FACTORS INFLUENCING THE RESPIRATION OF ERYTHROCYTES

II. Mammalian Reticulocytes

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Cohnstein and Zuntz (1) were the earliest investigators who clearly pointed out that certain types of blood exhibit a metabolism involving the disappearance of oxygen and reduction of the hemoglobin. This metabolism was most evident in fetal blood, which, they observed, gradually darkened in color upon standing and upon evacuation liberated less oxygen than before. They ascribed this loss of oxygen to the action of the red cells, many of which, at the stage of development of the blood which they examined, still possessed large vesicular nuclei.

Since then the metabolism of the blood has been very actively studied, notably by Warburg, by Morawitz, and by Harrop. The rather extensive literature has been well reviewed by Morawitz (2). In general, it may be summarized by stating that it is now agreed that the normal orthochromatic erythrocytes, which form the vast majority of the circulating red cells of mammals, have a negligible if any respiration (Daland and Isaacs (3)). On the other hand the polychromatic cells, which have been recently liberated from the bone marrow and which are found in large numbers in young animals and during active blood regeneration, exhibit an easily measurable consumption of oxygen and formation of carbon dioxide. The earlier nucleated cells, ordinarily present only in the bone marrow in mammals, and the nucleated circulating erythrocytes of birds and amphibia, also respire very actively.

With the exception of Warburg's studies (4), which were largely concerned with the manner in which respiration was inhibited by

various narcotics, the factors which influence this metabolism have not been extensively investigated. This paper deals with the variations in the respiration of the reticulocytes of the rabbit brought about by various alterations in the medium in which they are suspended.

Methods

The majority of the methods used in this investigation have already been described in the previous paper. The blood used was obtained from the ear vein and was defibrinated as it was collected by shaking with glass beads. Before use it was filtered through cotton wool to remove the coagulated fibrin and with it the large majority of leucocytes (Warburg (5)).

The rabbits were made anemic by intraperitoneal injections of phenylhydrazine hydrochloride (15 mg. per kilo) dissolved in normal physiological saline solution. The anemia reached its greatest severity in about 7 days. The blood used in these experiments was removed from animals at a time when there was a large proportion of reticulocytes and consequently a high rate of respiration.

The Respiration of the Blood during the Onset and Recovery Stages in Phenylhydrazine Anemia

An injection of phenylhydrazine into a rabbit is rapidly followed by the development of anemia. With suitable quantities of the substance (15 to 20 mg. per kilo) the red cell count may be caused to fall from its normal value of rather over five million to between one and two million red cells per c. mm. Regeneration, however, sets in very quickly, and by the time that the anemia has become most severe, between the sixth and eighth days, the blood may contain enormous numbers of reticulocytes. When the red cell count falls below two million per c. mm., more than 50 per cent of the cells may be reticulocytes, or more than one million per c. mm. More commonly the number lies between six and eight hundred thousand per c.mm. If the oxygen consumption of such a specimen of blood be determined it will be found to have a very much higher value than that from a normal rabbit. Harrop (6) has shown that a rough proportionality exists between the number of reticulocytes in, and the oxygen consumption of, various pathological human bloods. Similarly Derra (7) studied the oxygen consumptions of blood during the remissions resulting from the administration of liver extract in patients suffering from pernicious anemia. He found a similar relationship, though not so close as that

observed by Harrop, between the oxygen consumption and the reticulocyte content of the blood at various times in the remissions.

In the following experiment two rabbits were made anemic by injection with phenylhydrazine. Red cell counts, reticulocyte counts and oxygen consumption determinations were made every second day until the main wave of liberation of reticulocytes had subsided. The observations are to be found in Table I and Fig. 1.

TABLE I

The Oxygen Consumption of Reticulocytes at Various Phases in the Anemia Induced by the Injection of Phenylhydrazine

Rabbit No.	Day	Red cells in mills. per c.mm.	Retics. per cent	Retics. in mills. per c.mm.	C.mm. O_2 consumed	
					Per cc. blood per hour	Per billion retics. per hour
28	before inject.	5.49	1.5	0.083	5.1	63.0
	1st after inject.	6.01	3.2	0.192	11.1	57.8
	3rd " "	2.85	9.3	0.265	17.0	64.2
	5th " "	2.40	27.0	0.649	41.9	64.7
	7th " "	2.44	33.0	0.805	52.9	65.5
	9th " "	3.80	17.2	0.654	35.5	54.3
	11th " "	4.07	10.2	0.423	25.4	59.9
	13th " "	4.90	9.6	0.470	26.0	55.3
40	before inject.	4.97	2.0	0.099	5.3	53.6
	1st after inject.	4.96	3.0	0.149	8.9	59.7
	3rd " "	3.32	9.4	0.313	14.9	47.6
	5th " "	2.64	23.9	0.631	40.0	63.2
	7th " "	3.15	21.0	0.630	35.2	55.9
	9th " "	3.67	13.1	0.479	25.3	52.8
	11th " "	3.19	9.1	0.290	15.2	52.4
	13th " "	3.61	5.3	0.192	10.9	56.7

From Table I it can be seen that the actual number of reticulocytes per c.mm. of blood increased, in one animal to more than nine, in the other to more than six, times their initial figures, and that the oxygen consumption of the blood underwent a concurrent change. But in spite of the very large alteration in both, the oxygen consumption for every billion (thousand million) reticulocytes was, within reasonably close limits, the same throughout the course of the experiment.

There are at least two possible explanations for this rather strikingly uniform relationship between oxygen consumption and reticulocyte concentration. In the first place it is possible that the reticulum in the cell either promotes oxidation or is itself oxidized at the same rate at all stages in its gradual disappearance. When taken in conjunction with the cytological appearances of the reticulocytes with their very variable content of basophil filaments, such an assumption seems

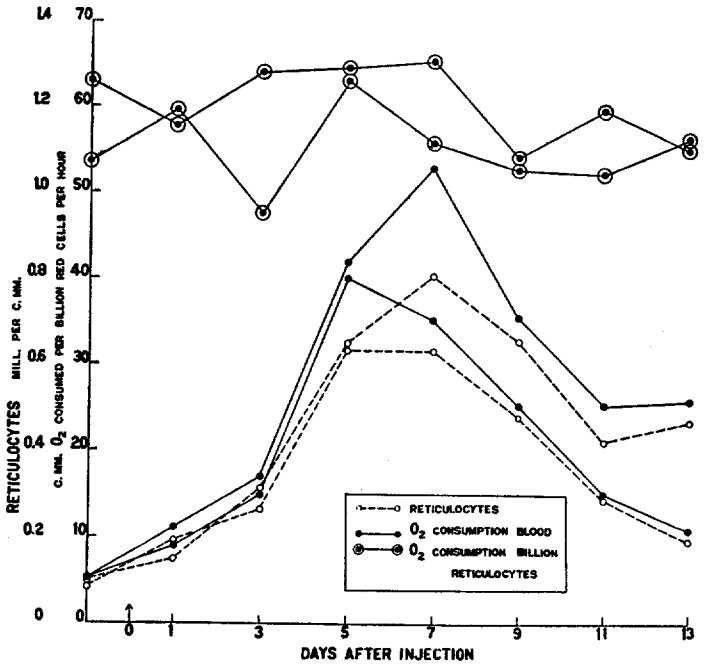

FIG. 1. Showing the oxygen consumption of reticulocytes related to their concentration in the blood at various stages of an anemia produced by phenylhydrazine.

hardly probable. A second and seemingly more likely explanation is that the reticulocyte stage of development is of comparatively short duration in comparison with the whole duration of the wave of reticulocyte production evoked by the phenylhydrazine. Under these circumstances the oxygen consumption curve for the blood would represent, at any one particular time, the summation of the oxygen consumption of reticulocytes at all stages in their life history. In this

event, though the total number of reticulocytes in the circulation might change greatly, the proportions of the various categories of cells, young and old, might not be very different at different times.

An accurate estimation of the duration of the reticulocyte stage of development would determine this point. Subject to some assumptions which will be discussed below, this period can be indirectly estimated from a consideration of the regeneration curves for the blood of rabbits recovering from anemia. In the course of this recovery the red cell count of the animal rises from the day of most severe anemia until regeneration is complete. If daily total red cell counts and reticulocyte percentage counts be made, the actual number of reticulocytes per c. mm. of blood can be calculated for any particular day. Should, on an average, each reticulocyte retain its reticulum for only 1 day before developing into an ordinary orthochromatic erythrocyte, it will, taken in the aggregate, be only counted once as a reticulocyte in the daily blood examinations made. Consequently, if the life of the reticulum once the cell has reached the circulating blood be only 1 day, the total daily calculated numbers of reticulocytes added together for the whole regeneration period should be equal to the rise in the red cell count during the same period. If the aggregate number of the reticulocytes so counted be greater than the number of new cells produced, as judged by the rise in the total red cell count, the average duration of the reticulum in the cells must be longer than 1 day, since they must have been counted more than once.

Such a calculation obviously makes several important assumptions. First, it presupposes that all the red cells added to the circulation in the period of regeneration are liberated from the bone marrow as reticulocytes and not as the normal adult orthochromatic variety of red cell. It is impossible at the present time to be certain whether this is so or not, since there are no satisfactory ways of recognizing new cells other than those of vital staining. If considerable numbers of non-reticulated red cells are liberated the calculation just outlined will estimate the duration of the reticular stage of development at too brief a period. It further disregards any alteration in blood volume and the normal or abnormal destruction of red cells, either adult or reticulated, during the regeneration period. How far these might affect the result it is clearly impossible to estimate.

With all these assumptions, only a very roughly approximate estimate is possible, yet the calculation seems to be worth the making. Table II contains data taken from a group of animals used in another experiment.

The agreement between the results on these animals is not good. Nevertheless, it would seem that the duration of the persistance of the reticulum in the red cell after it has left the bone marrow is probably not much greater than 2 days, and that in severe grades of anemia it may be even less. Such a period is much briefer than the duration of the entire reticulocyte wave following an injection of phenylhydrazine. Further it would support the view already suggested that the uniformity of the oxygen consumption per billion reticulocytes throughout the period of the anemia results from this consumption being largely

TABLE II

Rabbit No.	Red cell counts			Tota lnumber of retics. counted	Tota lretics.
	Lowest	On recovery	Rise		Red blood corpuscle rise
104	3.77	5.72	1.95	4.13	2.12
105	3.82	5.70	1.88	4.10	2.18
106	2.20	5.11	2.91	3.19	1.10
103	1.14	5.47	4.33	6.22	1.44

or even exclusively due to the metabolism of those cells which had first appeared in the blood stream in the previous 36 to 48 hours.

Estimations of the duration of the reticulocyte stage of development have been made by Pepper (8) and by Heath and Daland (9), both largely from observations upon the rate of disappearance of reticulocytes from regenerating blood incubated at 37°C. Pepper states that in no specimen of blood left in the incubator were fully formed reticulations found after 48 hours, but in one instance a few remnants of reticular substance were found after 66 hours incubation. Heath and Daland used a variety of methods, all of which gave consonant results, and showed that reticulocytes of the rabbit persist for a period varying from 48 to 72 hours, in incubated blood, after introduction into the pleural cavity of the rabbit and probably also after transfusion into a normal rabbit. A few persisted longer, but the large majority had

disappeared in that time. These results are in fairly good agreement with those calculated from the regeneration curves.

The Influence of Hydrogen Ion Activity upon the Respiration of Reticulocytes

The hydrogen ion activity in the interior of the mammalian red cell has been found to depend upon the reaction of the medium in which it is suspended. The divergencies between the reactions inside and outside the cell have been investigated notably by Warburg (10), by Van Slyke and his collaborators (11), and by Henderson (12). At reactions between pH 6.5 and pH 6.9 the hydrogen ion activity of the interior of the cell and of the surrounding medium are approximately the same, but, as the alkalinity of the surrounding medium increases, that of the cell increases less rapidly so that they become relatively acid. Through the region of physiological neutrality the difference between the two is not very considerable (at pH 7.4 there is a difference of about 0.1 pH), but the difference increases more than proportionately rapidly until at a serum pH of 8.0 the cell interior is at a reaction of about pH 7.75. It is evident that the contents of the mammalian red cell are capable of considerable variations in their reaction, and that these variations can be controlled by alterations in the reactions of the surrounding medium. The purpose of the present experiments was to determine how far the oxidation processes associated with the reticular substance were affected by changing the hydrogen ion activity of the medium in which the cells were suspended.

The reaction of the cells was varied by the addition of small quantities of sodium hydroxide or of hydrochloric acid to defibrinated blood. Two cc. of normal physiological sodium chloride solution containing the necessary acid or alkali were added slowly and with frequent shaking to 4 cc. of blood from an anemic rabbit. After mixing, 3 cc. of the blood saline mixture were placed in the flask of the Barcroft-Warburg apparatus and their oxygen consumption determined. Directly this had been ascertained the contents of the flask were rapidly transferred to centrifuge tubes and the cells and suspension medium separated. The reaction of the medium was determined with the hydrogen electrode. I am indebted to Dr. Arda A. Green for making most of the determinations.

TABLE III

The Influence of Hydrogen Ion Activity upon the Oxygen Consumption of Reticulocytes

Concentration of acid or alkali added	C.mm. O_2 per bill. retics. hourly	Per cent of value in serum saline	pH	C.mm. O_2 per bill. retics. hourly	Per cent of value in serum saline	pH	C.mm. O_2 per bill. retics. hourly	Per cent of value in serum saline	pH
NaOH 1/50 N.....	30.6	89					54.4	92	8.31
1/100 N.....	30.6	89	8.06				54.8	93	8.16
1/200 N.....	32.0	93	8.02				56.3	96	8.04
Neut. Sal.....	34.5	100	7.96	48.4	100	8.10	58.8	100	8.02
HCl 1/200 N.....	34.0	99	7.86						
1/100 N.....	34.1	99	7.81	46.8	97	7.89			
1/50 N.....	31.9	92	7.68	45.7	95	7.70	55.6	95	7.65
1/33 N.....				44.5	92	7.55			
1/25 N.....				39.4	82	7.34			
1/20 N.....				36.8	77	7.06	48.5	83	7.22

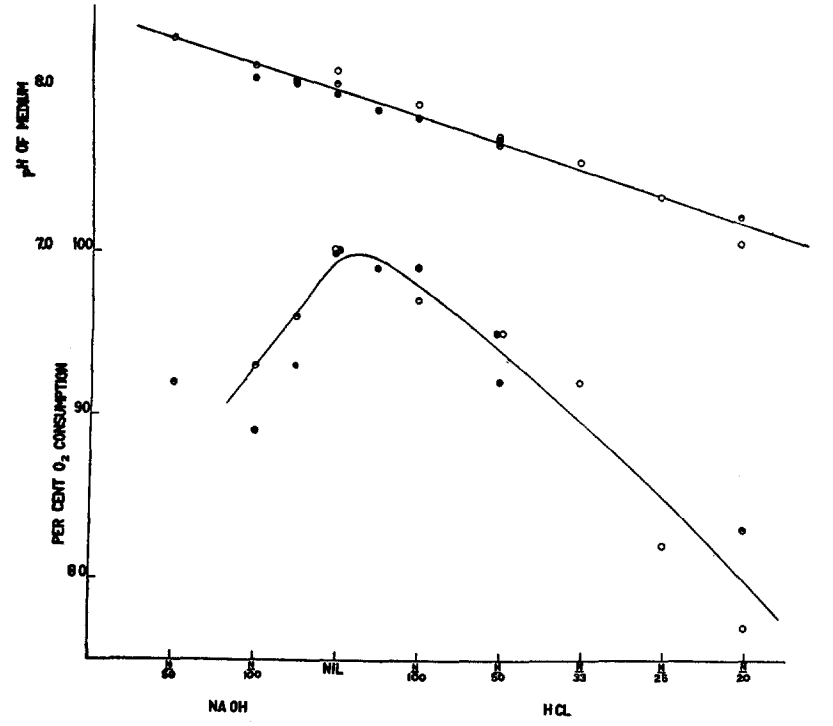

FIG. 2. Showing the influence of the addition of HCl and NaOH to blood upon its reaction and upon the respiration of the reticulocytes present in it.

The results of the experiments will be found in Table III and in Fig. 2. It will be seen that variations in the reactions of the medium have considerable influence upon the reaction of the cells. The maximal oxygen consumption occurred about pH 8.0, which would correspond to a reaction in the interior of the cells about pH 7.75. The metabolism at pH 7.4, or that normally to be found in the blood, would be about 85 per cent of this maximal value.

The Influence of Variations in Tonicity upon the Respiration of Reticulocytes

Determinations of the oxygen consumption of reticulocytes were made in solutions of sodium chloride of various concentrations ranging from 0.11 molar (0.6 per cent) to 0.28 molar (1.5 per cent). These solutions were added to the cells after they had been separated from their serum by centrifugation.

The results are given in the following table:

Red cells in mills. per c.mm.	NaCl: 0.6 per cent	0.9 per cent	1.2 per cent	1.5 per cent
	C.mm. O_2 consumed per billion reticulocytes per hour			
2.75 (14.2)*	32.5	39.4	34.9	31.2
2.37 (23.4)	38.5	44.2	39.6	37.8
1.96 (39.0)	38.9	42.9	42.5	37.8

* Reticulocyte percentages are given in brackets after the red cell counts.

It can be seen that in all three experiments the maximal respiration took place in a sodium chloride solution which was closely isotonic with normal blood. The figures, however, suggest that slight degrees of hypertonicity are less injurious to the cell respiration than the reverse condition of hypotonicity. The cells in 0.6 per cent saline have a metabolism 13 per cent below that in 0.9 per cent saline, while those in 1.2 per cent saline are only depressed to the extent of 7 per cent.

The Influence of Glucose, Glycine, and Alanine on the Respiration of Reticulocytes

In order to determine whether the presence of certain readily oxidizable substances in the surrounding medium resulted in any accelera-

tion of the rate of respiration of the reticulocytes, they were separated from their serum and added to saline solutions containing variable concentrations of these substances. Glucose was used in concentrations varying from zero to 400 mg. per hundred cc. Glycine and alanine were used in solutions containing from zero to 28 mg. of amino acid nitrogen per hundred cc. These amino acid solutions had no perceptible effect upon the rate of respiration, while the glucose solutions progressively depressed it, the respiration in saline containing 400 mg. per cent of glucose being on the average only 84 per cent of that in saline. Whether the glucose actually entered the reticulocytes is problematical. Kosawa (13), and Ege (14) found that the red cells of the rabbit are impermeable to glucose, but this may or may not be true of the reticulocytes.

Since reticulocytes have approximately the same rate of respiration in normal physiological saline solution as they have in their own serum, it is probable that the substances oxidized are present in the cells as a residue from an earlier stage of development, and are not gaining access to them continuously from the exterior. The disappearance of the reticulum during the period in which respiration takes place suggests that it is part if not the entire material consumed. The actual quantity of this substance oxidized may be approximately calculated. Since the reticulocyte stage of development is about 50 hours, and the average oxygen consumption about 60 cm. per billion reticulocytes per hour, the total quantity of oxygen used by every billion reticulocytes will be about 3 cc.

Unfortunately very little is known of the chemical nature of the reticular substance. Gawrilow (15), from a study of its reaction with vital stains, believes that it is partly protein, partly lipoid. Neither is there any consistent data upon the respiratory quotient of reticulocytes from which it might be possible to draw any conclusions as to its chemical constitution. Douglas (16) found an average respiratory quotient of 1.02 on three samples of citrated anemic rabbit's blood. This is much too high a value to be accounted for by the oxidation of either lipoid or protein, though there is no reason to believe that other substances may not be oxidized in addition to the vitally staining reticulum. Harrop and Barron (17) (1928) found values from which they suggested that the respiratory quotient was between 0.75 and

0.80, and such a value would of course be in closer agreement with Gawrilow's views.

Should the material consumed in the reticulocytes be a fatty acid, 3 cc. of oxygen would account for the oxidation of about 1.5 mg.; were it carbohydrate or protein, the weight would be greater. The weight of the dry material of one billion reticulocytes is about 40 mg., of which about 35 mg. is hemoglobin, so that the respiration of the reticulocyte must result in the destruction of a large proportion of its contents apart from the hemoglobin. This proportion is all the greater since more than 1 mg. of the residual 5 mg. is inorganic matter.

DISCUSSION

At the conclusion of this paper it is possible to compare the respiration of the mammalian reticulocytes and that of the normal nucleated and primitive red cells of the bird. Such a comparison is necessarily rough, but it may afford some indication of the solution of the problem which was raised at the commencement of the first paper of this series: the relative importance of the nucleus as the seat of cell oxidation. On the one hand, in the rabbit reticulocytes, we are concerned with a corpuscle entirely devoid of a nucleus; on the other, in the primitive avian erythroblast, a nucleated cell possessing an intensity of respiration not greatly inferior to that of a parenchymatous cell such as the liver cell.

Since all the determinations were made at the same temperature (37.5°C.) no allowance need be made for the temperature coefficients of the respiratory processes. There are, however, considerable differences in the sizes of the various cells whose respiration is to be compared. Consequently, in order to render the comparisons more satisfactory, the respirations should be considered not on the basis of cell number but rather upon one of cell volume. This is evident since the volume of the primitive red cell of the fowl is about twice as great as that of the rabbit's reticulocyte.

Hematocrit observations make it possible to determine the volume occupied by the various types of red cell. The volume of one billion red cells in the blood of anemic fowl is approximately 140 c.mm., in the blood of a normal fowl 100 c.mm., and of the reticulocytes of the rabbit 85 c.mm. In the blood of the anemic fowl about 50 per cent

of the cells were the normal variety of adult erythrocyte, so that the volume occupied by one billion of the more primitive cells would be about 180 c.mm. The following table shows the relationship between the respiration of these types of cells, together with their volumes, and allows a comparison to be made of their respiration on the basis of an arbitrary unit volume of 100 c.mm.

Type of cell	Volume occupied by one billion cells	Oxygen consumption per billion cells	Oxygen consumption per 100 c.mm. cells
	c.mm.	*c.mm.*	*c.mm.*
Fowl: Prim. Red Cell	180	250	140
Fowl: Norm. Red Cell	100	12	12
Rabbit: Retics	85	60	70

It is evident from this table that if the metabolism of the reticulocyte gives any indication of the intensity of oxidation taking place in the cytoplasm of a cell such as the primitive red cell of the fowl, that the oxidative processes of the cell are far from taking place exclusively in relation with the nucleus. The reticulum appears to be associated closely with the cell oxidation either as the material consumed or as the mechanism by means of which the process is effected. The possibility that the reticulum is in reality a nuclear remnant is now generally discredited. Both the primitive red cells of fowls and many of the normoblasts of mammals possess both a nucleus and a reticulum, and both are often present simultaneously in the cells as distinct structures. Further, their staining properties are dissimilar. It would seem probable, therefore, that in the primitive avian red cell oxidation processes are taking place both in the cytoplasm and in the nucleus and that the partition between the two may not be far from equal. Further, it is evident that though the definitive red corpuscles of the fowl are nucleated cells the respiration is small in comparison with the non-nucleated reticulocyte of the mammal with its residual cytoplasmic reticulum.

SUMMARY

1. The respiration of the reticulocytes of the rabbit has been measured during the period of an anemia produced by phenylhydrazine. Though the respiration increased greatly during the phase

of regeneration, the oxygen consumption per billion reticulocytes throughout the period remained approximately the same.

2. The respiration of the reticulocytes was affected by changes in the reaction of the medium in which they were suspended, and was at its maximum about a pH of 8, with a probable intracorpuscular pH of about 7.75.

3. Variations in the tonicity of the suspending medium did not produce any great change in the respiration of the reticulocytes.

4. The presence of glycine, alanine, and glucose in the suspending medium resulted in no acceleration in the respiration of the cells. At higher concentrations glucose tended to depress the respiration. The material oxidized appears to be mainly or entirely contained in the corpuscles at the time they are liberated from the marrow.

5. A comparison is made of the respiration of the reticulated nucleated red cells present in the blood of anemic fowls and the non-nucleated reticulated red cells of rabbits. On the basis of equal volumes of cells, the respiration of the former is about twice that of the latter, while this in turn is about six times as great as the nucleated but non-reticulated normal red cells of the fowl.

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