Hematologic Studies
of Severe Undernutrition of Infancy

II. Erythropoietic Response to Phlebotomy by Calorie-Deprived Pigs

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Extract

These studies have determined the erythropoietic response of the chronically calorie-deprived pig to the hypoxic stress of phlebotomy. Three animals were maintained on diets sufficiently restricted to prohibit weight gain from twenty-one days to six months of age. The animals were anemic, with hematocrit values of 35%, and were subjected to controlled phlebotomy of one-third the total erythrocyte mass. During a subsequent two-month period, two of the animals were given a caloric diet *ad libitum* and a seven-fold increase in weight, accompanied by active erythropoiesis and an increase in hematocrit, was observed. Phlebotomy studies were repeated after the period of *ad libitum* feeding. The erythropoietic response to phlebotomy was evaluated in both the undernourished and the rehabilitated pigs by study of erythropoietin excretion, circulating reticulocyte numbers, iron kinetics, Cr^{51} total RBC mass, bone marrow morphology, and changes in serum iron concentration.

When measured on day 2 preceeding phlebotomy, the calorie-deprived animals had very low concentrations of urinary erythropoietin (0.06 U/24 h) which, following blood loss, promptly increased to a concentration of 2.0 U/24 h. A similar prompt response was seen in the rehabilitated animals (fig. 1). Prior to phlebotomy, plasma iron turnover was <1.0 mg/100 ml whole blood/24 h and promptly increased to 1.5–2.0 mg/100 ml whole blood/24 h by four days after phlebotomy (fig. 2). There was a marked increase in iron utilization for erythrocyte production when measured six days following phlebotomy (fig. 3). A decrease in the myeloid:erythroid ratio of the marrow accompanied these other evidences of increasing erythropoiesis (fig. 4). The degree of response in the calorie-deprived and in the rehabilitated animals was similar. The calorie-deprived animals had a higher reticulocyte response to phlebotomy than the animals fed *ad libitum*, but reestablished the prephlebotomy hematocrit no more rapidly (figs. 5 and 6). The higher reticulocyte counts in the undernourished pigs was shown to be related to less mature reticulocytes entering the circulation following the bleeding stress, reflecting a greater degree of marrow 'shift' (fig. 8).

Calculation of total erythrocyte production in response to phlebotomy under conditions of severe caloric deprivation and *ad libitum* feeding demonstrates that a comparable response occurred under both experimental conditions.

Speculation

These studies support the hypothesis that anemia accompanying chronic caloric deprivation in the pig is not caused by a primary lack of essential nutrients, but represents an adaptive response to the decreased metabolic needs of the chronically undernourished organism. It is likely that the moderate anemia sometimes seen in marasmic infants is due to a similar mechanism. If such is the case, therapeutic measures such as blood transfusions and administration of various hematinics directed primarily at altering this adaptive phenomenon would appear contraindicated in the management of the marasmic infant.

Introduction

The use of young pigs for the study of the effects on erythropoiesis of prolonged caloric undernutrition in the growing animal has been described previously [6]. These studies were prompted by the knowledge that severe caloric undernutrition in growing infants is common in many parts of the world. Undernourished infants often have complicating infections and infestations and may have isolated specific nutritional deficiencies that make it difficult to define the effects of caloric deprivation on erythropoiesis. The development of an animal model free of such complications has been found to be particularly useful in studies of erythropoieses.

A diet that provided for normal growth in young pigs was fed in quantities so restricted that a failure to gain weight for as long a period as ten months resulted. Such animals were shown to develop a progressive decrease in total erythrocyte mass and a lower hemoglobin concentration than have pigs fed the same diet *ad libitum*. It was demonstrated that this anemia was the result of decreased erythropoiesis. Erythropoietin excretion was found to be low and it was postulated that the decreased production of erythrocytes was the result of decreased stimulation of erythropoiesis [6].

An attempt was made in the present study to determine whether, in the chronically undernourished pig, erythropoietin excretion was increased in response to a hypoxic stimulus and, if so, whether the rate of erythropoiesis was increased. The response to controlled acute phlebotomy was measured while the animals were being maintained on a severely restricted caloric intake. This response was compared with that of the same animals subjected to similar phlebotomy following rehabilitation with intake of the diet *ad libitum* for a period of two months.

Material and Methods

The method of dietary restriction and the management of the undernourished animals have been described previously [6]. Three animals were subjected to phlebotomy after six months of dietary restriction. There was little weight gain during this six-month period; at the time of phlebotomy, hematocrit values were 33.8, 35.0, and 37.0 %. The restricted diet was not changed, and the weight remained stable during the period of these studies. Two of the animals were then fed the diet *ad libitum*. After two months of such feeding, weights were increased seven-fold and active erythropoiesis was evident. Phlebotomy was again performed.

These animals continued to be fed *ad libitum* and continued to gain weight during the second period of study.

Peripheral blood counts were performed using standard methods [6]. Reticulocyte counts were expressed as percent after correction of all counts to a hematocrit of 43 %. Determination of Cr^{51} erythrocyte mass, Fe^{59} iron kinetics, erythropoietin excretion, serum iron and total iron-binding capacity were performed as previously described [6]. Bone marrow was aspirated from the sternum and cover slip smears prepared. The myeloid:erythroid ratio was determined by counting 500 marrow cells. All nucleated erythrocyte and myeloid precursors, excluding segmented cells, were included in these counts.

During a period of two to four hours, phlebotomy was performed using the anterior vena cava. Immediately following the procedure, the animals were given an infusion of 6 % Dextran equal to one-half of the volume of blood removed by phlebotomy.

In each of the experiments, the day on which phlebotomy was performed was designated as day 0. Phlebotomy bleeding was calculated to remove one-third of the estimated total circulating erythrocyte mass in an attempt to produce a comparable stimulus in each of the animals. One rehabilitated pig required a second phlebotomy on day 2 to achieve the desired level of blood loss. Quantitative 24-hour urine collections for erythropoietin assay were obtained three days and one day prior to phlebotomy, and on the first, third and fifth days following phlebotomy. Measurement of the plasma iron turnover and bone marrow aspirations were performed prior to phlebotomy, on day 0, and on days 2, 4, and 6. Frequent venous blood samples were taken following the injection of Fe^{59} on day 6 for measurement of the utilization of Fe^{59} for red cell production. Values for hematocrit and reticulocyte count were obtained daily for several days prior to phlebotomy and until the completion of the experiment. The total erythrocyte mass was measured before phlebotomy on day 0. A second determination of the total red cell mass was performed when the hematocrit had approached the level found prior to phlebotomy.

The amount of iron required for erythropoiesis following phlebotomy was estimated. The undernourished animals had received 150 mg of Imferon® on the third day of life and a daily dietary intake of approximately 8.5 mg of iron for a period of six months while on the restricted diet. Previous observations had demonstrated on accumulation of considerable iron stores during this regimen and no additional iron was given prior to or following phlebotomy.

Erythropoietin assays, using a polycythemic mouse, were performed and are expressed as units of erythropoietin standard B/24 hours.

The diet of the rehabilitated animals provided a daily intake of approximately 100 mg of iron, which was supplemented with an injection of 1200 mg of Imferon® 30 days prior to phlebotomy. Following phlebotomy, the diet was further supplemented with ferrous sulfate given orally in a dose of 175 mg of elemental iron three times each day from day 0 to day 11 and 75 mg three times each day from day 11 to day 30

Results

Erythropoietin Excretion

The results of assays of urinary erythropoietin are shown in figure 1. The assays of a single 24-hour urine collection or of two pooled 24-hour urine collections from the undernourished pigs failed to reveal any detectable erythropoietin activity prior to phlebotomy. When prephlebotomy urine collections from the three undernourished pigs were pooled for a total collection period of 110 hours, erythropoietin activity could be detected. The activity was equivalent to 0.06 U/24 h urine collection. An increase in erythropoietin activity was detected following phlebotomy in urine of the undernourished animals when measured on days 2, 4, and 6.

In the rehabilitated animals, erythropoietin activity was readily detected in 24-hour urine collections prior to phlebotomy. Following phlebotomy, there was an increase in the excretion of urinary erythropoietin.

Plasma Iron Turnover

Values for plasma iron turnover were calculated in the undernourished animals prior to phlebotomy and on days 2 and 4, and in one animal, on day 6. The results are shown in figure 2. Values calculated for the undernourished pigs prior to phlebotomy were similar to those previously reported in other pigs that had been subjected to prolonged caloric deprivation [6]. Following phlebotomy, a progressive increase in the plasma iron turnover was observed.

Plasma iron turnover determined in one of the rehabilitated animals showed a higher value prior to phlebotomy than had been observed in the undernourished pigs. An increase in iron turnover following phlebotomy in the rehabilitated animal was clearly evident by day 2 and was observed when measurements were made on days 4 and 6.

Fe^{59} Utilization for Erythrocyte Production

Studies of the utilization of iron for erythrocyte production following intravenous injection of Fe^{59} were initiated six days following phlebotomy in the undernourished and in the rehabilitated pigs (fig. 3). There was a prompt reappearance of the Fe^{59} in new erythro-
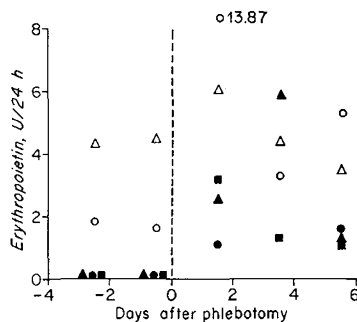

Fig. 1. Urinary erythropoietin excretion in three undernourished pigs (▲■●), and in two of the same pigs after two months of *ad libitum* dietary intake (○△).
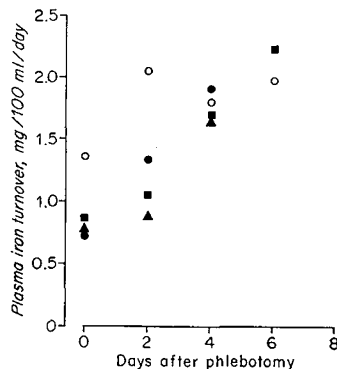

Fig. 2. Plasma iron turnover in three undernourished pigs (▲■●) and in one pig (○) after two months of *ad libitum* dietary intake as measured prior to and two, four, and six days following phlebotomy.
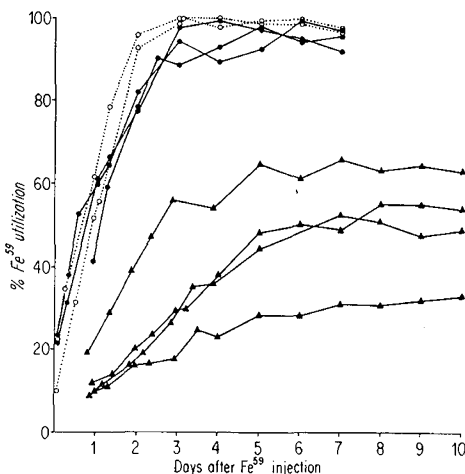

Fig. 3. Fe^{59} iron utilization in four undernourished pigs prior to phlebotomy (▲), in three undernourished pigs (●) six days after phlebotomy, and in two rehabilitated pigs (○) six days after phlebotomy.

cytes and a rapid rise to levels representing utilization of more than 90 % of the injected isotope in both groups. This response is compared with the pattern of iron utilization observed previously in four undernourished pigs after seven months of dietary restriction (fig. 3). The interpretation of the early portion of the post-phlebotomy curves was complicated by the appearance of measurable Fe^{59} in circulating erythrocytes immediately following the injection of the isotope. The appearance was due to the large number of reticulocytes capable of assimilating iron present in the circulation six days after phlebotomy.

Bone Marrow

The myeloid:erythroid ratios of aspirated marrow cells prior to phlebotomy and on days 2, 4 and 6 were calculated and the results are shown in figure 4. The ratios progressively decreased in the undernourished pigs. There was a marked increase in the proportion of erythroid elements by the sixth day following phlebotomy.

In the rehabilitated animals, there was a prompt decrease in the ratios by day 2; a decrease was still observed on days 4 and 6 following phlebotomy.

Cr^{51} Total Erythrocyte Mass

Measurements of Cr^{51} RBC mass were made prior to phlebotomy and again at the time when the hematocrit had returned to the level that was present prior to phlebotomy (table I). Total RBC mass returned to the prephlebotomy level somewhat earlier in the rehabilitated than in the undernourished pigs. The consistent relation between the hematocrit and the total RBC mass was not affected by differences or changes in body weight.

Reticulocyte Count and Hematocrit

The reticulocyte and hematocrit responses to phlebotomy in the chronically undernourished pigs are shown in figure 5. An almost identical response in reticulocytes and increasing hematocrit occurred in the three animals. By day 4 following bleeding, there was a detectable rise in reticulocytes from the prephlebotomy level of 1 %. The increase continued to day 10 and returned to a normal level by the time the total red cell mass had been reestablished at the prephlebotomy level.

The hematocrit response was progressive and was interrupted on days 2, 4, and 6 by the loss of blood associated with various studies performed on these days.

The changes in reticulocyte count and hematocrit following phlebotomy in the rehabilitated pigs are shown in figure 6. A prompt reticulocyte response occurred, reaching peak levels of 6 and 8 % on days 5 and 6, respectively, followed by a progressive increase in hematocrit. The peak reticulocyte counts were lower and the hematocrit response more rapid in the rehabilitated pigs than in the undernourished animals.

Serum Iron and Iron-Binding Capacity

Serum iron and the iron-binding capacity were measured prior to phlebotomy. Serum iron and, in some instances, the iron-binding capacity, were measured on days 0, 2, 4, and 6, and on certain subsequent days. The results of these measurements are shown in figure 7. There was a fall in the levels of serum iron in the two rehabilitated animals prior to phlebotomy and in two of the three undernourished animals following phlebotomy.

Table I. Cr^{51} RBC mass in undernourished and rehabilitated pigs before phlebotomy and after recovery of the hematocrit

	Pig No.	Day	Weight (kg)	Hematocrit %	Cr^{51} RBC mass Total ml	Cr^{51} RBC mass ml/kg
Undernourished	17	0	6.85	33.0	120.8	17.6
		15	7.15	34.0	116.8	16.3
	24	0	7.00	36.5	129.1	18.4
		23	7.10	34.0	114.0	16.1
	28	0	7.60	37.5	156.4	20.6
		20	7.80	37.0	150.0	19.2
Rehabilitated	24	0	42.00	34.0	738.0	17.6
		12	47.25	34.0	854.0	18.1
	28	0	48.00	33.0	895.0	18.6
		9	50.00	34.0	906.0	18.1

Bone marrow examinations on days 0, 2, 4, and 6 revealed the presence of stainable iron in erythroblasts (sideroblasts) in decreasing numbers from day 0 to day 6 in all animals. Sideroblasts were still present in bone marrow on day 6.

Discussion

A previous report described changes in erythropoiesis accompanying prolonged and severe caloric restriction in the young pig [6]. A reduction in hemoglobin con-

centration was present after several months of a dietary intake sufficiently restricted to arrest growth in the normal, rapidly growing animal. This reduction was shown to be the result of decreased production of erythrocytes and hemoglobin. The decrease of erythropoiesis was associated with decreased urinary excretion of erythropoietin. An attempt was made to determine whether the decreased excretion of erythropoietin and the reduced erythropoiesis were the primary results of nutritional deficiency or whether these changes might have been the result of a decreased demand for oxygen transport in the undernourished animal.
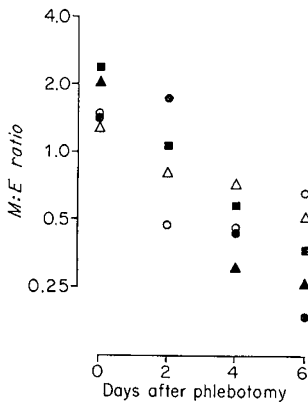

Fig. 4. The myeloid:erythroid ratio following phlebotomy in three undernourished pigs (▲■), and two of the same pigs (△○) after two months of *ad libitum* feeding.
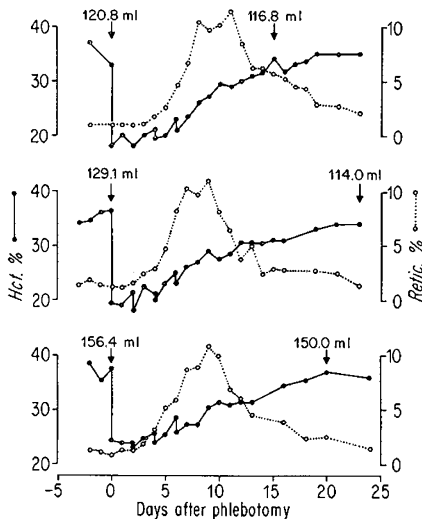

Fig. 5. The hematocrit (●) and reticulocyte (○) response to phlebotomy in three chronically undernourished pigs. Cr51 RBC mass determinations are indicated at the arrows (↓).
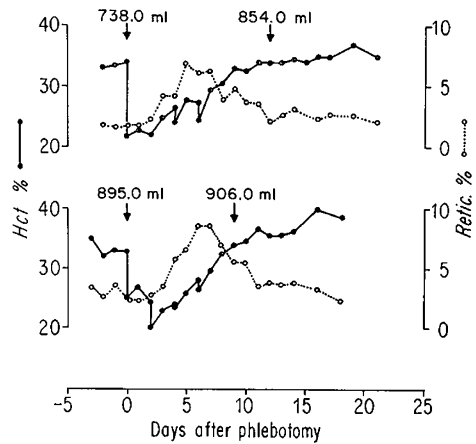

Fig. 6. The hematocrit (●) and reticulocyte (○) response to phlebotomy in two of the same pigs after two months of *ad libitum* dietary intake. Cr51 RBC mass determinations are indicated at the arrows (↓).
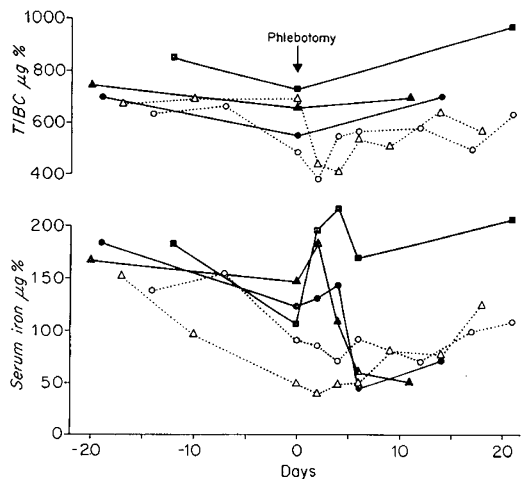

Fig. 7. The serum iron and iron binding capacity before and after phlebotomy in three undernourished pigs (▲●■), and two of the same pigs (△○) after two months *ad libitum* feeding of a normal diet.

The chronically undernourished animals studied were able to respond to phlebotomy. The increase in erythropoietin excretion was followed by an increase in the rate of erythropoiesis. The total erythrocyte mass returned to prephlebotomy levels within fifteen to twenty days.

Comparative evaluation of the erythropoietic response to phlebotomy in the undernourished animals was complicated by problems in the selection of a suitable control. A comparison with pigs of similar size would have necessitated use of control animals of a much younger age. Comparison with control animals of similar age would have necessitated use of a pig of a size over twenty times that of the undernourished animals. Thus, animals selected for comparison in these studies were the same animals that had been rehabilitated on a normal diet. An increase in erythropoietin excretion, rate of plasma iron turnover, iron utilization, and proportion of erythroid precursors in the marrow was observed in both the undernourished and the rehabilitated pigs following phlebotomy. The rehabilitated pigs demonstrated less increase in reticulocyte count, but reestablished the prephlebotomy level of total red cell mass somewhat earlier than did the undernourished pigs.

Calculation of the rate of erythrocyte production in both groups of pigs before and after phlebotomy was possible, since the following factors were known: the amount of blood loss, the total erythrocyte mass before phlebotomy and after recovery, the correlation of total erythrocyte mass to the hematocrit, changes in body weight, and the erythrocyte lifespan in normal and in undernourished pigs [7]. The erythrocyte lifespan in these species seems to be controlled mainly by a random process and, thus, would not be influenced by variation in the age of red cells [2].

The calculated rates of red cell production, expressed as average daily production before and during the ten days following phlebotomy, are listed in table II. The rapidly growing rehabilitated pigs had a greater rate of red cell production per kg of body weight than did the undernourished animals prior to phlebotomy. Both groups showed an increase in the rate of production of red cells following phlebotomy. The rehabilitated pigs had the largest increase in erythrocyte production per kg of body weight. The smaller increase in production of red cells per kg of body weight in the undernourished animals represented a greater relative increase in production. These differences in the degree of response in the two groups could have been due to the presence of a relatively smaller erythropoietic tissue mass in the undernourished pigs prior to phlebotomy.

The difference in reticulocyte response in the two groups of animals could have been due, in the case of the undernourished pigs, to the release into the circu-

Table II. Erythrocyte production in undernourished and rehabilitated pigs before and after phlebotomy

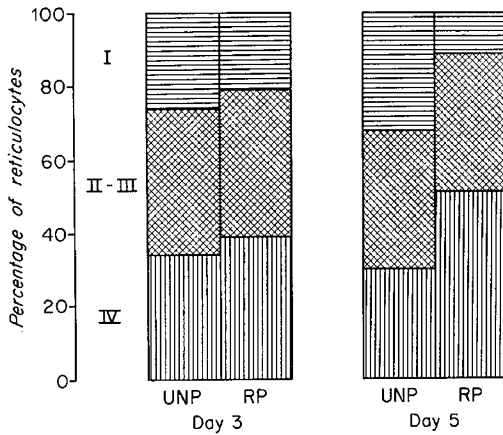| Pig number	Weight (kg) day 0	Total RBC mass (ml) day 0	Basal rate (day 0) RBC production ml/kg/d	Calculated RBC mass (ml) Day 10	Erythrocytes removed (ml) Day 0–day 10	Average RBC production ml/kg/d Day 0–day 10	Absolute increase in RBC production ml/kg/d	Relative increase in RBC production over basal rate
Undernourished								
17	6.85	120.8	0.20	100.1	56.5	0.67	0.47	3.3
24	7.00	129.1	0.20	103.0	64.3	0.70	0.50	3.5
28	7.60	156.4	0.23	133.6	72.1	0.83	0.60	3.6
Rehabilitated								
24	42.00	738.0	0.56	838.9	325.2	1.23	0.67	2.2
28	48.00	895.0	0.62	932.5	482.0	1.31	0.69	2.1

Fig. 8. Classification of reticulocyte maturity (according to HEILMEYER) in undernourished (UNP) and rehabilitated pigs (RP) three and five days after phlebotomy. I indicates least mature and IV most mature reticulocytes. The undernourished animals have larger numbers of less mature reticulocytes present, particularly on day five.

lation of less mature reticulocytes; these would have retained the reticulum for longer periods of time. The degree of maturity of reticulocytes of the rehabilitated and undernourished pigs on the third and fifth day after phlebotomy is shown in figure 8. The number of less mature reticulocytes present on day 5 was larger in the undernourished animals than in the rehabilitated pigs. This finding may explain the higher reticulocyte counts observed in the undernourished pigs. A greater degree of marrow 'shift' is suggested by this reticulocyte response in the undernourished pig, and may be the result of a relatively more intense degree of erythropoietin stimulation in these animals [3].

In these experiments, the response to phlebotomy could have been influenced by the amount of iron available for erythropoiesis. The serum iron level fell in two of the undernourished pigs by day 6; however, this fall was not progressive and the erythropoietic response in these animals was no different than that in the one animal in which a high serum iron concentration was maintained. Sideroblasts were present in the marrow of the three undernourished animals on day 6.

The large dose of iron given to the rehabilitated pigs thirty days prior to phlebotomy was not mobilized effectively enough to meet the demands of erythropoiesis associated with rapid growth. A fall in serum iron was demonstrated prior to the phlebotomy. The iron supplements given orally to these two pigs following phlebotomy appear to have been sufficient to meet

the needs of erythropoiesis. The fasting serum iron levels gradually increased during the postphlebotomy period of active erythropoiesis.

The demonstration that the chronically undernourished pig maintained on a severely restricted diet could respond to phlebotomy indicates that the reduced hemoglobin level present after prolonged caloric deprivation was not a primary consequence of an inadequate supply of nutrients essential for either erythropoietin production or for erythropoiesis.

MCCANCE [4] has shown that pigs similarly managed have low body temperature and reductions in oxygen consumption and metabolic rate. MÖNCKEBERG *et al.* have demonstrated that severely undernourished infants with marasmus have reduced oxygen consumption [5] and reduced thyroid function [1]. The observations reported in this study, when interpreted in relation to metabolic studies, lead to the speculation that the alterations in erythropoiesis and the reduced hemoglobin concentration found after prolonged restricted caloric intake represent only an appropriate adaptation of erythropoiesis to the decreased oxygen demands of a nutritionally induced hypometabolic state.

Summary

Young pigs, anemic after many months of caloric deprivation, responded to phlebotomy with a prompt increase in excretion of erythropoietin and in the rate of erythropoiesis while maintained on a severely restricted caloric intake. The prephlebotomy level of total red cell mass was rapidly reestablished.

The results of these studies support the hypothesis that anemia found after prolonged caloric deprivation is not caused by a primary lack of any essential nutrients, but represents an adaptive response to the decreased metabolic needs of the chronically undernourished organism.

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