RESPIRATION OF MAMMALIAN ERYTHROCYTES

By L. MICHAELIS and K. SALOMON

(*From the Laboratories of The Rockefeller Institute for Medical Research*)

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1. Respiration of Erythrocytes Induced by a Respiratory Supplement Prepared from Various Tissues

Lavoisier's fundamental interpretation of respiration as oxidation of organic compounds by free oxygen is valid even at the present time without restriction. His opinion was that this oxidation takes place in the blood. This hypothesis had to be given up, the respiration really taking place in the tissues. Red corpuscles, at least the non-nucleated corpuscles of mammals, do not participate in the reduction of oxygen to any appreciable extent.

Recently, however, Harrop and Barron (1, 2) showed that non-nucleated erythrocytes do respire in the presence of methylene blue and some other related dye stuffs, which can be designated, therefore, as catalysts for respiration. All of these artificial catalysts are alien to the animal body, and no component of any tissue has ever been shown to exhibit the same effect as methylene blue on erythrocytes. Never has a respiration of non-nucleated erythrocytes in any way comparable in extent to that of other tissues been observed prior to the experiments of Harrop and Barron.

It will be shown in this paper that a substance can be extracted by water from various organs which induces non-nucleated erythrocytes to respire just as methylene blue does. This substance will be shown not to be identical with Warburg's (3) respiration enzyme because it is not sensitive to carbon monoxide.

Methylene blue was used by Thunberg (4) as an oxidant instead of oxygen, for something that may be reckoned as respiration only when the meaning of this concept is considerably widened. In his experiments, methylene blue is a hydrogen acceptor, instead of oxygen,

which is absent throughout the experiment. Different from these experiments is the use of methylene blue in the presence of oxygen as catalyst for the consumption of oxygen by cells. Meyerhof (5) was the first to show that methylene blue sometimes acts as a catalyst for respiration in air. He observed this property of methylene blue, however, only in cell extracts the respiratory faculty of which had been artificially damaged, such as in heated extract of yeast previously treated with acetone. The respiration of the yeast cells themselves could not be improved by methylene blue under normal conditions. Not even the oxygen consumption of the extract of acetone yeast was altered by methylene blue unless the spontaneous oxygen consumption of such an extract was damaged by heating. Only a damaged respiration could be improved by methylene blue, this dye acting as a substitute for the destroyed respiration enzyme.

Later on it was shown by Harrop and Barron (2a), that certain cells which by nature exhibit a very low respiration, or none at all under natural conditions, can be stimulated to an intense respiration by methylene blue. This has been shown for erythrocytes and for unfertilized echinoderm eggs. The effect of methylene blue, according to Barron, is shown also for other dyes reversibly oxidizable and reducible. The magnitude of this effect appeared to depend on the potential range of the dye, methylene blue showing the optimum effect, whereas dyes with a more positive or a more negative range of potential have smaller effects or even no effect at all. As for the interpretation of this observation, we may refer to Barron's paper.

The reaction of a physiologist's mind to this observation may be, and has been, different. One point of view is that this effect of methylene blue is an accidental property of the dye, that no substance analogous to the dye exists in the living organisms, as seemingly shown by the fact that erythrocytes really do not respire. Another point of view is the hypothesis that methylene blue might be a model for some unknown substance in the organisms, and that this substance may play a part in respiration, which is not obvious as yet. This hypothesis is supported by the observation presented in this paper, showing that extracts of various organs induce erythrocytes to respire just as methylene blue does. The best effect was obtained with liver extract of various animals, and rat's liver seems to be the most suitable tis-

sue for the extraction of the active substance, to which we will refer in what follows under the term *respiratory supplement.* Then follows kidney, spleen, testicle, lymph-node, whereas brain, muscle, and blood serum showed no effect or at most a very slight effect. Ox organs showed similar effects, the action often being somewhat less intense, but the present method is not yet adapted for a quantitative extraction of the supplement or for the estimation of its original concentration in the living organ.

The respiration of blood corpuscles induced by methylene blue or by organ extract is not influenced at all by the presence of carbon monoxide. Rabbit's erythrocytes, in the presence of methylene blue or rat's liver extract, respire at the same rate in air or in a mixture of pure carbon monoxide containing a small amount of oxygen, varied from 1 to 2 per cent in different experiments. Such a gas mixture inhibited the rate of respiration of yeast or of nucleated erythrocytes of fowl by approximately 60 to 80 per cent depending on the conditions, in accordance with Warburg's observations. This difference in the behavior toward CO proves that the supplement is not identical with Warburg's respiration enzyme.

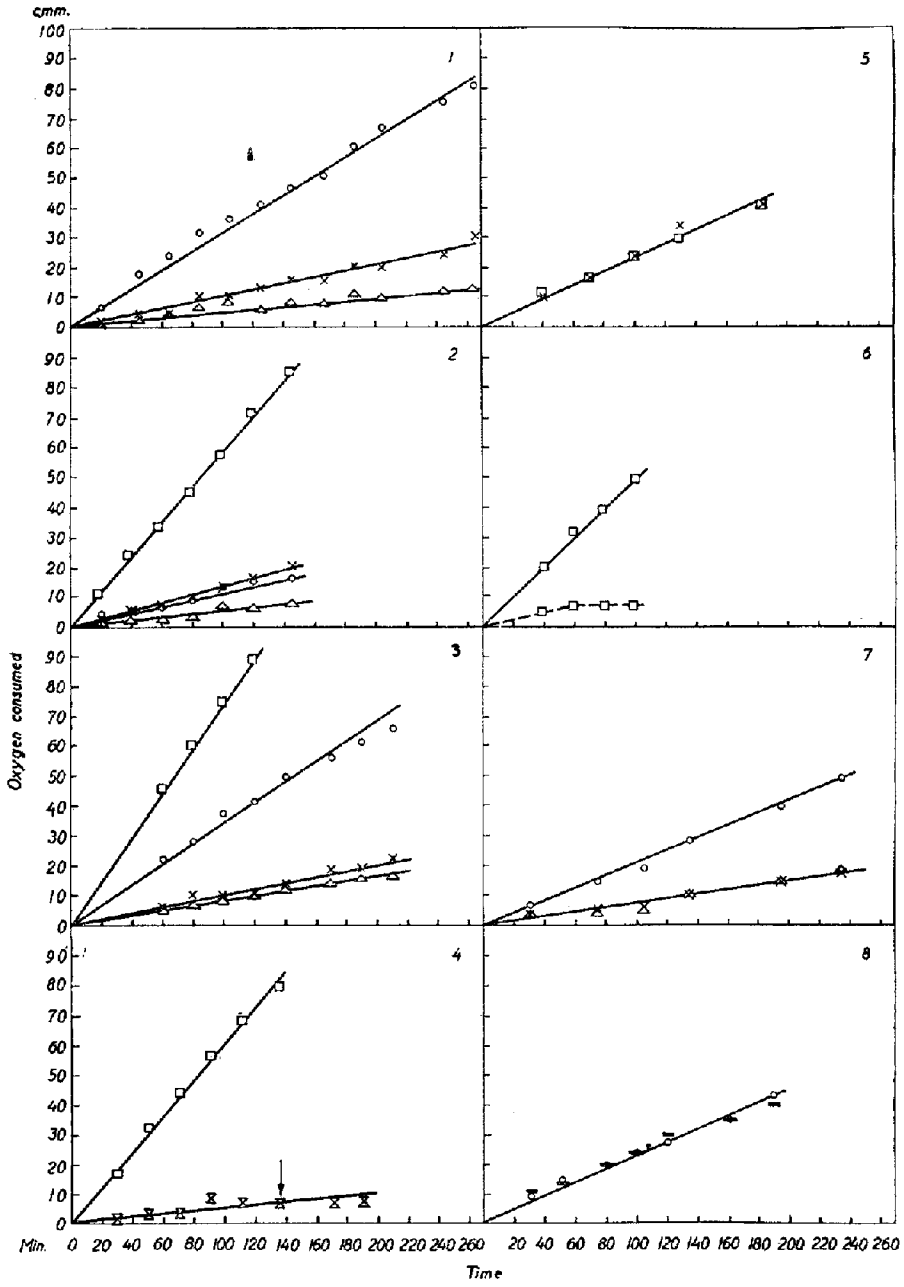
The end products of this respiration and the respiratory quotient will be investigated in a subsequent paper.

In varying the temperature from 23° to 37°, the rate of respiration of blood corpuscles induced by methylene blue showed practically no difference, whereas the respiration induced by organ extracts was two to three times as fast at 37° as compared with 23°. In general, the organ extracts, at 23°C., are less active than methylene blue in its optimum concentration (0.0006 molar), but are considerably stronger than methylene blue at 37°.

As a preliminary statement it may be added that the effect of methylene blue, though very strong even in the dark, is considerably increased by illumination. The effect of organ extracts is the same in the dark or when illuminated by a 150 watt bulb at a short distance.

Methylene blue had no influence upon that slight oxygen consumption exhibited by organ extracts alone in the concentrations used.

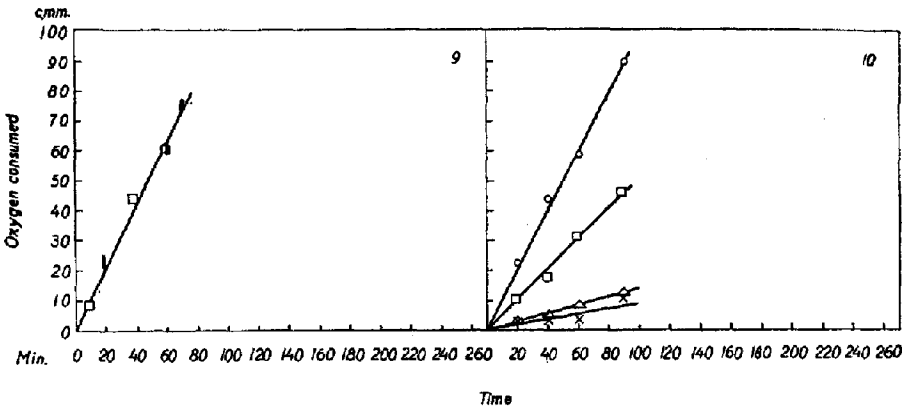
The influence of narcotics has been studied only for phenyl urethane as yet. This showed no influence at all upon the effect of methylene blue or of the supplement.



Figs. 1 to 8 at 23°C.

Common designations for all curves:

- ○ Experiments with blood, sugar and organ extract.
- × Control with blood and sugar.
- △ Control with organ extract and sugar, without blood corpuscles.
- □ Experiment with blood, sugar and methylene blue.
- ⧖ Control with blood plus methylene blue, without sugar.
- - - - Experiments with hemolyzed blood (Fig. 6).
- = Experiments with blood, sugar, organ extract in carbon monoxide containing 1.4 per cent oxygen (Fig. 8).



Figs. 9 and 10 at 37°C.

Common designations for all curves:—*continued.*

‖ Experiments with blood, sugar, methylene blue in carbon monoxide with 1.4 per cent oxygen.

FIG. 1. Effect of rat liver extract at 23°C. The respiration in presence of rat liver extract is far greater than the sum of the respirations in all controls.

FIG. 2. Effect of methylene blue (□) compared with that of rat muscle extract (○), at 23°C., in the presence of sugar. Muscle extracts show no effect in this case (a very slight effect in other cases, especially at 37°).

FIG. 3. Comparison of methylene blue (□) and rat kidney extract (○) at 23°C. At this temperature organ extracts as a rule act less intensely than methylene blue.

FIG. 4. Shows that methylene blue increases the respiration only in presence of glucose (□) whereas it has no influence in absence of sugar (▽△). When sugar is added after the methylene blue has been in contact with the blood corpuscles for 2 hours, subsequent addition of sugar induces no respiration. The arrow shows the time of addition of glucose.

FIG. 5. For chicken erythrocytes respiration is not influenced by the addition of methylene blue even in presence of sugar. Temperature 23°C. The spontaneous respiration of these nucleated erythrocytes in sugar is rather high alone even at 23°C., and cannot be increased by methylene blue.

FIG. 6. The effect of methylene blue is present only for intact erythrocytes but not after hemolysis.

FIG. 7. Effect of rat spleen on the respiration of rabbit erythrocytes in presence of sugar. 23°C. The effect is somewhat smaller than with liver.

FIG. 8. The respiration of rabbit erythrocytes induced by rat liver extract is equal in air (○) or in carbon monoxide containing 1.4 per cent oxygen (=).

FIG. 9. The respiration of rabbit erythrocytes in the presence of glucose induced by methylene blue, is equal in air (□) or in carbon monoxide containing 1.4 per cent oxygen.

FIG. 10. Effect of rat liver extract (○) or of methylene blue (□) on the respiration of rabbit erythrocytes with glucose at 37°. At this temperature the effect of organ extract is greater than that of methylene blue.

On discussing the chemical nature of this respiratory supplement it might be a suggestive assumption that glutathione or another sulfhydril body is the responsible substance of the tissue extracts, and to link these observations with Hopkins' interpretation of glutathione as a catalyst for respiration, owing to its property of being readily reduced and reoxidized. Meyerhof also showed such an effect for thioglycolic acid and thiolactic acid, though it was only exhibited on structure-free cell extracts. Such an idea was even more suggestive as just those tissues which contain no, or very little, glutathione, namely, blood serum and muscle tissue, show no, or a very small, effect on the respiration of blood corpuscles. Our experiments, however, do not as yet support this hypothesis. Reduced glutathione, or cysteine, added to blood corpuscles in Ringer solution plus glucose, consumed just as much oxygen as was required for the oxidation to the disulfide. When added in such small amounts that this oxygen consumption could be practically neglected, they had no effect whatsoever. When glutathione in the oxidized state or cysteine was added, no effect could be seen at all, with or without addition of an iron salt.

A second possibility was that the respiratory supplement might be identical with Harden and Young's (7) coenzyme. Meyerhof showed that the coenzyme enhances not only fermentation but also oxidation. However, this idea has to be abandoned too, because muscle contains by far the greatest amount of the coenzyme among all tissues, according to Meyerhof, whereas our effect is exhibited by muscle extract almost to the least degree among all tissue extracts. The fact that muscle extract contains so little supplement is also contradictory to the suggestion that Keilin's cytochrome be identical with the supplement.

On searching for another explanation, one may think of the fact that it is just cells without a nucleus (erythrocytes) or cells with a nucleus in an obviously resting state (unfertilized eggs) which can be induced to respire by methylene blue. Perhaps the respiratory supplements of organ extracts, analogous to methylene blue, is some substance derived from the cell nucleus. In fact, tissues with no nucleus (serum) or with very few nuclei per unit of mass (muscle, brain), have almost no effect. This idea is, at the present time, a mere suggestion.

The respiration of erythrocytes as described before, either under the

influence of methylene blue or of the supplement, is shown only by intact erythrocytes. Erythrocytes hemolyzed by distilled water, or by repeated freezing, show no respiration, or when the hemolysis is incomplete there is a diminution of the respiration according to the extent of hemolysis.

The respiration of chicken erythrocytes is not influenced by methylene blue.

There is in the literature on respiration one observation which may have a connection with ours. Battelli and Stern (8) showed that what they call the accessory respiration of a tissue or a tissue extract (such as liver or kidney) is noticeably, though not strongly, increased by addition of washed blood corpuscles. This observation, however, is made under rather different experimental conditions and differs from ours by the fact that the effect described by Battelli and Stern is the same whether the blood corpuscles are added in the intact or in the hemolyzed state, whereas our effect is strictly confined to the intact state of the erythrocytes.

2. The Influence of the Medium upon the Action of the Respiratory Supplement

The medium in which most of the experiments were performed is Ringer solution with 0.18 per cent of glucose. In sugar-free Ringer solution methylene blue shows no effect, at least when the erythrocytes are used in not too fresh a condition, where the natural sugar content of the blood corpuscles may be supposed to be not yet consumed. But even in very fresh condition, when perhaps not all of the natural sugar content of the blood corpuscles may have been destroyed, the respiration of blood corpuscles under the influence of methylene blue is very much smaller in sugar-free Ringer solution than it is in a medium containing sugar. When sugar-free blood corpuscles suspended in Ringer solution containing glucose are mixed with methylene blue, a strong oxygen consumption starts immediately and goes on at a constant rate over several hours; indeed over a period of time such an experiment of this sort can reasonably be extended, 5 to 6 hours at 23°, or 2 to 3 hours at 37°. Methylene blue does not harm the blood corpuscles within this period. When, however, methylene blue is added to a suspension of blood corpuscles in a sugar-free

medium, the dye seems to exhibit a damaging influence to the blood corpuscles. For not only is the respiration missing under this condition, but also the respiration is not established on adding glucose after an interval of 1 to 2 hours. One may think of the following interpretation: If there is no sugar the oxidation catalysed by methylene blue is directed toward some other oxidizable substance in the cell which is important for its physiological structure. This substance is protected from oxidation in the presence of sugar.

Experiments of the same kind but with organ extracts instead of methylene blue did not lead to any interpretable result because such extracts cannot be obtained free from sugar or glycogen.

The effect of methylene blue can be shown to the same extent within the limits of error by substituting fructose or saccharose for glucose. In contrast, there was no respiration with mannite. Ringer solution with mannite behaves like sugar-free Ringer solution.

On attempting to replace Ringer solution in the methylene blue experiments by various isotonic salt solutions, the following results were obtained. The oxygen consumption in 0.9 per cent NaCl solutions was somewhat smaller than in Ringer solution. Omitting only $CaCl_2$ from the Ringer solution the respiration was a little smaller too, when compared with the control with complete Ringer solution. By substituting for the very small amount of $NaHCO_3$ of the Ringer solution an equivalent amount of alkali phosphate, no distinct difference could be found, no matter whether this small amount of phosphate was added in the form of primary or secondary phosphate or of a mixture of both. Phosphates at a higher concentration, however, have a decidedly inhibiting effect.

When the blood corpuscles are hemolyzed by dissolving them in distilled water and salt solution is added, such as to give the final mixture the composition of a Ringer solution, no respiration takes place at all either with or without methylene blue, either in the presence or in the absence of sugar, provided the hemolysis is complete. With incomplete hemolysis the result may be intermediary. Hence, the respiration faculty of red blood corpuscles is linked with what is called the structure of the cell just as much as is the glycolytic faculty. None the less, phenyl urethane has no influence on the respiration induced by liver extract.

3. Preliminary Attempts to Characterize or Isolate the Catalyst Supplement

As yet we have not succeeded in isolating the efficient substance from the tissue extract or in separating by any means an active fraction of the extract from an inactive one. The following attempts were made:

a) Heating the extract at 80° destroys the active substance, at least the filtrate of the heated extract is without any effect. When, however the boiled turbid suspension as a whole was used the experiments were obscured by the fact that boiled extracts containing the coagulated particles show a very considerable oxygen consumption themselves which overshadows any oxygen consumption which they might induce.

b) When rat liver is ground in a mortar and extracted with acetone, neither the acetone-insoluble residue nor the residue of the acetone extract showed any effect comparable to that of the fresh tissue extract.

Exposure to light does not influence the effect of tissue extract, no matter whether the respiration takes place in air or in a mixture of 98 per cent CO + 2 per cent O_2. On the other hand, there is a remarkable effect of illumination upon the action of methylene blue. The effect of methylene blue, though it is very marked even in the dark, is strongly increased in the light. This preliminary observation will be elaborated in a subsequent paper.

4. Experimental Part

The oxygen consumption was measured by Warburg's micro-respiration apparatus with Haldane micro-manometer. The volume of the vessels was about 25 cc., and 7 per cent KOH was used as absorbent for CO_2. The temperature was in the majority of the experiments 23°C., in other experiments 37°, and constant within ±.01°. As a rule, rabbit erythrocytes were used, prepared by repeatedly washing citrated blood with the wanted salt solution, and suspending in a volume of the liquid equal to the original volume of the blood sample. In other experiments human erythrocytes were used with the same result. As a rule, fresh blood corpuscles were used. For especial cases, namely, when a strict absence of any trace of sugar was wanted, the corpuscles were used after 24 hours' standing in the ice box. No noticeable difference could be found, as a rule, whether the corpuscles were fresh or 1 day old, disregarding that very slight effect of a residue of glucose which is supposed to be left in fresh corpuscles.

The organ extracts were prepared by grinding the fresh organ in the mortar and gradually adding Ringer solution (or another medium), ten times the weight of the organ. This emulsion was filtered through ordinary filter paper and the turbid liquid free from coarse particles was used. These extracts were always used in fresh condition.

A selection of the experiments is reproduced in the following diagrams. The time "0" in these diagrams, plotted on the abscissa, is usually 20 to 30 minutes after closing the stop-cock of the manometer, because the very first stage sometimes shows irregularities and the linear course of the change of the manometer reading with time is often reached only after this period. From here the linear course is maintained over several hours. The ordinates give the oxygen consumption in cubic millimeters.

Each experimental mixture is composed as follows:

a) 2 cc. of a suspension of blood corpuscles in Ringer solution (containing all corpuscles of 2 cc. pure blood); or, in control experiment, without blood, the equal volume of pure Ringer solution instead.

b) 2 cc. organ extract, or the same volume of pure Ringer solution instead.

c) In the experiments with methylene blue instead of *b*), methylene blue is added to a definite concentration of 0.0006 molarity. (The variation of this concentration has a relatively small effect, down to about a tenfold dilution of the above concentration.)

d) In all experiments containing sugar the concentration is 0.18 per cent in the final mixture.

e) 7 per cent KOH was used as absorbent for CO_2.

SUMMARY

Non-nucleated mammalian erythrocytes do not respire even in the presence of sugar, but they do respire after addition of a small amount of methylene blue.

It is shown in this paper that aqueous extracts of various organs, especially liver, act in the same way as methylene blue. The respiration of erythrocytes induced by an organ extract is not altered in the presence of carbon monoxide.

The content of this respiratory supplement in extracts of organs varies according to the organ: liver and kidney show the best effect; muscle, brain, and blood serum the least.

With hemolyzed erythrocytes no respiration can be induced either by methylene blue or by organ extracts.

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