CCLXIII. OBSERVATIONS ON THE RESPIRATION OF RED BLOOD CORPUSCLES OF NORMAL SUBJECTS AND IN VARIOUS FORMS OF ANAEMIA.

By WALTER DEUTSCH.

*From the Department of Physiology and the Department of Clinical Investigations
and Research, the University and Royal Infirmary, Manchester.*

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BARRON AND HARROP [1928] discovered that non-nucleated red blood corpuscles in the presence of carbohydrate and a small amount of methylene blue have a marked respiration. Warburg *et al.* [1930] pointed out that the "methylene blue respiration" takes place in three stages:

- (1) methylene blue forms methaemoglobin from haemoglobin and is itself reduced to leucomethylene blue;
- (2) the methaemoglobin formed oxidises the carbohydrate by means of the "structure" of the red cells and is itself reduced to haemoglobin;
- (3) the leucomethylene blue is reoxidised to methylene blue by the oxygen of the air.

Previous to this Michaelis and Salomon [1930, 1, 2] found that Ringer-extracts of fresh liver and other tissues increased the respiration of red blood corpuscles in the presence of carbohydrate. They called the unknown principle, contained in the tissues, the "respiratory supplement."

Then followed the discovery of a class of dyestuffs, the so-called lyochromes in liver, kidney, egg-yolk, whey *etc.*, designated [Wagner-Jauregg and Ruska, 1933] likewise as the "methylene blue of the cell" from their capacity to act as hydrogen acceptors from suitable substrates and then to be reoxidised by the oxygen of the air.

Presumably there is a connection between the respiratory supplement and the lyochromes, and its mode of action may be similar to that of methylene blue. The following experiments were carried out to test whether methaemoglobin is actually formed when the respiratory supplement or when therapeutically efficient extracts of liver are used. Further the effect of the respiratory supplement was examined in different types of anaemias and during different stages of these anaemias.

METHAEMOGLOBIN AND THE ACTIVE PRINCIPLE OF LIVER EXTRACTS EFFECTIVE IN THE TREATMENT OF PERNICIOUS ANAEMIA.

On account of the fact that methaemoglobin is sometimes found in the serum of patients suffering from pernicious anaemia, Duesberg and Koll [1931] and Felix and Frühwein [1933] have regarded the transformation of haemoglobin into methaemoglobin by means of extracts of liver as a sign that the active principle

effective in the treatment of pernicious anaemia is present. They therefore prefer to regard the formation of methaemoglobin instead of the inconvenient clinical examination as a test for the efficiency of those extracts.

In our opinion methaemoglobin does not arise through the action of the active anti-anaemic principle of the liver, and its presence is purely a coincidence. This becomes evident from the following experiment. In the case of methylene blue the formation of methaemoglobin as pointed out above leads to an increased consumption of oxygen in the presence of carbohydrate. No such increased consumption of oxygen however was observed (see Table I) and no band of methaemoglobin was seen in the spectroscope when commercial mammalian liver extract was used in the place of methylene blue, although clinically the extract was very efficient in the treatment of pernicious anaemia. (For the technique see pp. 2005–2006.)

Table I. *Oxygen consumption.*

	Time mins.	Without extract μl.	Time mins.	With extract (0.5 ml.) μl.
A. Pernicious anaemia during reticulocyte crisis	30	6.0	70	13.6
B. Pernicious anaemia 7 days after reticulocyte crisis	30	4.6	60	6.0
C. Normal person	30	5.0	60	9.5

In this experiment an active commercial mammalian liver extract was kept in the side chamber for 30 minutes during the determination of the respiration without extract and subsequently tilted into the main chamber of the respirometer. The extract did not contain any preservative, and since its reaction was acid it had to be neutralised.

On the other hand a number of liver extracts that were haemopoietically inactive in the treatment of pernicious anaemia have been found to give very marked stimulation effects on the respiration of the red cells. These and other results will be reported elsewhere.

METHAEMOGLOBIN AND THE RESPIRATORY SUPPLEMENT.

Quite apart from the relationship between methaemoglobin and the therapeutically active anti-anaemic principle in liver the question arises whether saline liver extracts containing the respiratory supplement increase the respiration of red blood cells by forming methaemoglobin. Stern and Greville [1933] claimed that the dyestuff uroflavin which can be isolated from urine is able to increase the respiration of red blood cells by formation of methaemoglobin. This result however was not confirmed by Wagner-Jauregg and Wollschitt [1934] in the case of uroflavin when it was purified further. These workers also found that lactoflavin, the lyochrome from milk, was unable to act as producer of methaemoglobin.

As the mode of action of the respiratory supplement is still unknown, an attempt was made to determine whether or not the methaemoglobin acts by limiting the oxygen capacity of the blood concerned.

If one supposes a cycle of reactions similar to that occurring with methylene blue, arrangement must be made to interrupt the cycle so as to enable the methaemoglobin formed to accumulate. Such an arrangement would be to use a haemoglobin solution free from stromata, so that the methaemoglobin formed cannot be reconverted into haemoglobin. The other possibility, *viz.*, the removal

of carbohydrate, is not possible as in the methylene-blue experiments because the liver extract itself contains carbohydrate which is not removable without interfering with the respiratory supplement.

Human red blood corpuscles were washed three times with Ringer solution on the centrifuge and after centrifuging to a small volume partly haemolysed by adding distilled water; the haemolysis was completed by freezing and then thawing, and the product was finally freed from stromata by centrifuging at a high speed for half an hour. Determination of the oxygen capacity was carried out according to the method of Haldane-Barcroft as modified by Warburg [1931] in the Warburg apparatus. This haemoglobin solution was mixed with fresh Ringer extract of liver (for technique see pp. 2005–2006) and the oxygen capacity of this mixture was determined before and after incubation in a water thermostat at 37° (Table II).

Table II.

Manometer...	(1)	(2)	(3)	(4)	(5)
	ml.	ml.	ml.	ml.	ml.
Main chamber	1.0 Hb solution 0.5 liver extract 2.0 piperazine	2.0 Hb sol. 1.0 liver extr. —	2.0 Ringer 1.0 liver extr. 0.1 glucose	2.0 blood susp. 1.0 liver extr. 0.1 glucose	1.5 from manometer (2) 2.0 piperazine 0.2 $K_3Fe(CN)_6$
Side chamber	0.2 ml. $K_3Fe(CN)_6$	—	—	—	0.2 $K_3Fe(CN)_6$
Small inner receptacle	0.15 ml. KOH	0.15 ml. KOH	0.15 ml. KOH	0.15 ml. KOH	0.15 KOH
Oxygen capacity at the time: 0 min., μ l.	67	—	—	—	—
Oxygen capacity after 170 mins. in thermostat, μ l.	—	—	—	—	50.5
Oxygen consumption after 170 mins. in thermostat, μ l.	—	59.5	12.5	299	—

A decrease in the oxygen capacity of 16.5 μ l. was found (manometers 1 and 5), indicating that some of the oxyhaemoglobin was converted into a substance which either had a smaller oxygen capacity or was unable to give up its oxygen. The washed red blood corpuscles together with the same liver extract took up 299 μ l. oxygen (manometer 4) showing the high efficiency of the liver extract. The respiration of the liver extract itself being very small (manometer 3) is slightly increased by the haemoglobin solution (manometer 2). This effect is probably due to the stimulation of the respiration of tissue extracts by haemoglobin solutions according to Battelli and Stern [1909].

Examination with the spectroscope at the end of the experiment did not show the presence of methaemoglobin, whereas in an artificial mixture of haemoglobin and methaemoglobin in the expected proportion the spectrum of the latter was easily visible.

No difference in the decrease of oxygen capacity was noticed on keeping the mixture in the thermostat during 170 minutes or during 18 hours. In other words the loss of oxygen capacity is a limited one.

If the haemoglobin solution and the liver extract were kept separate from one another during 24 hours and then mixed, the determination immediately after mixing had the same value as on the previous day, thus indicating that no change in the solutions themselves could be responsible for the loss of oxygen capacity in the foregoing experiment.

When using washed red cells instead of haemolysed blood no loss of oxygen capacity was found to occur.

Further evidence against the formation of methaemoglobin is shown by the next experiment. The haemoglobin solution and the liver extract kept separate

in the flask during 10 mins. took up 4.5 μ l. oxygen and during 15 mins. after mixing 18 μ l. Thus no development of gas but absorption took place.

Had methaemoglobin been formed, development of oxygen would have occurred after mixing a methaemoglobin solution with fresh liver extract.

A decrease in the oxygen capacity is the only point in favour of the formation of a substance similar to methaemoglobin.

Discussion.

The fact that extracts of liver shown to be very efficient in the treatment of pernicious anaemia are at times unable to form methaemoglobin, suggests that no causal relation exists between the formation of methaemoglobin and the activity of the haemopoietic liver principle. Substitution, therefore, of the methaemoglobin test in place of the present clinical method of determining haemopoietic potency does not appear to be justified.

With regard to the mode of action of saline extracts of liver on haemoglobin solutions it is noteworthy that there is a decrease in the oxygen capacity without any indication of the presence of methaemoglobin when examined spectroscopically. The explanation would therefore appear to be that the haemoglobin molecule in contact with extracts of liver is changed to haematin or to a lower product of catabolism. This catabolism can easily take place under the influence of the enzymes contained in liver. Asher and Ebnöther [1916] for instance found a catabolism of haemoglobin to a stage lower than haematin by adding extracts of liver and spleen, and Doljanski and Koch [1933] found haematin under the same circumstances.

RESPIRATORY SUPPLEMENT AND ANAEMIAS.

In previous investigations concerning the respiratory supplement use was made of normal red blood corpuscles of different species of animals as a substrate and only the tissues from which the extracts were prepared were changed.

Continuing previous work [Deutsch and Wagenfeld, 1931; see also Damblé, 1933] on a larger scale, investigation has now been made of the influence of fresh Ringer extracts of rat liver on red blood corpuscles obtained from anaemias of various types and at different stages.

These workers came to the conclusion that in cases of pernicious anaemia, contrary to the theory of a peripheral toxin, erythropoiesis itself is disturbed, in consequence of which the red cells are of a poorer quality than the cells of secondary anaemia. In severe cases of pernicious anaemia the increasing effect of the respiratory supplement on the cell respiration rate was found to be low, in secondary anaemia high. They attributed this to an alteration in the structure of the membrane of the red cell.

Method. The blood was taken from the vein of the patient by syringe (12 ml. in the case of severe anaemia) and mixed at once with 1.5 ml. of sodium citrate (3.8 %) brought to the p_H of the blood by the addition of hydrochloric acid. After the first centrifuging and removal of the plasma, 1 ml. sodium citrate solution was added and the volume of the cell suspension made up to about 15 ml. by adding Ringer-bicarbonate solution, vigorously blown out from a pipette to secure thorough mixing. It was then again centrifuged and was given two consecutive washings with the Ringer-bicarbonate solution used in the experiment (96 ml. 0.9 % NaCl; 2 ml. 1.15 % KCl; 2 ml. 0.11 *M* CaCl₂; 2 ml. 1.3 % NaHCO₃). After sucking off the supernatant liquid sufficient Ringer-bicarbonate solution was added to the suspension of red blood corpuscles until

the final concentration of the cells was about 4.2 millions per μl . The amount required can be calculated easily from the red blood count of the patient and the volume of blood taken. After thoroughly mixing, a count of the corpuscles was made in a counting chamber and, according to the result obtained, such a volume was taken that the concentration of the cells was 4 millions per μl ., when the volume was increased to 2 ml. by adding Ringer-bicarbonate solution. The specimen of normal blood used for comparison was made up to the same concentration.

The consumption of oxygen was determined in the Warburg apparatus. The volume of the flasks having the shape of a cone was about 15 ml., the temperature of the thermostat $38^{\circ} \pm 0.01^{\circ}$. The space above the liquid contained air. The extracts were always made from the fresh livers of rats. The liver was ground with sand in a mortar and mixed with ten times its weight of Ringer-bicarbonate solution. The mixture was allowed to stand for about 2 hours, occasionally stirred, then centrifuged and the supernatant, opalescent, cell-free liquid (liver extract, *LE*) used. In rare cases the extract was found to be inactive.

For the purpose of the experiment the solution was prepared as follows:

2 ml. suspension of blood corpuscles (4 million cells per μl .) saturated with air, 1.0 ml. liver extract, 0.1 ml. glucose (10 %) and 0.5 ml. Ringer-bicarbonate solution were placed in the main chamber while 0.15 ml. KOH was placed in the small inner receptacle of the flask.

The experiment consisted of:

- (a) determination of the O_2 consumption of pathological blood under the influence of the liver extract;
- (b) the same as (a) with normal blood (for comparison);
- (c) determination of the O_2 consumption of pathological blood without extract;
- (d) the same as (c) using normal blood;
- (e) determination of the O_2 consumption of the liver extract alone (using here instead of blood 2.0 ml. Ringer-bicarbonate solution).

The results of some experiments are plotted below, in order to make clear the results given later in tabulated form.

- (1) Patient A.K., pernicious anaemia, treated with intramuscular injections of liver extract. The experiment was carried out during a period of reticulocyte crisis. Reticulocytes on the day of the experiment 20.1 %; on the previous day 4.3 %; on the day after the experiment 33.7 % and 2 days later the peak of the crisis was 53 %.

The curves demonstrating the increase of the respiration by the respiratory supplement run in the case of the pernicious blood (*RPE*) somewhat above the curve of the normal blood (*RNE*). But the respiration of the pernicious anaemia blood itself (*RP*) is far higher than that of the normal blood (*RN*). Thus the effect of stimulation on the pernicious blood is far less than that on the normal blood used for comparison. Cases of this type are marked by a — sign. When the increases of the respiration of the pathological blood and of the normal blood are alike, the respiration of both types of blood being taken into consideration, the sign 0 is used. When the increase of the respiration of the pathological blood is greater than that of the normal blood, it is marked with the sign +.

- (2) Patient T.E., untreated pernicious anaemia.

In this case the respiration of the pernicious anaemia blood (curve *RP*) was greater than that of the normal blood (curve *RN*) but under the influence of the added liver extract it was less (curves *RPE* and *RNE* respectively); consequently it will be seen that the stimulating effect of the liver extract was less in the case of the pernicious anaemia blood than in the normal control.

(3) Patient E. W., pernicious anaemia at beginning of reticulocyte crisis.

The effect of stimulation on the pernicious anaemia blood ceased after a short time. Had records of the respiration started 30 mins. after the established equilibrium of the temperature, as was done by Michaelis and Salomon, the *RPE* and the *LE* curves would have coincided.

Fig. 1.

RPE = respiration of pernicious anaemia blood with extract.
RNE = " normal blood with extract.
RP = " pernicious anaemia blood without extract.
RN = " normal blood without extract.
LE = " liver extract.

Fig. 2.

Fig. 3.

These curves were not the same in all cases of pernicious anaemia. Examples were also obtained of 0 and + cases in addition to these - cases. Table III summarises some of the results obtained.

In general the effect of stimulation is strongest at the time of the peak of the crisis and subsequently decreases. After the conclusion of the treatment it approaches the behaviour of the normal blood.

Table III.

[illegible]

Secondary anaemia. In the series of cases of mild and severe secondary anaemia examined only one case of chronic haemolytic anaemia with 3·6 % reticulocytes was found in which the increase of the respiration was less than that of normal blood. In one case the respiration was extraordinarily high, in another case not quite so high, but still very considerable.

Table IV. *Secondary anaemias.*

Patient	Disease	Stimulating effect	Million red cells μ l.	Hb %
W. H.	Gastric. carc. ?	+ +	2·24	27
A. F.	Carc. cervix	+(+)	2·04	24
G. D.	Achlorh. anaem.	+	2·63	34
E. H.	Achlorh. anaem.	+	4·27	28
E. Ch.	Achlorh. anaem.	+	5·80	61
I. K.	Carc. breast	+	2·55	—
B. T.	Fibro-myoma uteri	0	3·97	61
A. H.	Achlorh. anaem.	0	3·98	28
E. C.	Carc. cervix	0	3·31	52
J. C.	Carc. of mouth	0	—	—
F. St.	Epithel. of skin	0	2·55	53
E. I.	Carc. cervix	0	2·06	36
W. L.	Haemolytic anaem.	—	3·57	84

In order to show that two samples of blood of two normal persons are stimulated at an equal rate by the respiratory supplement, the following experiment may be cited (Table V).

Table V.

Oxygen consumption of the blood alone.

Time min.	Subject S. C. O_2 , μ l.	Subject G. S. O_2 , μ l.
15	3·9	3·2
30	6·7	5·4

After addition of liver extract.

15	15·1	15·6
30	28·4	27·6
40	35·5	34·5
80	52·4	51·1

Discussion.

It is difficult at present to give an unequivocal explanation of the different behaviour of anaemias in relation to the respiratory supplement and the mode of action of the latter. It has been known for a long time that young red cells have greater respirations than ordinary ones. The stimulation effect, however, is an additional one, brought about by the presence of the respiratory supplement. At the peak of a reticulocyte crisis the stimulation effect is large. A reasonable conclusion therefore would be that a relationship exists between the unknown substance of which the reticulum of the reticulocyte consists and the respiratory supplement. But the validity of this inference is weakened by the variety of steps which may be involved in the course of reaction. The structure of the cell surface on which the oxidation takes place may be altered, as was assumed in the previous paper, or the oscillation of the stimulation effect may be due to a corresponding oscillation in the enzymes of the red cells discovered by Warburg and Christian [1931]. These enzymes being a link in the breakdown of glucose act through the formation of hexosephosphoric acid and the succeeding activation

of this ester. Therefore a smaller amount of these enzymes could cause a decrease of the stimulation effect.

It is important to stress that the amount of haemoglobin contained in the red cells has no influence on the stimulation effect. Variations in the amount of haemoglobin are accounted for as follows: in order to have an equal number of red cells in all specimens of blood examined (4 millions) the red cells of the anaemias had to be distributed in less solution than those of the normal blood for comparison. The amount of haemoglobin present in the samples is bound to rise correspondingly with the colour index which is high in the case of pernicious anaemia, but low in secondary anaemia. At an equal number of red cells the haemoglobin content of a sample of blood of a secondary anaemia is therefore less and of pernicious anaemia more than of the normal blood used for comparison. In spite of these great differences in the content of haemoglobin the stimulation effect can be large in secondary anaemias and small in pernicious anaemia.

SUMMARY.

1. No formation of methaemoglobin was observed when red blood cells were mixed with an active commercial liver extract (used for the treatment of pernicious anaemia) and examined either spectroscopically or by measurement of the respiration of the red cells.
2. Substitution of the methaemoglobin test for the present clinical method of determining haemopoietic potency does not appear to be justified.
3. Saline extracts of fresh rat liver containing the "respiratory supplement" produce a slight loss of oxygen capacity when mixed with a haemoglobin solution, without any indication of the presence of methaemoglobin.
4. This decrease in oxygen capacity is limited, being the same after 3 hours and after 18 hours.
5. When a haemoglobin solution has been mixed with a Ringer-bicarbonate extract of rat liver no evolution of oxygen but a disappearance takes place indicating that methaemoglobin has not been formed.
6. The stimulation effect on the respiration of red blood cells brought about by the "respiratory supplement" (Michaelis and Salomon) has been examined during different stages of pernicious anaemia and in secondary anaemias.
7. The effect of stimulation is usually the strongest at the time of the peak of the reticulocyte crisis, decreases subsequently and approaches that given with normal blood after the conclusion of the treatment. Some cases show a small effect before the peak of the crisis but it is not possible to make any generalisation.
8. Secondary anaemias show a stimulation effect greater than or equal to that of the normal blood, with the exception of one case of haemolytic anaemia which showed a lower effect than that of the normal blood.
9. The stimulation effect is independent of the haemoglobin content of the red cells.

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