

Studies on the Production of Erythropoietin by Isolated Perfused Organs

By Z. KURATOWSKA, B. LEWARTOWSKI AND E. MICHALAK

IT IS generally accepted that in mammals under hypoxic conditions an erythropoiesis stimulating substance is produced. This substance, called the humoral erythropoietic factor or erythropoietin, has been isolated in recent years from the plasma, and its chemical nature partially elucidated.¹⁻³

The site of the production of erythropoietin is uncertain. Some earlier authors⁴ suggested that erythropoietin was produced by the reticuloendothelial system (RES). Jacobson et al.^{8,9} have suggested that the kidney may be the organ responsible for the production of erythropoietin. According to Osnes⁵ the mechanism of erythropoietin release is more complex, nervous and hormonal (ACTH) factors being involved in the production of erythropoietic principles in the kidney and liver.

In this study, a new approach was utilized in order to examine whether isolated organs are able to produce an erythropoietic factor under hypoxic conditions.

MATERIAL AND METHODS

Isolated organs—kidneys, livers, lungs and spleens—of the rabbits were examined. Rabbits of both sexes weighing 2000–2600 Gm. were bled under light ether or chloralose anesthesia. The perfusion apparatus was filled with heparinized blood in the amount of 70–90 ml. The cannula was inserted into the renal, pulmonal or splenic artery, and the organs were quickly isolated and placed in the apparatus. The renal, pulmonic, splenic and hepatic veins were cut and left opened. In the case of liver perfusion the cannula was inserted into the portal vein and the hepatic artery and ductus choledochus were tied. Separate cannulas were placed in the ureter and gall bladder for urine and bile collection. The perfusion method was based on the closed circuit perfusion technic described by Briscoe and Burn.⁶

The blood flowed down from the reservoir into the organ through the glass or plastic tubing surrounded by a jacket in which warm water was circulating (fig. 1). Its temperature was thermostatically controlled. The perfusion pressure was regulated by the height of the upper reservoir. It amounted for the kidney to about 120 mm. Hg., for the spleen to 80 mm. Hg., for the lung to 30 mm. Hg., and for the liver to 20 mm. Hg., respectively. If the kidney and spleen were perfused, the organs were immersed in the blood flowing out from their veins and then collected in a small container immersed in a warm water bath. The lungs and livers were closed in a thermostatically controlled wet chamber and the blood was collected in the funnel placed below the organ. From this container or funnel the blood flowed down through a plastic tubing about 1 M. long to another vertical tubing, through which it was driven back to the upper reservoir by the gas stream. The blood in the vertical tubing and in the upper reservoir achieved equilibrium with the gas used. The upper reservoir was open to air, but it was filled with the gas used for the blood driving. Thus, using oxygen and nitrogen in various proportions, a different degree

From the Laboratory of Clinical Biochemistry, Institute of Hematology, and from the Department of Human Physiology, School of Medicine, Warsaw, Poland.

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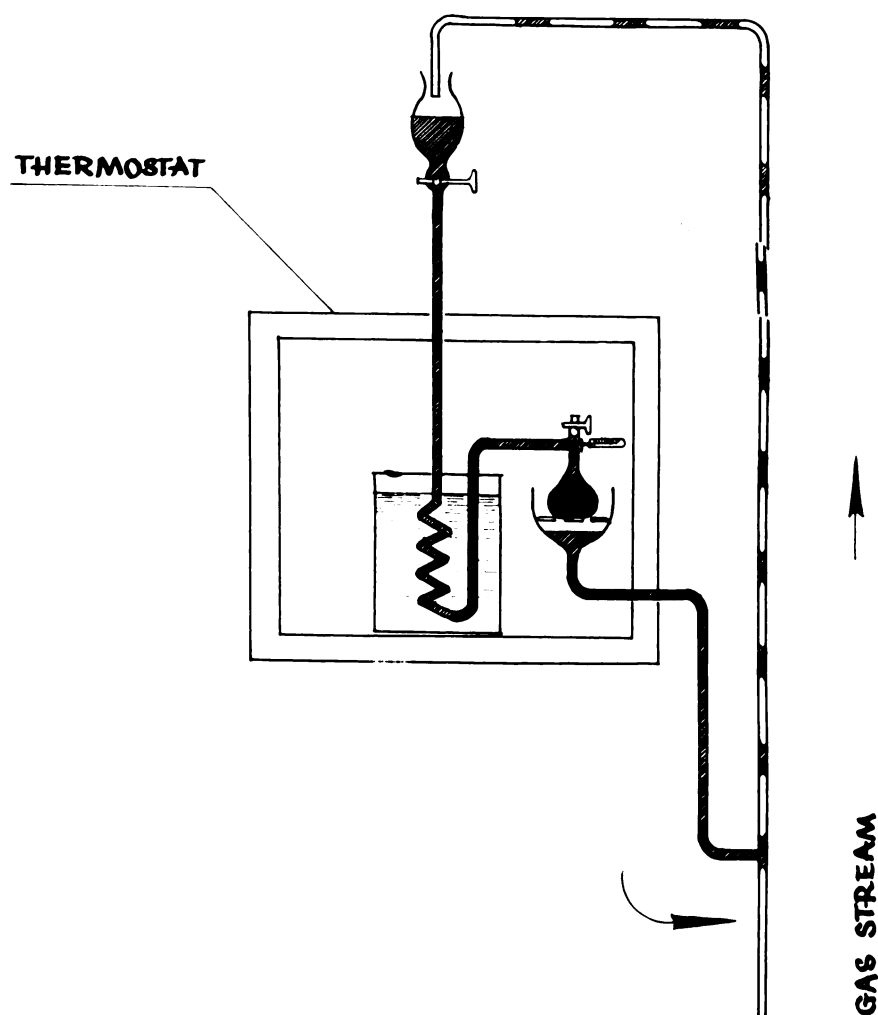


Fig. 1.—Diagram of perfusion apparatus.

of blood oxygenation could be obtained. Hypoxia was elicited by using 5 per cent CO_2 in 95 per cent nitrogen for 15 minutes. After each 15 min. period this mixture was substituted by 100 per cent oxygen for 5 minutes. The perfusion was continued for three hours. After each experiment the survival of each respective organ was controlled by perfusion of fully oxygenated blood and the colors of arterial inflow and venous outflow blood were compared. In experiments with the kidneys a continuous flow of urine was observed (about 15 ml. per experiment). In experiments with the livers a slow flow of the bile (1–1.5 ml. per experiment) was noted. Subsequently, the blood was let out of the apparatus and was centrifuged.

The plasma was boiled at pH 5.5 for 5 minutes after the modified Borsook method,⁷ and the protein precipitate was separated by centrifugation and discarded. The supernatant was adjusted to pH 7.3–7.4 with 1 n. NaOH and stored in the frozen state.

The extract from each perfusion was injected subcutaneously to five white, male mice for three days in a dose of 1 ml. for every injection per animal; 1.5 ml. of original plasma was represented by 1 ml. of extract.

Reticulocytes were determined by the direct smear method using 1 per cent alcoholic solution of brilliant cresyl blue or 1 per cent Nil blue solution in 0.9 per cent NaCl. The blood for reticulocyte determinations was drawn from the tail vein at the day of first injection of tested material (it is referred to as 0 day) and then at the 5th, 7th, and 10th days of experiment. The reticulocytes were counted in at least 2000 red cells.

Between the 10th and 12th day the bone marrow was examined. Both femurs were cut out immediately after the animals were killed. The femoral marrow was removed and the smears were made. Preparations were stained with Giemsa stain. The number of nucleated erythroid cells per 100 nucleated cells of marrow was counted.

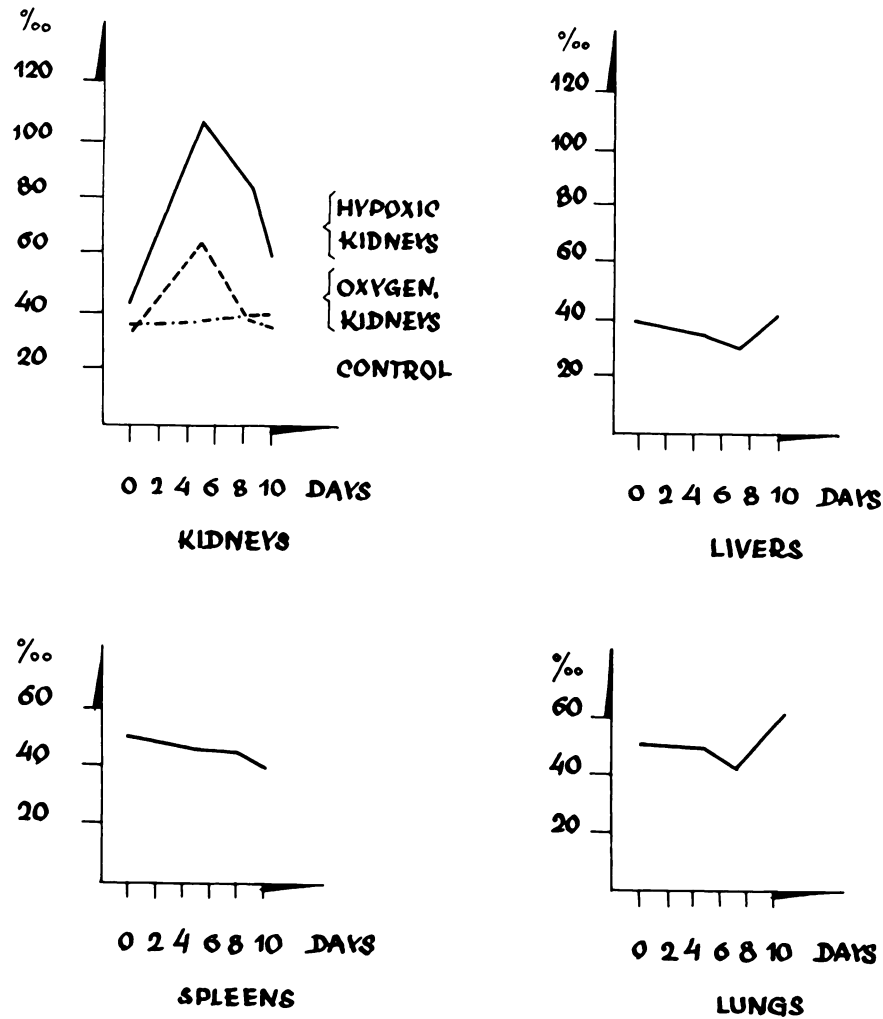


Fig. 2.—Mean reticulocyte counts following three successive injections (at 0, 1st, and 2nd days) of extracts of perfusates of isolated organs. The data obtained in all experiments on hypoxic organs and oxygenated kidneys is included. Number of experiments and test animals as in appropriate tables. Controls as in table 1. Ordinates = mean values of reticulocyte level per 1000. Abscissae = days of experiment.

Table 1.—*Effect of Plasma Extracts Obtained from Perfusates of 3 Hypoxic and 3 Oxygenated Kidneys, Compared with a Control Group**

Days	Mean values of reticulocyte counts (in %)				
	Control	Hypoxic kidneys	p	Oxygenated kidneys	p
0	36.5 ± 6.5†	43.0 ± 9.7	0.5 > p > 0.4	34.9 ± 10.9	0.7 > p > 0.6
5	36.4 ± 7.6	105.0 ± 43.9	p < 0.001	63.3 ± 19.5	p < 0.001
7	35.3 ± 6.9	87.5 ± 12.0	p < 0.001	39.5 ± 9.3	p > 0.1
10	38.5 ± 10.2	59.8 ± 17.1	p < 0.001	42.2 ± 12.2	p > 0.7

*Five mice were used for testing of each extract. Twelve mice injected with saline and 10 mice injected with extracts of normal rabbit plasma are included in the control group.

†Standard deviation of the mean.

RESULTS

Experiments were carried out in the following groups:

I. *Controls.* Twelve mice were injected with 1 ml. of 0.9 per cent NaCl for three successive days. Extracts of fresh plasma of two rabbits were administered to ten mice in the same manner. Since there were no statistically significant differences in reticulocytes and bone marrow counts between the mice injected with plasma extracts as compared with those injected with saline, all these animals are included in a common control group.

II. *Kidney.* (1) The kidneys of three rabbits were perfused with hypoxic blood. The administration of this blood extract to mice caused a marked increase in the reticulocyte count from the initial level of 43 per 1000 to an average of 105 per 1000, reaching the peak on the 5th day. Thereafter, the number of reticulocytes diminished gradually. However, even on the 10th day, they remained at an elevated level (mean value 59.8 per 1000). As may be seen from figure 2 and table 1, the mean reticulocyte level on the 5th day of the experiments amounted to about 250 per cent of the control. The differences between the control mean and the mean of reticulocytes on 5th, 7th, and 10th days are highly significant statistically ($p < 0.001$). An increase in the number of erythroblasts was observed in the bone marrow. The percentage of erythroblasts in the bone marrow of extract-injected mice was 42.3 as compared to 25 in the control group. (2) The kidneys of 3 rabbits were perfused with fully oxygenated blood. The administration of extracts of this blood plasma into mice caused a slight increase in reticulocyte number (fig. 2 and table 1), averaging 63 per 1000 on the 5th day. During the following days their number fell to a normal level.

In bone marrow smears a slight increase in the erythroblasts count was found (29.9 per cent, as compared to control, 25 per cent).

III. *Liver.* The livers of five animals were perfused, one with oxygenated blood, four with "hypoxic" blood. The results are presented in figures 2 and 3 and in table 2. It can be seen that the reticulocyte count is not above the control values ($p > 0.1 - p > 0.7$) and the percentage of bone marrow erythroblasts does not escape the order of magnitude of the control records.

IV. *Lung.* The results of perfusion of four hypoxic lungs are presented in table 3 and figures 2 and 3. It can be seen that there is no erythropoietic response to blood extracts obtained from lung perfusates. The differences between the initial level of reticulocytes and the levels on the 5th, 7th and 10th

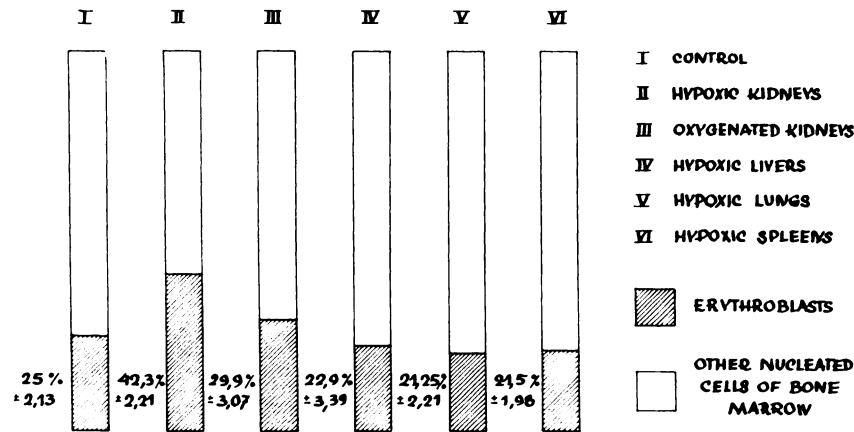


Fig. 3.—Percentage of erythroblasts in mice bone marrow after injections of extracts of perfusates of isolated organs. The results of three experiments on each organ are included. In each experiment the bone marrow of three test mice was examined. The counts of bone marrow of seven mice injected with saline and seven mice injected with extracts of fresh blood plasma are included in the control group. I/II: $p < 0.001$. I/III: $0.01 > p > 0.001$. I/IV, V, VI: $p > 0.7$.

days are statistically insignificant. Furthermore, there was no increase of the erythroblasts percentage in the bone marrow.

V. *Spleen*. Perfusates of all the three hypoxic spleens did not stimulate erythropoiesis (table 4 and figs. 2 and 3).

VI. *Hypoxic blood*. In three experiments, hypoxic blood obtained from normal rabbits was circulated in the perfusion apparatus in which no organs were placed. Extracts from this blood did not stimulate erythropoiesis in 12 test mice.

DISCUSSION

The results of experiments presented in this paper seem to confirm the hypothesis concerning the predominant role of the kidney in the production or activation of erythropoietin. A slight erythropoietic activity was observed in the extracts of blood obtained after kidney perfusion even in conditions of full oxygenation. Jacobson, Goldwasser and their co-workers⁶⁻¹¹ found that bilateral nephrectomy in rabbits eliminated any measurable increase of erythropoietin

Table 2.—Effect of Plasma Extracts Obtained from Perfusates of 4 Isolated Hypoxic Livers, Compared with Reticulocyte Level at 0 Day*

Reticulocyte counts in % (mean values in 19 mice)	Days			
	0	5	7	10
	43.3 ± 12.2†	40.3 ± 10.1	37.3 ± 6.3	43.5 ± 12.2
p		0.4 < p < 0.5	0.05 < p < 0.1	0.6 < p < 0.7

*Five or four mice were used for testing of each extract.

†Standard deviation of the mean.

Table 3.—Effect of Plasma Extracts Obtained from Perfusates of 4 Isolated Hypoxic Lungs, Compared with Reticulocyte Level at 0 Day*

	Days			
	0	5	7	10
Reticulocyte counts in % (mean values in 20 mice)	50.3 ± 20.7†	49.7 ± 18.6	44.8 ± 18.1	60.3 ± 25.2
p		p > 0.9	0.4 < p < 0.5	0.2 < p < 0.3

*Five mice were used for testing of each extract.

†Standard deviation of the mean.

Table 4.—Effect of Plasma Extracts Obtained from Perfusates of 3 Isolated Hypoxic Spleens, Compared with Reticulocyte Level at 0 Day*

	Days			
	0	5	7	10
Reticulocyte counts in % (mean values in 14 mice)	49.8 ± 12.2†	48.0 ± 15.2	42.0 ± 13.8	42.2 ± 21.3
p		0.6 < p < 0.7	0.8 < p < 0.9	0.5 < p < 0.6

*Five or four mice were used for testing of each extract.

†Standard deviation of the mean.

level as a response to the routine stimuli (hypoxia, hemorrhagia, cobalt). Ligation of both ureters reduced, but did not eliminate, the capacity of the animals to produce an increased plasma level of erythropoietin, in spite of a high degree of azothemia. A similar observation was made by Mirand et al.¹² Naets described similar findings^{13,14} and observed a slight erythropoietic activity in the extracts of homogenates of kidney tissue.¹⁵ The erythropoietic activity of kidney homogenates was also demonstrated by Lagrue et al.¹⁶ Ardaillou et al. showed a great increase of Fe⁵⁹ incorporation into red cells in nephrectomized rats with auto-implanted kidney.¹⁷

Many clinical data support this concept. Decreased erythropoietin level was observed in the plasma of anemic patients suffering from kidney insufficiency.¹⁸ Several cases of tumors of the kidneys with polycythemia were described.¹⁹⁻²¹ Hewlett²² and Jones²³ found that the extracts from some renal tumors possess strong erythropoietic activity. It should be noted that other authors were not able to confirm these results.^{24,25}

In our experimental conditions the liver, spleen and lung did not produce or activate erythropoietin. Since, under the same conditions, production or activation was observed in the experiments involving the kidney, it is possible that this organ is the main site of release of erythropoietin. However, it must be borne in mind that the kidney is not the only source of the erythropoietic factor. Gurney and Jacobson,²⁶ for instance, are of the opinion that about 10 per cent of circulating erythropoietin is of extrarenal origin.

In the present state of our knowledge it is not possible to establish what kind of tissue or cell may be responsible for erythropoietin production. Our experi-

ments seem to eliminate RES, because the organs rich in reticuloendothelial cells such as the spleen did not produce or activate erythropoietin under experimental conditions.

The results obtained in our experiments with the blood made anoxic are contradictory to those of Bonsdorff and Hirsjarvis. These authors claimed that anoxic blood itself may stimulate erythropoietin production.²⁶⁻²⁸

It is probable that in the organism as a whole the kidney is an important link in a complex mechanism of erythropoietin release, in which the central nervous system and endocrine system may be involved as well. However, under our experimental conditions, it was shown that isolated kidney can produce or activate erythropoietin factor in hypoxic conditions.

SUMMARY

(1) Isolated organs of rabbits (kidney, liver, spleen and lungs) were perfused with rabbit blood. Experimental conditions of hypoxia and full oxygenation were applied.

(2) It could be shown that under these conditions only the kidney produces the erythropoietic factor.

SUMMARIO IN INTERLINGUA

1. Isolate organos de conilio—ren, hepate, splen, pulmon—esseva perfusionate con sanguine de conilio.

2. Esseva trovate que sub iste conditiones solmente le ren produce le factor erythropoietic.

ACKNOWLEDGMENT

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Z. Kuratowska, M.D., Senior Research Fellow in Department of Health Protection, Institute of Nuclear Research, Warsaw, Poland.

B. Lewartowski, M.D., Senior Research Fellow in Department of Human Physiology, School of Medicine, Warsaw, Poland.

E. Michalak, M.D., Research Fellow in Department of Health Protection, Institute of Nuclear Research, Warsaw, Poland.