

A COMPARATIVE STUDY OF ERYTHROCYTE METABOLISM¹

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NINE FIGURES

It has been known for many years (Warburg, '09; Ramsey and Warren, '30, '34; Michaelis and Salomon, '30) that the nucleated erythrocytes of birds consume oxygen at a much higher rate than the non-nucleated erythrocytes of mammals. A number of investigators including Laskowski ('42), Dounce and Lan ('43) and Hunter and Banfield ('43) attempted to find a basis for this difference by measuring the oxidative activity of isolated nuclei. The respiratory activity of these nuclei, however, was not appreciably higher than that of whole cells. The difference in oxygen consumption might be due to one of the following factors: (1) Quantity of specific enzymes available; (2) Quantity of specific substrates available; (3) Quantity of cofactors or inhibitors present; or (4) Local differences in pH, temperature, etc. The present investigation is an attempt to test the first of these 4 possibilities.

Such a study takes on added significance in view of the present interest in the mechanism involved in cation exchanges in erythrocytes. Several groups of workers including Weller and Taylor ('51), Solomon ('52), Maizels ('51) and others have suggested that metabolic energy is involved in maintaining the imbalance of Na and K between erythrocytes and their environment. Enzyme studies might indicate the energy source for this process. Also, from the viewpoint of compara-

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tive physiology and biochemical evolution it would be valuable to have information about the enzymic constitution and general metabolism of these highly specialized and important cells.

Before undertaking this investigation it was necessary to determine whether the generalization could be made that erythrocytes of all the lower vertebrates respire at a level higher than that of mammalian red cells. It is difficult to make comparisons using the values in the literature since the measurements of the activities of fish, frog, turtle and snake bloods were made at 20° or 25°C., while the measurements with avian and mammalian bloods were made at 37–38°C. (Albritton, '51).

The present investigation consists of two parts: (1) measurements of the respiration of erythrocytes of 6 different vertebrates at 20°C. and 37°C., and (2) assays of the activity of several enzymes in these cells. Enzymes which might be responsible for differences in respiration such as succinoxidase and malic oxidase from the aerobic Krebs cycle were studied. As regards glycolysis, the nucleated avian erythrocytes show negligible aerobic lactic acid production but appreciable anaerobic glycolysis (Negelein, '25), while non-nucleated cells show a high glycolytic rate both aerobically and anaerobically (Bird, '47). From glycolysis, the terminal enzyme, lactic dehydrogenase was chosen as a convenient one to study.

MATERIALS AND METHODS

The blood from humans was obtained by venipuncture and that from rabbits, chickens, frogs (*Rana pipiens*) and sting ray (*Dasyatis*) by cardiac puncture. Fish (*Haemulon plumieri*, white grunt) blood was collected by cutting the branchial arteries which may have resulted in some contamination by body fluids and sea water. Heparin was a satisfactory anti-coagulant in all cases except for fish blood which frequently clotted during collection. The clots redissolved, however, before the blood was used. Defibrinated and heparinized bloods were compared and similar results were obtained, indicating that heparin did not affect the enzyme activity.

In some experiments the whole ray blood was stored on ice for about 2 hours, and the fish blood for 3–4 hours since the laboratory is some distance from the coast.

The whole blood was centrifuged at $2000 \times g$. for 10 minutes and the plasma and buffy layer were removed by aspiration. The erythrocytes were washed in isotonic saline or Ringer-Locke solution twice. They were immediately frozen in dry ice, and thawed at 37°C . This was repeated twice and the resulting hemolysate made up to the approximate dilution desired with phosphate buffer or water. It was frequently necessary to use a glass Ten-Broeck homogenizer in order to disperse the ghosts through this mixture.

Respiration measurements and enzyme assays were made at 37°C . in a standard Warburg apparatus. In general, assays previously described in the literature were used. To determine optimum concentration of substrates and added cofactors, the linearity between activity and homogenate (enzyme) dilution was tested for each system.

Routine C. P. chemicals were used except for DPN, which was purchased from Schwartz Co., and cytochrome c from Sigma Chemical Co. The purity of these latter two materials was tested spectrophotometrically. All the solutions used were adjusted to pH 7.4 with KOH or HCl.

RESULTS

Respiration of whole cells

The oxygen consumption of whole washed red blood cells was determined at 20°C . and 37°C . In some experiments the respiration of 2–3 samples of the same blood was determined for 60–90 minutes at 20°C . and then at 37°C . following a 30 minute interval for temperature adjustment. In other experiments, simultaneous measurements of oxygen consumption in two different baths at these temperatures were made. The blood was equilibrated with oxygen before measuring the respiration to insure oxygenation of the hemoglobin. The data are shown in table 1. Here, as in all the following tables,

the Q_{O_2} refers to the microliters of oxygen consumed per milligram of dry weight per hour. The weight was determined on 0.7–1.0 ml duplicate samples dried to constant weight at approximately 100°C.

In general the respiration data are comparable to those previously reported. The mean for chicken cells is lower than that in the literature, but additional data collected during other experiments in this laboratory give a mean of 0.142 and indicate that this is a reliable figure. As was expected, the oxygen consumption of the nucleated erythrocytes

TABLE 1
Oxygen consumption of whole erythrocytes

| KIND OF BLOOD | Q_{O_2} at 20°C. | | | Q_{O_2} at 37°C. | | | Q_{10} (27–37°C.) |
|--------------------------|--------------------|-------|-------|--------------------|-------|-------|------------------------|
| | N | Mean | S.E. | N | Mean | S.E. | |
| Human | | | | 5 | 0.020 | 0.008 | |
| Rabbit | 3 | 0.023 | 0.004 | 3 | 0.037 | 0.007 | 1.37 |
| Chicken | 6 | 0.050 | 0.003 | 6 | 0.136 | 0.007 | 1.60 |
| Frog | 8 | 0.068 | 0.005 | 9 | 0.139 | 0.047 | 1.43 |
| Fish | 5 | 0.067 | 0.008 | 6 | 0.141 | 0.009 | 1.45 |
| Sting ray (D. sp.) | 3 | 0.127 | 0.003 | 3 | 0.266 | 0.003 | 1.45 |
| Sting ray (D. centroura) | | | | 10 | 0.130 | 0.017 | |
| Sting ray (D. centroura) | | | | 9 ¹ | 0.184 | 0.020 | |

¹ Urea in the isotonic suspending medium.

is significantly higher than that of the non-nucleated ones when compared at the same temperature. A possible explanation for the high respiratory rate of the sting-ray erythrocytes might be found in the work of Dawson ('33), who demonstrated that elasmobranch blood has a large number of immature red cells. In mammalian blood, at least, these immature cells consume oxygen at a much higher rate than do mature erythrocytes. As Ponder ('48) points out one must keep in mind the large quantity of inert hemoglobin present in red blood cells and when a correction is made for this by multiplying the values obtained by a factor of at least 6, erythrocytes have oxidative activity comparable with other tissues.

It is interesting to note that the Q_{10} 's for all of the erythrocytes are of the same order of magnitude. This is what one would predict on the assumption of a similar series of chemical reactions in each type of erythrocyte.

The malic oxidase system

Malic dehydrogenase and the associated hydrogen carriers, DPN, cytochrome reductase, and the cytochrome oxidase system constitute the "malic oxidase" system. The overall activity of this complex of enzymes was assayed as follows (modified from Potter, '46):

0.1 ml of 20% potassium hydroxide in the center well
0.3 ml of 0.5 M potassium malate in the side arm
0.3 ml of 0.06 M DPN
0.3 ml of 2.0 M niacin
0.3 ml of 1×10^{-4} M cytochrome c
0.3 ml of 0.5 M potassium glutamate
1.4 ml of homogenate

The niacin is included to inhibit the enzymatic breakdown of the DPN (Mann and Quastel, '41). The glutamate serves to transaminate the oxaloacetic acid formed from the malate (Straub, '41) and thereby prevents product inhibition. This of course depends on the presence of transaminase in the homogenate.

Although in Potter's study the activity fell off very rapidly, this is not true with the erythrocyte homogenates as shown in figure 1 where the rate is constant for 40–50 minutes after tipping in the malate. The calculations of Q_{O_2} are based on 5 readings at 10 minute intervals after tipping. It is not feasible to add the DPN from the side-arm to initiate the reaction, since there is already considerable DPN in some of the erythrocyte homogenates (Leder and Handler, '51) and the reaction starts immediately on addition of the malate.

Of the nucleated erythrocytes, the chicken was studied in the most detail and figure 2 shows the linearity of the dilution curve over a wide range of homogenate concentration. In general about 100–200 mg dry weight of homogenate were

used per vessel. This may appear to be a very concentrated preparation to use in the homogenate technique but it should be remembered that of the dry weight of the nucleated red cell 80–85% is inert hemoglobin and for the non-nucleated cell this value is about 95% (Ponder, '48).

The data in table 2 show that the malic oxidase activity of the homogenates is higher in chicken, fish and one species of sting-ray than in human and rabbit.

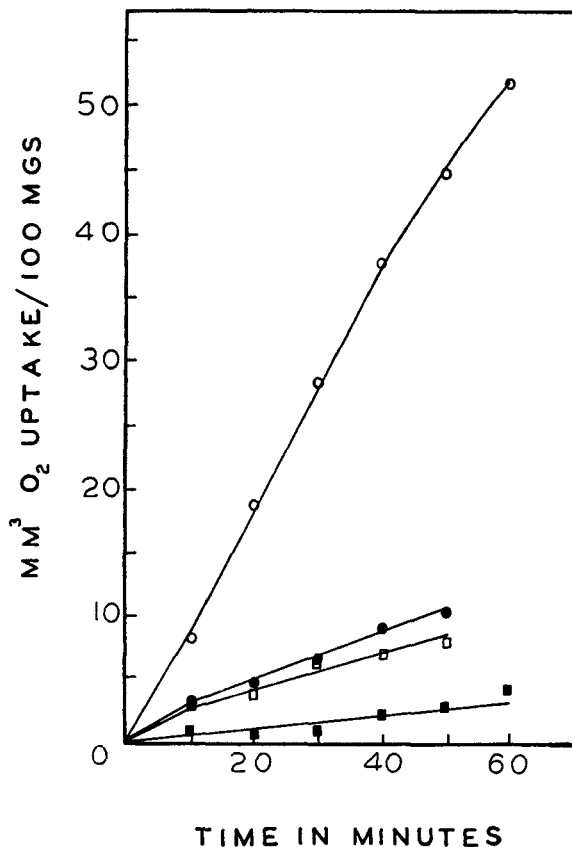


Fig. 1 Malic oxidase activity of erythrocyte homogenates
○ chicken, in the presence of 0.05 M sodium malate;
● chicken, no malate added;
□ human, in the presence of 0.05 M sodium malate;
■ human, no malate added.

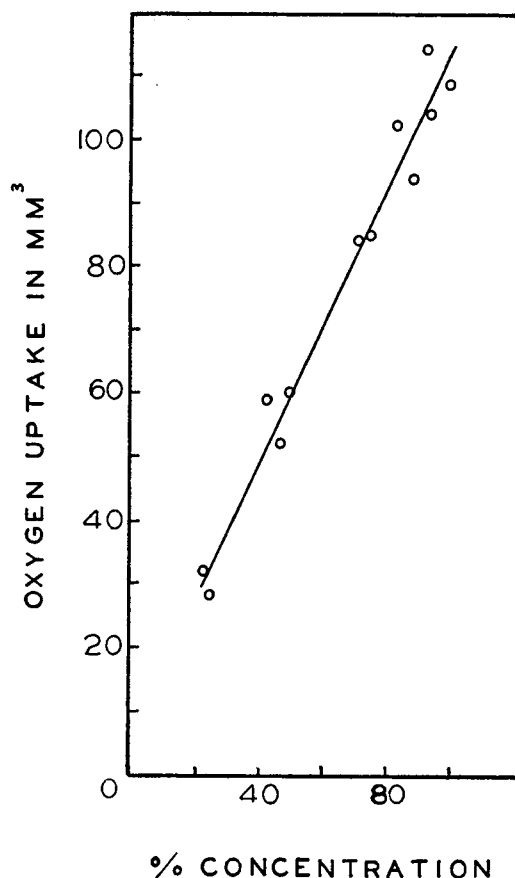


Fig. 2 Dilution curve of malic oxidase activity of chicken erythrocyte homogenates. One hundred per cent homogenate contains 0.2100 gm dry weight. Values are for 60 minutes (calculated on the rate for the first 50 minutes), obtained from three different homogenates.

TABLE 2
Malic oxidase activity expressed as Q_{O_2}

| KIND OF BLOOD | NUMBER OF SAMPLES | MEAN | S.E. |
|--------------------------|-------------------|-------|-------|
| Human | 5 | 0.057 | 0.010 |
| Rabbit | 10 | 0.167 | 0.034 |
| Chicken | 14 | 0.544 | 0.028 |
| Frog | 5 | 0.104 | 0.015 |
| Fish | 8 | 0.313 | 0.020 |
| Sting ray (D. sp.) | 5 | 0.745 | 0.042 |
| Sting ray (D. centroura) | 5 | 0.178 | 0.040 |

The succinic oxidase system

Succinic dehydrogenase, the "Slater Factor" and the cytochrome oxidase system constitute the succinoxidase system. The technique of assay used is that described by Schneider and Potter ('43). The course of the reaction for a chicken and a human erythrocyte homogenate is shown in figure 3

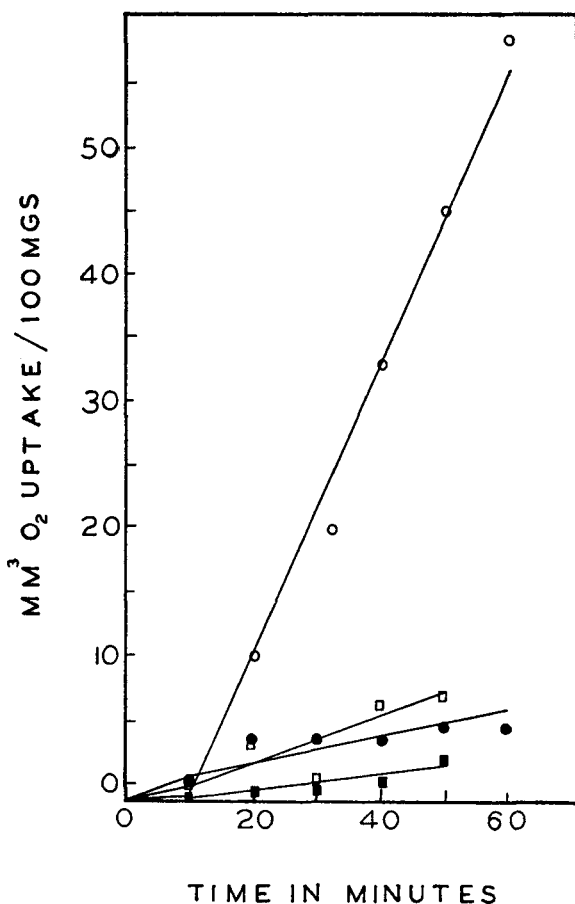


Fig. 3 Succinoxidase activity of erythrocyte homogenates

- chicken, in the presence of 0.05 M sodium succinate;
- chicken, no succinate added;
- human, in the presence of 0.05 M sodium succinate;
- human, no succinate added.

and it can be seen that the rate of oxygen consumption is linear for at least 60 minutes. The relationship between homogenate concentration and enzyme activity for chicken erythrocytes is shown in figure 4 and is linear. The Q_{O_2} values presented in table 3 were determined from 6 readings

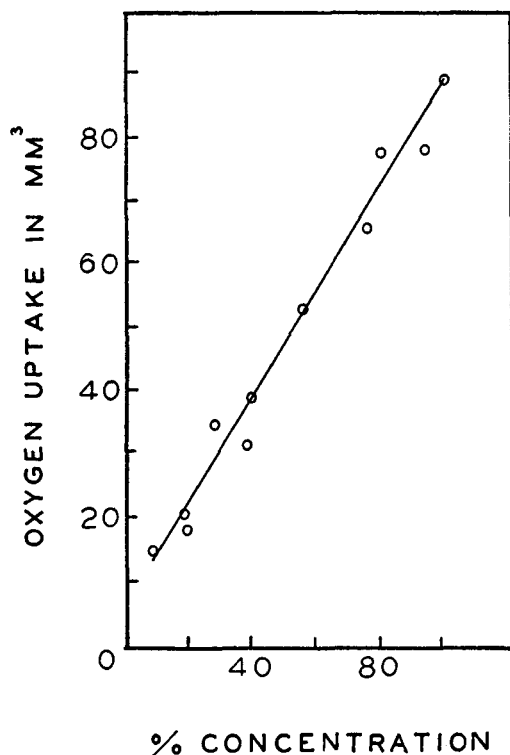


Fig. 4 Dilution curve of succinoxidase activity of chicken erythrocyte homogenates. One hundred per cent homogenate contains 0.1409 gm dry weight. Values are for 60 minutes, obtained from three different homogenates.

at 10 minute intervals after tipping. These data show that except for chicken erythrocytes there is very little succinoxidase activity in either nucleated or non-nucleated red cells. To test whether this actually represented succinoxidase activity, experiments were performed using two-side arm vessels with malonate in the second side arm. Addition of this

substance completely inhibited the reaction thus validating the assay.

The initial experiments in this series were performed on homogenates of human and chicken erythrocytes (Baker and Hunter, '52). These preliminary data suggested that both the malic and succinoxidase systems had low activity in the non-nucleated erythrocyte but considerably higher activity in the nucleated cells. The subsequent data, however, do not allow one to generalize in this regard.

In assaying any "system" such as these two which have just been described, it is necessary to determine which individual step is limiting the overall rate of the reaction. It is

TABLE 3
Succinoxidase activity expressed as Q_{O_2}

| KIND OF BLOOD | N | MEAN | S.E. |
|--------------------------|----|-------|-------|
| Human | 4 | 0.040 | 0.014 |
| Rabbit | 13 | 0.151 | 0.027 |
| Chicken | 21 | 0.534 | 0.031 |
| Frog | 5 | 0.119 | 0.051 |
| Fish | 8 | 0.033 | 0.019 |
| Sting ray (D. sp.) | 5 | 0.166 | 0.041 |
| Sting ray (D. centroura) | 13 | 0.151 | 0.027 |

usually assumed that cytochrome oxidase is present in homogenates in sufficient excess so that it is not rate-limiting. In most tissues studied this seems to be the case so that the activity of the succinoxidase and malic oxidase systems is a measure of succinic and malic dehydrogenase respectively. On theoretical grounds, one might postulate that a cell, such as a mammalian erythrocyte, which has a low rate of respiration might also have low cytochrome oxidase activity. Consequently, an attempt was made to assay for cytochrome oxidase in these erythrocytes.

The usual assay procedure includes the use of ascorbic acid for a substrate, and cytochrome c as an electron carrier. When this technique was tried with erythrocyte homogenates

it was found to be unsatisfactory because the ascorbate reacted with the hemoglobin to form choleglobin. To date it has been impossible to obtain hemoglobin free homogenates with cytochrome oxidase activity.

Krebs cycle dehydrogenases

Since it was not possible to assay directly for cytochrome oxidase activity, an indirect approach was used. By sub-

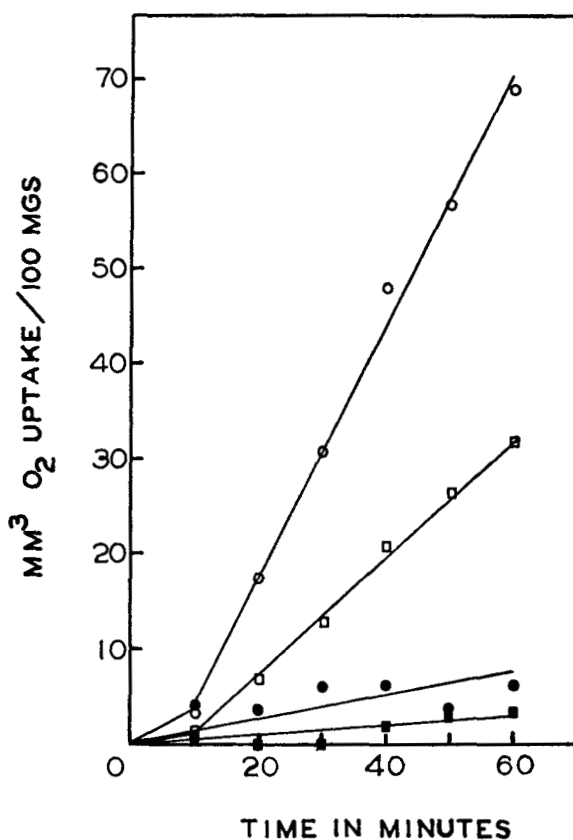


Fig. 5 Malic dehydrogenase activity of erythrocyte homogenates

- chicken, in the presence of 0.05 M sodium malate;
- chicken, no malate added;
- human, in the presence of 0.05 M sodium malate;
- human, no malate added.

stituting methylene blue as the terminal electron carrier, cytochrome oxidase was bypassed and the activity of the dehydrogenase could be determined directly. Malic dehydrogenase was assayed according to the system described above with the exception that 0.3 ml of a 1:10,000 solution of methylene blue was substituted for the cytochrome c.

It can be seen from the typical curves shown in figure 5 for the course of the reaction that the rate is constant for

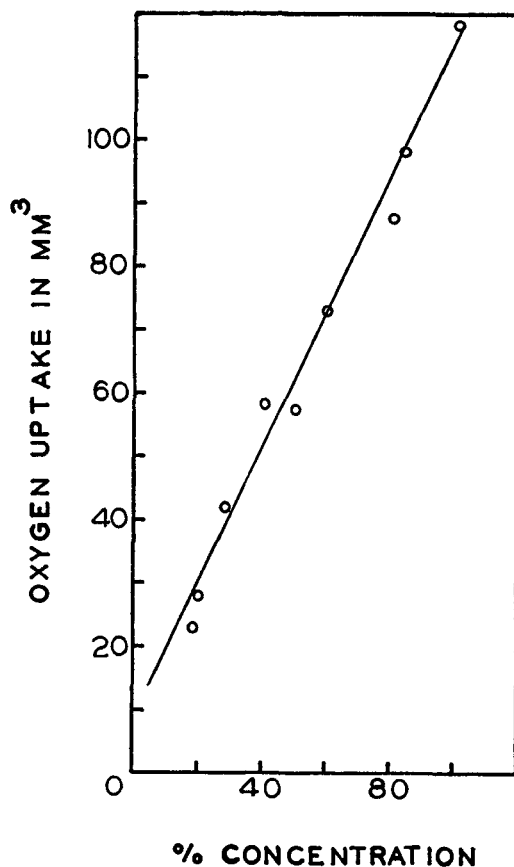


Fig. 6 Dilution curve of malic dehydrogenase activity of chicken erythrocyte homogenates. One hundred per cent homogenate contains 0.2048 gm dry weight. Values are for 60 minutes, obtained from three different homogenates.

60 minutes after tipping in the substrate. With chicken cell homogenates the clear-cut linear relationship between activity and homogenate concentration is shown in figure 6. Homogenates of rabbit cells, however, did not dilute linearly because of the presence of DPN-ase. Consequently it was necessary to use smaller amounts of homogenate (40 mg dry weight per vessel) and to use approximately the same amount of homogenate from one experiment to the next.

The data presented in table 4 show some malic dehydrogenase activity for all types of erythrocytes with no sharp differences between the nucleated and non-nucleated cells.

TABLE 4

Malic dehydrogenase activity expressed as Q_{O_2}

| KIND OF BLOOD | N | MEAN | S.E. |
|--------------------------|----|-------|-------|
| Human | 13 | 0.291 | 0.031 |
| Rabbit | 15 | 1.320 | 0.070 |
| Chicken | 12 | 0.709 | 0.032 |
| Frog | 5 | 0.370 | 0.043 |
| Fish | 10 | 0.770 | 0.063 |
| Sting ray (D. sp.) | 6 | 0.794 | 0.064 |
| Sting ray (D. centroura) | 9 | 0.374 | 0.045 |

Therefore it can be concluded that it is not the malic dehydrogenase which is responsible for the difference in malic oxidase activity shown in table 2. It is interesting to note that in all of the erythrocytes studied there is more malic dehydrogenase activity than there is malic oxidase activity. This comparison indicates that in the malic oxidase assay some hydrogen carrier with a more positive oxidation-reduction potential than methylene blue is limiting the rate of the reaction. If methylene blue picks up the electrons from diaphorase (Slater, '49), then cytochrome oxidase and/or the "Slater Factor" would be the rate-limiting step in these experiments. The difference between the malic oxidase and malic dehydrogenase

activities is much greater in the mammalian than in the nucleated cells, which means that cytochrome oxidase (or the "Slater Factor") limits at a considerably lower level in the non-nucleated cells.

When a similar series of experiments was performed using methylene blue (or cresyl blue) to determine the activity of succinic dehydrogenase in chicken erythrocytes, no activity could be measured. The reason for this is not clear, and will be investigated further.

Lactic dehydrogenase

For a glycolytic enzyme, lactic dehydrogenase was chosen, and the following system, which is a modification of that described by Straub ('40) was used for assay:

0.05 ml of 0.002 M potassium hydroxide in the center well
0.05 ml of 2.0 M potassium cyanide in the center well
0.3 ml of 10% sodium lactate in the side-arm
0.3 ml of 0.06 M DPN
0.3 ml of 0.1 M niacin
0.3 ml of 1: 10,000 methylene blue
0.3 ml of 0.5 M potassium cyanide
1.4 ml of homogenate

The cyanide in this system inhibits further breakdown of the end product, pyruvic acid. It has been reported (Colowick, Kaplan and Ciotti, '51) that pyridine nucleotide reacts with cyanide and interferes with the spectrophotometric measurements, but at the pH used in most manometric experiments this reaction is negligible. Glutamate was tested for removing the pyruvic acid with chicken erythrocyte homogenates and was found to give similar values to those obtained using the cyanide. The activity, however, fell off sooner using glutamate (see fig. 7) and consequently only data obtained using the Cyanide technique are reported (fig. 8).

Figure 9 shows the linear relationship between the activity and concentration of chicken erythrocyte homogenates. The DPN-ase activity of homogenates of rabbit erythrocytes was

so high that maximum values for lactic dehydrogenase could be obtained only by using very dilute homogenates. Erythrocytes of the other species studied did not have enough DPN-ase activity to interfere with the determinations.

In general the data in table 5 show a wide range of lactic dehydrogenase activity with no clear-cut differences between nucleated and non-nucleated erythrocytes.

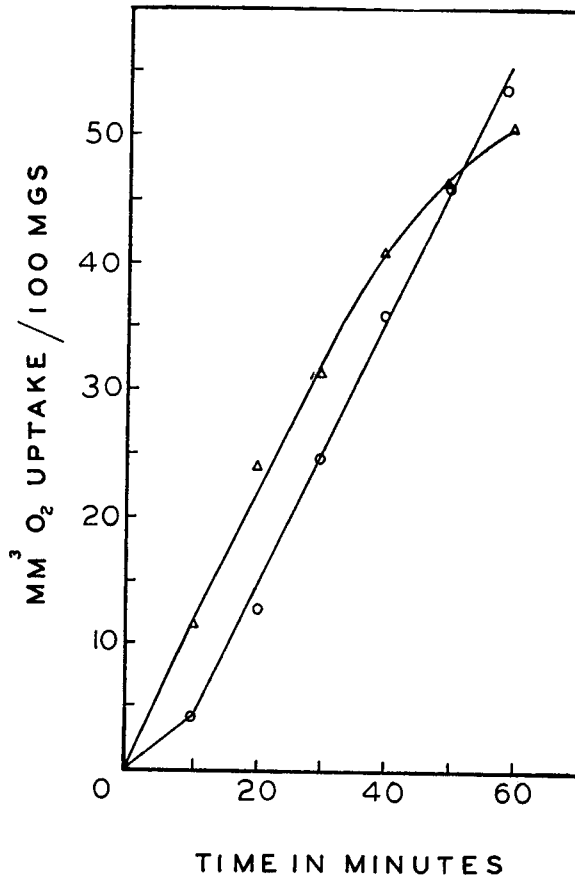


Fig. 7 Lactic dehydrogenase activity of chicken erythrocyte homogenates comparing the effectiveness of glutamate and cyanide in the assay.

- △ chicken, in the presence of lactate and glutamate;
- chicken, in the presence of lactate and cyanide.

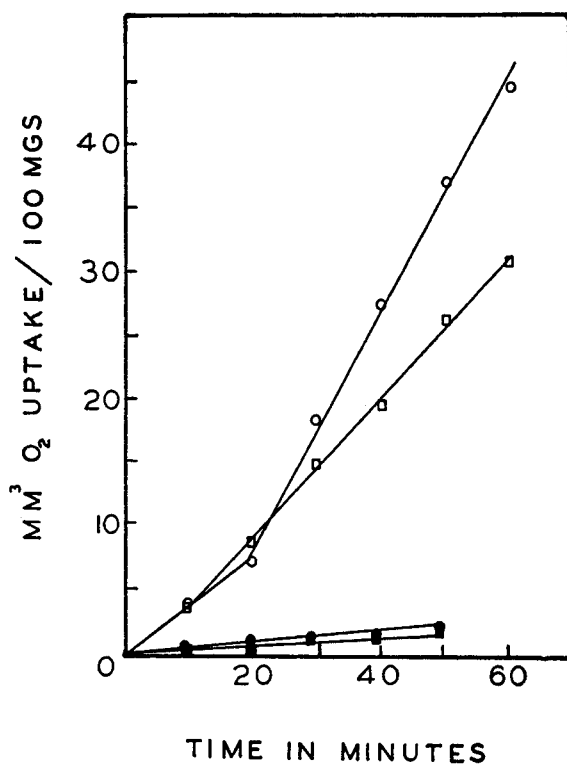


Fig. 8 Lactic dehydrogenase activity of erythrocyte homogenates

- chicken, in the presence of 10% lactate;
 ● chicken, no lactate added;
 □ human, in the presence of 10% lactate;
 ■ human, no lactate added.

TABLE 5

Lactic dehydrogenase activity expressed as Q_{O_2}

| KIND OF BLOOD | N | MEAN | S.E. |
|--------------------------|----|-------|-------|
| Human | 9 | 0.310 | 0.015 |
| Rabbit | 8 | 3.590 | 0.232 |
| Chicken | 13 | 0.531 | 0.035 |
| Frog | 5 | 0.440 | 0.100 |
| Fish | 13 | 0.441 | 0.046 |
| Sting ray (D. sp.) | 5 | 1.894 | 0.078 |
| Sting ray (D. centroura) | 5 | 1.016 | 0.102 |

DISCUSSION

The experiments measuring the rate of oxygen consumption of whole erythrocytes of various species definitely establish the fact that at the same temperature the nucleated erythrocytes of the lower vertebrates have a much higher activity than the non-nucleated mammalian red cells. These experiments were necessary since the values in the literature were obtained from experiments run at different temperatures.

The next step was to compare the activity of certain Krebs cycle and glycolytic enzymes in a nucleated and non-nucleated erythrocyte. A comparison was first made between the activities in homogenates of chicken and human red cells. The level of activity of malic, succinic and lactic dehydrogenase was relatively high in the chicken cells but only lactic dehydrogenase activity was found in the human cells. This led to the tentative suggestion that possibly the difference in levels of activity of the Krebs cycle enzymes in the two types of cells might account for the difference in respiratory rates (Baker and Hunter, '52). When erythrocytes of additional lower vertebrates were studied it was found that succinoxidase and malic oxidase activity was not uniformly high. Consequently, no generalization could be made concerning a difference in activities of Krebs cycle enzymes in nucleated and non-nucleated erythrocytes.

A number of interesting speculations suggest themselves from a further analysis of the data. If one compares the succinoxidase activity with whole cell respiration, only in rabbit and chicken cells is the Q_{O_2} for succinoxidase considerably larger than the Q_{O_2} for respiration. In fact, in frog cells and particularly in fish cells there is less of the former than the latter. Since it is usually assumed that the homogenate technique gives a value for the maximum activity of an enzyme system and since this is usually much higher than the regulated rate in the intact cell these data are unexpected. It might be that (a) the usual assay procedures were not satisfactory in the present experiments, (b) some alternate

metabolic pathway functions in the nucleated cells, (c) some inhibitor of a portion of the succinoxidase system is present in some of the preparations, or (d) some portion of the succinoxidase system actually is rate limiting at a level which in turn limits the rate of respiration.

Several attempts were made to determine which of these alternative suggestions was correct. Since (d) seemed to be

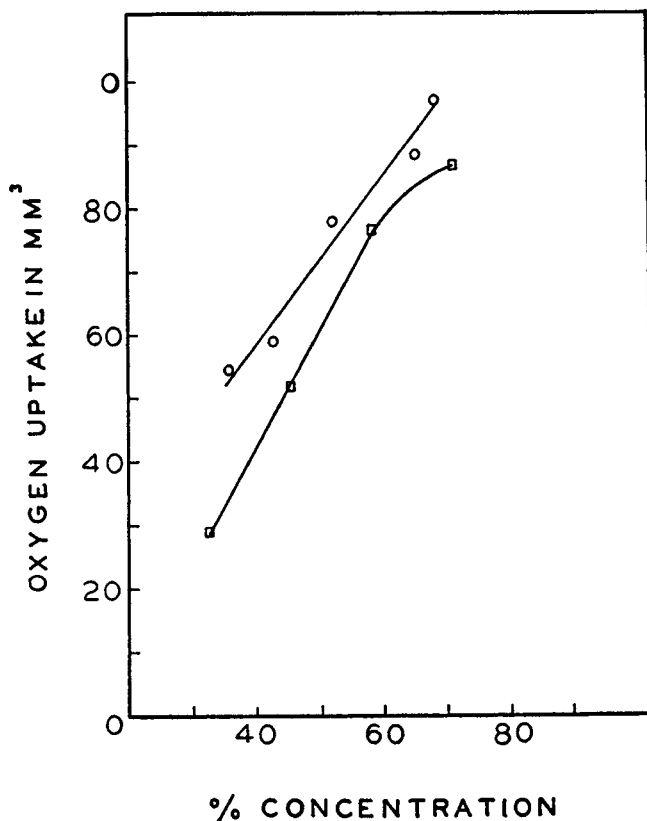


Fig. 9 Dilution curve of lactic dehydrogenase activity of erythrocyte homogenates.

○ chicken

□ human

One hundred per cent chicken erythrocyte homogenate contains 0.2200 gm dry weight and 100% human erythrocyte homogenate contains 0.2210 gm dry weight. Each curve was obtained from a single homogenate.

the easiest to check, experiments were set up to measure the activity of succinic dehydrogenase and of cytochrome oxidase. In assaying for the activity of the succinoxidase system it is usually assumed that the enzymes terminal to the dehydrogenase are present in excess. The possibility exists, however, that in an erythrocyte where another iron porphyrin is present in such large amounts, there might be a limiting quantity of cytochrome oxidase (see, for example Duncan et al., '53). Unfortunately, we were not able to assay either cytochrome oxidase or succinic dehydrogenase. The ascorbate, which is used as a substrate in the assay of the former reacted with the hemoglobin in our preparations making it impossible to carry out the manometric determinations. Neither methylene blue nor cresyl blue acted as satisfactory electron acceptors in our attempts to measure the activity of the dehydrogenase.

Turning our attention to the data on the malic system proves more rewarding. A comparison of tables 2 and 4 shows that in every case except one type of sting ray the malic dehydrogenase activity is considerably greater than that of the malic oxidase system. In other words, when an electron carrier such as methylene blue is substituted for cytochrome c and cytochrome oxidase, considerably more oxygen is consumed. This would suggest that cytochrome oxidase is rate limiting in these cells. In the sting ray which had the highest rate of respiration (0.266) the ratio of malic dehydrogenase to malic oxidase is approximately 1. In man and in rabbit with the lowest respiratory rates (0.02 and 0.037 respectively) the ratios of dehydrogenase to oxidase are 5.1 and 7.9 respectively. The others with respiratory rates ranging from 0.130 to 0.141 have ratios between 1.3 and 3.6. These data would tend to substantiate the hypothesis that cytochrome oxidase is rate limiting in all these cells and in general, the lower the rate of respiration, the lower is the level of cytochrome oxidase activity.

Such a conclusion is different from that reached by Rubinstein and Denstedt ('53) following their analysis of tricarb-

oxylic acid cycle enzymes in chicken erythrocytes. They state: "The ability of the avian erythrocyte to respire, in contrast to the mammalian red blood cell, appears to be due to the presence of a nucleus which contains many of the enzymes of the Krebs cycle." Our data on the two Krebs cycle enzymes in chicken erythrocytes certainly confirm their observations that these enzymes are present. There may be some question, however, about their localization in the nucleus. We have previously reported (Hunter and Baker, '53) that following freezing and thawing and centrifuging the enzymes in mammalian erythrocytes are present in the supernatant fluid while those of chicken red cells are present in the cellular debris. Microscopic observation of these "ghosts" shows that each nucleus is surrounded by a remnant of the cell membrane.

Although it is quite possible that the nuclei are responsible for the enzymatic activity of such preparations, we would prefer to reserve judgment until we have preparations in which the nuclei are more completely separated from the stroma. We have been unable to date to obtain such preparations from chicken erythrocytes. Preliminary experiments with erythrocytes of the sting ray suggested that in those cells, at least, a most interesting situation prevailed. Under certain experimental conditions it was possible to separate three fractions of these hemolyzed cells. There was no enzyme activity when the fractions were tested separately but recombination gave activity. These experiments suggested that in certain nucleated erythrocytes at least different components of some of the enzyme system were located on different portions of the cell. These could be separated by differential centrifugation. The whole problem of enzyme localization in erythrocytes needs clarification and experiments are being carried out in this laboratory in an attempt to do this.

In general our data compare qualitatively with those presented by Rubinstein and Denstedt. A detailed quantitative comparison is difficult, however, since different methods of assay were used in the two series of experiments. Usually the ferricyanide technique which they used gives higher values

than the techniques we used (see, for example, Alivisatos and Denstedt, '51). Their value for succinoxidase for the whole hemolysate as shown in Table IV was $-Q_{O_2} = 0.13$ which is considerably lower than our figure of $-Q_{O_2} = 0.534$. The difference here may have resulted from the fact that they did not add cytochrome c whereas we did. Using their technique they were able to assay for succinic dehydrogenase. In their Table III they give a $Q_{O_2} = 0.91$ with an endogenous value of $Q_{CO_2} = 0.41$, in Table IV they give a Q_{CO_2} value of 0.49 for the whole homogenate. As they point out, the ratio of this Q_{CO_2} for the dehydrogenase to the C_{O_2} for the oxidase is close to the expected 4:1.

Another line of evidence which might suggest that low activity of cytochrome oxidase could not explain all of our observations comes from experiments in which respiratory rates of whole erythrocytes are measured in the presence of added substrates. Rubinstein and Denstedt include such data in Table II and we have done considerable work along this line particularly with rabbit reticulocytes (Hunter, Baker and Burr, '54). In some instances, added succinate increases whole cell respiration to a higher level than would be predicted on the basis of the amount of succinoxidase activity of homogenates.

It should be evident from this discussion that in our opinion considerably more work must be done before we can state with certainty the basis for the difference in metabolic activity of nucleated and non-nucleated erythrocytes. We do not believe that the suggestion made by Rubinstein and Denstedt can be reconciled with all our data. Some of our results are consistent with the suggestion that cytochrome oxidase is rate limiting in all erythrocytes studied and is limiting at a much lower level in mammalian than in nucleated red cells. Some of our other observations, however, might lead to the conclusion that using the standard methods for enzyme assays in this material leads to erroneous conclusions. It is hoped that additional experiments now being carried on in this and other laboratories may resolve this apparent paradox.

The theoretical implications of the determinations of the activity of lactic dehydrogenase are less complex. In general, all of the erythrocytes studied have a considerable amount of this enzyme present. This is what might have been predicted since there are no sharp differences in the glycolytic activity of these cells with the exception of the presence or absence of the Pasteur effect. One would certainly predict that both nucleated and non-nucleated red cells would have the enzymes necessary to form lactic acid. The determination of the absolute amount of lactic dehydrogenase present is complicated in some of these cells by the presence of large amounts of DPNase. This is particularly true of rabbit erythrocytes.

CONCLUSIONS

1. Measurements were made of oxygen consumption of whole cells and the activity of malic oxidase, succinoxidase, malic dehydrogenase and lactic dehydrogenase in homogenates of erythrocytes of sting ray, fish, frog, chicken, rabbit and human.

2. At a given temperature, the nucleated red blood cells of lower vertebrates respire at a level considerably above that of the non-nucleated, mammalian erythrocytes.

3. The succinoxidase activity of homogenates of chicken erythrocytes and the malic oxidase activity of homogenates of chicken, fish, and some sting rays are considerably higher than in other erythrocytes studied. The differences in activity cannot be correlated with the presence or absence of a nucleus.

4. There is considerable malic dehydrogenase activity in homogenates of all types of erythrocytes studied with no sharp differences between the nucleated and non-nucleated cells. There is more malic dehydrogenase than malic oxidase activity in all of the erythrocytes studied. This might be interpreted as meaning that cytochrome oxidase and/or the Slater Factor is rate limiting and limiting at a lower level in mammalian cells.

5. All of the erythrocytes studied had considerable lactic dehydrogenase activity with no consistent differences between nucleated and non-nucleated cells.

6. Certain possible interpretations of these data are discussed.

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