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Blood Oxygen Transport in the Early Avian Embryo

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I. INTRODUCTION

The avian embryo for a long time has been a favorite object of study for the respiratory physiologist, since more than any other embryo of higher vertebrates it allows easy access to well-specified stages of development. The first attempt to study properties of embryonic hemoglobin of developing avian embryos was carried out on chick embryos by Hall (70).

The special problems imposed on late embryos by the diffusion limitation of gas transfer across the eggshell and shell membranes have been systematically analyzed in the chick embryo, and corresponding data for the oxygen and carbon dioxide transport properties have been obtained by several groups from approximately *day 8* of incubation through development. Thus the general physiology of blood gas transport of the avian embryo, as studied in the chick, is well documented for the later period of development, and the interested reader can profit from several extensive monographs covering almost any aspect in this field (33, 90, 104, 118).

Strictly speaking, those studies dealt with gas transport in avian fetuses, since the period of embryogenesis ends at about *day 6* of development.

However, functional data about the properties of primitive red blood cells of early embryos are needed to understand why the blood of vertebrates contains a unique set of embryonic hemoglobins and why such a complicated pattern of erythropoiesis and hemoglobin synthesis prevails during embryonic development.

With the advent of micromethods for the study of blood oxygen-binding properties and the application of microelectrode techniques to determine parameters such as pH, oxygen partial pressure (P_{O_2}), and carbon dioxide partial pressure (P_{CO_2}) in situ, attempts have been made in recent years to bridge the gap in knowledge. With the help of these techniques, gas transport in

the chick embryo has now been characterized from *day 3* of development onward.

Moreover, the primary structure of the specific embryonic hemoglobins of the chick is now completely known; it has thus become possible to some extent to analyze functional properties in terms of the molecular structure of embryonic hemoglobin.

It is the aim of this review to give a concise description of the current state of knowledge. In addition we emphasize those areas where data are still scarce in the hope of attracting other developmental physiologists into this somewhat neglected field.

Of necessity the view presented is somewhat biased, since in several cases we have had to rely only on our own experimental data.

A. Ontogeny of Hemoglobin Types and Red Blood Cell Populations in the Avian Embryo

Similar to all other vertebrate embryos analyzed so far, the avian embryo produces a specific set of embryonic hemoglobins during the first stage of development. They are synthesized by the first population of red blood cells (primitive red blood cells) that arises in the yolk sac, which is the principal site of erythropoiesis in early development (31, 54).

In the chick embryo visibly hemoglobinized red blood cells appear at ~26–38 h of incubation in the blood islands that are found in a horseshoe-shaped area caudal from the embryo proper (31). A closed circulatory system is established after ~48 h of incubation, allowing convective transport between the embryo and the yolk sac. The vascularized part of the yolk sac (area vasculosa) is the principal gas exchange organ during early development, i.e., until the beginning of the second week of incubation. Analyses of the hemoglobin pattern of the primitive red blood cells of the chick embryo revealed two major and two minor hemoglobins (30) whose chain compositions as well as amino acid sequences have been completely worked out during the last 10 years (36–39, 56, 58, 114, 115). The hemoglobin types and their chain compositions are given in Table 1.

TABLE 1. *Hemoglobin types of embryonic blood*

Hemoglobin	Chain		Location
HbP	π_2 ρ_2	75%	Primitive RBCs
HbP′	π'_2 ρ_2		
HbE	α_2^A ϵ_2	25%	Primitive RBCs
HbM	α_2^D ϵ_2		
IIbA	α_2^A β_2		Definitive RBCs
HbD	α_2^D β_2		
HbH	α_2^A β^H		Transient minor component in definitive RBCs of prehatch, chick embryo, and hatchling

Hb, hemoglobin; RBCs, red blood cells. Nomenclature follows Brown and Ingram (30).

Comparative studies on other avian embryos suggest that the principal pattern of embryonic hemoglobin types and hemoglobin synthesis is the same among the species (41, 123).

In the chick embryo, primitive red blood cells are the principal red blood cell population until *day 6* of development. By that time a second generation of red blood cells, definitive red blood cells, enters the vascular bed (31). These cells are unable to produce embryonic hemoglobin; instead they synthesize the two adult hemoglobins A and D (HbA and HbD), as well as a minor hemoglobin component HbH. In adult chicken red blood cells the ratio of HbA to HbD is ~3:1. In contrast the first population of definitive red blood cells produces HbD in excess of HbA (31), and the same has been observed for duck embryos (41). It is only during the later course of development that the final ratio of HbA to HbD is established. As shown in section III*D*, the high amount of HbD found in early definitive red blood cells may have significant consequences for their oxygen-binding properties and function in oxygen transport.

In contrast to mammalian embryos where embryonic and adult hemoglobins may be produced in the same cell, hemoglobin synthesis in avian embryos is strictly lineage specific (19, 44, 45, 120). By an ingenious set of experiments with xenogenic and allogenic chimeras constructed of yolk sac and embryo proper, the group of Dieterlen-Lievre (20, 51, 52–54, 84, 87) has demonstrated that the stem cells for permanent definitive erythropoiesis are of intraembryonic origin, presumably arising in the wall of the aorta (48).

Primary yolk sac erythropoiesis is responsible for the production of all primitive red blood cells and the first definitive cell population. Primary yolk sac erythropoiesis lacks, however, a stem cell compartment with the capacity for self-renewal (53, 99); thus the final size of the primitive and first definitive red blood cell population is arising from primary yolk sac erythropoiesis determined by the number of cells initially committed to the erythroid pathway.

An unsettled question is the molecular mechanism controlling the switch in hemoglobins. Work in this area has primarily concentrated on nuclear factors that control the stage-specific expression of the globin genes. The principal alignment of the genes coding for the α-chains follows their sequential expression during development (58). A specific switching activity has not yet been demonstrated. Available evidence indicates that the regulation of globin gene expression involves *1*) nuclear transcription factors that confer the ability for constitutive expression of globin genes in general, *2*) stage-specific selectors responsible for the choice of globin type expressed at a given time, and *3*) possibly *trans*-acting cytoplasmic factors having a pronounced influence on globin chain expression (85, 102, 108). With regard to epigenetic influences, there is some evidence that in avian embryos the timing of the switch can be altered in response to changes in ambient oxygen pressure (17).

Furthermore, the correct timing process of the

switch from primitive to definitive erythropoiesis apparently depends on a minimum of structural integrity of the yolk sac environment; although even parts of yolk sac kept in organ culture produce the correct sequential pattern of erythropoiesis, i.e., first primitive red blood cells and then the definitive red blood cells, dispersion of yolk sac tissue in suspension culture invariably causes both cell types and hemoglobins to develop at the same time (68, 69, 138). This strongly suggests that the micromilieu of the yolk sac and short-range interactions between cells are important for the timing process (138).

Unlike red blood cells of adult animals that, excepting pathological states, enter the circulation as postmitotic cells, the primitive red blood cells enter the circulation as immature erythroblasts and complete their terminal differentiation, including several mitoses, inside the circulation. This is especially pertinent for those cells that enter the circulation directly after the fusion of the circulatory system; these cells mature as a cohort and are an excellent model for the study of ongoing differentiation processes. There is some evidence that primitive red blood cells are also recruited from extravascular erythropoietic sites in the yolk sac from *day 4* of development onward. Support for this comes from the study of the cell population kinetics that demonstrate that the rate of growth of the red blood cell population after *day 4* of development cannot be explained by the mitotic frequency of circulating red blood cells (43).

Thus immature primitive red blood cells, unlike all other red blood cells, have functional characteristics that allow them to proliferate rapidly and at the same time carry out their oxygen transport function in an adequate way.

II. BOUNDARY CONDITIONS FOR BLOOD OXYGEN TRANSPORT IN THE EARLY EMBRYO

A. Basic Structure of Extra- and Intraembryonic Circulatory System

In the chick embryo, blood islands appear toward the end of the first day of incubation. The endothelial cells form lumina enclosing primitive blood corpuscles and anastomose with neighoring blood islands, thus forming the primary extraembryonic blood vessel system. Concomitantly, primary endothelial tubes develop in the intraembryonic tissue. When these vessels join the extraembryonic vessels, continuity of circulation is established.

In the chick embryo, blood cells begin to circulate at *day 2* of incubation. The driving force for blood circulation is generated by the primitive U-shaped heart. It pumps the blood through the short ventral aorta and the aortic arches into the dorsal aorta and the carotid arteries. From the dorsal aorta arise the omphalomesenteric arteries and the umbilical arteries that supply the vitelline and the allantoic circulation, respectively. The venous blood, which is returned by the extraembryonic and intraembryonic veins, is collected in the sinus venosus before it enters the heart at the atrial side.

The vitelline circulation supplies the embryonic tissue with food material absorbed from the yolk and up to about *day 6* of incubation provides for the respiratory gas exchange with the environment. When the allantoic sac develops and fuses with the chorion (around *day 8*) respiratory function is successively transferred to the blood vessels of the newly formed chorioallantoic membrane. These stages of development are, however, not considered in this paper.

The vascularized area of the yolk sac is roughly circular. It spreads rapidly in a radial direction and reaches a diameter of ~4 cm at *day 4*. The major blood vessels are the paired lateral vitelline arteries and the paired lateral vitelline veins, as well as the unpaired anterior and posterior vitelline veins. The area vasculosa is enclosed by the terminal sinus that collects blood from distal capillaries and that is drained by the anterior and posterior vitelline veins and by branches of the lateral vitelline veins.

The branching pattern of arteries and veins is dichotomous, thus giving rise to a treelike topology, whereas the capillaries, in particular near the terminal sinus, form a frequently meshed network.

In contrast to the adult, both the circulatory system that takes up oxygen from the environment (extraembryonic) and the circulation supplying the tissue (intraembryonic) share the same passage through the heart. This parallel arrangement of blood vessel loops acts as an arterial-to-venous shunt, setting special conditions for oxygen transport to tissue that are considered particularly in the following section.

B. Determination of pH and Carbon Dioxide and Oxygen Partial Pressures in Vitelline and Intraembryonic Circulation

1. Technique and technical problems

Oxygen transport and acid-base status have been documented extensively from *day 8* of incubation (28, 50, 126) but not in earlier stages of development, since conventional techniques of acid-base analysis cannot be applied due to the small sample volumes. Instead these parameters have to be determined with microelectrodes.

The measurement itself can only be performed after partial removal of the eggshell and membranes, thereby eliminating part of the diffusion resistance, creating the possible error that too high P_{O_2} values and too low P_{CO_2} values are measured. As shown in section II*E*, the major diffusion resistance is located inside the area vasculosa itself, which substantially reduces the magnitude of the error.

Lomholt (86) was the first to measure P_{O_2} in the early embryo. He removed the eggshell and the outer shell membrane just above the air space and inserted

the microelectrode through the intact inner shell membrane and recorded P_{O_2} profiles in the area adjacent to the embryo proper. Although this method leaves the extraembryonic diffusion barrier partially intact, it has, due to the opaqueness of the inner shell membrane, one major disadvantage; it is not possible to determine the exact location of the site of measurement. Nevertheless, the major finding from this study was that the P_{O_2} values close to the embryo were exceedingly low at *day 3* of development and sometimes near zero.

The most extensive study of P_{O_2}, pH, and P_{CO_2} inside the embryo and attached extraembryonic circulation has used a slightly different approach, insofar as no attempt was made to preserve the integrity of the outer diffusion barrier, i.e., eggshell and shell membranes. This disadvantage of removing the diffusion barrier was taken into account to be able to make measurements at precisely located sites. Aside from a possible reduction of the diffusion resistance, a second source of error to be taken into account is a change in the oxygen consumption under conditions where the eggshell is removed. This error, however, can be excluded, since Hoiby et al. (72) found that removal of the eggshell had no immediate effect on the oxygen uptake. Finally, one has to consider the error of the P_{O_2} measurements that is introduced by the aerobic metabolism of the red blood cells and that could cause a substantial difference between the end-capillary P_{O_2} and the P_{O_2} measured in the anterior and posterior vitelline veins.

Calculations based on the data of Grima et al. (65) have shown that the maximum error introduced is in the range of 1 Torr/s; given the fact that transit times from capillaries to the vitelline vein are only a few seconds (Meuer, unpublished observation), this does not cause a substantial deviation of vitelline vein P_{O_2} versus end-capillary P_{O_2} (92).

Three types of electrodes have been successfully used for the blood gas measurements. Recessed-tip microelectrodes of the Whalen type were used to determine P_{O_2} (92). Tissue and blood pH were determined with microelectrodes that use a liquid exchanger for the detection of protons (1). Microelectrodes for determination of P_{CO_2} were constructed according to the description of Bomsztyk and Calalb (26) but were adapted to be able to measure P_{CO_2} at values of <20 Torr (97). A survey of the extra- and intraembryonic sites chosen for the measurements of pH, P_{O_2}, and P_{CO_2} is given in Figs. 1 and 2.

2. Oxygen partial pressure in embryonic blood

The mean values of the P_{O_2} in extraembryonic and intraembryonic blood vessels are summarized in Table 2 (92). In the 4-day-old embryo the P_{O_2} of the extraembryonic blood vessels ranges from 45.4 Torr in the vitelline arteries to 80.9 Torr in the anterior and posterior vitelline veins. These numbers increase significantly by *day 6* of incubation.

Although the eggshell and the shell membranes had been removed before the measurements, the highest P_{O_2} value determined in the anterior and posterior vitelline veins at *day 6* was only about two-thirds of the environmental P_{O_2} (mean value 149 Torr). These substantial P_{O_2} differences between environment and blood suggest that the oxygen uptake by the embryo is diffusion limited. There is also a significant difference between the P_{O_2} values measured in the anterior and posterior vitelline veins and the lateral vitelline veins. In the lateral vitelline veins P_{O_2} is significantly lower than in the anterior and posterior vitelline veins. This could be due either to a difference in the diffusion resistance or to morphological and/or rheological heterogeneity of the capillary network that effects the amount of admixture of blood with low oxygen saturation from vitelline arteries. This point is referred to in section II*E*.

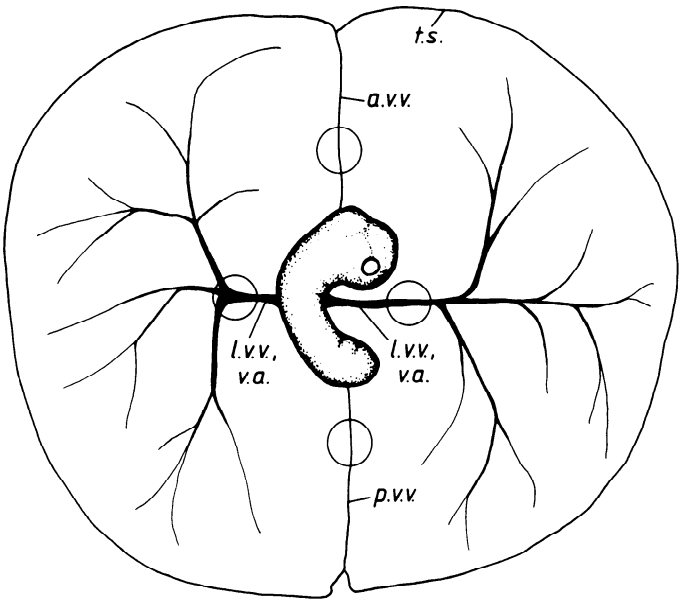

FIG. 1. Schematic of principal vessels of yolk sac vascular system in early chick embryo. O_2 partial pressure (P_{O_2}), pH, and CO_2 partial pressure (P_{CO_2}) were measured in lateral vitelline veins (lvv), vitelline arteries (va) (which are mostly covered by lateral veins and therefore not drawn separately), and in anterior and posterior vitelline veins (avv and pvv), which drain terminal sinus (ts). Sites of measurement are marked by circles.

The mean P_{O_2} in the dorsal aorta is nearly the same as in the vitelline arteries. This is to be expected, because the vitelline arteries originate from the dorsal aorta.

Mean venous P_{O_2} values in the intraembryonic circulation range from 16.1 to 34.6 Torr, displaying a gradient from cranial to caudal. Because at this stage of development the caudal region is less vascularized than the head region (95), it seems that the lower P_{O_2} in the caudal veins may reflect a lower oxygen supply of the caudal region rather than higher oxygen consumption.

Because of the parallel arrangement of the intra- and extraembryonic circulation, the blood from the vitelline veins, which is rich in oxygen, is mixed with nearly desaturated intraembryonic venous blood before entering the intraembryonic circulation. Therefore the

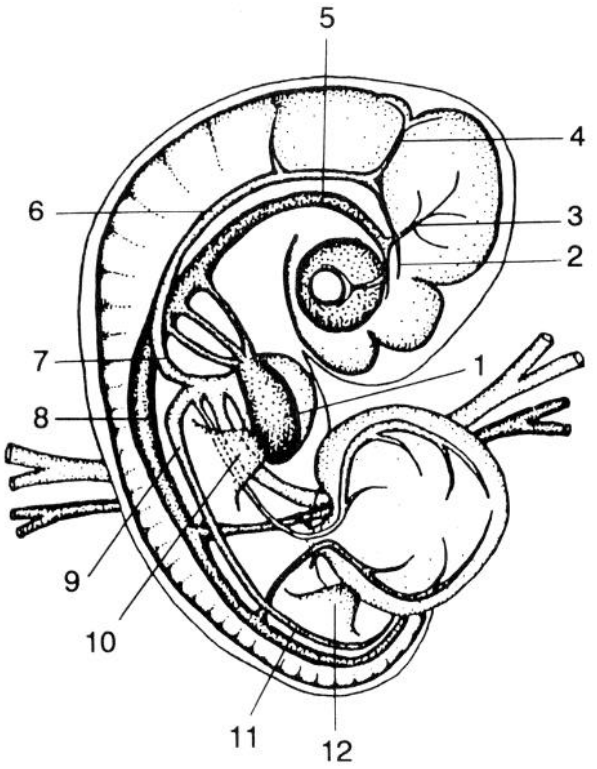

FIG. 2. Schematic of 4-day-old chick embryo. Approximate sites of P_{O_2} and pH measurements in intraembryonic blood vessels are indicated. *1*) heart, *2*) anterior cerebral vein, *3*) bigerminal artery, *4*) middle cerebral vein, *5*) internal carotid artery, *6*) internal jugular vein, *7*) anterior vena cava, *8*) dorsal aorta, *9*) posterior cardinal vein, *10*) wing bud, *11*) vein of tail, *12*) leg bud. [From Meuer and Tietke (97).]

P_{O_2} of the arterial blood that supplies the embryonic tissue with oxygen is substantially lower than the vitelline venous P_{O_2}. Mean arterial P_{O_2} values of <50 Torr were measured. Consequently, one must expect that the P_{O_2} in the embryonic tissue is also very low, which is confirmed by the measurements described in section II*C*.

3. Blood pH

The pH was measured in the lateral vitelline veins, in the vitelline artery, in the jugular vein, and in the heart (96; Table 3). In the first series of measurements the embryo was exposed to air. In a second series of measurements ambient carbon dioxide concentration was increased stepwise from 0 to 3% to evaluate the effect of hypercapnia on blood and interstitial pH.

The highest pH values were found in the vitelline veins (pH 8.0 at *day 4* and 7.89 at *day 6*). These high pH values are caused by the low P_{CO_2} of the vitelline blood, which can be extrapolated from the P_{CO_2} measurements in the air cell (82) of the early embryo. Direct measurements of blood P_{CO_2} (97) have confirmed these values.

The pH values measured in the vitelline arteries are ~0.2 pH unit lower than in the vitelline veins. Similar venous-to-arterial pH differences have been reported by Tazawa (124) and Tazawa et al. (127) for the blood of the chorioallantoic vein and artery from *day 10* onward through development. Because the vitelline arteries originate from the dorsal aorta, the pH in intraembryonic arteries should be close to the pH determined for the vitelline arteries.

The lowest pH is present in the intraembryonic veins. Mean values determined in the jugular vein were pH 7.64 and 7.42 at *days 4* and *6*, respectively.

The pH data obtained in the heart vary much more than the other blood pH values. This is probably due to incomplete mixing of intraembryonic venous blood of low pH with blood of the vitelline veins, which has the highest pH. This was demonstrated using dye injection

TABLE 2. *Mean P_{O_2} in vitelline and intraembryonic blood vessels of 4- and 6-day-old chick embryos*

Vessel Type	Mean P_{O_2}, Torr	
	Day 4	*Day 6*
Extraembryonic		
Vitelline arteries	45.4 ± 4.7	49.1 ± 6.3*
Lateral vitelline veins	63.7 ± 9.5	80.9 ± 11.9*
Anterior/posterior vitelline veins	80.9 ± 13.4	102.4 ± 16.5*
Intraembryonic		
Dorsal aorta	44.9 ± 6.9	48.2 ± 7.2
Internal carotid artery	41.3 ± 2.9	46.5 ± 3.4*
Bigerminal artery	41.6 ± 4.9	45.4 ± 4.9
Anterior cerebral vein		34.6 ± 4.8
Middle cerebral vein	33.9 ± 7.0	28.8 ± 8.2
Internal jugular vein	29.4 ± 7.2	32.2 ± 4.8
Anterior vena cava	26.0 ± 2.2	29.6 ± 7.1
Vein of wing bud		28.3 ± 6.9
Posterior cardinal vein	21.8 ± 5.6	25.3 ± 4.8
Vein of leg bud		20.9 ± 8.0
Vein of tail	16.1 ± 4.5	

Values are means ± SD. Mean ambient O_2 partial pressure (P_{O_2}) during the measurements was 149 Torr. * Significant difference ($P < 0.05$) between *days 4* and *6*.

TABLE 3. *Change in blood pH, P_{CO_2}, and HCO_3^- concentration in chick embryos between days 4 and 17*

Location	*Day 4*	*Day 6*	*Day 10*	*Day 17*
Extraembryonic vein				
pH	8.00 ± 0.05	7.89 ± 0.08	7.636	7.461
P_{CO_2}, Torr	4.2 ± 0.49	7.0 ± 0.9	11.8	33.0
$[HCO_3^-]$, mmol/l	12.3	15.3	12.4	23.3
Extraembryonic artery				
pH	7.80 ± 0.04	7.66 ± 0.07	7.430	7.305
P_{CO_2}, Torr	6.9 ± 0.4	10.6 ± 1.0	18.2	44.5
$[HCO_3^-]$, mmol/l	11.9	12.9	11.9	21.8
Jugular vein				
pH	7.64 ± 0.07	7.42 ± 0.06		
Heart				
pH	7.68 ± 0.10	7.67 ± 0.17		

Values are means or means ± SD. At *days 4* and *6*, values refer to blood of vitelline circulation (96, 97); values for *days 10* and *17* refer to chorioallantoic blood (27). $[HCO_3^-]$ was calculated using the Henderson-Hasselbach equation ($pK' = 6.1$). P_{CO_2}, CO_2 partial pressure.

into different vitelline veins, which indicated that the flow through the heart tube is laminar and that the preferential direction of the dye stream varies with the site of injection (136). Therefore the measured pH of the blood in the heart will depend on the position of the microelectrode tip within the lumen of the heart.

Previous measurements of the blood pH of the chorioallantoic vein have shown that there is a progressive decrease of the blood pH with increasing age of the embryo (127). As the increase in aerobic metabolic rate is not accompanied by a corresponding increase of the diffusion capacity, it results in accumulation of carbon dioxide (and in consequence a reduction of pH), as well as in a reduction of the P_{O_2} in the blood. The present results combined with those of Tazawa et al. (127) show that between *days 4* and *17* of development there is a decrease in the pH of the arterialized blood leaving the gas exchange area of >0.5 pH unit from ~8.0 at *day 4* to <7.5 in the prehatching period.

The measured pH differences between the venous and arterial parts of the early embryonic circulation are also quite substantial at both *days 4* and *6*. The maximum difference experimentally recorded amounts to 0.47 pH unit (7.89 in the vitelline vein vs. 7.42 in the jugular vein at *day 6*). Therefore the Bohr effect (i.e., the pH-dependent decrease of the oxygen affinity, with increasing concentration of protons) plays an important role in the oxygen supply of the embryo. The very alkaline pH in the vitelline veins is essential for a high oxygen saturation of the arterialized blood, and the low pH in intraembryonic veins allows a desaturation of the blood at a fairly high P_{O_2} (12% oxygen saturation at a P_{O_2} of 32 Torr in the jugular vein at *day 6*), facilitating oxygen transfer to the embryonic tissues.

An increase of the environmental P_{CO_2} is followed by a substantial fall of blood pH by as much as 0.3–0.5 pH unit with 3% CO_2 (96), which interferes directly with oxygen uptake in early development. The high pH values found in the vitelline vein under physiological conditions are necessary for sufficient oxygenation of the embryonic blood. The arteriovenous difference in oxygen saturation between vitelline vein and artery is 52% at *day 4* and 70% at *day 6*. In the presence of 8 Torr P_{CO_2} the arteriovenous difference in oxygen saturation is reduced to 31% at *day 4* and 58% at *day 6* (96). This substantial reduction reflects the fact that the cooperativity of oxygen binding to embryonic chick hemoglobin is much higher than to adult hemoglobin (16, 82), with maximum n values, which are a measure of that binding, of ~7 in the upper saturation range. Even though this behavior is very advantageous to counter the effects of the central venous shunt present in the embryonic heart, inasmuch as the ensuing fall in P_{O_2} is minimized (16, 82), it also leads to a considerable decrease of the oxygen saturation of the vitelline vein if only a moderate decrease of the oxygen affinity is encountered. Thus "acidosis" of any kind can jeopardize oxygen supply to the early embryo, particularly since at this stage the embryo is unable to counter hypoxia by an increase in red blood cell mass (17) or cardiac output (64).

4. Blood carbon dioxide partial pressure and acid-base status

The mean blood P_{CO_2} values (97) determined under normoxic conditions at *days 4–6* of development range between 4.2 and 10.6 Torr. These data are compatible with the previously measured low P_{CO_2} in the air space of the egg [2–3 Torr (82)] and with the high blood pH values.

From measurements in elevated environmental carbon dioxide concentrations the in ovo relationship between log P_{CO_2} and pH has been obtained (Fig. 3).

Estimates of the carbon dioxide dissociation curves of the blood were obtained from the sum of the bicarbonate concentration and the respective concentration of physically dissolved carbon dioxide (Fig. 4). Carbon dioxide bound to hemoglobin was neglected, because the hemoglobin concentration is low [20 g/l at *day 4* and 35 g/l at *day 6* (17, 111)]. For the same reason, carbon dioxide concentration differences between oxygenated and deoxygenated blood are negligible. This is also supported by the data of Tazawa (126), which demonstrated that even at *day 10* the haldane effect was insignificant.

Standard bicarbonate concentrations determined from the carbon dioxide dissociation curves were 29.4 and 24.4 mmol/l at 4 and 6 days, respectively. The respective buffer values obtained from the bicarbonate versus pH relationship were 28.8 and 21.4 mmol/l.

Between *days 4* and *6* pH decreases from 8.0 to 7.83 in the vitelline veins and from 7.8 to 7.66 in the vitelline arteries. These changes are not only caused by the rise in P_{CO_2} but also by a metabolic acidification, which is indicated by the drop in the standard bicarbonate concentration. This tendency seems to continue to *day 10*, since at this stage the acid-base status is characterized by increased blood P_{CO_2} values and negative base excess (124).

C. Determination of Oxygen Partial Pressure and pH in Embryonic Tissue

1. Oxygen partial pressure in embryonic tissue

Tissue P_{O_2} was measured in seven different embryonic locations (brain, lens, neck, wing bud, back, leg bud, tail) using oxygen microelectrodes (95). The electrodes were inserted randomly within the natural limits of the respective location at a depth of ~1 mm. The mean and median values for head (brain, lens, neck) and trunk (wing bud, back, leg bud, tail) are summarized in Table 4.

The P_{O_2} data exhibit a large scatter between the sites of measurement. Furthermore, in each site of measurement the readings range between zero and arterial P_{O_2} values. The frequency distributions are asymmetric, with maximum frequency near zero P_{O_2} (Fig. 5). The highest median value was found in the head at *day 4* (8.4 Torr). The median decreases with embryonic age and

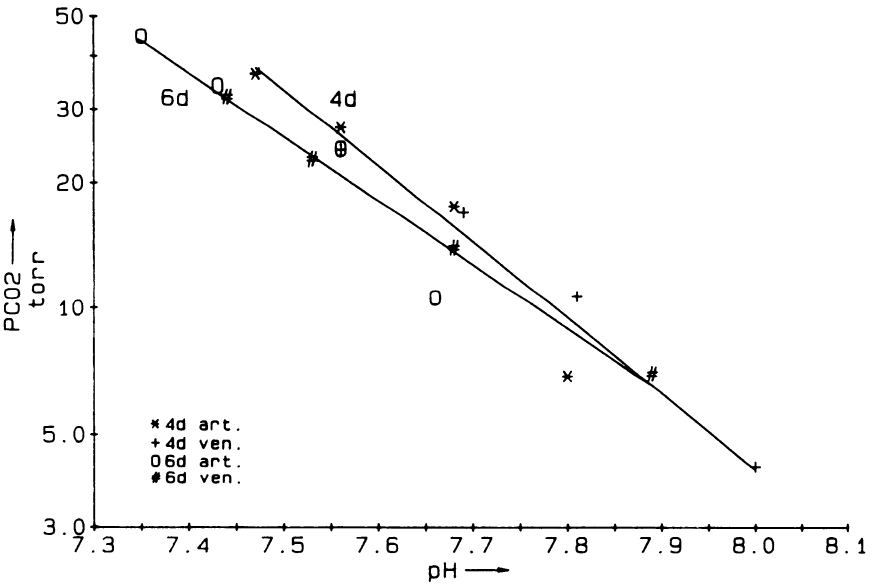

FIG. 3. CO_2 titration curves of blood of vitelline arteries (art) and veins (ven) of 4- and 6-day-old chick embryos obtained by stepwise increase of ambient CO_2 concentration. Symbols, measured mean values; solid lines, calculated by linear regression (4 days (4d): $\log PCO_2 = 15.1 - 1.81 \times pH$; 6 days (6d): $\log PCO_2 = 14.3 - 1.72 \times pH$). [From Meuer and Tietke (97).]

each day is significantly lower in the trunk than in the head.

A wide range in the tissue P_{O_2} data was expected, since because of the random selection of the site of measurement the electrodes were located at different distances from a blood vessel. However, it is remarkable that despite these uncertainties, those P_{O_2} values that appear with the highest frequency (i.e., 40–60% of all values) are found in the lowest P_{O_2} group (0–5 Torr; Fig. 5).

The P_{O_2} gradient between capillary blood and tissue determines the rate of oxygen diffusion from blood to tissue. For a given blood P_{O_2} this gradient is defined by the tissue P_{O_2}. Therefore the extremely low P_{O_2} values found in the embryonic tissue indicate that the embryo extracts as much oxygen from the capillary blood as possible. This implies that even under conditions of normal oxygen supply the utilization of available oxygen is maximized. This suggests that the aerobic metabolic rate is indeed limited by the oxygen availability during early development. Consequently, a diminished oxygen supply due to lowering blood P_{O_2} will directly affect the growth rate of the embryo. On the other hand, increases in blood P_{O_2} will accelerate growth. This is in accord with experimental observations (3).

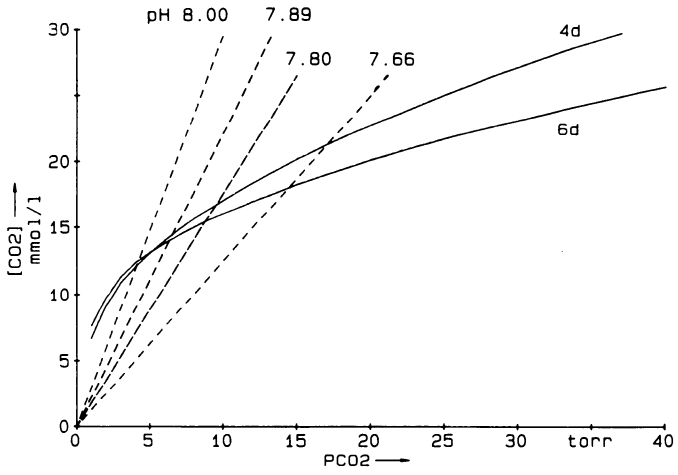

FIG. 4. Calculated CO_2 dissociation curves for chick embryonic blood at 4 and 6 days of incubation. Dashed lines, iso-pH curves. [From Meuer and Tietke (97).]

2. *Measurements of tissue pH*

The tissue pH values display a significant craniocaudal gradient at *day 4* with pH falling from 7.68 (cranial) to as low as 7.47 in the caudal region (96; Table 5). At *day 6*, however, this gradient no longer exists, and values range from pH 7.52 to pH 7.61.

Interstitial pH has been measured in one other study by Gillespie and McHanwell (62), who investigated early developmental stages (up to 22 somites, which corresponds to ~53 h of incubation). They reported interstitial pH values of 7.8–8.1 at 34–35°C, and they also found a sizeable craniocaudal pH gradient.

Our measured interstitial pH values are lower, and

TABLE 4. *Tissue P_{O_2} measured with oxygen microelectrodes in different locations of chick embryo at 4, 5, and 6 days of incubation*

Location	P_{O_2}, Torr		
	Day 4	*Day 5*	*Day 6*
Head			
Mean	11.4	10.8	8.4
Median	8.4	6.4	4.2
Trunk			
Mean	8.3	9.3	6.6
Median	4.8	3.6	2.6

At each day the differences between head and trunk are significant (U-test, $P < 0.05$).

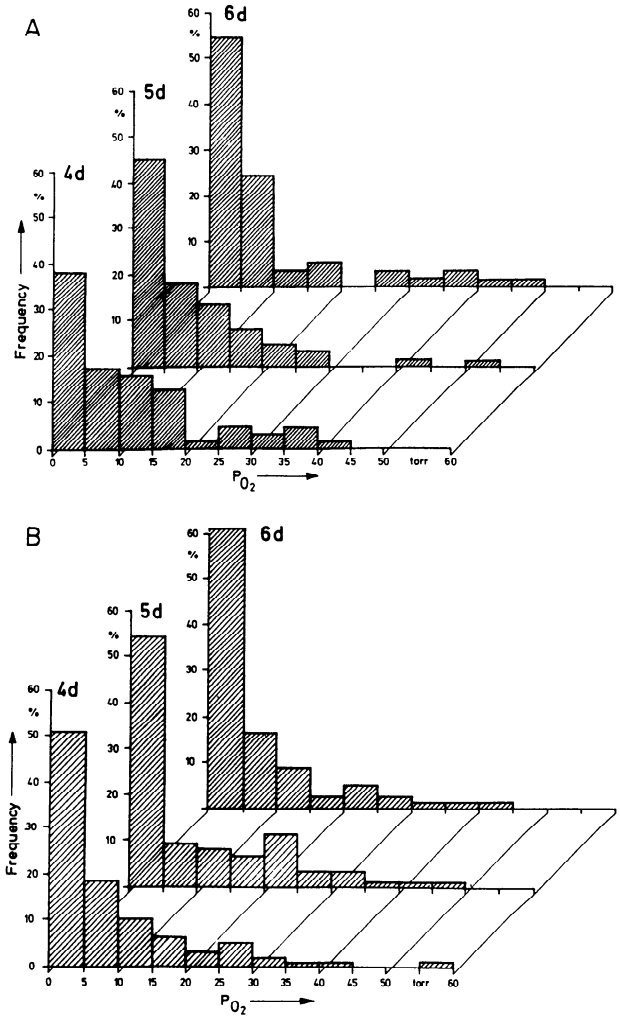

FIG. 5. Frequency distribution of tissue P_{O_2} measurements in head (brain, lens, neck; *A*) and in trunk (tail, back, wing, bud, leg bud; *B*) at 4, 5, and 6 days of incubation.

a craniocaudal gradient is apparent only at *day 4*. Therefore the existing results suggest that the highest interstitial pH is present around *day 2* of development.

It is perhaps no coincidence that there is also a craniocaudal gradient for P_{O_2} in the intraembryonic veins (92). In the 4-day-old embryo the P_{O_2} of the veins decreases from cranial to caudal, but at *day 6* this gradient is no longer apparent. The presence of pH and P_{O_2} gradients presumably reflects significant regional differences in oxygen supply and/or utilization, which have also been reported for very early development (106). This is also supported by the finding that between *days 4* and *6* the dry mass of the head region increases from 34% of total embryonic dry mass to 48% (95), which indicates a substantial rise of the metabolic rate in the cranial region. Judging by the measured pH values the collapse of the craniocaudal pH gradient between *days 4* and *6* seems predominantly due to a progressive acidification of the cranial region.

An increase in the ambient P_{CO_2} causes an immediate fall of the tissue pH similar to the pH decrease observed in the blood. With 3% ambient carbon dioxide, tissue pH decreases by ~0.4 unit (96).

It is known that incubation in elevated P_{CO_2} leads to increased mortality and malformations in young embryos even when a normal P_{O_2} is maintained (cf. Ref. 112). One reason for this phenomenon may be the effect of decreased pH on cell proliferation.

Several studies have demonstrated a permissive role of cell pH in the promotion of cell division (cf. Ref. 66) or a direct increase of cell pH after stimulation of cells with mitogens. Furthermore, it has been demonstrated that the cellular binding of insulin and insulin-like growth factors, which are present early in avian development, is maximal at alkaline pH values and is substantially decreased by an increase of the proton concentration. (6). A pH-dependent reduction of growth factor binding may also lead to arrested or disturbed development.

D. Dependence of Red Blood Cell pH on Extracellular pH

The pH values for embryonic blood cover a range of nearly 0.5 pH unit between various parts of the embryonic circulation at *day 4* or *6*. The pH of the arterialized blood leaving the gas exchanger via vitelline vein/chorioallantoic vein decreases from pH 8.0 at *day 4* (96) to ~7.46 at *day 18* (125).

This raises the question as to how the red blood cell controls its cell pH in the face of the constantly changing external conditions. Red blood cell pH plays a crucial role in the regulation of hemoglobin function. In the first place protons by themselves are allosteric effectors of the hemoglobin molecule (namely the Bohr effect). Second, the binding of organic phosphates to hemoglobin (they are the most important allosteric effectors) is profoundly pH dependent, decreasing with increasing pH (21-23), and, finally, red blood cell pH affects the metabolic pathways connected with the synthesis of organic phosphate cofactors, especially 2,3-diphosphoglyceric acid (2,3-DPG) (cf. Ref. 32).

It is not clear if the ongoing proliferative activity of circulating primitive erythroblasts requires specific adjustments of cell pH during the cell cycle.

In adult red blood cells, chloride, bicarbonate, and proton distributions are in equilibrium because of the

TABLE 5. *Mean tissue pH values of 4- and 6-day-old chick embryos*

Location	*Day 4*	*Day 6*
1	7.68 ± 0.15	7.60 ± 0.14
2	7.63 ± 0.14	7.56 ± 0.13
3	7.51 ± 0.09	7.61 ± 0.09
4	7.47 ± 0.13	7.57 ± 0.06
5	7.50 ± 0.13	7.52 ± 0.02

Locations of measurements were spaced evenly in craniocaudal direction along dorsolateral part of the embryo (*location 1*, head; *location 5*, tail). Depth of insertion of microelectrode was ~1 mm.

presence of the band 3 anion exchanger (cf. Ref. 71). Rapid readjustment of pH after perturbations is achieved via the Jacobs-Stewart cycle, which in addition to band 3 requires the presence of intracellular carbonic anhydrase to be effective under physiological conditions.

At equilibrium the distribution ratios of Cl^-, HCO_3^-, and OH^- calculated from red blood cell and plasma concentrations are such that $r_{Cl} = r_{HCO_3} = r_{OH}$, and in essence red blood cell pH can be calculated from the chloride distribution ratio, which is primarily dependent on the net charge and osmotic activity of the impermeant anions inside the red blood cell, i.e., phenomenologically it follows the Donnan distribution.

Because the electrogenic conductance of chloride exceeds that of other ions, its distribution also sets the membrane potential (83). If one compares the values of red blood cell pH of various species of birds and mammals under resting conditions (i.e., with arterial pH between pH 7.4 and 7.5), there is remarkably little divergence; most cell pH values are in the region of 7.2 (7, 10). The measured values for the membrane potential of adult human and chick red blood cells are also very similar (−10 to −15 mV) (80, 83).

In the early chick embryo, carbonic anhydrase activities of embryonic red blood cells have been measured from *day 4* of incubation through hatching (15). At this stage the activity of carbonic anhydrase is <10% of the adult value, and even lower activities occur during midterm incubation (15). Electrophoretic analysis of red blood cell membranes of chick embryos first detected band 3 in small amounts at *day 3* (34, 35), which then rapidly increased during the succeeding days, reaching adult values by *day 6* of development. Thus both components required for rapid pH equilibrium across the red blood cell membrane are present early in development.

Direct measurements of red blood cell pH have been carried out at *days 4* and *6* of development. The results demonstrated that under physiological conditions, i.e., extracellular pH = 7.9, the cell pH is ~0.6 unit lower than the plasma pH, whereas the pH difference calculated from the chloride distribution was much less, i.e., only 0.3 pH unit (12, 16, 121).

Moreover, the distribution of chloride across the red blood cell membrane could be altered in the expected manner when external chloride was replaced by impermeable anions, whereas the distribution of protons was only slightly affected (12, 121). Subsequent measurements of the membrane potential of primitive red blood cells from 4- and 6-day-old chick embryos demonstrated that the membrane potential is apparently dominated by a proton conductance. Consequently the membrane potential is much more negative than observed for adult red blood cells [about −38 to −44 mV at *days 6* and *4*, respectively (59)]. These results imply that the heteroexchange (bicarbonate-chloride exchange) mediated by band 3 protein is impaired, since with a normal mode of bicarbonate-chloride exchange a disequilibrium between protons and chloride ions cannot be maintained. Indeed, preliminary measurements of the kinetic properties of band 3 in primitive embryonic red blood cells indicate that bicarbonate transfer is much slower in early development compared with adult red blood cells, whereas chloride transfer is much less affected (U. Sieger, J. Brahm, and R. Baumann, unpublished observations). Determinations of the membrane potential and cell pH as functions of developmental age demonstrate that equilibrium of chloride distribution, membrane potential, and proton distribution are achieved by the end of the second week of incubation, presumably due to normalization of band 3 function and a decreased importance of the proton conductance (8, 12). Because all of these measurements were carried out in whole blood, it is possible that substantial differences exist between the various red blood cell subpopulations.

Nevertheless, the continuous change of the proton distribution ratio that causes a decrease of the pH unit difference between red blood cell and plasma from 0.6 to ≈0.2 pH between *days 6* and *16* of development helps to maintain cell pH nearly constant, i.e., between 7.2 and 7.3, despite the continuing acidification of the extracellular compartment.

Several questions arise from these findings regarding *1*) the nature of the proton conductance of primitive red blood cells and its regulation, *2*) the kind of mechanism responsible for the altered bicarbonate transport characteristics of band 3 protein during early development, and *3*) the transport mechanisms that keep red blood cell chloride concentration in primitive red blood cells above electrochemical equilibrium. In conclusion, it can be said that the unusual blood acid-base conditions under which early embryonic red blood cells have to carry out their function are mirrored by a red blood cell pH regulation that is completely different from the one present in adult red blood cells.

E. Assessment of Diffusion Conditions for Oxygen Transfer

Diffusion plays an important role for the respiratory gas exchange between embryo and environment. After the egg is laid, the embryo is directly exposed to the gas partial pressures in the ambient atmosphere that are ~150 Torr for oxygen at sea level and close to 0 Torr for carbon dioxide. The gas fluxes between embryo and environment depend on *1*) the gas partial pressures in the blood of the extraembryonic circulation, *2*) the effective gas exchange area, and *3*) the thickness and diffusive properties of the material separating the red blood cells from the environment. In the early embryonic stages when respiratory gas exchange takes place via the blood vessels of the yolk sac membrane, the diffusion pathway is mainly formed by the eggshell, the outer and inner shell membrane, and a layer of egg albumen on top of the area vasculosa.

A large number of publications deal with the morphology and diffusive properties of the eggshell and the shell membranes (e.g., see Refs. 25, 79, 81, 103, 107, 119,

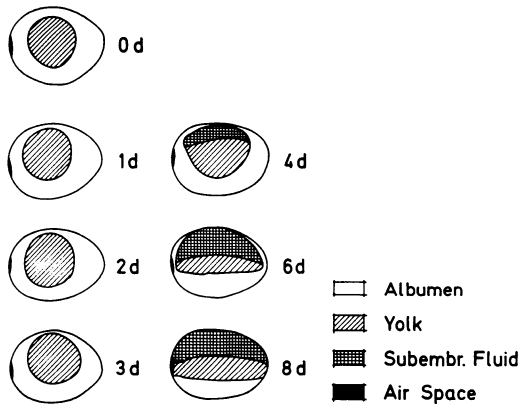

FIG. 6. Position and shape of yolk within egg during incubation drawn from cross sections of frozen eggs. Subembryonic fluid was not distinguishable from yolk before *day 4*.

128–132, 134). Mentioning all would be beyond the scope of this review.

Eggshell and outer shell membrane exhibit a low resistance to gas diffusion, since the pores of the eggshell as well as the space between the fibers of the outer membrane are gas filled. The inner shell membrane is moist at the onset of incubation so that gas diffusion through it can be treated as diffusion through an aqueous solution. However, the water content of the inner membrane decreases rapidly between *days 2* and *6* (81, 119), and consequently the resistance to gas diffusion is reduced accordingly. Thus Kayar et al. (79) determined the oxygen permeability of the eggshell and shell membranes and found a mean value of 0.12×10^{-6} $ml \cdot s^{-1} \cdot cm^{-2} \cdot Torr^{-1}$ at *day 3* that increased to 0.68×10^{-6} $ml \cdot s^{-1} \cdot cm^{-2} \cdot Torr^{-1}$ by *day 6*.

The resistance caused by the albumen layer on top of the area vasculosa is also subject to change. Up to about *day 6* of incubation there is no tight connection between the embryo and the inner shell membrane, which alone would guarantee an even thickness of the albumen layer. Close apposition of the area vasculosa to the inner shell membrane is caused by movement of egg yolk during the first week of incubation (94).

The basis for the movement of egg yolk is buoyancy, caused by different densities of albumen and yolk; there is also evidence for biochemical processes weakening the gel structure of the thick albumen (94).

As a result of these forces, the yolk sphere, which is initially located in the center of the egg, moves to the upper pole and in its upper part approaches the shape of the egg shell (Fig. 6). As a consequence of the change in the yolk position and shape, the blood vessels of the yolk sac membrane come into close contact with the inner shell membrane, which facilitates the gas exchange with the environment. The distance between blood capillaries and inner shell membrane has not been directly measured, but estimates range between 20 and 30 μm at *day 4* (109).

From *day 2* of incubation onward the area vasculosa of the yolk sac spreads along the yolk sac surface. The shape of the vascularized area is circular, with a diameter of ~27 mm at *day 3*, increasing to 69 mm by *day 6*. The increase of the size of the area enclosed by the terminal sinus is listed in Table 6. The area covered by the embryo is <5% of the area surrounded by the terminal sinus (estimated from photographs; Meuer, unpublished observations). Therefore the data given in Table 6 are about equal to the vascularized area involved in respiratory gas exchange.

TABLE 6. *Area of yolk sac surface included by terminal sinus of area vasculosa between days 3 and 6 of incubation*

	Day 3	*Day 4*	*Day 5*	*Day 6*
Mean area ± SD, mm^2	558 ± 102	1,389 ± 219	2,753 ± 398	3,750 ± 208
Sample size	17	28	20	20

Measurements were performed by the following method: in a transilluminated egg the area of shade of the terminal sinus was drawn on the eggshell with water-resistant ink. After boiling the egg the marked area of the eggshell including the membranes was cut off by a fraise, and the membranes were gently stripped from the eggshell. To obtain a plain surface the membranes were cut radially. Then the area was measured by a semi-automatic planimeter. (From H.-J. Meuer and C. Bertram, unpublished data.)

Mean capillary diameter in the area vasculosa is 23 μm at *day 4*. This parameter has been evaluated for randomly selected locations using fluorescent video microscopic techniques. To visualize the vessel lumen a solution of fluorescein-labeled dextran was injected into the circulation (101). The diameters were measured using digital video image analysis (93).

The capillary length per unit area has been determined in the same series of experiments described in section II*F*, also using video image analysis. Assuming that the projection plane of the capillaries is the effective area for respiratory gas exchange and using the measured value for the vascularized area, one calculates a total diffusion area of 216 mm^2 at *day 4* (Table 7).

With these data one can estimate the diffusion capacity of the area vasculosa for oxygen, i.e., the oxygen flux per unit pressure difference. The total oxygen up-

TABLE 7. *Parameters defining respiratory gas exchange between environment and extraembryonic circulation in a 4-day-old chick embryo*

Parameter	Sample Size	Value
Capillary diameter, μm	541	23.2 ± 6.0
Capillary length per unit area, mm/mm^2	20	6.69 ± 1.3
Capillary projection plane per unit area, mm^2/mm^2		0.155*
Vascularized area, mm^2	28	1,389 ± 219
Capillary gas exchange area, mm^2		216*

Values are means or means ± SD. * Calculated numbers. (From H.-J. Meuer and C. Bertram, unpublished data.)

TABLE 8. *Microcirculatory parameters of vitelline capillary system of 4-day-old chick embryos*

Parameter	Count	Mean ± SD	Median
Red blood cell velocity, μm/s	893	193	169
Segment length, μm	893	117 ± 66	
Length of arteriovenous channel, μm	802	434	386
Capillary transit time, s	821	3.27 ± 2.9	2.5

From H.-J. Meuer and C. Bertram, unpublished data.

take of the egg at *day 4* is 117 nl/s (61). The major part of this flux is utilized by the extraembryonic structures, whereas only 8% will serve for the oxygen supply of the embryo (Meuer, unpublished observations).

The driving force for the diffusion of oxygen can be estimated from the Po_2 measurements in the extraembryonic circulation. If it is assumed that the mean capillary Po_2 is the mean of the vitelline venous and vitelline arterial Po_2, the mean pressure gradient between ambient air and capillary is calculated to be 95 Torr. With these data one arrives at a minimum estimate of the diffusion capacity of 0.0985 $nl \cdot s^{-1} \cdot Torr^{-1}$. It should be pointed out that this result is a very rough estimate calculated under the assumption that the effective gas exchange area is identical with the morphologically determined capillary area. In section II*F*, however, we show that the capillary transit time varies to such an extent that there are, on the one hand, capillary pathways functioning like arteriovenous shunts and, on the other hand, arteriovenous connections with redundant length.

F. Oxygenation in Vitelline Microcirculation: Determination of Capillary Transit Times and Kinetics of Oxygenation

The capillaries of the vitelline microcirculation form a highly meshed network. The entrances and exits of this network are connected by a multitude of capillary segments, meshed by nodes of three or four vessels. Because of this anatomic pattern, a variety of pathways through the network exists, which differs with respect to the number of segments, total length, transit time, and red blood cell flux.

Capillary transit time and other microcirculatory parameters were determined by Meuer and Bertram (93) after injection of fluorescent (fluorescein)-labeled red blood cells into the embryonic circulation (Table 8). Measurements were carried out on video images using an on-line digital video analyzing system. To describe the rheologic properties of the arteriovenous channels segment length, red blood cell velocity and labeled cell flux were determined for each capillary segment of randomly selected areas of the yolk sac circulation. These data were used to calculate the respective parameters of each arteriovenous channel of the area under investigation.

1. Microcirculatory properties of vitelline capillary system

It seems to be a general characteristic of the vitelline capillary system that the values of each microcirculatory parameter are widely distributed. The arteriovenous channels are formed by between 1 and 13 capillary segments, the blood cell velocity ranges between 17 and 664 μm/s (mean value 193 μm/s), and the red blood cells require transit times between 0.09 and 17 s for passage through the capillary network (Table 8; Fig. 7).

2. Kinetics of oxygenation

To get an estimate of the transit time required for complete oxygenation of the red blood cells, the kinetics of blood oxygenation were calculated in the following way. The physiological hemoglobin oxygen dissociation curve of the capillary blood was determined from the dissociation curves for vitelline arterial and venous blood at actual pH (96; see sect. III*E*). Because we know the Po_2 and oxygen saturation at the arterial end of the capillaries and the ambient Po_2, the diffusive oxygen uptake of the blood was calculated by Bohr's integration method.

However, this calculation requires knowledge of the diffusive capacity of the capillary system. Because this number has not been measured, we took for a first trial the minimum estimate value of 0.0985 $nl \cdot s^{-1} \cdot Torr^{-1}$.

To check the calculation, the oxygen saturation of the mixed vitelline venous blood was computed and compared with the oxygen saturation determined from Po_2 measurements (92). Because blood flow and capillary transit time are heterogeniously distributed among the arteriovenous channels, the contribution of each channel to total vitelline venous oxygen saturation varies substantially. Furthermore, because of the nonlinear relationship between transit time and end-capillary oxygen saturation, the mean capillary transit time is an unsuitable figure to calculate the mean venous saturation. Therefore the oxygen flux of each arteriovenous

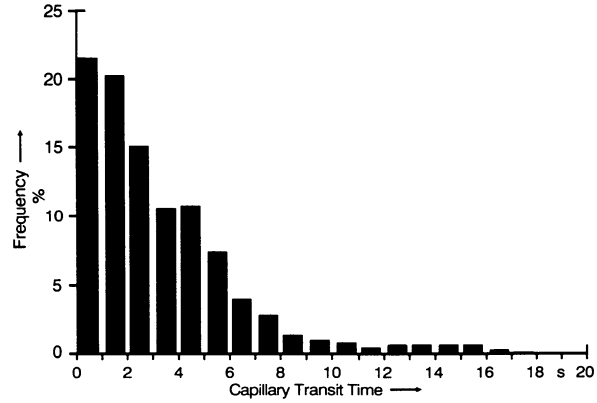

FIG. 7. Frequency distribution of capillary transit time in vitelline circulation of 4-day-old chick embryo.

channel was computed separately and then added for the total flux.

With the use of the value of 0.0985 nl · s^{-1} · $Torr^{-1}$ for the diffusion capacity, the calculated vitelline venous Po_2 was 52 Torr (55% oxygen saturation), whereas the measured Po_2 was 64 Torr (89% oxygen saturation). Further calculation demonstrated that a value of 89% for vitelline venous oxygen saturation requires a diffusion capacity of at least 0.538 nl · s^{-1} · $Torr^{-1}$.

For a diffusive capacity of 0.0985 nl · s^{-1} · $Torr^{-1}$, a capillary transit time of 8.3 s is required for 99% oxygen saturation of the blood. This number was reduced to 1.5 s when a value of 0.538 nl · s^{-1} · $Torr^{-1}$ was used in the calculation.

Because the measured capillary transit times of the red blood cell range up to 17 s (Fig. 7), there is a considerable number of arteriovenous channels of which only a part of the total length is used for oxygen uptake. In the remaining section of the capillary pathway where the transit time is longer than required, hemoglobin is completely oxygen saturated, i.e., the diffusion gradient is zero. For the calculation of the first estimate of the diffusive capacity (0.0985 nl · s^{-1} · $Torr^{-1}$) it was assumed that the mean capillary Po_2 is the mean value between arterial and venous Po_2. This obviously overestimates the mean diffusion gradient, since it was not taken into account that in a substantial portion of the capillary network the blood Po_2 is near ambient Po_2. Therefore we believe that a diffusive capacity of 0.538 nl · s^{-1} · $Torr^{-1}$ and the ensuing transit time for complete oxygenation of 1.5 s are fairly reasonable estimates.

Whatever the true oxygenation time may be, there is in every case also a substantial number of arteriovenous channels in which the transit time is shorter than required for complete oxygenation of the blood. This means that in the vitelline veins completely oxygenated blood is mixed with blood that is less oxygenated, thus reducing the vitelline venous oxygen saturation. This seems to be the main reason for the relatively low saturation of 89% determined for the vitelline venous blood at *day 4*.

G. *Effect of Central Shunt on Intraembryonic Oxygen Supply*

The highly oxygen-saturated blood of the vitelline veins is shunted with the intraembryonic venous blood before it supplies the embryonic tissue with oxygen. Because the intraembryonic venous blood is nearly completely desaturated (40) but contributes the major part of the blood flow into the heart, the mixed arterial oxygen saturation is substantially lower than the vitelline venous saturation. For the same reasons the arterial Po_2 is also reduced but not to the same extent, because the admixture of intraembryonic venous blood also affects the intraembryonic arterial pH. Direct measurements show that the arterial pH is ~0.2 unit lower than the pH in the vitelline veins (96; Fig. 8). Because of the Bohr effect, the arterial Po_2 is kept at a fairly high level.

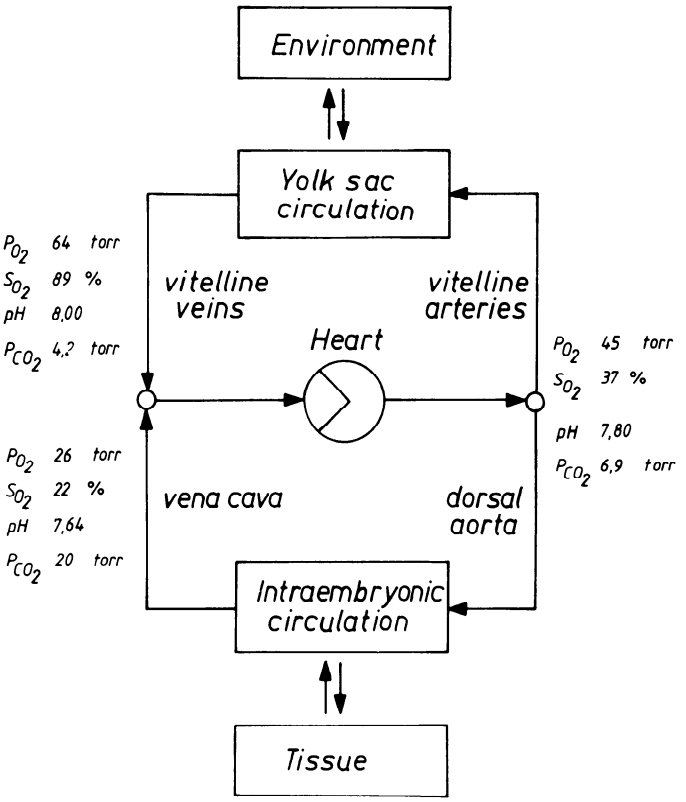

FIG. 8. Circulation block diagram and schematic of oxygen transport in early embryo with representative Po_2 values (Torr) measured in 4-day-old chick embryo and corresponding oxygen saturation (%) of blood obtained from oxygen hemoglobin curves (96). So_2, O_2 saturation.

This seems to be essential for a sufficient oxygen supply to the embryonic tissue, since the arterial Po_2 determines the diffusion gradient between blood and tissue as tissue Po_2 is near zero.

The parallel arrangement of the intra- and extraembryonic circulation makes it possible to calculate the relative contributions of intra- and extraembryonic blood flow to total flow using the values for the oxygen saturation in different parts of the circulation (92). Because the oxygen flow through the heart (total flow) is equal to the sum of intra- and extraembryonic venous oxygen flow, the ratio of intraembryonic venous and total blood flow ($\dot{q}_{iv}/\dot{q}_t$) is given by the equation

$$\dot{q}_{iv}/\dot{q}_t = (S_{ev} - S_a)/(S_{ev} - S_{iv})$$

where S_a, S_{ev}, and S_{iv} are the oxygen saturation of the blood in the arteries, the extraembryonic veins, and the intraembryonic veins, respectively. If we use the oxygen saturation in the lateral vitelline veins, the jugular vein (these vessels carry the main portion of venous blood flow), and the dorsal aorta, the percentage of blood flow from intraembryonic veins is calculated to be 77 and 84% of the total blood flow into the heart at *days 4* and *6*, respectively (92). This result has recently been confirmed by Cirotto and Arangi (40).

III. OXYGEN TRANSPORT FUNCTION OF EMBRYONIC BLOOD

The physiological function of hemoglobins and the effect of allosteric cofactors on their properties are commonly assessed through the measurement of the oxygen equilibrium curve from which two key functional parameters are extracted, namely, the oxygen pressure necessary for half saturation (P_{50}) as an indicator of hemoglobin oxygen affinity and the n value as a measure for the cooperativity of oxygen binding. Both can be determined with Hill's empirical formula for the oxygen-binding curve, $y = k(PO_2)^n$, where y is the fractional saturation and k is the dissociation constant. In its linearized version, $\log[y/(1 - y)] = \log k + n\log PO_2$, it usually gives a satisfactory plot for the experimental data of many hemoglobins except at the upper and lower limits of the oxygen-binding curve where $n = 1$. For most mammalian hemoglobins n is in the range of 2.6–3 for the middle part of the oxygen equilibrium curve. The theoretical maximum n value for a tetrameric protein like hemoglobin is $n = 4$. Thus n values >4 must result either from ligand-dependent association/dissociation phenomena of tetramer complexes or alternately from formation of a stable complex of several tetramers with intrinsically unchanged cooperativity where higher cooperativity can be induced via the action of allosteric effectors bound to this complex in a ligand-dependent fashion. For tetrameric hemoglobins, it has been shown that the n value is decreased whenever the conformational transition on ligand binding is impaired or when the conformational equilibrium between high- and low-affinity forms of hemoglobin is drastically shifted in favor of one conformation (cf. Ref. 32).

With regard to the hemoglobin affinity, it can be said that all known allosteric effectors of vertebrate hemoglobin, i.e., protons, carbon dioxide, inorganic anions such as chloride, as well as the organic phosphates [2,3-DPG, ATP, GTP, and inositol pentakisphosphate$^-$ (IP_5)], cause a reduction of the oxygen affinity (usually expressed as an increase of the P_{50}) due to preferential binding to deoxyhemoglobin. Detailed description of the molecular mechanisms involved can be found in references 23, 32, and 110.

A. In Vitro Studies of Isolated Specific Embryonic Hemoglobins: Effects of pH, Carbon Dioxide, and Adenosine Triphosphate

Studies of the functional properties of isolated embryonic hemoglobins of higher vertebrates are scarce. In addition to the technical difficulties arising from the need to sample a large amount of primitive red blood cells for quantitative separation, the embryonic hemoglobins are also characterized by a greater instability than the corresponding adult hemoglobins with regard to autoxidation, which requires that separations are carried out in the presence of carbon monoxide, which subsequently has to be removed. Additionally, the solubility of the embryonic hemoglobins at low temperature in media of low ionic strength is much lower than that of adult hemoglobins so that concentration of separated samples has to be carried out in media of moderate ionic strength, i.e., 0.1–0.15 M NaCl. We also observed that ultrafiltration under high pressure with the systems from various suppliers caused massive denaturation of the early embryonic hemoglobins, and therefore we have usually concentrated the hemoglobins using systems that rely on centrifugation to provide the necessary filtration pressure.

TABLE 9. *P_{50} and Bohr effect of embryonic and adult hemoglobin and 6-day embryonic blood*

	P_{50}, Torr				$\Delta\log P_{50}/\Delta pH$ (pH 6.7-7.2)	
	pH 6.7		pH 7.2			
	−ATP	+ATP	−ATP	+ATP	−ATP	+ATP
HbP	2.7	15.5	2.50	9.4	−0.05	−0.44
HbP′	3.10	14.8	2.80	7.3	−0.08	−0.61
HbM	2.3	15.5	1.90	6.8	−0.38	−0.72
HbE	6.0	50.1	3.4	20.4	−0.49	−0.78
Hb adult	12.4	87.3	7.4	35.62	−0.44	−0.78
6-Day embryonic blood	98.1		43.8		−0.70	

Conditions for hemoglobin solutions: 0.31 mmol Hb_4, total Cl^- 160 mmol, temperature = 37°C. ATP was added in 10-fold excess over Hb_4. O_2 half-saturation pressure (P_{50}) in embryonic blood is related to red blood cell pH.

Continuous registration of the oxygen equilibrium curves of either separated embryonic hemoglobins or red blood cell preparations has been possible by using thin-film methods that need only a few microliters to complete one oxygen equilibrium curve (cf. Ref. 82).

Storage of the isolated hemoglobins is best at temperatures of −80°C and below, and repeated freeze-thawing should be avoided, since it leads to artifacts. Because of the tendency for autoxidation the shelf life of carbon monoxide-free hemoglobin solutions at 4°C is again very limited; storage at this temperature should not extend the period necessary for completion of individual measurements.

The first systematic study of the properties of the major embryonic chick hemoglobins (HbP/P′) was carried out by Cirotto and Geraci (42), who found that the embryonic hemoglobins had a higher oxygen affinity than adult hemoglobin both in the absence and presence of organic phosphates. Their measurements were carried out at 20°C. Our subsequent studies were carried out at 37°C to allow better comparison with the oxygen-binding properties of embryonic blood (16). The relevant data are presented in Table 9. At pH 7.2 and 37°C the P_{50} of the four hemoglobin components is very high when only chloride (0.16 mol) is present as allosteric effector. The cooperativity is normal (n values were between 2 and 3 for all 4 hemoglobins), and the P_{50} values ranged from 1.9 Torr for HbM to 3.7 Torr for HbE. The Bohr

effect ($\Delta\log P_{50}/\Delta\log pH$) determined in the physiological pH range is nearly absent for the two major embryonic hemoglobins, being −0.05 and −0.06, respectively. The effect of carbon dioxide on the embryonic hemoglobins is very small (16) and in vivo is probably negligible, since *1*) P_{CO_2} is low (4–10 Torr in arterial and venous parts of the circulation), and *2*) the binding of carbon dioxide to the α-chain NH_2 terminus is impossible due to acetylation (39) and at the β-chain NH_2 terminus carbon dioxide has to compete with organic phosphates (cf. Ref. 32).

When the oxygen affinity is determined in the presence of a 10-fold excess of ATP over hemoglobin there is a 3-fold decrease of the oxygen affinity of HbP and HbP′ compared with a 5-fold increase for adult hemoglobin solutions.

Nevertheless, even in the presence of excess ATP the P_{50} is <10 Torr at pH 7.2 and 37°C and thus substantially lower than the corresponding value for whole blood, which is 43.8 Torr. This large discrepancy strongly suggests that additional factors are involved in the control of embryonic blood oxygen affinity. The pH dependence of ATP binding to embryonic hemoglobin results in a substantial increase of the Bohr effect. Overall the in vitro results for the embryonic hemoglobins from chick are in good qualitative agreement with corresponding data for hemoglobin from mammalian embryos (29, 78, 135).

Phylogenetic analysis has shown that the embryonic α-type chains diverged more than 300 million years ago, and all embryonic α-type chains have maintained a high degree of homology (49). In contrast, the embryonic β-type chains are of recent origin and show close homology to the respective adult β-chains. Thus it seems reasonable to attribute the specific functional properties of embryonic hemoglobins to the specific α-chains.

Indeed, a comparison of the oxygen affinity of the major embryonic chick hemoglobins with the minor embryonic HbM and HbE demonstrates that the high oxygen affinity is predominantly due to the specific type of α-chain. Of the two adult chick α-chains, it is the presence of the α^D-chain that confers a higher oxygen affinity on HbM and HbD. Czelusniak et al. (49) have pointed out that the α^D-chain is more closely related to the embryonic π-chain than to the adult α^A-chain.

A comparison of the published sequences for the embryonic α-chains from chicks, pigs, and humans with adult human hemoglobin and the HbA and HbD from chicks reveals consistent divergance of several residues at important functional domains (Table 10). All embryonic α-chains have blocked, i.e., acetylated, NH_2-terminal amino groups, which abolishes the contribution of this site to the alkaline Bohr effect and reduces the oxygen-linked binding of chloride and carbon dioxide (110).

Nevertheless, the magnitude of the reduction of the Bohr effect of embryonic hemoglobins, which is close to zero, is not consistent with the proposed role of the NH_2 terminus in adult hemoglobins, where blocking of these groups reduces the Bohr effect usually by <50%. Thus additional structural changes have to be involved.

TABLE 10. *Comparison of published sequences of embryonic α-chains*

	Human α	α^A	α^D	π	ζ-Pig	ζ-Human
20	His	His	His	Gln	Gln	Gln
23	Glu	Glu	Glu	Ser	Thr	Thr
38	Thr	Ser	Gln	Gln	Gln	Gln
50	His	His	Pro	Gln	Pro	Pro
82	Ala	Lys	Glu	Lys	Lys	Lys
138	Ser	Ala	Glu	Glu	Glu	Glu
Na1	Val	Val	Met	*X*-Ala	*X*-Ser	*X*-Ser

Some substitutions common to embryonic α-chains from birds and mammals compared with human α-chain and the α-chains of chick hemoglobin A and D. Na1, site of Bohr effect and CO_2- and chloride-binding site; α20 and α50, residues involved in external salt bridges to Glu α23 and Glu α30, respectively; α38, important α_1/β_2 contact in oxy- and deoxyhemoglobin. α138 is adjacent to COOH-terminal HC region. Data for α^A/α^D from Schnek et al. (116) and Dodgson et al. (55); for chicken π-chain from Engel et al. (58); for pig ζ-chain from Weber et al. (135); for human ζ-chain from Clegg and Gagnon (47). [Modified from Clegg and Gagnon (47).]

At the sliding contact $\alpha_1\beta_2$, one finds a replacement of serine or threonine (human HbA and chick HbA) by glutamine not only in all embryonic α-chains but also in all α^D-chain sequences published so far (116). It has been suggested that this replacement may in part account for the higher intrinsic oxygen affinity of all embryonic hemoglobins; the fact that the intrinsic oxygen affinity of avian HbD is also in general higher than that of HbA supports this idea.

Two residues that allow the formation of stabilizing external salt bridges in adult α-chains are also missing in the embryonic α-chains. These are α20 His (which is replaced by glutamine) and α50 His (which is replaced by either proline or glutamine); this substitution is also found in all α^D-sequences.

Finally, all embryonic hemoglobins and all α^D-chains share an unusual substitution in the COOH-terminal region at position H21, where neutral amino acids (serine in human HbA and alanine in chick HbA) are invariably replaced by glutamic acid. It is likely that replacement of a neutral by a charged residue in this crucial region significantly disturbs the tertiary structure of the COOH terminus as well as that of the NH_2 terminus of the adjacent α-chain, which may be relevant for the creation of intermolecular contact sites necessary for the aggregation of HbD and embryonic hemoglobin (see sect. III*C*). In addition, the π-chain has an unusually large number of substitutions at the $\alpha_1\beta_1$-interface where 11 amino acids are exchanged compared with the adult α^A-chain; this may have resulted in a different packing of the subunits compared with adult hemoglobin and may have consequences both for the Bohr effect and for the affinity for organic phosphates.

B. Red Blood Cell Organic Phosphate Pattern

All avian embryos progress through rather complicated changes of their red blood cell organic phosphate

metabolism (5, 27, 76), and the ontogenetic pattern is remarkably consistent, as demonstrated by comparative investigations (4, 77). During the first part of development ATP is the principal organic phosphate both of the primitive and the emerging definitive red blood cells (4, 16). Between *days 4* and *7* peak concentrations of ATP are found, resulting in a stoichiometric ratio of ATP to Hb of ≥5:1, corresponding to ~17–20 mmol ATP/ kgH_2O. From *day 7* the ATP concentrations gradually decline until *day 14* of development. At this stage there is a rapid drop of ATP matched by a concomitant increase of 2,3-DPG (76).

The potency of organic phosphates to decrease the oxygen affinity of both adult and embryonic chick hemoglobins is such that $IP_5 > IP_4 >> ATP > 2,3\text{-DPG}$, which explains why substitution of ATP by an equimolar amount of 2,3-DPG causes an increase of hemoglobin oxygen affinity (16, 27).

The binding sites for organic phosphates are identical in both embryonic and adult chick hemoglobin except for the fact that one site involved in the binding of IP_5 (β135 Arg) is replaced by Asn in the ϵ-chain and by Ser in the ρ-chain (39). The consistently lower effect of organic phosphate on embryonic hemoglobin phosphate compared with HbA therefore cannot be attributed to direct changes in the amino acid composition of the binding site but may result from a general rearrangement of the subunits caused by the large number of exchanges at the $\alpha_1\beta_1$ contact (39).

Nevertheless, the affinity of avian embryonic hemoglobin for organic phosphates is quite high, and it is therefore puzzling why ATP is present in such excessively high concentrations in the early embryonic red blood cells.

There are indications that such high concentrations may have a significant influence on the regulation of the Donnan equilibrium. Direct measurements of the distribution of chloride ions have shown that they follow the Donnan distribution in early embryonic red blood cells (12) such that under physiological conditions the distribution ratio $r = Cl_i/Cl_e$ is 0.6 (where subscripts i and e represent intracellular and extracellular, respectively). Although in human red blood cells hemoglobin is the major source of impermeable anion (cf. Ref. 57), this does not hold for the embryonic hemoglobins, which have high isoelectric points (pI for HbP = 8.1 at 20°C) and in addition a much lower buffer capacity due to the decreased histidine content of both the embryonic α- and β-chains (38, 39).

Consequently the buffer power β is only 4.6 mol H^+/ mol Hb_4 in the range of pH 7–8 compared with 9.3 mol for chick HbA and 7.3 mol for HbD. At physiological intracellular pH of 7.2–7.4, embryonic hemoglobin will bear a slight positive charge of <5 mmol/kgH_2O (R. Baumann and E. A. Haller, unpublished observation). Using the data of Duhm (57) for the dissociation constant of ATP at physiological pH, one calculates a net anionic charge of ~78 mmol/l H_2O, which together with the osmotic contribution explains the chloride distribution ratio. Because of the low buffer power of embryonic hemoglobin, it is also reasonable to assume that ATP and not hemoglobin is the predominant intracellular buffer of early embryonic red blood cells. High concentrations of organic phosphates (ATP or 2,3-DPG) have also been demonstrated for the embryonic red blood cells of some mammalian species (63, 78), and although much less is known about the charge properties of the mammalian embryonic hemoglobins, published sequences show that they also are characterized by a notably low content of histidine compared with adult hemoglobin.

C. *Influence of Organic Phosphates on Aggregation of Hemoglobin P and Hemoglobin D*

In 1974 Morrow et al. (100) observed that chick HbD rapidly crystallized when deoxygenated at room temperature. This process was reversed by oxygenation and inhibited by increased ionic strength. These results were subsequently extended to HbD from other species as well (13), and it could be shown that the binding of the organic phosphate cofactors, namely, ATP and IP_6, increases solubility substantially; similarly, addition of chick HbA (but not human HbA) increased solubility. Corresponding observations were made with purified hemolysates from 6-day chick embryo. Again solubility of the deoxygenated sample was very low but increased to the physiological range in the presence of organic phosphates.

Thus organic phosphates not only regulate oxygen affinity but are apparently also necessary for maintenance of adequate solubility of embryonic hemoglobin. This is especially pertinent in view of the fact that hemoglobin oxygen saturation may be as low as 10% (or less) in the venous part of the intraembryonic circulation. The regulation of hemoglobin solubility by organic phosphates may also explain why in contrast to several mammalian fetuses, where the oxygen affinity of fetal blood is increased by reducing the intracellular concentration of ATP or 2,3-DPG to very low values (cf. Ref. 32), this strategy is never employed by avian embryos. Here changes of hemoglobin oxygen affinity are caused by changes in the red blood cell organic phosphate pattern (76). Because the allosteric effectivity is different for ATP, 2,3-DPG, and inositolphosphate, substitution of one effector, for example, ATP, by either a less potent (2,3-DPG) or a much stronger (IP_5) effector molecule will automatically cause an increase or a decrease of hemoglobin oxygen affinity.

Further investigations were carried out to establish the presence or absence of hemoglobin aggregates composed of several tetramers by measuring the colloid osmotic pressure of solutions of HbA, HbD, purified HbP, and fresh hemolysate of red blood cells from 6-day embryos. These data demonstrated that both oxy- and deoxy-HbD as well as HbP form aggregates composed of at least two to three tetramers under physiological conditions (14). However, no difference could be observed in the aggregation status of oxygenated or deoxygenated

solutions at physiological hemoglobin concentrations; likewise there was no difference in the aggregation behavior of purified HbP compared with fresh hemolysate despite the fact that hemoglobin oxygen affinity and cooperativity are markedly different. The oxygen equilibrium data show that unpurified hemolysate from primitive red blood cells has a saturation-dependent change of cooperativity, with $n > 4$ in the upper saturation range, and in addition the oxygen affinity is significantly lower than that of a purified solution of HbP to which identical amounts of ATP were added. Also the latter specimen had normal cooperativity, with n well below 4 throughout (14, 16).

Thus, irrespective of the oxygenation state, both embryonic hemoglobin as well as HbD can exist as stable compounds composed of several tetramers under physiological conditions. This rules out ligand-linked association-dissociation phenomena as the principal cause for elevated cooperativity, as is indeed indicated by the normal oxygenation properties of both HbD and HbP when analyzed under controlled conditions, i.e., in the presence of ATP and chloride as allosteric effectors (11, 16).

Nevertheless, the aggregation creates the possibility that cooperativity can be altered via binding of yet another effector molecule that associates with the aggregate in a ligand-dependent manner.

As adult HbD as well as HbA share the same β-chain, it is reasonable to assume that the sites responsible for the aggregation reside on the α-chain of HbD and on the π-chain of embryonic HbP.

From the structural information available one substitution that is uniformly shared by all embryonic α-chains and α^D-chains is the introduction of the negatively charged glutamic acid residue at position 138, which due to its location close to the structurally extremely important COOH-terminal region must create significant perturbations. Clearly it would be desirable to analyze this point by methods yielding more direct information about the three-dimensional arrangement of this domain.

Nevertheless, even in the absence of this information the functional neccessity to maintain the sites for intermolecular aggregation must have created a substantial structural constraint, explaining perhaps in part why there exists such a pronounced homology between the embryonic α-chains of various vertebrate species and classes (49).

D. Oxygen-Binding Properties of Embryonic Blood Between Days 3-8 of Development

Although the oxygen affinity of isolated embryonic chick hemoglobins is high (even in the presence of excess concentrations of ATP), measurements of embryonic blood oxygen affinity performed at a stage when predominantly primitive red blood cells are present invariably demonstrated that the blood oxygen affinity is much lower than expected. This was first observed by Faroqui and Huehns (60), who measured oxygen binding to chick embryo red blood cells suspended in phosphate buffer pH 7.13 from *day 3* onward. They found a rapid increase of P_{50} to values exceeding those for adult blood, and they noted that the slope of the oxygen equilibrium curve at *day 7* was biphasic. Subsequent measurements were carried out under conditions more closely approaching the physiological state. Lapennas and Reeves (82) used their thin-film method to determine the oxygen-binding properties of embryonic blood from 4 days of development onward.

They equilibrated the blood with the P_{CO_2} of the air cell to achieve conditions similar to those in the vitelline vein or chorioallantoic vein but did not measure actual pH.

Their study demonstrated a decrease of the oxygen affinity between *days 4* and *6* when P_{50} was found to increase from ~38 to 49 Torr, and they observed a continuous saturation-dependent increase of the n value of the oxygen-binding curve of primitive red blood cells from $n = 1$ to ~6.5 in the upper saturation range.

Our own data were recorded on red blood cell suspensions equilibrated with buffer solutions of pH 7.4–8.1 (16, 17, 91, 92). The P_{50} values obtained from these measurements when related to the physiological pH of the vitelline vein at *days 4* and *6*, respectively, are 40 and 52 Torr, which is in good agreement with the above-mentioned results. When compared at constant pH of 7.75, P_{50} increased from 46.5 Torr at *day 3* to 62.2 Torr at *day 6*. We also observed a strong saturation dependence of the n value, with maximal n value (n_{max}) > 7 in the upper saturation range. Because Lapennas and Reeves (82) kept P_{CO_2} rather than pH constant in their measurements and because pH decreases with increasing oxygen saturation, this technique tends to underestimate the apparent n value. This explains the difference between n_{max} in the two sets of data. With regard to the Bohr effect, from *day 4* to *6* there is a substantial change of the Bohr effect ($\Delta \log P_{O_2}/\Delta pH$). At *day 4* it is absent in the low saturation range, i.e., at oxygen saturations of <30%, $\Delta \log P_{O_2}/\Delta pH = 0$, and it increases slowly with increasing oxygen saturation to a maximum value of −0.30 at 80% saturation. At *day 6* the Bohr effect is present over the whole oxygen saturation range and increased to about −0.50 at oxygen saturation >20% (16).

The above-mentioned results obtained in red blood cell suspensions and in whole blood differ in several parts from those obtained in purified solutions either with the separated hemoglobins or total hemolysate. In the first place, the results obtained in purified hemoglobin solutions never gave n values >3; moreover, when the oxygen affinity at P_{50} is compared under conditions where the hemoglobin concentration and buffer pH, as well as ATP concentration, all mimic physiological conditions, there remains a substantial difference between the P_{50} in solution and in cells, which amounts to >20 Torr when data are compared at pH 7.2–7.4 (16).

Likewise, there is as yet no explanation for the drastic changes of the Bohr effect of whole blood between *days 4* and *6*. The in vitro results demonstrate that the Bohr effect is substantially increased by ATP,

but the marginal ATP concentration changes between *days 4* and *6* cannot account for the changes of the Bohr effect. When unstripped freshly prepared hemolysate is investigated the resulting oxygen equilibrium, as well as the Bohr effect, mimics the results obtained for embryonic red blood cells (16). In other words, the available experimental evidence strongly suggests that additional factors are involved in the regulation of the oxygen-binding properties of the early embryonic red blood cells.

In mammalian embryos such a clear-cut increase of the n value to >4 has recently been demonstrated for embryos from the marsupial tammar wallaby (73). In addition, published oxygen equilibrium curves for embryonic rat blood and embryonic mouse blood also indicate a marked nonlinearity of the oxygen equilibrium curve, with n approaching 4 in the upper saturation range (29, 63, 105).

1. *Effect of replacement of definitive red blood cells on embryonic blood oxygen affinity*

From *day 6* of incubation definitive red blood cells enter the circulation of the chick embryo, and by *day 8* of development ~75% of all circulating red blood cells belong to the definitive population (19). The life span of the first populations of definitive red blood cells is short, i.e., <1 wk, and there is a rapid expansion of the total red blood cell volume by a factor of 10 between *days 7* and *14*.

As the definitive red blood cells usually enter the circulation after their terminal mitosis, this expansion is almost entirely due to rapid influx of cells from the extravascular erythropoietic sites in the yolk sac and in the intraembryonic mesoderm. When one analyzes the whole blood oxygen affinity at fixed extracellular pH one sees that between *days 6* and *8* of development there is very little change of the oxygen affinity measured as P_{50}, and furthermore the n value still remains elevated above 4, although not as high as in blood containing only primitive red blood cells (17, 82). After *day 8* there is a substantial increase of the hemoglobin oxygen affinity. One may therefore ask if these changes are due predominantly to replacement of primitive red blood cells or if there are changes within the definitive red blood cell population. As the most immature red blood cells are always those with the lowest hemoglobin concentration and lowest density, red blood cells can be fractionated according to their age using density gradient centrifugation, since mature red blood cells will be in the bottom fractions. When the oxygen-binding curves of immature definitive red blood cells were analyzed as a function of age it was found that their P_{50} decreased from 85.3 Torr (pH 7.4, *day 7*) to 59.6 Torr at *day 14*, whereas in an older fraction the corresponding changes were from 80 to 55 Torr (11).

Thus the major part of the oxygen affinity change occurs within the definitive red blood cell population per se. This is also evident from a comparison of the oxygen-binding curves of young definitive red blood cells from *day 7* with cells from *day 6* (95% primitive). There was hardly any difference between both curves except that the data for definitive red blood cells indicated that cooperativity is slightly decreased in the upper saturation range compared with primitive red blood cells but still n is >4 (11).

Thus, although both cell types contain a different set of hemoglobins, this initially has no functional consequence whatsoever. However, compared with adult blood there is one significant difference with regard to the relative amount of HbA and HbD inside the cell. In adult chicks the ratio of HbA to HbD is ~3:1, whereas at *day 7* of incubation it is ~0.64:1 and increases to 1.05 at *day 9* (17). Thus in this transitional period HbD rather than HbA is the major adult hemoglobin component, and consequently the intracellular concentration of HbD is high enough to result in the formation of stable aggregates. Other avian species show the same developmental pattern for adult hemoglobin synthesis (41, 123). However, the oxygen-binding curve of concentrated (200 g Hb/l) HbD solutions in the presence of physiological amounts of ATP does not display saturation dependence of cooperativity or n values >4 (11, 12). It therefore seems possible that regulation of the in vivo oxygen affinity of both primitive and early definitive red blood cells (i.e., up to *day 9/10*) involves a common but as yet unidentified allosteric effector that either disappears or becomes too low in concentration during later stages of development to have an effect.

The large changes of both oxygen affinity and cooperativity seen between *days 8* and *14* have several causes; the best understood is certainly the gradual fall of the red blood cell ATP concentration (4), whereas the contributions made by the reduction of intracellular HbD in conjunction with the missing effector have to await further experimental analysis.

In conclusion, however, it seems as if HbD plays a major role during embryonic development as a transitory hemoglobin, which because of the greater flexibility of funtional regulation (compared with either embryonic or adult Hb) allows a smooth transition from primitive to definitive erythropoiesis. In this context it would be interesting to see if those avian species that as adults possess no HbD express HbD during embryonic development.

E. *Calculation of In Situ Blood Oxygen Saturation and Content in Various Parts of Embryonic Circulation*

Oxygen hemoglobin equilibrium curves for different pH values covering the physiological pH range and the Po_2 values of the early embryonic blood in ovo have been determined by Baumann and co-workers (16, 17). Using the Bohr coefficients obtained from the oxygen equilibrium curves, we interpolated the equilibrium measurements at actual pH for the blood of the vitelline vein, the vitelline artery, and the jugular vein (Fig. 9). The actual oxygen saturation of the blood was estimated

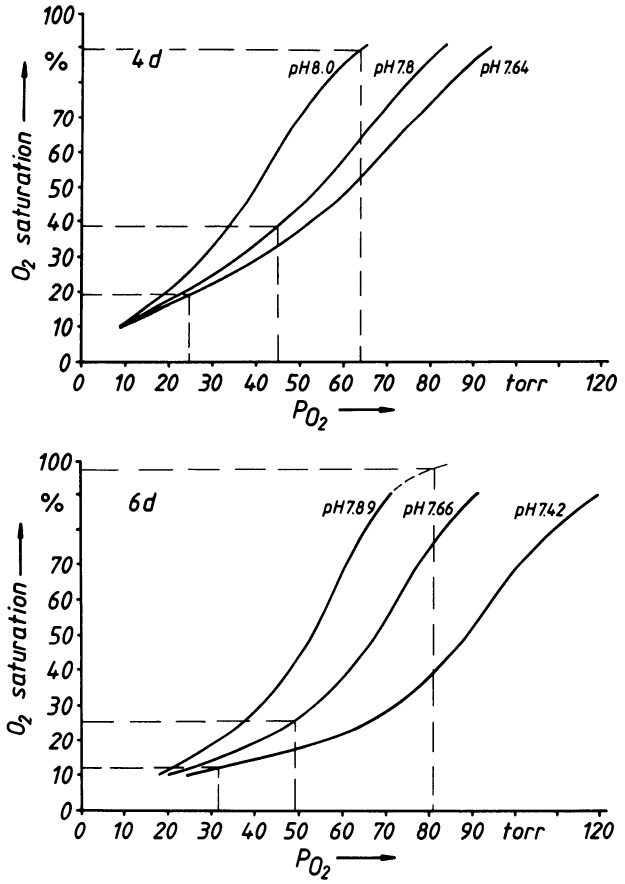

FIG. 9. Oxygen equilibrium curves of blood of 4- and 6-day-old chick embryos at actual pH. Curves refer to blood of vitelline vein, vitelline artery, and jugular vein (from *left* to *right*). [From Meuer et al. (96).]

from these curves using the measured intravascular Po_2 (96). The results are summarized in Table 11.

The venous-to-arterial difference in oxygen saturation between vitelline vein and vitelline artery is 52% at *day 4* and ~70% at *day 6*, which results in respective arterial oxygen saturations of 37 and 25%. Compared with the adult animal these values are extremely low. When the blood passes through the intraembryonic circulation, oxygen saturation is further reduced by ~15% of saturation, yielding a venous saturation of as low as 12% at *day 6*. This extreme extraction of oxygen is only possible because the arterial Po_2 is fairly high (between 45 and 49 Torr) and tissue Po_2 is near 0 Torr, demonstrating that tissue oxygenation depends critically not only on oxygen availability but also on the low oxygen affinity of the blood and a large Bohr effect.

Because of the low hemoglobin concentration of the blood of early embryos, the oxygen content of the blood offered to the embryo proper (remember that data for the vitelline artery are identical with those of the dorsal aorta) is no more than 1.4 μl/100 μl at *day 6* and even less at *day 4*. About 10% of the arteriovenous difference in oxygen content (see Table 11) is covered by physically dissolved oxygen. Moreover, the oxygen equilibrium curve is more or less a straight line when related to actual acid-base conditions. In other words, there is a direct linear relationship between changes in intravascular Po_2 and oxygen release ($\Delta\mu l\ O_2/\Delta Po_2$) from embryonic blood.

The principal effect of the higher cooperativity of oxygen binding to embryonic hemoglobins is not to safeguard oxygen content but to maintain a fairly high Po_2 after mixing in the heart. Using the data for the oxygen affinity and Bohr effect of adult chicken blood (7), we calculated the Po_2 of the dorsal aorta for the pH and oxygen saturation values that are actually present. These results show that if chick embryos had blood with adult properties the Po_2 in the dorsal aorta would drop to 16 Torr at *day 4* and 24 Torr at *day 6* (9). Thus the high cooperativity of embryonic blood results in a net gain for the Po_2 of the intraembryonic arterial blood of ~25–30 Torr.

TABLE 11. *Po_2, pH, oxygen saturation, and oxygen content of the blood in intra- and extraembryonic vessels*

	Day 4	*Day 6*
[Hb], g/l	20	35
O_2 capacity, ml (STP)/l	27.2	47.6
Vitelline vein		
Po_2, Torr	64	81
pH	8.00	7.89
Saturation, %	89	>95
[HbO_2], ml/l	24.2	>45.2
$[O_2]_{diss}$, ml/l	2.1	2.7
Total [O_2], ml/l	26.3	>47.9
Vitelline artery		
Po_2, Torr	45	49
pH	7.80	7.66
Saturation, %	37	25
[HbO_2], ml/l	10.1	11.9
$[O_2]_{diss}$, ml/l	1.5	1.6
Total [O_2], ml/l	11.6	13.5
Jugular vein		
Po_2, Torr	29	32
pH	7.64	7.42
Saturation, %	22	12
[HbO_2], ml/l	6.0	5.7
$[O_2]_{diss}$, ml/l	0.96	1.1
Total [O_2], ml/l	6.96	6.8
$VADO_2$, ml/l	14.7	>34.4
Physically dissolved, %	4.1	<3.2
$AVDO_2$, ml/l	4.6	6.7
Physically dissolved, %	11.7	8.2

Hemoglobin concentration [Hb] was taken from Refs. 17 and 111. Oxygen saturation was determined from oxygen hemoglobin equilibrium curves (Fig. 9) and measured mean Po_2 values (92). Hüfner number, 1.36 ml O_2 STP/g Hb, was used to calculate the concentration of oxygen bound to hemoglobin at 100% saturation (oxygen capacity). For the solubility coefficient of oxygen in blood the value 0.033 ml O_2 STP/1 Torr determined for water at 38°C (67) was taken. Total oxygen content (total [O_2]) is the sum of oxygen bound to hemoglobin ([HbO_2]) and physically dissolved oxygen ($[O_2]_{diss}$). $VADO_2$ and $AVDO_2$ are the differences in total oxygen content between vitelline vein and vitelline artery and between vitelline artery and jugular vein, respectively.

The working range of the oxygen saturation curve utilized for the supply to the embryo proper and those parts of the extraembryonic circulation that may depend on the vitelline artery is the lower part of the oxygen equilibrium curve where cooperativity approaches $n = 1$. The fact that the relationship between Po_2 and blood oxygen content is almost invariant between *days 4* and *6* suggests that the Po_2 gradients themselves are subject to tight regulation. Furthermore, the data make it abundantly clear that any derangement of the oxygen supply to the embryo proper automatically endangers embryonic survival, since there is no oxygen "reserve" present in the embryo as oxygen contents range from at most 2 μl/100 μl blood to <1 μl/100 μl blood. Thus the principal function of the early embryonic hemoglobin seems to be less one of oxygen transporter with high oxygen-carrying capacity but rather its presence helps to create and maintain Po_2 gradients inside the embryo that guarantee adequate supply. As it is the lower part of the oxygen equilibrium curve that is used for the oxygen supply of the embryo proper, the absence of a large Bohr effect in this saturation range guarantees that within certain limits oxygen supply will not be affected by local changes in pH.

F. *Estimation of Intraembryonic Oxygen Transport Rates*

The total oxygen uptake of the egg that has been repeatedly determined (61, 72, 113) is not an adequate measure of the embryonic oxygen uptake, since a substantial amount is required to supply the extraembryonic structures (2). With the use of Fick's principle the embryonic oxygen uptake rate can be determined either from the intraembryonic venous flow or the extraembryonic blood flow. Blood flow data for early chick embryos were reported first by Clark and Hu (46) and by Stewart et al. (122). Both groups determined blood flow velocity of the dorsal aorta using a pulsed Doppler meter. A summary of blood flow and blood pressure measurements in 2- to 6-day-old chick embryos is given by Hu and Clark (74). Wispé et al. (136) studied the change of dorsal aortic blood flow in hypothermia. Because the dorsal aorta not only supplies embryonic tissue but also the extraembryonic circulation, it is impossible to estimate intraembryonic venous or extraembryonic blood flow from the dorsal aorta blood flow. Therefore we determined the total extraembryonic blood flow by a different method.

1. *Determination of extraembryonic blood flow*

Because of the small blood vessels of the yolk sac vasculature, volumetric blood flow was assessed by measuring the velocity of circulating fluorescent-labeled blood cells using the video frame-by-frame method (Meuer, unpublished observations). From mean blood cell velocity and vessel diameter the mean blood flow was calculated (140). Summarizing the blood flow of all veins entering the embryo gave the total extraembryonic blood flow.

TABLE 12. *Mean total blood flow in yolk sac circulation of 4- and 6-day-old chick embryos and calculation of cardiac output and embryonic oxygen uptake rate*

	Day 4	*Day 6*
Extraembryonic blood flow, μl/s	0.66 ± 0.22	1.2 ± 0.41
Percentage of cardiac output, %	23	16
Calculated cardiac output, μl/s	2.9	7.4
Dorsal aortic blood flow, μl/s	1.14	3.56
$VADO_2$, ml/l	14.7	34.4
Embryonic O_2 uptake, nl (STPD)/s	9.6	40.2
Total egg O_2 uptake, μl (STPD)/min	7	20
Sp. embryonic O_2 uptake, %	8	12

Extraembryonic blood flow data are means ± SD. The percentage of extraembryonic venous blood flow on cardiac output was previously calculated from the oxygen saturation of intra- and extraembryonic blood (92). Dorsal aortic blood flow was taken from Zahka et al. (139). $VADO_2$, vitelline venous-to-arterial difference of oxygen concentration of blood (see sect. III*E*); sp. embryonic oxygen uptake, percentage of specific embryonic oxygen uptake on total egg oxygen uptake. Values for total egg O_2 uptake were taken from Freeman and Vince (61).

Mean total extraembryonic blood flow was found to be 0.66 μl/s at *day 4*, increasing nearly twofold by *day 6*. From these data and the estimated percentage of blood flow of the vitelline veins as percent total blood flow into the heart (92) one calculates a cardiac output of 2.9 μl/s at *day 4*, which increases 2.5-fold by *day 6* (Table 12).

Clark and Hu (46) determined a mean blood flow in the dorsal aorta of 0.77 μl/s at *day 4*. Zahka et al. (139) reported values of 1.14 μl/s at *day 4* and 3.56 μl/s at *day 6*, and Hu and Clark (74) reported values of 0.85 and 2.4 μl/s at *days 4* and *6*, respectively.

Because the dorsal aorta does not only supply the yolk sac circulation via the vitelline arteries but also the allantoic circulation via the umbilical arteries and furthermore supports the trunk of the embryo, it was expected that the dorsal aorta blood flow would be higher than the blood flow through the yolk sac circulation. On the other hand, the dorsal aorta blood flow underestimates cardiac output, because the dorsal aorta does not supply the head of the embryo. Estimations of the share of blood flow to the head on cardiac output revealed values of <10% (74). If this is true, the cardiac output calculated from our measurements is about twice as high as the respective value estimated from dorsal aorta blood flow.

2. *Embryonic oxygen uptake rate*

From the extraembryonic blood flow and the venous-to-arterial differences in oxygen saturation one calculates embryonic oxygen uptake rates of 9.6 and 40.2 nl/s at 4 and 6 days, respectively. These numbers are only ~10% of the total oxygen upake of the egg, which

TABLE 13. *Mean Po_2 in vitelline blood vessels of 4- and 6-day-old chick embryos incubated in normoxic and hypoxic environments*

	Po_2, Torr			
	Normoxia		Hypoxia	
Location	*Day 4*	*Day 6*	*Day 4*	*Day 6*
Vitelline arteries	45 ± 5	49 ± 6	45 ± 6	46 ± 9
Vitelline veins	64 ± 10	81 ± 12	59 ± 8*	61 ± 10*

Values are means ± SD. Eggs were either incubated in air (mean ambient Po_2 = 150 Torr) or in a hypoxic gas mixture (mean ambient Po_2 = 100 Torr). Measurements were performed in the same ambient gas composition. * Significant differences ($P < 0.05$) between hypoxia and normoxia.

demonstrates that in the early embryo the major part of the total oxygen uptake is utilized by the extraembryonic structures. The data also show that the percentage of embryonic oxygen consumption tends to increase with increasing age. This is consistent with the finding of Ar et al. (2), who reported that the percentage of embryonic oxygen utilization is 69% at *day 12* and increases up to 94% by *day 20*.

IV. ADAPTIVE CHANGES OF OXYGEN TRANSPORT AND RED BLOOD CELL DEVELOPMENT DURING EXPOSURE TO SHORT-TERM AND CHRONIC HYPOXIA

It has been repeatedly shown that embryonic growth and development is strongly influenced by oxygen availability. In general, exposure to hypoxia causes retarded development (17, 18, 112, 132), increased mortality, and a fall in the embryonic respiratory rate (18, 132).

Conversely, exposure to elevated ambient Po_2 accelerates growth rate and rate of oxygen consumption of early as well as late chick embryos (3, 89). Thus available experimental evidence indicates that up to a point the embryo can adjust its metabolic rate to changes in oxygen availability. It is important to know how this is effected and to which extent the embryo is able to adapt to oxygen deficiency. Results demonstrating adaptive changes of blood gas transport properties are presented next.

A. Effect of Hypoxia on Oxygen Pressure in Embryonic Circulation

The Po_2 in the vitelline blood vessels of chick embryos incubated in moderate hypoxia (13.5% O_2-86.5% N_2) was determined by Meuer and Baumann (91). Table 13 compares the results with the blood Po_2 measured in normoxic incubated embryos.

As expected, the Po_2 in the vitelline veins that collect the blood from the extraembryonic gas exchange vessels is lower in hypoxia than in normoxia. Surprisingly in hypoxic incubation the arterial Po_2 values do not differ significantly from the normoxic group. It has been shown that the vitelline arterial Po_2 is approximately equal to the intraembryonic arterial Po_2 (92). Furthermore, it is very likely that tissue Po_2 values in hypoxia are also close to zero, as in normoxic incubation (sect. II*C*). This suggests that the Po_2 difference between intraembryonic arterial blood and embryonic tissue that determines tissue oxygen uptake is the same in hypoxia and normoxia. On the other hand, the differences in embryonic dry weight between normoxia and hypoxia indicate a lower metabolic rate in hypoxic incubated embryos.

It is not known how the embryo adapts to reduced oxygen supply. The diminished metabolic rate could be due to several reasons: reduced capillary blood flow in hypoxia due to reduced cardiac output, diminished tissue vascularization, and/or reduced aerobic metabolic rate per unit tissue.

The measured data suggest that in early chick embryos an arterial Po_2 of ~45 Torr is the lower limit tolerated by the embryo. Consequently, if hypoxia develops to an extent that cannot be countered by adaptive mechanisms, the embryo is no longer able to survive.

B. Estimates of Blood Volume in Normoxia and Hypoxia

The blood and red blood cell volumes of chick embryos exposed to experimental hypoxia (13.5% O_2) have been determined between *days 4* and *8* of incubation (17, 117) to find out if hypoxia causes an adaptive expansion of blood and red blood cell volumes. Table 14 contains the results of these investigations. The total blood volume as well as the red blood cell volume of control and hypoxic embryos are not different until *day 6* of development. At *days 7* and *8* values for hypoxic embryos are lower than in the control group. This reflects the general retardation of the hypoxic embryos that becomes more prominent with increasing age. When blood volume is instead correlated with embryonic weight (to allow comparison at similar developmental stages) the difference is no longer observed (17).

In conclusion it can be said that during early development the chick embryo is unable to counter hypoxia by an increased production of red blood cells and/or ex-

TABLE 14. *Blood and red blood cell volume in normoxic and hypoxic chick embryos*

Day of Incubation	Total Blood Volume, μl		Total Red Blood Cell Volume, μl		Hematocrit	
	Air	13.5% O_2	Air	13.5% O_2	Air	13.5% O_2
4	50.0	43.3	10.8	8.7	21.6	22.0
5	110.0	91.9	24.9	18.9	22.5	20.5
6	144.0	150.0	36.4	36.3	25.3	24.0
7	312.5	202.0	75.4	47.0	24.0	23.1
8	422.5	313.5	100.9	80.7	24.0	25.8

Data are from Sender (117).

pansion of total blood volume. This indicates that the expansion of both total blood and red blood cell volumes occurs at maximum speed during normal development.

C. *Influence of Oxygen Pressure on Timing of Switch From Embryonic to Adult Hemoglobin*

As stated in section I, the mechanisms that control the change from embryonic to adult hemoglobin production in the embryo are not yet identified at the molecular level. The question as to what extent epigenetic factors may be involved in the timing of the process has been analyzed in chick embryos grown under different Po_2 (17, 117). The results of these investigations conclusively demonstrated that ambient Po_2 can modulate the timing of the switch.

When embryos are grown under hypoxic conditions definitive red blood cells and consequently adult hemoglobins appear ~24 h earlier in the circulation than they do in normoxic controls. Conversely, when embryos are incubated under hyperoxic conditions definitive red blood cells are first observed by *day 7* of incubation (17, 117), as shown in Figure 10.

Furthermore, it has been shown that these changes are entirely due to alterations of primary yolk sac erythropoiesis (117). The results are most compatible with the idea that the yolk sac harbors two subpopulations of erythroid precursors: one turning invariably into primitive cells, whereas the other can produce definitive as well as primitive red blood cells, depending on the ambient Po_2. The total size of the red blood cell population produced from yolk sac precursors is, however, invariant.

In hypoxia one observes an increased production of definitive red blood cells, whereas in hyperoxia all cells turn into primitive red blood cells and the onset of definitive erythropoiesis is linked to the activation of intraembryonic erythropoiesis, which is fed from the permanent stem cell pool (117). In any event the premature appearance of definitive red blood cells in hypoxic embryos has some bearing on the oxygen transport function of the blood, as shown in the next section.

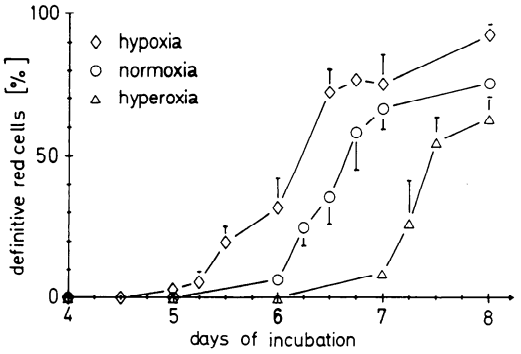

FIG. 10. Influence of ambient Po_2 on amount of circulating definitive red blood cells of 4- to 8-day-old chick embryos (hypoxia, 13.5% O_2; hyperoxia, 100% O_2).

D. *Influence of Hypoxia on Red Blood Cell-Phosphate Pattern, Carbonic Anhydrase Activity, and Oxygen Affinity*

Unlike the mammalian embryo or fetus where changes in placental blood flow at the maternal side may partially compensate for environmental hypoxia, the avian embryo has no such mechanism at its disposal. This has raised the question as to whether the chick embryo exposed to hypoxia can alter its blood oxygen affinity as an adaptive measure.

Oxygen equilibrium curves determined between *days 4* and *9* of development (17) demonstrated that already at *day 6* of development the oxygen affinity of the chronically hypoxic embryos was significantly increased compared with normoxic controls. This difference was accentuated during the next few days so that hypoxic chick embryos analyzed at *day 9* of development had the same high oxygen affinity as normal embryos after *day 14* (8).

It was also recognized that although definitive red blood cells appear earlier in the circulation of hypoxic embryos, there was no convincing correlation between the percent of circulating definitive cells and the oxygen affinity of hypoxic embryos (17). However, analysis of the phosphate pattern demonstrated that under hypoxic conditions there is a rapid reduction of ATP from *day 6* of development onward and a concomitant increase of red blood cell 2,3-DPG (12, 15, 17), which explains at least part of the increase of blood oxygen affinity.

Furthermore, it was observed that the regulation of red blood cell carbonic anhydrase synthesis is coordinated with the change in red blood cell phosphate pattern (15). Thus experimental hypoxia apparently activates a program in definitive red blood cells, which is usually only realized after *day 14* of development when progressive hypoxia and hypercapnia develop due to diffusion limitations (125). That blood Po_2 has a controlling influence on red blood cell metabolism was also shown by Ingermann et al. (75), who found that they could suppress the switch from ATP to 2,3-DPG and the concomitant increase of the oxygen affinity when late chick embryos were incubated in hyperoxia. Moreover, the repression was entirely reversible. Recent experiments have demonstrated that hyperoxia can also suppress the increase of red blood cell carbonic anhydrase synthesis normally observed after *day 14* (98).

The Po_2-dependent changes of red blood cell metabolism observed after *day 6* of development are not due to a direct effect of oxygen on the red blood cell metabolism but are mediated via circulating plasma factors (98) that under in vitro conditions are able to induce de novo synthesis of carbonic anhydrase and stimulation of 2,3-DPG synthesis at the expense of ATP. The plasma factor is present from *day 7* of incubation. Moreover, there seems to be a Po_2-dependent control system that can suppress the action of the factor under normoxic conditions that involves phospholipase C and protein kinase C (98). These data are the first evidence that the avian embryo is able to actively respond to hypoxia us-

ing an P_{O_2}-dependent system for the regulation of red blood cell metabolism that is usually (i.e., during normal development) activated after *day 14* when P_{O_2} of the chorioallantoic vein progressively decreases. Future work will have to clarify the molecular base of this mechanism and will have to establish if this is a control system universally present in avian embryos. The presence of such a system may have contributed to the fact that birds are able to breed at higher altitudes than all other terrestrial vertebrates.

V. CONCLUDING REMARKS

The preceding presentation of the various factors that determine the oxygen transport properties of the embryonic blood and the state of tissue oxygenation is by necessity imperfect. Thus, although the last decade has seen considerable progress in the analysis of embryonic hemoglobin structure, we are still unable to explain the unique oxygen-binding curves found in early embryos in particular with regard to the control of hemoglobin cooperativity. We need to know the intermolecular contact sites involved in the formation of tetramer-tetramer aggregates of HbP/P′ as well as HbD, and we need to find the elusive metabolites of primitive and early definitive red blood cells that as additional allosteric effectors induce changes in cooperativity and oxygen affinity.

The demonstration of a P_{O_2}-regulated system of plasma factors that apparently controls red blood cell metabolism and function after *day 6* of development raises a host of questions with regard to cellular mechanism, hormonal transducers, and not least the structures that are responsible for monitoring the P_{O_2}.

Finally, more data are needed to analyze the processes involved in the formation and optimization of structure and function of the capillary network of the area vasculosa, the first gas exchange area of the avian embryo.

Against these unresolved questions there is a new body of experimental data that at least at the phenomenological level demonstrates the necessity for the persistance of embryonic hemoglobins, as they possess indeed functional properties uniquely fitted to the needs of the embryo. Furthermore, the results presented once again stress the critical role played by oxygen for the development of the early embryo during the period of organogenesis. There is no doubt that during this period the gas transport system works at the limit of its capacity; thus even small malformations of the circulatory system inside or outside the embryo could have the same consequences for the oxygen availability to tissue as a reduced environmental P_{O_2}. Thus the rate of development of the gas transport and circulatory system determines embryonic survival and may at this stage serve as one principal factor of early embryonic selection. This conclusion can be extended to mammalian embryos as well. Meegdes et al. (88) determined the degree of the chorionic vascularization of early human embryos. They found that in spontaneous abortion the incidence of vascularized villi and the vascular density of villi was significantly lower than in legal abortions.

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