STUDIES ON BLOOD CELL METABOLISM.

I. The Effect of Methylene Blue and Other Dyes upon the Oxygen Consumption of Mammalian and Avian Erythrocytes.*

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Although a considerable number of studies have been published on various phases of the metabolism of blood cells, the relative importance of fermentation and of respiration in their normal activities is still very imperfectly understood. Still more obscure is the metabolic behavior of pathological blood cells, although it may be of considerable significance. It is of particular interest, for example, to examinethe activities of the cells of leucemic blood in the light of the recent advances made in our knowledge of the metabolism of cancer cells.

A question which at once attracts attention in this field is that of the cause of the great variations which are found in the respiration of various types of normal erythrocytes. Several years ago it was shown by one of us (1) that normal human erythrocytes, separated from leucocytes and blood platelets by appropriate methods, have a scarcely demonstrable oxygen consumption when incubated at 38°C. for some hours under sterile conditions. The utilization of oxygen was considerable, however, when large numbers of reticulocytes were present in the blood, such as are found in hemolytic jaundice or in other conditions with active blood regeneration. These findings in general were in harmony with the previous studies of Morawitz and Itami (2) upon the oxygen consumption of anemic blood, and of Warburg (3), who found, using the Barcroft-Haldane manometer, that although normal adult mammalian erythrocytes in general had a very low oxygen consumption, the respiration was much increased in the blood of

* The second paper of this series will appear in a forthcoming number of *The Journal of Biological Chemistry.*

young rabbits and appeared to be roughly proportional to the amount of polychromasia present. In contrast to the slight oxygen consumption of non-nucleated mammalian erythrocytes, Warburg showed that the nucleated erythrocytes of birds (geese) have a very large oxygen consumption.

By the use of the technique previously described (1), but with the more accurate Van Slyke constant volume blood gas apparatus now available, it can be shown that non-nucleated adult mammalian erythrocytes have an exceedingly small, but measurable oxygen consumption when incubated at 38°C. This is accompanied by the production of carbon dioxide.

The interesting question now arises as to the cause of this difference in the metabolism of mature mammalian erythrocytes, in contrast to that of the young or immature forms and that of the nucleated cells of avian blood. To state that the oxidative activities of the different types of red blood cells seem to be related in some intimate way to the presence or absence of nuclear material furnishes no satisfactory clue as to the actual mechanism involved. It is conceivable that in the course of its development some inhibiting substance is formed in the mammalian erythrocyte which almost stops the respiratory metabolism of the cell. On the other hand, it is also conceivable that in the course of development, some essential link in the respiratory mechanism is lost in the cell so that oxidations no longer take place. We wish to present evidence for the latter view. We have found that the oxygen consumption of non-nucleated mammalian erythrocytes can be greatly increased, and can approach the magnitude of that of the nucleated cells of avian (goose) blood when one of a number of vital dyes, and particularly methylene blue in low concentration (0.005 to 0.0005 per cent), is added to the blood before its incubation.* The amount of oxygen absorption when compared to that in ordinary defibrinated blood may be increased many times. This rise occurs in the blood of all mammals tested—dog, beef, sheep, and man, and is accompanied by a corresponding evolution of carbon dioxide (Table I).

* The methylene blue used in these experiments was Purified Methylene Blue from Leopold Cassella and Co., Frankfurt a.M.

TABLE I.

The Effect of Methylene Blue upon the Respiration of Mammalian Erythrocytes.

	Content carbon dioxide	Content oxygen	Carbon dioxide output	Oxygen consumption	Oxygen consumed
	vol. %	*vol. %*	*vol. %*	*vol. %*	*per cent*
1. Man—defibrinated blood					
Control before incubation	36.79	19.24			
after incubation	38.57	18.94	1.78	0.30	1.6
+M.B. after incubation	48.01	6.82	11.22	12.42	64.6
2. Man—washed cells suspended in Locke's solution					
Control before incubation	4.22	17.01			
after incubation	4.57	16.53	0.35	0.48	2.8
+M. B. after incubation	14.64	3.39	10.42	13.62	80.1
3. Dog—defibrinated blood					
Control before incubation	13.91	23.20			
after incubation	19.45	22.77	5.54	0.43	1.9
+M.B. after incubation	22.17	12.64	8.26	10.56	45.5
4. Dog—defibrinated blood					
Control before incubation	9.99	27.95			
after incubation	19.35	27.80	9.36	0.15	0.05
+M.B. after incubation	25.00	14.40	15.01	13.55	48.5
5. Sheep—defibrinated blood					
Control before incubation	21.64	16.32			
after incubation	26.92	16.10	5.28	0.22	1.5
+M.B. after incubation	32.17	7.94	10.53	8.38	51.4
6. Beef—defibrinated blood					
Control before incubation		22.62			
after incubation		22.03		0.59	2.6
+M.B. after incubation		12.84		9.78	43.3
7. Dog*—defibrinated blood					
Control before incubation	13.59	22.01			
after incubation	15.74	20.02	2.15	1.99	9.2
+M.B. after incubation	21.35	10.09	7.76	11.92	54.1

The incubation time in all of these experiments was 3 hours at 37°. Methylene blue (M.B.) 0.005 per cent.

* Blood taken from a dog that had been previously subject to large bleedings. Examination of the blood for immature forms was not made.

TABLE I—*Concluded.*

	Content carbon dioxide	Content oxygen	Carbon dioxide output	Oxygen consumption	Oxygen consumed
	vol. %	*vol. %*	*vol. %*	*vol. %*	*per cent*
8a. Man—defibrinated blood					
Control before incubation	11.11	20.45			
after incubation	16.47	19.47	5.36	0.98	4.8
+M.B. after incubation	25.60	11.32	14.49	9.13	44.6
8b. Man—washed cells of above (8a) suspended in Locke's solution					
Control before incubation	5.28	19.95			
after incubation	5.92	19.24	0.64	0.71	3.6
+M.B. after incubation	12.80	10.93	7.52	9.02	45.2
9. Man†—washed cells suspended in Locke's solution					
Control before incubation	2.94	22.95			
after incubation	5.30	20.35	2.36	2.60	11.3
+M.B. after incubation	13.68	8.44	10.74	14.51	63.4
10. Dog—washed cells suspended in Locke's solution					
Control before incubation	3.36	22.58			
after incubation	4.35	21.62	0.99	0.96	4.3
+M.B. after incubation	13.56	9.58	10.20	13.00	57.6

† Blood from a patient with polycythemia vera. A good deal of polychromasia and some immature cells present. It may be seen that in this experiment the percentage acceleration of respiration on the addition of methylene blue is much less than with normal human erythrocytes.

The blood used in these studies was freshly drawn and carefully defibrinated with glass beads or a stirring rod. It was then passed with very gentle suction once or twice through a column of several centimeters of sterile cotton wool. The filtration was carried out in the cold (10°C.) to prevent glycolysis. This proved a most effective method of removing the leucocytes, as demonstrated by numerous control counts, which rarely exceeded 1000 per c. mm. The blood was then thoroughly saturated with oxygen and a control sample taken for immediate gas analysis. A portion was then treated with the dye or other reagents whose effect was to be studied, and another portion, untreated, was taken for a control. These samples were incubated at once either under a thick layer of paraffin oil, or, when carbon dioxide analyses were also desired, in small stoppered bottles, provided with a long side arm capillary, essentially as described in the earlier paper(1). They contained a few glass beads and were well paraffined. Certain control analyses were also done by means of a microrespiration apparatus.

The microrespiration apparatus used was slightly modified from the design of

that described by Winterstein (4). Our vessels were made in the shape of tiny Erlenmeyer flasks with very broad bases. Two small chambers were blown out from the vessel wall at opposite sides to carry other fluids, whose effect on the respiration it was desired to test. In the present instance these were methylene

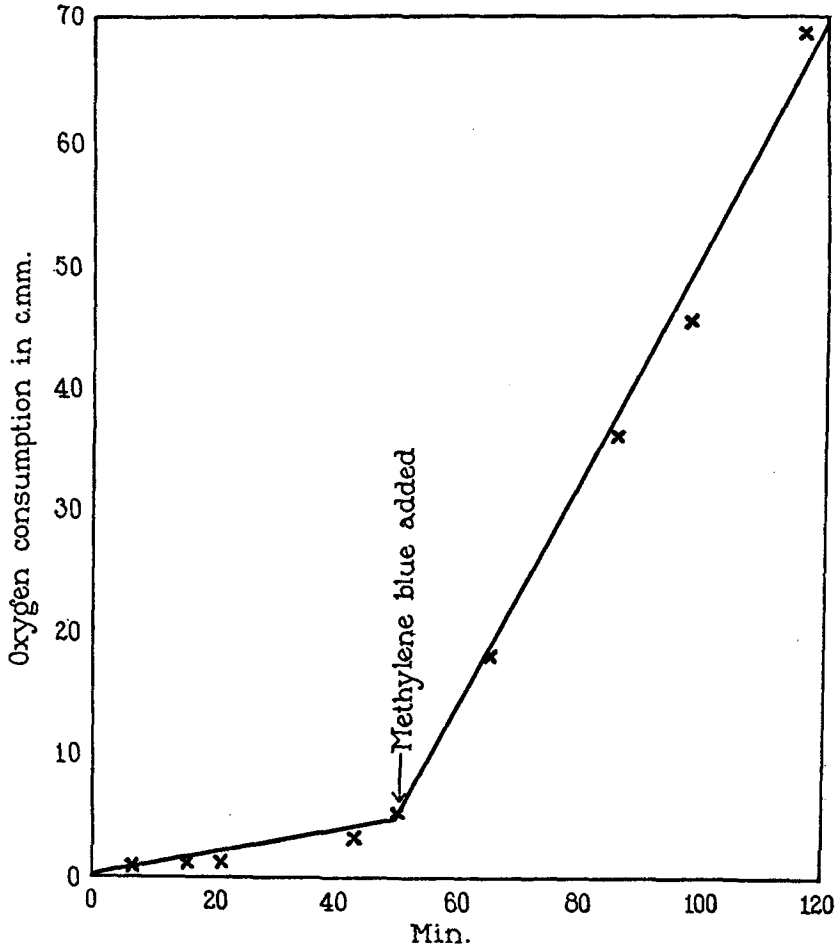

CHART 1. Microrespiration experiment. The effect of methylene blue upon the oxygen consumption of human erythrocytes (1.0 cc.).

blue or other dyes, and potassium cyanide, or other agents which inhibit cell oxidations. A special shaking device was constructed, and the apparatus was shaken at a rate of 75–100 per minute. A large rubber bulb with set screw attachment was used to regulate the mercury burette level. When two essential

precautions were observed—absolute cleanliness of the capillary tubes which contained the bubble, and care in the maintenance of a constant temperature of the water bath—the apparatus proved very useful in our hands. It presents certain advantages over the Barcroft-Warburg method in that elaborate calibration and calculations are avoided, since the oxygen consumption is found by direct readings on the mercury burette.

The increased oxygen consumption of human erythrocytes upon the addition of methylene blue in the microrespiration apparatus is indicated in the experiment shown in Chart 1.

We have excluded the possibility of methemoglobin formation in these studies by spectroscopic examination and particularly by a

TABLE II.

The Effect of Temperature upon the Oxygen Consumption of Mammalian (Dog) Erythrocytes.

	Oxygen content before incubation	Oxygen content after 3 hrs. incubation	Oxygen content after 24 hrs. incubation
	vol. %	*vol. %*	*vol. %*
Control at 21°C.	19.45	19.20	18.84
+M.B. 0.05%		17.40	13.92
Control at 38°C		18.80	12.72
+M.B. 0.05%		9.60	Completely reduced

Methylene blue (M.B.) concentration 0.005 per cent.

second estimation of the oxygen capacity of the blood after incubation, upon resaturation with air or oxygen. The latter is the more reliable test. Methylene blue alone gives a prominent line between the characteristic bands of methemoglobin in the spectroscope which may readily cause confusion on casual inspection.

The recent study of Wales, Munch and Schwartze (5) is of interest in regard to the question of methemoglobin formation. The authors made intravenous injections into rabbits and cats of very large amounts of certain dyes used for the coloring of foods and then made spectrophotometric determinations upon blood samples withdrawn at various intervals. With all of the dyes studied, reduced hemoglobin was formed within an hour. Complete reduction occurred when the specimens were permitted to stand for 24 hours or more. No evidences of methe-

moglobin formation could be detected in any of their studies. It seems likely that these dyes produced an effect similar to that of methylene blue upon the respiration of the erythrocytes.

The respiration takes place in fresh red blood cells carefully washed with Ringer's solution at approximately the same rate as in defibrinated blood alone and the increased respiration on addition of methylene blue is of about the same order of magnitude for both as is indicated in Experiments 8a and 8b, Table I.

The accelerated oxygen consumption in the presence of methylene blue proceeds even at room temperature, but its optimum appears to be at 37°C. (Table II).

TABLE III.

The Effect of Varying the Methylene Blue Concentration upon the Respiration of Mammalian Erythrocytes.

Nov. 21—dog blood	Before incubation		After incubation		Output carbon dioxide	Consumption oxygen
	Content carbon dioxide	Content oxygen	Content carbon dioxide	Content oxygen		
	vol. %	*vol. %*	*vol. %*	*vol. %*	*vol. %*	*vol. %*
Control—no M.B.	17.75	29.25	19.86	28.93	2.11	0.32
M.B. 0.005%			29.88	14.45	12.13	14.80
M.B. 0.0025%			30.00	14.21	12.25	15.04
M.B. 0.001%			28.75	14.80	11.00	14.45
M.B. 0.0005%			27.98	18.06	10.23	10.19

Methylene blue (M.B.) concentration 0.005 per cent.

The final concentration of methylene blue used in these studies has been 0.005–0.0005 per cent. Higher concentrations produced no marked increase in the respiration and at 0.0005 per cent concentration there was a diminution in the effect of the added methylene blue (Table III).

It is known that potassium cyanide, even in very dilute solution, stops cell oxidations. We have therefore studied the effect of potassium cyanide upon methylene blue respiration in the experiments presented in Table IV. It will be seen that the increased oxygen consumption upon the addition of methylene blue was nearly as great in the presence of the cyanide as in the untreated control. The effect

of methylene blue upon the respiration of mammalian blood cells is not therefore inhibited by this concentration of potassium cyanide (N 0.001).

The effect of methylene blue upon the active respiration of the nucleated red blood cells of birds was found to be entirely different from the effect which it had on the non-nucleated mammalian red blood cells. As is indicated in Table V and in the microrespiration experi-

TABLE IV.

Effect of Potassium Cyanide upon Methylene Blue Respiration.

	Content oxygen		Content oxygen
	vol. %		*vol. %*
Beef			
Blood before incubation	22.61	After 3 hrs. incubation	22.03
Blood + N/5000 KCN		After 3 hrs. incubation	21.95
Blood + 0.005% methylene blue		After 3 hrs. incubation	12.84
Blood + N/5000 KCN + 0.005% M.B		After 3 hrs. incubation	12.74
Dog			
Blood before incubation	23.80	After 3 hrs. incubation	23.40
+ N/5000 KCN		After 3 hrs. incubation	23.58
+ 0.005% methylene blue		After 3 hrs. incubation	14.63
+ N/5000 KCN + 0.005% M.B		After 3 hrs. incubation	15.10
Man			
Blood before incubation	19.42	After 3 hrs. incubation	19.18
+ N/1000 KCN		After 3 hrs. incubation	19.09
+ 0.005% methylene blue		After 3 hrs. incubation	9.42
+ N/1000 KCN + 0.005% M.B		After 3 hrs. incubation	11.70

ment shown in Chart 2, the respiration of goose erythrocytes, either from normal blood or from animals rendered anemic by bleeding, is increased by methylene blue, but to a much smaller extent than in the case of non-nucleated mammalian cells. Whereas in the latter the respiration is accelerated by methylene blue on the average twenty to fiftyfold, in avian blood it is never increased more than twice that of the simple defibrinated blood to which dye has been added.

Chart 3 illustrates the results obtained from a microrespiration experiment and shows that the respiration of goose erythrocytes which

TABLE V.

The Effect of Methylene Blue upon the Respiration of Avian (Goose) Erythrocytes.

	Content carbon dioxide	Content oxygen	Carbon dioxide output	Oxygen consumption	Oxygen consumed	Time of incubation	Condition of animal
	vol. %	*vol. %*	*vol. %*	*vol. %*	*per cent*		
1. Dec. 6—defibrinated blood							
Control before incubation....	11.59	21.60					
after incubation.....	18.77	11.63	7.18	9.97	46.2	3 hrs.	Normal
+M.B. after incubation.....	30.98	1.01	19.39	20.59	95.5		
2. Dec. 16—defibrinated blood							
Control before incubation....	12.00	16.55					
after incubation.....	20.46	7.84	8.46	8.71	52.6	1½	Anemic
+M.B. after incubation.....	24.28	1.36	12.28	15.19	91.6	hrs.	
+T.B. after incubation.....	23.51	1.76	11.51	14.79			
3. Feb. 1—defibrinated blood							
Control before incubation....	22.64	11.24					
after incubation.....	26.20	7.05	3.56	4.19	37.2	40	Anemic
+M.B. after incubation.....	28.42	5.03	4.78	6.21	55.2	min.	
4. Feb. 6—defibrinated blood							
Control before incubation....	22.40	22.00					
after incubation.....	32.05	16.92	9.65	5.08	23.1	40	Normal
+M.B. after incubation.....	35.50	14.16	13.10	7.84	35.6	min.	
The blood for Experiments 1, 2, and 3 was taken from the same animal during the course of several large periodical bleedings.							
5. Dec. 21—washed cells in Locke's solution							
Control before incubation....	4.00	19.65					
after incubation.....	10.66	11.35	6.66	8.30	42.2		
+M.B. after incubation.....	12.66	8.44	8.66	11.21	57.1		Anemic

M.B. means methylene blue 0.005 per cent solution.

T.B. means toluylene blue 0.005 per cent solution.

has been inhibited by the addition of potassium cyanide may be again restored when methylene blue is added.

We have extended our studies to an examination of the effect of

several other dyes whose oxidation-reduction potentials have been determined, including Bindschedler's green, indigo disulfonate, *o*-chlorophenol indophenol, phenol indophenol, and toluylene blue. Some

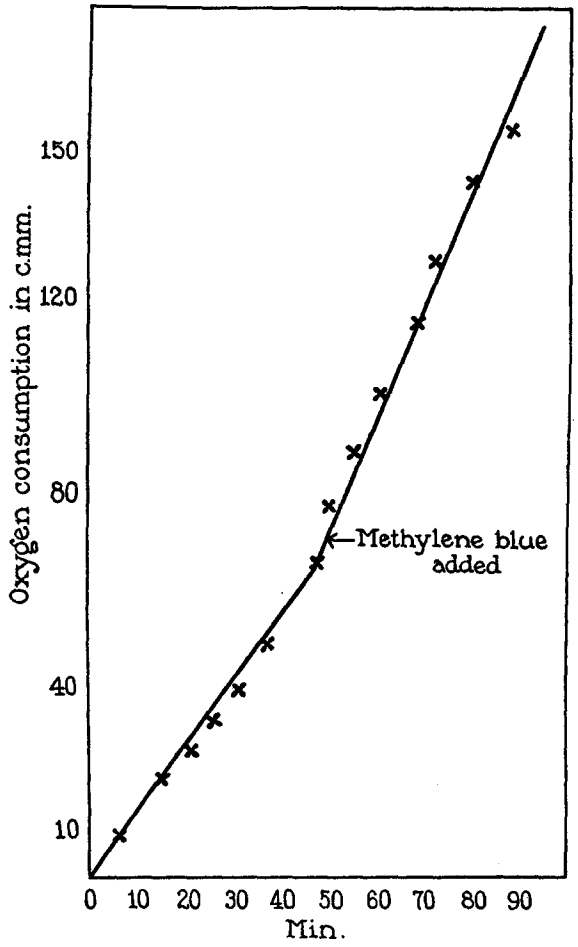

CHART 2. Microrespiration experiment. The effect of methylene blue upon the oxygen consumption of goose erythrocytes.

of these were kindly supplied to us by Professor W. Mansfield Clark. As seen in Table V, Experiment 2, and in Table VI, none of these with the exception of toluylene blue is as effective as methylene blue in

stimulating respiration at this range of concentration. The fact that no direct correlation can be shown between the oxidation-reduction potential of these dyes and the kinetic phenomena involved in the

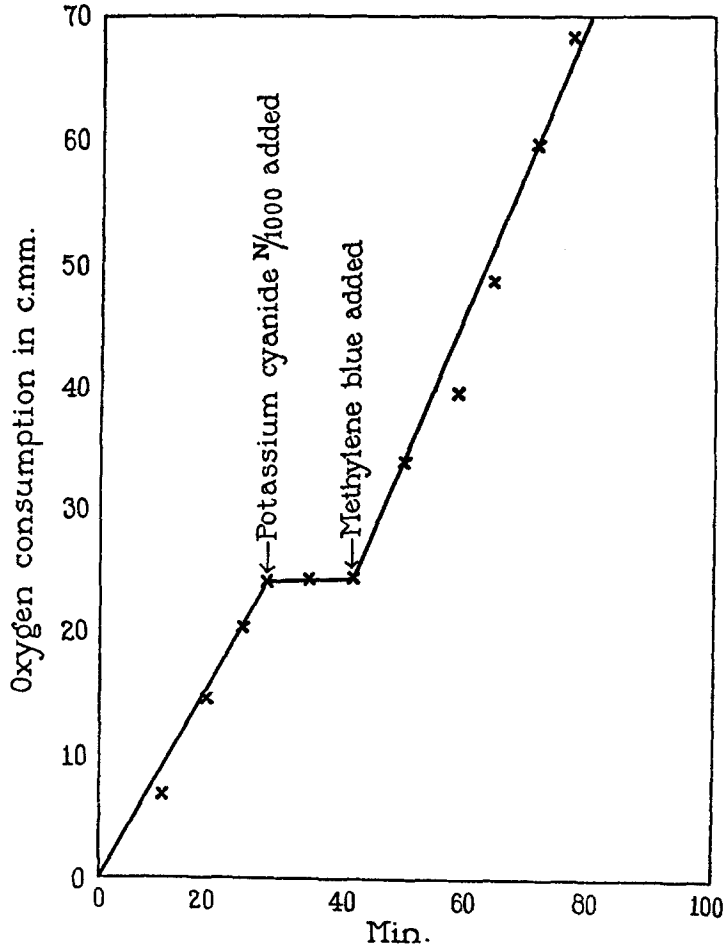

CHART 3. Microrespiration. The effect of methylene blue upon the oxygen consumption of goose erythrocytes in the presence of potassium cyanide.

accelerated cellular oxidations is not surprising. The question of the variable permeability of the cell membranes to the several dyes and the possibility of their variable toxic action must be taken into considera-

tion. It is at least of some significance that the potentials of the dyes which activate these oxidations fall within a rather restricted range.

The increased red blood cell metabolism reported in the present paper recalls the work of Heymans and Heymans (6), who produced fever in dogs ranging as high as 43° or 44°C., together with a marked increase in the output of carbon dioxide, by the intravenous injection of methylene blue, 0.05–0.10 per kilo of body weight.

TABLE VI.

The Effect of Various Dyes upon the Respiration of Mammalian Erythrocytes.

Oct. 28—beef blood; 3 hrs. incubation	Oxygen content before incubation (A)	Oxygen content after incubation (3 hrs. 38°C.) (B)	Oxygen consumption (A–B)	Percentage oxygen consumption $\frac{A-B}{A} \times 100\%$
	vol. %	*vol. %*	*vol. %*	
Control blood alone	18.50	18.11	0.39	2.1
Methylene blue 5 mg. % added		11.70	6.80	36.8
Indigo disulfonate 5 mg. % added		17.75	0.75	4.1
o-Chlorophenol indophenol 5 mg. % added		16.45	2.05	11.1
Phenol indophenol 5 mg. % added		16.28	2.22	12.0
Toluylene blue 5 mg. % added		10.00	8.50	46.0
Dec. 21—goose blood; 1 hr.'s incubation				
Control blood alone	19.65	11.35	8.30	42.2
Methylene blue 0.0025%		8.44	11.21	57.1
Bindschedler's green 0.0025%		9.04	10.61	54.0
Dec. 22—human blood (polycythemia vera); 3 hrs. incubation				
Control blood alone	23.58	22.35	1.23	5.2
Methylene blue 0.0025%		8.44	15.14	64.2
Bindschedler's green 0.0025%		15.52	8.06	34.2

In seeking an explanation of the effect of methylene blue upon erythrocyte respiration our attention was drawn to the studies reported by Meyerhof (7) upon staphylococci, precipitated by acetone in the cold, dried over phosphorus pentoxide, and subsequently heated *in vacuo* at 100–107°C. Under such circumstances, and by proper selection of the heating time, he was able to suppress to a large extent the oxygen absorption of this material. Upon the addition of methyl-

ene blue, 2.5 or 5 mg. per cent, the oxygen utilization again increased, although never to the same extent as in the unheated material. The methylene blue, by reason of its oxygen carrying properties was considered to take the place of some component of the respiration system which had been destroyed by the previous manipulations. The usual inhibiting effect of potassium cyanide upon the oxygen consumption of the killed staphylococci was much less marked in the presence of the methylene blue. The respiratory quotient lay within physiological limits -0.6 to 1.0.

Meyerhof reports a similar effect upon the oxygen consumption of acetone yeast, although here the optimal concentration of methylene blue was higher (0.02 – 0.1 mg. per cent). It was found to be unnecessary to heat the acetone yeast because the respiration of the unheated yeast, after the acetone-ether treatment, had already fallen to about 2 per cent. The respiration of the living cells, which are much less affected by methylene blue, was not inhibited in the presence of 0.0015 N KCN when 0.05 per cent methylene blue was added. Acetone yeast respiration in the presence of methylene blue is only affected to the extent of 10–20 per cent even in the presence of 0.01 N KCN. Macerated yeast juice (Lebedew) was found to behave in a similar manner in the presence of methylene blue.

The oxidation mechanism in the experiments which we report in the present paper appears to be analogous to that encountered by Meyerhof in his experiments. The effect of methylene blue occurs even in the presence of cyanides in both sets of experiments. The rate of oxidation in the mammalian erythrocytes in the presence of methylene blue is increased to about the rate of oxygen consumption found in the untreated nucleated erythrocytes of birds. Mammalian erythrocytes can be regarded as old or altered cells, in which oxidation processes have been largely suppressed through the disappearance of some link of the oxidation system. This link, however, is present in the younger cells. Methylene blue appears to be able to take the place of this substance and to act essentially as an oxygen carrier enabling the erythrocytes to utilize in their metabolism the oxygen stored in them as oxyhemoglobin. The respiration then resumes a rate similar to that present in the nucleated erythrocytes of avian blood.

The demonstration of proper respiratory quotients in studies on the

metabolism of blood has been difficult. Most authors content themselves with the statement that "physiological" respiratory quotients are obtainable, but actual figures are seldom given. At the time of our previous paper (1) we were unable to obtain agreement in this regard between oxygen consumption and carbon dioxide production in human blood. Consequently no figures on the carbon dioxide

TABLE VII.

The Respiratory Quotient of Mammalian and Avian (Goose) Erythrocytes with and without the Presence of Methylene Blue. Washed Cells Suspended in Locke's Solution.

	Content carbon dioxide (A)	Content oxygen (B)	Carbon dioxide output (C)	Oxygen consumption (D)	Respiratory quotient $\frac{C}{D}$
	vol. %	*vol. %*	*vol. %*	*vol. %*	
*Man**					
Control before incubation	4.22	17.01			
after incubation	4.57	16.53	0.35	0.48	0.73
+M.B. after incubation	14.64	3.39	10.42	13.62	0.77
Dog†					
Control before incubation	3.36	22.58			
after incubation	4.35	21.62	0.99	0.96	1.00
+M.B. after incubation	13.56	9.58	10.20	13.00	0.79
Goose‡					
Control before incubation	4.00	19.65			
after incubation	10.66	11.35	6.66	8.30	0.80
+M.B. after incubation	12.66	8.44	8.66	11.21	0.78

M.B. means methylene blue 0.005 per cent.

* Experiment 2 Table I.

† Experiment 10 Table I.

‡ Experiment 5 Table V.

production were published. The difficulty in metabolism studies upon incubated mammalian erythrocytes lies in the excessive production of carbon dioxide compared to the oxygen consumption, as may readily be observed by an inspection of Table I. A large initial formation of carbon dioxide in shed blood appears to occur in the serum in the presence of the red cells. It does not occur when the cells are

washed and suspended in Locke's solution (Table I, Experiments 8a and 8b). The amount of extra carbon dioxide thus produced in the 1st hour of incubation may amount to 2 millimols per 100 cc. of blood. The source of this extra carbon dioxide is under investigation. As seen from Table VII, the respiratory quotient of both avian and mammalian erythrocytes usually lies between 0.75 and 0.80 both in the presence of methylene blue and in its absence, when the serum is removed and the cells are suspended in Locke's solution.

TABLE VIII.

Effect of Hemolysis (Freezing and Thawing) upon Oxygen Consumption of Goose Blood.

	Oxygen content before incubation	Oxygen content after incubation	Oxygen consumption	
	vol. %	*vol. %*	%	
Control blood	16.55	7.84	52.6	1 hr.
Control blood + 0.005% M.B.		1.76	89.4	incubation

After hemolysis by freezing and thawing, the material was centrifuged at high speed and the top and bottom layers separated. The top material contained only a small amount of cellular debris and no nuclear fragments when examined microscopically. The bottom layer contained cell fragments and nuclei and a very few intact erythrocytes.

Top layer control	10.84	10.40	4.0	2 hrs.
Top layer control + 0.005% M.B.		9.13	15.8	incubation
Bottom layer control	13.67	10.90	20.3	2 hrs.
Bottom layer control + 0.005% M.B.		7.94	41.9	incubation

Effect of Destruction of the Cell Membrane (Freezing and Thawing) upon Oxygen Consumption and the Effect of Methylene Blue.

Warburg has shown that the cell membranes of erythrocytes (goose) can be destroyed by freezing and thawing. When the material is then centrifuged at high speed, two layers may be obtained, the top containing only hemolyzed cell contents, while the lower contains nuclear material and the remains of the formed elements. He found that the respiratory activity was then confined to the lower layer which contained the nuclear material. We have repeated these experiments,

and have found (Table VIII) that the oxygen consumption is greatly reduced in both top and bottom layers, as compared to the consumption in untreated defibrinated blood. Although the oxygen consumption of the material in the top layer is much less than it is in the bottom layer, it is still appreciable. The addition of methylene blue produces the usual increase in oxygen consumption in both layers, but it is interesting to note that the effect is much more marked in the very slowly respiring top layer which contains no nuclear material.

CONCLUSIONS.

1. The respiratory metabolism of non-nucleated mammalian erythrocytes is enormously accelerated and approaches the magnitude of the metabolism of the nucleated erythrocytes of birds on the addition of methylene blue (and certain other dyes), to a final concentration of 0.005–0.0005 per cent.

2. In the presence of methylene blue the respiration is accelerated even when M/1000 KCN is also present.

3. The accelerated respiration due to methylene blue occurs at room temperature but it is most active at 38°.

4. Methylene blue in the above concentration accelerates the respiration of avian (goose) erythrocytes to a much smaller extent than it does the respiration of the erythrocytes of mammalian blood, while the effect upon anemic goose blood seems to be less than it is upon cells of normal goose blood.

5. Owing to a rather large initial carbon dioxide formation in defibrinated blood on incubation, which may not be related to the immediate respiratory process, proper respiratory quotients cannot be obtained in whole blood. When the cells are separated from the serum and suspended in Locke's solution, respiratory quotients are obtained upon incubation comparable to those of other resting mammalian cells, as well as of the actively respiring erythrocytes of birds.

6. The hypothesis is advanced that methylene blue acts in the rôle of an oxygen carrier, supplying a substance which has disappeared from adult mammalian non-nucleated erythrocytes and restoring their metabolic activity to an extent comparable to that of the young immature forms, or to that of the actively respiring avian (goose) blood.

We wish to acknowledge the kindness of Professor Michaelis in drawing our attention to the analogy between our findings and those reported by Meyerhof.

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