FACTORS INFLUENCING THE RESPIRATION OF ERYTHROCYTES

I. Primitive Avian Erythrocytes

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INTRODUCTION

The function of the nucleus as the principal seat of the oxidative processes in the cell was first suggested by the investigations of Spitzer (1) upon the location of oxidizing enzymes. These observations were given considerable support by Lillie (2), who studied the problem with the aid of dyes that underwent color changes upon oxidation. He found that "the coloured oxidation products are seen to be deposited chiefly in and about the nucleus, especially at the surface of contact between nucleus and cytoplasm." These studies were followed up by Warburg (3) in a series of observations upon the comparative rates of reduction of the hemoglobin in the blood of mammals and birds, incubated for some hours in sealed vessels. He found that there existed a very marked difference between the two: the non-nucleated cells of the mammals had a comparatively small consumption of oxygen, whilst the nucleated ones in avian blood possessed a vigorous oxidative metabolism. He further showed that in both mammals and birds the oxidative metabolism proceeded at a much greater rate in blood which contained many young cells staining in a basophilic manner with methylene blue. In fact it appeared doubtful whether the fully developed normally staining mammalian red blood cells consumed oxygen to any appreciable extent. The whole of the slight oxygen consumption of the normal blood of adult mammals would seem to be due to the small proportion of young reticulated and basophilic cells ordinarily in the circulation.

The very large oxygen consumption of avian blood, especially after

hemorrhage when the proportion of primitive red cells in the circulation was high, led Warburg to a study of the factors which influence its metabolism. Many of the experiments were concerned with those factors that reduce or inhibit oxidation, and culminated in his well known theory of the importance of the part that structural integrity plays in the processes of cell oxidations (4).

The following experiments were carried out with the intention of determining rather more fully the effects that certain changes in environment produce upon the metabolism of the primitive avian red cells.

Methods

The blood used in all the experiments was removed by cardiac puncture from fowls made anemic by intraperitoneal injections of phenylhydrazine hydrochloride. 10 cc. of a one half per cent solution in normal physiological saline was given to each bird. The blood was used from the third to the seventh day following the injection, and the removal, either daily or every second day, of 30 cc. required for each experiment assisted in maintaining the anemia for this period. Immediately the blood was drawn it was placed in small bottles and shaken with glass beads until defibrinated. It was then filtered through a small wad of cotton wool to remove the clot and was then ready for use.

The blood was generally removed immediately before the experiment was set up. Occasionally, when kept for a few hours, it was placed in the cold room. Harrop (5) has shown that at low temperatures the viability of the cells is well maintained. In his experiments there was only a small reduction in the oxygen consumption of cells incubated at 38°C., after having been kept for 24 hours at 7°C., as compared with cells from the same blood which were incubated as soon as they were removed from the animal. My own observations are in agreement with his finding.

The oxygen consumption of the blood was determined in the respiration apparatus devised by Barcroft and modified by Warburg (6). 2 cc. of blood or of cell suspension were invariably used for the estimations. The oxygen consumption was determined at a temperature of 37°C.

The apparatus proved very satisfactory for this purpose; the oxygen consumption of this quantity of blood in flasks of about 15 cc. capacity usually causing a movement of the fluid meniscus in the manometer tube of some 10 to 20 cm. in the 90 minutes duration of the experiment. After the blood was placed in the apparatus the flasks were shaken about eighty times a minute for half an hour in the thermostat tank for the purpose of raising the temperature of the blood to 37°C. and also of fully oxygenating the red cells. The carbon dioxide given off was absorbed by normal sodium hydroxide placed in the small central cups in the flasks.

The possible sources of error in determining the oxygen consumption of blood have been discussed by Morawitz (7), by Warburg (3), and by Harrop (5) and

consequently need little consideration here. The duration of the experiments carried out by these investigators frequently extended over a period of many hours and consequently necessitated an aseptic technique. These observations were completed within 2 hours of the commencement of incubation and consequently no especial precautions were taken to ensure absolute sterility.

The question of the applicability of the Warburg method to this type of experiment requires more consideration. Two possible sources of error in the determinations need to be discussed. First, the evolution of carbon dioxide from the blood might possibly have some effect in causing the hemoglobin to take up small quantities of oxygen by a change in its affinity and so giving a spurious appearance of true oxidation by the red cells themselves. Secondly, all the hemoglobin may not be present in the form of oxyhemoglobin by the time the actual metabolism measurements are started. That no serious error from either of these causes occurs is shown by experiments upon the blood of normal rabbits containing only a small number of reticulocytes. Such blood, after the usual equilibration period of 30 minutes, shows a practically negligible oxygen consumption, indicating that by that time all the hemoglobin in the cells has become completely oxygenated. Further, this small consumption of oxygen continued with only slightly diminishing intensity for the ensuing 90 minutes, the duration of the experiment.

The degree of accuracy of the actual determinations may be judged from the following measurements of the oxygen consumption of the same samples of blood carried out simultaneously in six manometer tubes:

Tube Numbers	1	2	3	4	5	6
	C.mm. oxygen consumed per cc. blood per hour					
Expt. 1	123.8	122.2	122.4	122.0	124.0	123.6
Expt. 2	64.0	63.5	64.6	63.6	64.3	63.5

I

Metabolism in Phenylhydrazine Anemia

Phenylhydrazine has been extensively used in the production of experimental anemias in animals. It was first made use of in birds by Hirsch and Edel (8). Later it was employed by Kasarinoff (9) in his study of the primitive blood cells in fowls and his paper contains several excellent illustrations of the early red cell forms. When fowls are injected intraperitoneally with phenylhydrazine in medium doses, (20 mg. per kilo) in the course of a few minutes there is a striking

change in their appearance. Their combs assume a dusky brown color, and if blood be drawn at this stage it exhibits the same discoloration. This chocolate brown color of the blood persists for 48 to 72 hours before finally passing off, its termination being roughly coincident with the point of greatest severity in the anemia. The recovery from the anemia takes place fairly rapidly at first and then more slowly, being characterized by somewhat considerable oscillations in the red cell counts, a condition previously observed in the phenylhydrazine anemia of rabbits by Morawitz (7).

TABLE I

Fowl No.	Days after injection	Red cell count in millions per c.mm.	Oxygen consumption		
			C.mm. per cc. blood per hour	C.mm. per billion red cells per hour	Ratio of anemic to normal blood
24	0	3.27	29.7	9.1	—
	2	1.12	118.2	105.4	11.6
	4	1.98	163.8	82.8	9.1
	6	2.34	72.3	30.9	3.4
	8	1.76	29.8	15.9	1.8
	10	1.98	30.2	15.2	1.7
	12	1.80	31.1	17.3	1.9
25	0	3.53	26.9	7.6	—
	2	1.49	129.1	86.7	11.4
	4	2.27	164.2	72.3	9.5
	6	2.37	64.8	27.3	3.6
	8	2.27	37.3	14.5	1.9
	10	1.82	24.4	13.4	1.7
	12	2.23	37.7	16.9	2.2

Both fowls were injected with phenylhydrazine hydrochloride 0.5 per cent solution in saline (20 mg. per kilo) immediately after the blood was taken for the oxygen determinations on the first day of the experiment.

In this experiment observations were made both on the red cell counts and on the oxygen consumption of the blood. Two fowls were injected intraperitoneally with a one half per cent solution of phenylhydrazine in normal physiological saline (20 mg. per kilo). Immediately previous to the injection 12 cc. of blood were removed from each bird by cardiac puncture and its oxygen consumption determined. These latter were 9.1 and 7.6 c.mm. of oxygen per billion (one thousand

million) red cells per hour. Every second day afterwards 12 cc. of blood were similarly removed and the oxygen consumption and red cell count determined. The results are found in Table I and in Fig. 1.

The onset of the anemia was accompanied by a striking rise in the oxygen consumption of the cells, which at its peak attained a value of more than ten times its initial figure. At the same time the morpho-

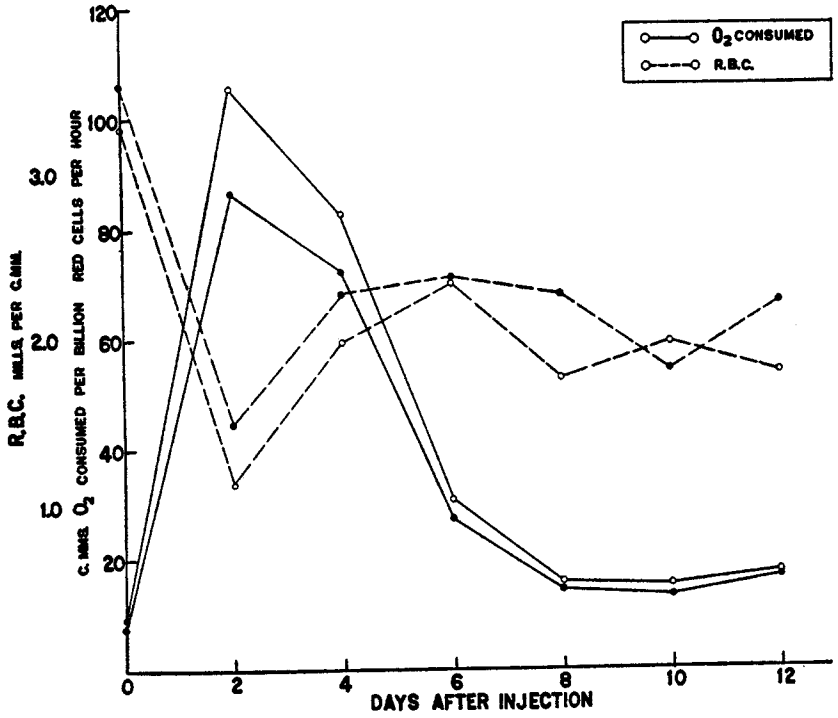

FIG. 1. Showing the oxygen consumption of avian red cells at various stages of an anemia produced by injection of phenylhydrazine.

logical appearances of the blood showed the marked changes described by Kasarinoff (9). Large numbers of the primitive hematoblasts were present together with many cells at all stages in the development of erythrocytes.

The enumeration of the various forms of primitive cells proved difficult on account of the many transitional varieties, and no attempt at division into specific categories was made other than into two large

groups: erythrocytes of normal appearance, and "primitive red cells" inclusive of all cells from the hematoblast to the large hemoglobin-containing cells still exhibiting vitally staining reticulum. Differential counts carried out in this way upon blood films, stained vitally with brilliant cresol blue followed by Wright's stain, showed that, at the peak of metabolism on the second day, in one blood 40 per cent and in the other 38 per cent, of the circulating cells fell into the category of "primitive red cells." With the decline in the intensity of the metabolism there was a corresponding fall in the proportion of these cells, development into the more advanced forms taking place in the blood stream. If the cells classified as normal merely possess the same oxygen consumption as similar cells present in the normal blood studied before the onset of the anemia, it is evident that the "primitive red cells" as a group have a metabolism some twenty to twenty-five times as great as the adult erythrocytes. Should the metabolism of normal blood be partly accounted for by the circulation of a small proportion of cells newly delivered from the bone marrow, and comparable with the reticulocytes of mammals, the difference between the oxygen consumptions of the two groups would be even greater.

The red cells which circulate in the blood of normal birds, though they retain their nuclei, are in an advanced state of degeneration so far as the normal cellular activities are concerned. Their respiration, which affords a measure of their metabolism, is low as judged by the respiration of other organs, while the process of pyknosis of their nuclei, which takes place in the later states of their development in the marrow, is indistinguishable from that taking place in the cells of other degenerating tissues. On the other hand the "primitive red cells" in the process of their transformation in the circulation into the normal adult erythrocytes consume oxygen at a rate of approximately 0.5 to 1.0 c.mm. per mg. per hour. While such an oxygen consumption is still somewhat smaller than that observed by Barcroft and Shore (10) in the cells of the liver of the cat, and by Usui (11) in those of the liver of the mouse, it must be recalled that the erythrocyte has included in its cytoplasm a relatively large quantity of oxyhemoglobin which has no oxygen consumption in the ordinarily accepted sense of that term. If allowance be made for this cellular inclusion the metab-

olism of the "primitive red cells" of the fowl becomes comparable with that of the cells of a parenchymatous organ like the liver. This relatively high metabolism, together with the resistance of the red cell to mechanical injury and its life in a fluid matrix of well determined composition, combines to render it a very suitable type of cell for the study of the influence of various factors upon cellular metabolism.

In the course of 8 days from the time of injection of the phenylhydrazine into the fowl the oxygen consumption of its blood had risen to a peak and returned nearly to its previous normal level. The persistent anemia was almost certainly due to the frequent removal of small quantities of blood for the purposes of the experiment. The continuous blood regeneration which just compensated for these losses was at the same time responsible for the somewhat elevated metabolism which was observed in the last few determinations made.

II

Effect of Variation in Tonicity upon Metabolism

The following experiments were carried out to determine how greatly metabolism was affected by variations in the tonicity of the saline solutions in which cells were suspended. The blood was obtained from fowls made anemic by an injection of phenylhydrazine. After centrifuging the blood for 5 minutes the supernatant serum was removed with a pipette and the cell suspension made up to its original volume with sodium chloride solutions of varying concentrations. Only one centrifugation was employed in order to minimize the inevitable injury to the cells caused by their being packed tightly together.

The observations are given in the following table:

Red cells in millions per c.mm.	NaCl: 0.6 per cent	0.75 per cent	0.85 per cent	0.95 per cent	1.10 per cent
	C.mm. O_2 consumed per billion red cells per hour				
2.05	32.2	32.0	32.0	33.2	32.8
1.33	36.7	37.9	39.7	39.6	32.8
0.92	41.7	42.6	44.8	44.6	41.1

While the effects of varying the tonicity of the saline solutions in which the cells are suspended are comparatively slight within the limits

employed in these experiments, it can be seen that the maximum metabolism occurs at some point between 0.85 per cent and 0.95 per cent of sodium chloride. Hematocrit observations made by the method described by Hirota (12) demonstrated that between these concentrations the size of the suspended red cells both in normal and anemic blood approximates most closely to that when they are present in serum.

Red cells in millions per c.mm.	Serum	0.75 per cent	0.85 per cent	0.90 per cent	0.95 per cent	1.05 per cent
	Hematocrit readings in per cent in NaCl solutions					
2.76	28.5	31.3	29.3		27.6	27.2
1.36	24.3			24.2		

These results are similar to those obtained by Ray (13) for the reticulocytes of the dog. He made observations on the oxygen consumption of these cells in the presence of M/12.5 solutions of sodium lactate to which sufficient sodium chloride was added to render them isotonic with the following concentrations of sodium chloride: 0.5, 0.7, 0.9, 1.1, 1.3 per cent. He found that the oxygen consumption attained its maximum in solutions isotonic with 0.9 per cent sodium chloride and that it declined rather more rapidly if the solutions were made hypertonic than if they were made hypotonic.

III

The Influence of Hydrogen Ion Activity upon Metabolism

The extent to which the hydrogen ion activity of the surrounding medium influenced metabolism in the avian red cell was determined in this series of experiments. The method employed here differed somewhat from that adopted in the other studies. The oxygen consumption of the cells was measured in their own serum to which certain known quantities of acid had been added. In order to examine whether any specific ionic effects were involved both hydrochloric and phosphoric acids were used.

To 2 cc. of blood from an anemic fowl 1 cc. of 0.87 per cent sodium chloride solution containing known amounts of these acids was added. The acidity of these saline solutions varied between N/200

TABLE II

The Effect of Variations in Hydrogen Ion Activity upon the Metabolism of Avian Red Cells

Concentrated (mols per l.) HCl in 0.87 per cent NaCl solution added to cells	C.mm. O_2	Per cent of max. O_2 consumption	pH	C.mm. O_2	Per cent of max. O_2 consumption	pH	C.mm. O_2	Per cent of max. O_2 consumption	pH
C.									
0.000	45.0	86	—	34.1	85	8.65	33.6	87	8.79
0.005	45.6	88	—	36.1	90	8.53			
0.010	48.9	94	—	37.6	94	8.03	36.4	94	8.02
0.020	52.1	100	—	40.0	100	7.80	38.7	100	7.90
0.030							38.8	100	7.64
0.040							35.2	91	7.44
0.050	45.3	87	—	35.2	88	7.27	28.7	74	7.22
0.100	27.1	52	—	19.0	48	5.85			
Red cell counts in millions.....	1.75			1.96			2.15		

Concentrated (mols per l.) H_3PO_4 in 0.87 per cent NaCl solutions added to cells	C.mm. O_2	Per cent of max. O_2 consumption	pH	C.mm. O_2	Per cent of max. O_2 consumption	pH	C.mm. O_2	Per cent of max. O_2 consumption	pH
C.									
0.000	29.6	92	8.34	47.4	91	8.26	45.0	84	8.46
0.005							45.9	86	8.19
0.010	30.8	96	8.04	51.9	100	8.10	49.8	93	8.08
0.015							53.4	100	7.88
0.020	32.1	100	7.65	52.0	100	7.53	53.5	100	7.62
0.025							45.1	84	7.30
0.030	24.9	78	7.28	40.3	77	7.18			
0.040	21.0	65	6.91	32.3	62	6.79			
0.050	18.6	58	6.66	29.8	57	6.56			
Red cell counts in millions.....	2.12			1.44			1.18		

* Oxygen consumption given in units of c.mm. O_2 per billion red cells per hour.

and N/10. 2 cc. of this cell suspension were used for the determination of its oxygen consumption. Immediately after the measurement of the metabolism was completed the suspension of cells was removed from the flasks of the Barcroft-Warburg manometers, rapidly centri-

fuged and the supernatant fluid removed. This separation of the cells was effected as quickly as possible to minimize any change in reaction of the solutions by the further metabolism of the red cells.

The reaction of the supernatant fluid was measured with the quinhydrone electrode. I am indebted to Dr. Arda Alden Green for checking a number of the more important points on the hydrogen

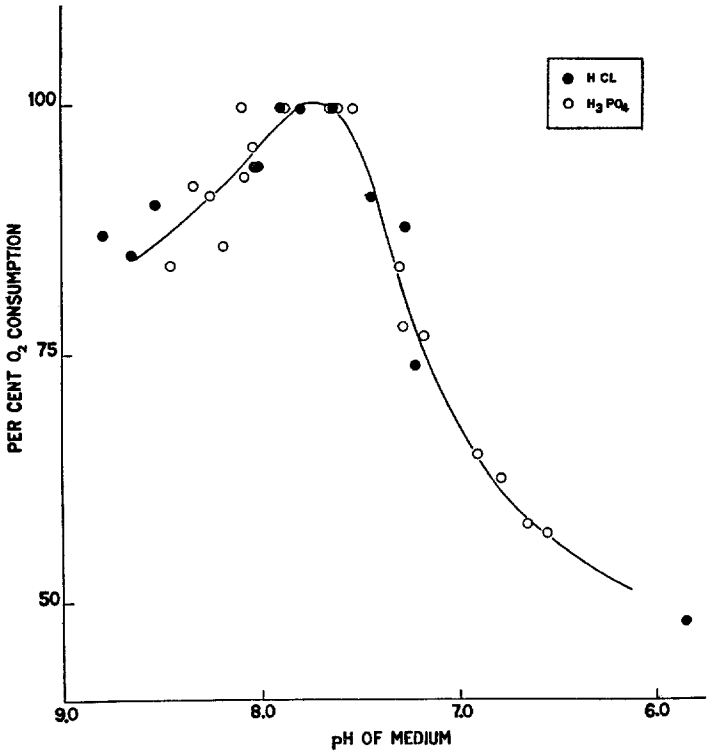

FIG. 2. Showing the influence of changes in hydrogen ion activity upon the oxygen consumption of primitive avian red cells.

electrode. The agreement between the two methods proved satisfactory up to a reaction of about pH 8.4.

The relative alkalinity of the sera, to which saline only was added, was due to the loss of a large part of its carbon dioxide in the first few minutes of shaking in the apparatus. This carbon dioxide was absorbed in the sodium hydroxide which was placed in the central cups

of the flask in order to combine with the carbon dioxide given off by the cells in the course of their metabolism.

The results are presented in Table II and in Fig. 2.

These experiments demonstrate that the addition of small quantities of acid to the serum in which the cells are suspended exercises a very considerable influence upon the oxygen consumption of the red cells themselves. This effect appears to be dependant upon the hydrogen ion concentration of the resulting mixture, it being immaterial whether this reaction be reached by the addition of phosphoric acid or hydrochloric acid. In the case of certain other acids their presence in concentrations necessary to bring about the range of reaction investigated here would most probably introduce specific ionic effects other than that from the dissociated hydrogen ion.

The range of variation studied was considerable: the lowest point being pH 5.85, and the highest pH 8.79. The maximum oxygen consumption took place at pH 7.75. In solutions of this reaction the contents of the corpuscles would probably have a considerably more acid reaction. Taylor (14) has estimated that at this reaction the corpuscular contents in goose blood have a pH value nearly 0.3 lower than the serum. On either side of this reaction the metabolism rapidly declined, until on the acid side at a pH of 6.5 it was only half of its value at the maximum. On the alkaline side the falling away did not seem to be so steep, but since the quinhydrone electrode is inaccurate at reactions more alkaline than pH 8.4 the figures in this range are less dependable. It is of interest that Fischer (15) in his investigations of the influence of hydrogen ion activity upon the growth and emigration of fibroblasts in tissue cultures found that these took place at their best between pH 7.4 and pH 7.8. He also observed that repeated cultivation in a medium on the alkaline side of pH 7.4 was much less injurious to the cells than cultivation in one more acid.

There exists the possibility that the hydrogen ion concentration of the media might undergo change during the course of the experiment and that the final determinations might give a false indication of the reaction at which the maximum oxygen consumption took place. Should the reaction change appreciably within the 90 minutes of the experiment it might be expected that, since the red cells are evidently

quite sensitive to such a change, the maximal metabolism in the last 45 minutes would be observed in a different observation flask from that of the experiment as a whole. This did not take place. In every case the flask of blood which registered the highest oxygen consumption for the whole experiment also registered the highest if the last 45 minutes were alone considered. It is consequently highly improbable that the reactions in the individual flasks varied appreciably in the period of observations.

IV

Effect of Variations of Glucose Concentrations upon Metabolism

A series of observations was made to determine the effect of the presence of small quantities of glucose upon the oxygen consumption of the red blood cells in the blood of anemic birds. The technical procedures were similar to those employed in the studies of the influence of tonicity. The blood from anemic fowls was defibrinated by shaking with glass beads and centrifuged for a period of 5 minutes in a series of graduated tubes. After the supernatant serum was removed the cell suspensions were restored to their original volumes by the addition of sodium chloride solutions (0.87 per cent) containing varying quantities of glucose. In the last experiment, where the glucose was added in rather high concentration, that of the sodium chloride was correspondingly reduced. It was found, by hematocrit determinations, that the cells both of normal and anemic fowls' blood had the same volume in 4.8 per cent glucose solutions as they had in their own serum and the concentrations of the sodium chloride present in the last experiment were adjusted upon this basis. No adjustment was made for variations in tonicity in the earlier experiments where lower concentrations of glucose were used. Previous observations have however shown that greater alterations in tonicity than were involved here have little influence upon the oxygen consumption of the cells, and this influence would in any case have been in the direction of reduction rather than acceleration of metabolism.

Table III and Fig. 3 show the results obtained.

It is evident that small increments in the concentration of the glucose present are accompanied by corresponding acceleration in the oxygen

consumption of the cells. With further increase in the amount of glucose, however, the metabolism was stimulated less and less until from 0.6 per cent to 1.2 per cent no change in the consumption of oxygen took place. The metabolism between these two points was steady at a level some 15 per cent above that in the corresponding glucose-free saline solution. We have not determined how far this condition of steady oxygen consumption continues with rising glucose concentration. Warburg (3) states that the blood cells in anemic geese consume the same quantity of oxygen in a 5 per cent glucose

TABLE III

C.mm. O_2 Consumed per Billion Red Cells Per Hour in Varying Concentrations of Glucose

Red cells in millions per c.mm.	0.91		1.66		1.98		1.98	
Concentration glucose	C.mm. O_2	Per cent of saline value	C.mm. O_2	Per cent of saline value	C.mm. O_2	Per cent of saline value	C.mm. O_2	Per cent of saline value
per cent								
nil	77.1	100	122.9	100	96.0	100	72.4	100
0.1	81.4	105	130.4	106	102.0	106		
0.2	83.6	108	132.0	107	102.0	106		
0.3	84.8	110	132.5	108	104.5	109	81.6	113
0.4			133.8	109				
0.5			134.3	110	106.5	111		
0.6							83.6	115
0.75					109.6	114		
0.9							83.4	115
1.2							83.4	115

solution as they do in Ringer's solution, which would imply a decline beyond a maximal plateau. I have previously carried out determinations (16) of the sugar concentration present in the serum of normal starved fowls and found it to be from 0.20 to 0.22 per cent. It is clear therefore that the maximal oxygen consumption of the red cells takes place at a glucose concentration several times that normally present in the serum.

The permeability of erythrocytes to glucose has been extensively studied, but so far as I have been able to find, in every case upon the normal fully developed cells and in nearly all cases upon those of

mammals. However since the metabolism measured in this study is almost exclusively that of the "primitive red cells" these observations upon permeability do not necessarily apply to them. It would seem improbable that the oxygen consumption of cells could be increased unless the glucose actually entered the cells. A control experiment showed that no interaction, involving the consumption of oxygen,

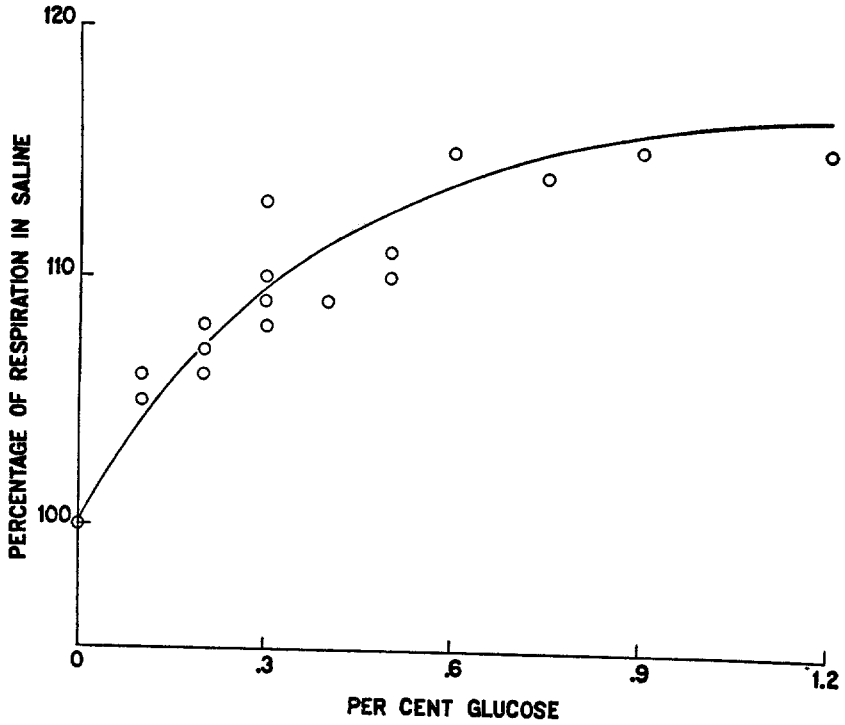

FIG. 3. Showing the influence of glucose concentration upon the oxygen consumption of primitive avian red cells.

took place when small quantities of cell-free serum were added to the glucose solutions used.

A further consideration indicating that the cells are permeable to glucose is provided by observations on the gradual decline in the oxygen consumption of the red cells as the duration of the experiment is extended. Ordinarily the period over which the observations were made was 2 hours and even in the course of that time a gradual de-

crease in the rate of consumption of oxygen could be observed. If this period were much extended the decline would become quite obvious. This decrease however was much less rapid in those solutions containing much glucose than in those with none. It would consequently seem that the oxidative processes involve the gradual depletion of some constituent of the cell which the presence of glucose in the surrounding medium could in some degree replace. This confirms the results of Grafe (17) who found that in Locke's solution containing glucose the cells respired almost as vigorously in a second experimental period of 90 minutes as they did in the first one of the same length of time.

The nature of the substances oxidized in the red cell is not known. Harrop and Barron (18) have carried out a few experiments upon the respiration of avian (goose) red cells, both in normal and anemic blood, in which they determined both the oxygen consumption and the carbon dioxide production. The resulting respiratory quotients were however too variable to give any indication of the character of the materials consumed. That these materials are, however, mainly within the cells themselves is shown by the fact that the oxygen consumption of cells suspended in an isotonic saline solution is not greatly inferior to that of cells from the same source in their own serum.

V

The Influence of Certain Amino Acids on Metabolism

Studies upon the metabolism of man and animals have shown that proteins and products of protein hydrolysis possess an especially great specific dynamic action. The following group of experiments was carried out to determine whether this stimulation of metabolism by amino acids could be reproduced on avian red blood cells *in vitro*.

In these experiments, as in those in which glucose was studied, the serum was separated from the cells by centrifugalization and replaced by solutions of various amino acids in 0.87 per cent sodium chloride. The amino acid solutions were neutralized with sodium hydroxide to pH 7.6. The buffering power of such solutions is very slight compared to that of the cells to which they were added. In each experiment the amino acid solutions added to the cells varied in concentration from

zero upwards to 50 mg. amino acid nitrogen per 100 cc. In a previous series of determinations I have found that serum of normal starving fowls contains from 14 to 18 mg. amino acid nitrogen per 100 cc. as estimated by the colorimetric method of Folin and Wu. Consequently the maximum concentration of amino acid used was approximately three times as great as in the serum of the starving fowl.

TABLE IV

Effect of Amino Acids upon the Oxygen Consumption of Red Cells

Amino acid	Millions per c.mm.	Concentration of amino acid (mg. amino N per cent)							
		nil.	1 mg.	2 mg.	3 mg.	5 mg.	10 mg.	25 mg.	50 mg.
		C.mm. O_2 consumed per billion red cells per hour							
Glycine	1.82	34.7 (100)				33.5 (97)	33.5 (97)	30.2 (87)	29.2 (84)
	1.39	92.8 (100)				90.9 (98)	85.3 (92)	72.0 (78)	66.1 (71)
	1.67	34.7 (100)	34.8 (100)	34.7 (100)	34.2 (99)	33.3 (96)			
Alanine	1.47	44.3 (100)	44.8 (101)	45.9 (103)	44.7 (101)	44.8 (101)	44.5 (100)		
Histidine	1.58	48.1 (100)				47.0 (98)	47.1 (98)	46.4 (97)	
l-aspartic acid	1.17	55.3 (100)				55.2 (100)	55.5 (100)		

The figures in brackets represent the oxygen consumption in percentage of that taking place in the saline medium.

The actions of four amino acids were investigated: glycine, alpha alanine, histidine and *l*-aspartic acid. The latter was studied because Burge, Wickwire, Estes, and Williams (19) had found that certain optically active amino acids, amongst them *l*-aspartic acid, exerted a stimulating effect upon the metabolism of *Paramecium*, which was not present in the optically inactive ones. Glycine and alanine on the other hand are of interest since Lusk (20) has shown them to have

especially high specific dynamic actions when tested on the metabolism of dogs.

The results are shown in Table IV.

It is clearly evident from these experiments that none of the amino acid solutions employed caused any elevation of the oxygen consumption of the fowls' red cells in any way comparable to their specific dynamic action in the intact animal. At low concentrations their effect is negligible; alanine being the only one to produce any increment and that of an order little greater than the experimental errors of the determinations. At higher concentrations than 5 mg. of amino acid nitrogen per 100 cc., glycine caused a very definite and increasing reduction in the oxygen consumption of the cells. The other three amino acids, alanine, histidine, and *l*-aspartic acid were without effect in concentrations up to 10 mg. of amino acid nitrogen per 100 cc., a concentration which it is unlikely that they ever exceed in the serum of the intact animal even at the height of digestion.

The particular component of the amino acids responsible for the specific dynamic action has long been a subject of controversy. Grafe (17) (21) has contributed to the problem from two aspects. Working originally with Warburg and employing his methods, he made a study of the action of ammonia and of a number of amines upon the oxygen consumption of the red cells of the blood of anemic geese. He observed that if 10 cc. of a neutralized saline solution containing 1/100 N NH_4Cl were added to 2 cc. of a concentrated suspension of red cells, their metabolism became approximately 60 per cent greater than those in a similar cell suspension to which saline only had been added. If, however, the cells were washed three times with this ammonium chloride solution before the oxygen consumption determinations were made the metabolism of the cells fell to approximately one-fifth of those in saline. This he accounted for by the specific absorption of ammonia by the cells, each washing contributing an additional quota to the cell contents. Consequently after three washings the cells contained considerably more ammonia than they would have absorbed from a single immersion in a solution of the same concentration. Provided the concentration of ammonia were not raised to a point at which hemolysis occurred, the inhibition of metabolism was reversible, for washing the cells from the ammonia

solutions in normal physiological saline restored the oxygen consumption to its original level. Apparently immersion in a saline solution containing 1/100 N NH_4Cl (14 mg. ammonia nitrogen per cent) raised the metabolism very considerably while the higher concentrations consequent upon repeated washings in the same solution resulted in a very considerable though reversible inhibition of the oxygen consumption.

Grafe's second contribution to this question was his study of the relative specific dynamic actions of various amino acids and ammonium chloride upon both men and animals. From the combination of his own observations with those of Lusk, Grafe comes to the conclusion that the specific dynamic action of the various amino acids is closely related to their nitrogen content. This, taken in conjuncture with the observation that, though containing no calorie content available to the body, ammonium chloride produces a pronounced stimulation of metabolism in man, makes him suggest that all these substances produce their action by reason of the amino groups which they contain. His experiments with ammonium chloride on rabbits and dogs were inconclusive.

I have carried out certain observations upon the effects of ammonium chloride at various concentrations upon the oxygen consumption of the red cells in the blood of anemic fowls. The solutions of ammonium chloride were made up and neutralized to a pH of 7.6 in exactly the same way as for the amino acids. The solutions in various concentrations were added to the concentrated cell suspensions to replace the serum removed after centrifugalization. In this way they resemble the experiment of Grafe in which he observed a very considerable stimulation of metabolism. The results are to be found in Table V.

The results of these experiments are somewhat equivocal. In all five the effect of the ammonium chloride was to produce a rise in the oxygen consumption of the cells. In two fowls, one examined twice, this stimulation was slight and attained a maximum at concentrations of between 1 and 2 mg. of nitrogen per 100 cc. In higher concentrations the metabolism of these bloods showed a progressive decline. Even at the maximum the elevation of metabolism was slight, being only approximately 5 per cent. In one fowl, also examined twice,

increase in the concentration of the ammonium chloride produced a rise in the oxygen consumption that continued as far as the maximum concentration used in experiments. The cause of the divergency in the reaction of the red cells to the ammonium chloride solutions is not evident and has not been further investigated. One difference between the blood of this animal and the others was the low oxygen

TABLE V

Effect of Ammonium Chloride upon the Oxygen Consumption of Red Cells

Fowl Number	Red blood corpuscles. Millions per c.mm.	Concentration of ammonium chloride (mg. N per cent)					
		nil.	1 mg.	2 mg.	5 mg.	10 mg.	25 mg.
		C.mm. O_2 consumed per billion red cells per hour					
2	2.36	17.8 (100)	19.0 (107)	20.1 (113)	20.5 (115)	20.1 (113)	21.1 (119)
4	1.54	37.0 (100)	38.1 (103)	38.8 (105)	36.3 (98)	35.1 (95)	34.7 (94)
5	1.91	35.4 (100)	37.2 (105)	37.2 (105)	35.6 (100)	33.2 (94)	33.0 (93)
2	1.98	15.7 (100)	17.2 (109)	17.9 (114)	18.6 (118)	18.9 (120)	19.4 (123)
5	1.74	47.4 (100)	49.0 (105)	48.6 (104)	46.3 (98)	42.3 (89)	40.7 (86)

The figures in brackets represent the oxygen consumption in percentage of that taking place in the saline medium.

consumption per billion red cells per hour, a figure only about twice that of the blood of normal fowls.

Grafe (17) in his experiment used a solution of ammonium chloride to which a molecular equivalent of sodium hydroxide had been added, so releasing free ammonia in the solution. The concentration of this ammonia was 14 mg. of ammonia nitrogen per 100 cc. Such a solution must have had an alkaline reaction at the time of its addition to the cells, though the presence of carbon dioxide in the cell suspension would ensure its rapid conversion into ammonium carbonate. In

his experiment with the red blood cells of anemic geese he observed a rise in oxygen consumption of 60 per cent. Such an elevation is far greater than any observed in the bloods of the fowls examined here. At concentrations of ammonia nitrogen comparable to his, two of the fowls' bloods showed a decrease of about 7 per cent, while the other showed an elevation of about 17 per cent.

CONCLUSIONS

1. The oxygen consumption of normal and "primitive red cells" of fowls' blood has been determined at intervals in the course of an anemia produced by the injection of phenylhydrazine. The "primitive red cells" have an oxygen consumption at least twenty to twenty-five times greater than the normal red cells.

2. Suspension of the cells derived from the blood in anemia in sodium chloride solutions of various concentrations has comparatively little effect upon the oxygen consumption of the cells.

3. The red cells from anemic blood are sensitive to variations in the reaction of the medium in which they are suspended. The maximum oxygen consumption, after addition of a saline solution containing variable amounts of acid to the blood, took place at pH 7.75. They appeared somewhat more sensitive to variations on the acid side of this reaction than on the alkaline.

4. Addition of glucose to the medium increased the oxygen consumption of the cells. Their metabolism in a physiological saline solution containing 0.6 per cent of glucose was 15 per cent higher than in one in which no glucose was present.

5. Certain amino acids in low concentrations had little effect on oxygen consumption, though at higher concentrations some of them definitely diminished it.

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