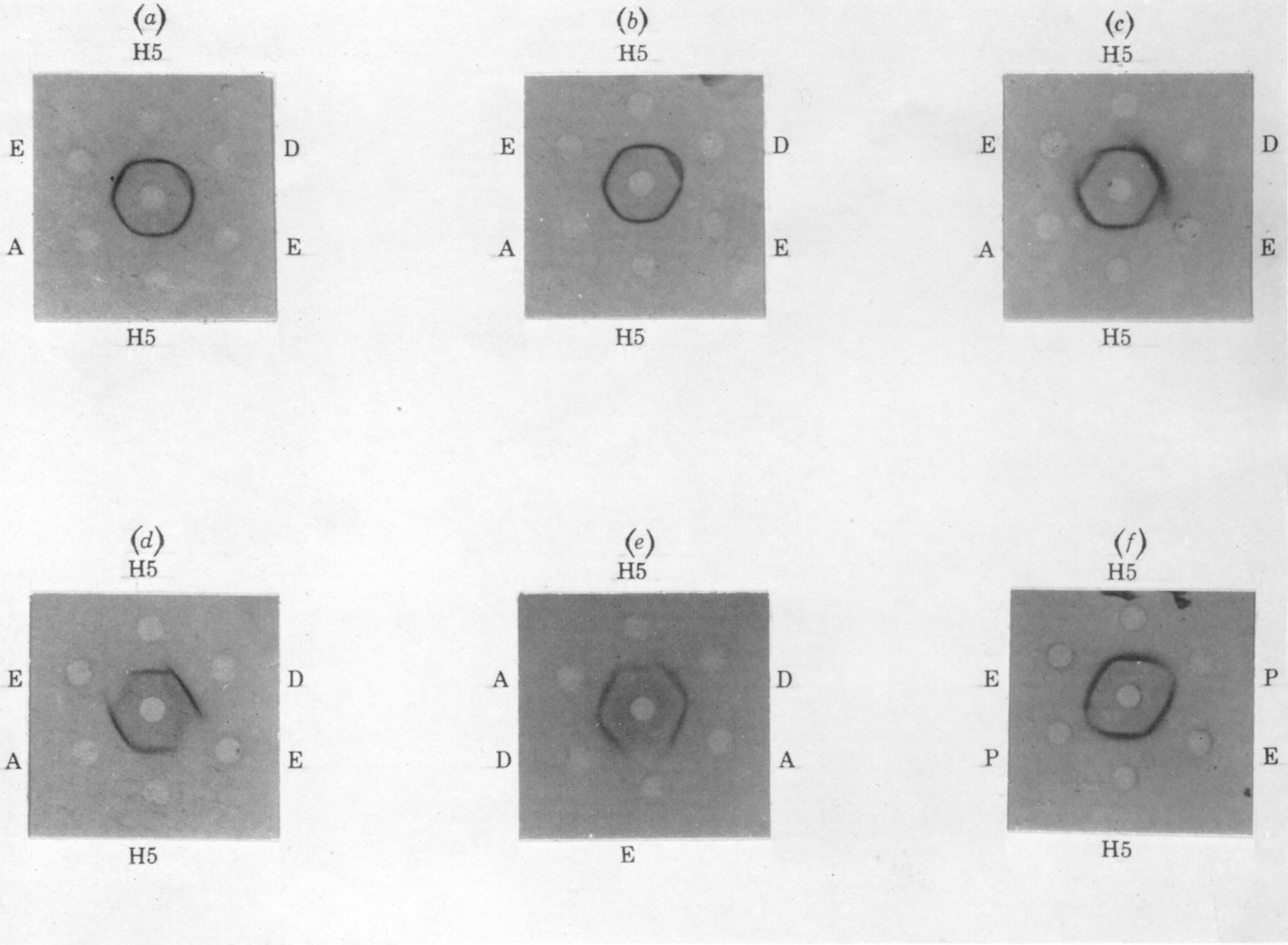

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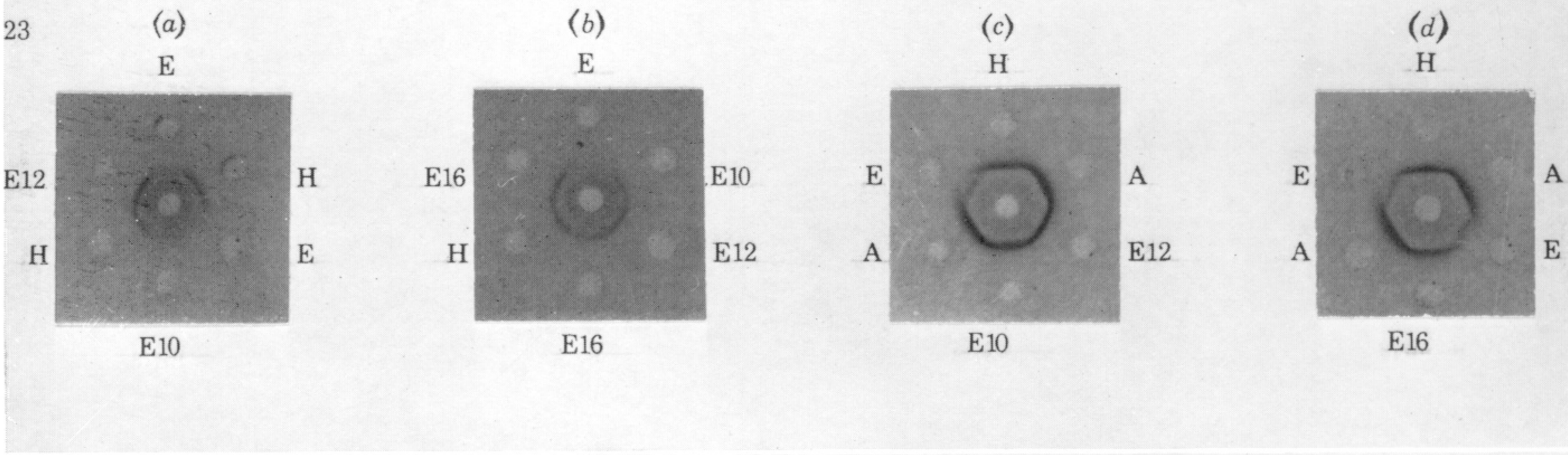

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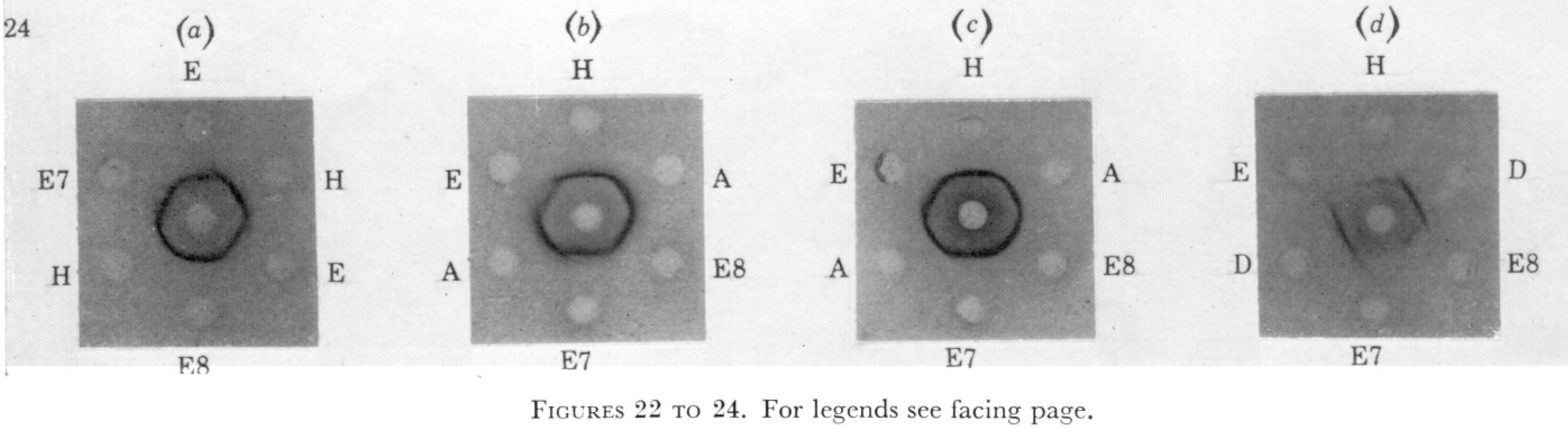

FIGURES 22 TO 24. For legends see facing page.