Iron Deficiency in the Rat

PHYSIOLOGICAL AND BIOCHEMICAL STUDIES
OF MUSCLE DYSFUNCTION

C. A. FINCH, LOUISE R. MILLER, A. R. INAMDAR, RICHARD PERSON,
KATHERINE SEILER, and BRUCE MACKLER

*From the Division of Hematology, Department of Medicine, and Department
of Pediatrics, School of Medicine, University of Washington,
Seattle, Washington 98195*

ABSTRACT Work performance on a treadmill has
been evaluated in normal and iron-deficient rats. Anemia
was removed as a variable by adjusting the hemoglobin
of all animals to the same concentration. At a hemo-
globin compatible with normal work performance, iron-
deficient animals showed a marked impairment of run-
ning ability as compared to control animals. Iron
therapy corrected the disability within 4 days. Concen-
trations of the cytochrome pigments and myoglobin,
and rates of oxidative phosphorylation with pyruvate-
malate, succinate, and α-glycerophosphate as substrates
were all reduced in mitochondrial preparations from
skeletal muscle of iron-deficient rats, but only the rate
of phosphorylation with α-glycerophosphate as substrate
increased significantly and in parallel with the recovery
in work performance of the iron-deficient rats treated
with iron dextran.

INTRODUCTION

Iron deficiency anemia limits the work capacity of the
experimental animal (1) and of man (2). Since car-
diac output is increased with exercise in the iron-
deficient individual to levels comparable to those of
the exercised normal (3), it has been assumed that the
decreased work performance was due to the limiting
effect of anemia on oxygen delivery to the musculature
(1, 4–7). On the other hand, it seemed possible that
a lesion in the muscle might exist but be obscured by
the presence of anemia. For some time fatigue and
weakness have been attributed to iron deficiency (8),
but a causal relationship has not been established (9).

A variety of intracellular enzymes are known to be
reduced in iron deficiency and structural changes have
been described (10); however, it has been difficult to
demonstrate a causal relationship of these changes to
the impaired function of nonerythroid tissues. The ob-
jective of the present study was to separate the possible
effects of tissue iron deficiency on work performance
from those of anemia, and, if such a functional lesion
was found to exist, to examine its biochemical nature.

METHODS

Male Sprague-Dawley rats were obtained at 4 wk of age,
1 wk after weaning. The control animals were given Purina
Laboratory Chow (Ralston Purina Company Inc., St. Louis,
Mo.) containing 382 mg of iron/kg. The mean plasma iron
(nonfasting) was 195 µg/100 ml, and iron binding capacity
averaged 463 µg/100 ml. Rats to be made iron deficient
were given a low iron diet (ICN Pharmaceuticals Inc., Life
Sciences Group, Cleveland, Ohio) which contained 8 mg
iron/kg. By the end of the 1st mo on this diet the hemo-
globin concentration of those animals receiving it had
fallen to a mean of 6.4 gm/100 ml. Their serum iron and
total iron binding capacity averaged 67 and 851 µg/100 ml,
respectively (mean of 25 animals). Another group of rats
was given the identical low iron diet but received weekly
intraperitoneal injections of 5 mg of iron dextran (Imferon
iron, kindly supplied by Lakeside Laboratories, Inc., Mil-
waukee, Wisc.). All animals were allowed to eat and drink
ad libitum. Only rats whose weights were between 150 and
200 g were used in these studies.

In preparation for measurements of running ability, rats
were trained for 3 days on a small animal treadmill (model
42-15, Quinton Instruments; Seattle, Wash.). The slope was
set at 12.5°, and the belt was run at rates of 13.4, 16.16,
and 18.73 m/min on successive days. On the 4th day, under
Inovar-vet anesthesia (Pittman-Moore, Inc., Washington
Crossing, N. J.) (0.15 ml/kg), a PE-50 polyethylene
catheter (Clay Adams, Inc., Division of Becton, Dickin-
son & Co.; Parsippany, N. J.) was placed into the superior

vena cava via the jugular vein. To prevent clotting, the dead space was filled with heparin (1,000 IU/ml) followed by a well-fitting nylon fishing line (Velux Monofilament, Les Davis Fishing Tackle Company, Tacoma, Wash.) as an obturator. The catheter was tunnelled around to the back of the neck by threading through a 15-gauge needle, and the free end was buried subcutaneously along the rat's back. A 1-cm length of catheter was left outside the skin but was covered with a metal clip. The rat was allowed to rest 2 days after surgery. On the 7th day, 3 days after the catheter was inserted (designated day 0 of the study), the end of the catheter was pulled out, the obturator was removed, and a small amount of blood was aspirated for determination of hemoglobin concentration. Calculations based on hemoglobin concentration and an estimated blood volume of 6% body weight were made to determine the amount of packed erythrocytes or plasma necessary to adjust the hemoglobin concentration to the desired level by exchange transfusion. In this exchange, which took 10–20 min, the blood volume was held constant by an equal volume exchange of plasma or erythrocytes. 5 min after completion of the exchange the hemoglobin concentration was again determined. The working ability of the rats was periodically determined by using the graded treadmill test at a speed of 18.76 m/min (6). The presence of an inlying venous catheter through the study and the procedure of exchange transfusion per se had been shown previously to have no significant effect on running ability (6).

Two studies were undertaken on treadmill-trained animals with venous catheter in place. In the first study there were four groups of rats, each containing six animals. On day 0 the hemoglobin of all animals in all groups was adjusted to 6 g (±0.5 g/100 ml) by exchange transfusion with plasma to lower, and with erythrocytes to increase, the hemoglobin concentration. Rats in group A were maintained on a normal diet before the study. Animals in group B were maintained on a low iron diet but received weekly injections of iron dextran. Animals of group C had been on a low iron diet and were so maintained during the experiment; however, after the initial treadmill test they were given 5 mg of iron dextran intraperitoneally, and this was repeated 4 days later. Animals in group D had been on a low iron diet and were maintained on this diet throughout the experiment. They were transfused on days 2, 4, and 7 to hemoglobin concentrations equivalent to those found in rats in groups A through C. During this study, the animals of the four groups gained an average of 4, 5, 6, and 2 g/day, respectively. Treadmill tests were carried out in all 24 animals on days 0, 1, 2, 3, 4, 7, and 9. Hemoglobin determinations were made on days 0, 2, 4, 7, and 9.

In the next study there were five groups of six rats each. Groups A and B were similar to the previous study. Group C animals were iron deficient but were given 5 mg of iron dextran after the initial run on day 0. Groups D and E were on an iron-deficient diet and remained iron deficient through the study. Group D had been on an iron-deficient diet for 4 wk, and group E received the diet for 3 wk before the study. Animals of the five groups gained an average of 5, 6, 5, 4, and 5 g/day, respectively. The hemoglobin of all animals on day 0 was brought to 10 g/100 ml by exchange transfusion, and the hemoglobin was maintained at this level by exchange transfusion on days 2 and 4 as required. All animals in the study were run on the treadmill on days 0, 1, 2, 3, and 4.

In a third study, a group of 30 rats was trained on the treadmill. A mean basal running time for each rat was established on the basis of six daily measurements. Six pairs of animals with similar running times were selected, and half of the animals were injected intraperitoneally with 5 mg of iron dextran. Animals were run daily for 5 days. On the 5th day a second injection of iron dextran was given, and all animals were run 4 days of the 2nd wk. The investigator who supervised the exercise experiment was unaware of which animals had been treated with iron.

Skeletal and heart muscle mitochondria were prepared and assayed as previously described by Makinen and Lee (11). Differential spectra (dithionite reduced-minus-oxidized) of the cytochromes of the mitochondrial respiratory chain were determined by means of a sensitive wavelength-scanning DW-2 spectrophotometer (American Instrument Co., Inc., Silver Springs, Md.) at 25°C, and the concentrations of the cytochromes were calculated by use of the following millimolar extinction coefficients: cytochromes $a +$ a_3 (605–630 nm), 24.0 (12); cytochromes $c + c_1$ (550–540 nm), 19.1 (13); total cytochrome b (562–575 nm), 20.0 (14). Concentrations of cytochromes b_T and b_K were determined as described by Wilson and Erecinska (15). Concentrations of myoglobin in samples of skeletal muscle were determined by the method of Åkeson and Theorell (16).

Oxidative phosphorylation was studied polarographically at 25°C with a Clark oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio) fitted to a closed glass thermostated chamber of 1.6–1.9-ml capacity (Gilson Medical Electronics Inc., Middleton, Wisc.). Determinations were of two types: ADP:O ratios and respiratory control indexes were determined as described by Chance and Williams (17) and Inamdar et al. (18), and P:O ratios were determined by measurement of ^{33}P incorporation into ATP as described previously by Mackler et al. (19). ADP content of standard solutions was determined by the method of Adam (20). Protein was determined by the method of Lowry et al. (21).

Crystalline bovine serum albumin (fraction V), ADP, ATP, rotenone, Tris, and EDTA were obtained from Sigma Chemical Co., St. Louis, Mo. Nagase was obtained from Biddle Sawer Corp., New York, and ^{33}P was obtained from New England Nuclear, Boston, Mass.

Hemoglobin was determined as cyanmethemoglobin with standards employed for calibration (Hycel, Inc., Houston, Tex.). Plasma iron and total iron binding capacity were done by established methods (22, 23), except that it was necessary to double the amount of iron added in the measurement of iron binding capacity due to the high transferrin concentration in iron-deficient rats.

Statistical analyses were performed by standard methods as described by Goulden (24).

RESULTS

Physiological studies. In preliminary studies on iron-deficient animals, marked impairment of running ability was demonstrated. After 1 wk of an iron-deficient diet, hemoglobin concentration had fallen to 7.6 g/100 ml (SE±0.17) and running time was 18.9 (±1.3) min. After 2 wk on an iron-deficient diet, hemoglobin averaged 7.0 (±0.23), and running time averaged 16.0 (±1.5) min. By 3 wk, hemoglobin was 6.2 (±0.33) g/100 ml with a running time of 4.7 (±0.92) min. By 4 wk, mean hemoglobin was 5.5 (±0.30) g/100 ml with
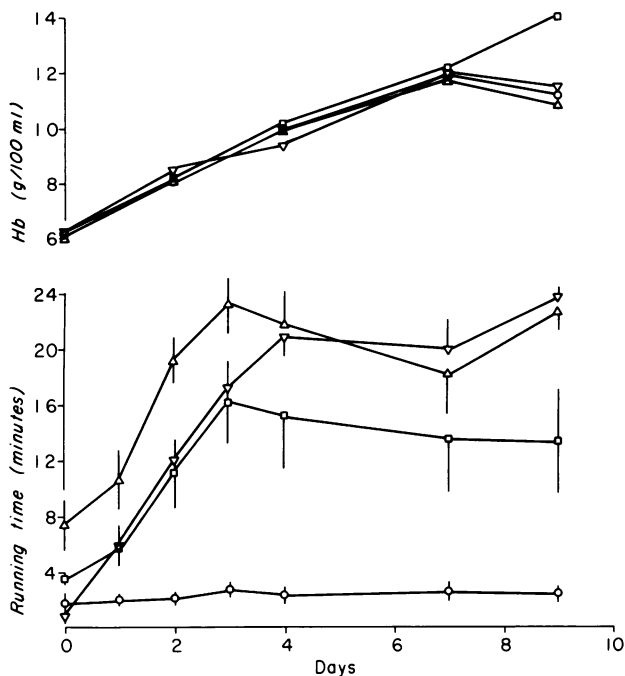
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FIGURE 1 Work performance in normal and iron-deficient animals during recovery from anemia. Group A animals (□) on a normal diet and group B animals (△) on a low iron diet, but given weekly injections of iron dextran, were exchange transfused to 6 g/100 ml hemoglobin and were then allowed to increase to a normal hemoglobin concentration. Group C animals (∇) were on an iron-deficient diet and were anemic, but were given an injection of iron dextran on day 0. Group D animals (○) were on an iron-deficient diet and remained iron deficient throughout the study. Their hemoglobin, however, was increased at a rate similar to that of the other groups. Standard error for running time of each group of six animals is shown by the verticle lines.

running time 2.8 (±0.31) min. While the hemoglobin fall seemed to precede the impairment in running ability, it was necessary to carry out further studies to separate the effect of anemia from other possible effects of iron deficiency on muscle function.

In the first controlled study, muscle performance was evaluated in four groups of animals whose hemoglobin was adjusted to 6 g/100 ml and then allowed to increase at a rate of about 1 g/day. Results of this study are shown in Fig. 1. The hemoglobin of all groups increased simultaneously from 6 g on day 0, to 8 g on the 2nd day, to 10 g on the 4th and to 12 g on the 7th day. Running time was markedly reduced in all groups at the beginning of the study. The mean values in the iron-replete animals (groups A and B) were 3.5 and 7.4 min, respectively, whereas the mean value in the two iron deficient groups was < 2 min. The two groups with adequate iron increased their work performance to near maximum values by the 3rd day despite the fact

that the hemoglobin was only 9 g/100 ml at that time. The iron-deficient animals treated with iron (group C) showed a maximal running time (mean > 20 min) on day 4. The animals with iron deficiency whose hemoglobin was raised by exchange transfusion at the same rate as the other group showed no significant change over a period of 9 days from the original mean value of 2 min.

In the second study the hemoglobin of all animals was maintained at 10 g/100 ml (Fig. 2). Iron-replete animals on a normal diet and on an iron-deficient diet plus iron (groups A and B) had mean running times of 17.2 and 20.1 min. Animals of group C, who were iron deficient but received iron at the beginning of the experiment, showed an improvement from a mean running time on day 0 of 4.1 min to a mean running time of 19.2 min on day 3. Animals of group D, who had been on an iron-deficient diet for 4 wk and who were transfused to 10 g, showed a consistently depressed running time of about 3 min. Animals of group E who had been on an iron-deficient diet for 3 wk began with running times of about 9 min (the mean of the first two days' runs) and decreased to a mean of 3.8 min on the last run.
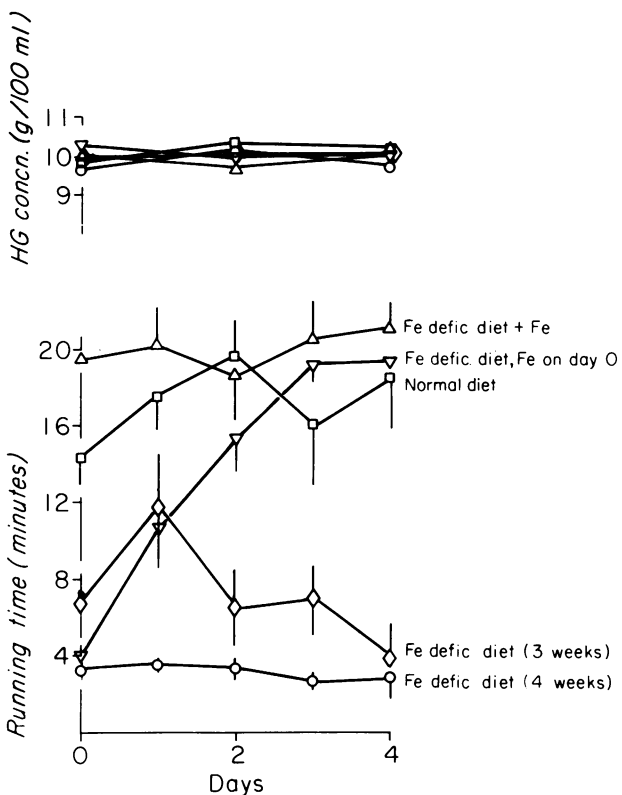

FIGURE 2 Work performance at 10 g/100 ml hemoglobin by normal and iron-deficient animals.

TABLE I

Effects of Iron Deficiency on Cytochrome Concentrations in Mitochondria from Skeletal Muscle and Heart Muscle

Tissue	Cytochrome $c + c_1$			Total cytochrome b $(b_T + b_K)$			Cytochrome $a + a_3$		
	Control	Iron deficient	Iron treated	Control	Iron deficient	Iron treated	Control	Iron deficient	Iron treated
	nmol/mg protein			*nmol/mg protein*			*nmol/mg protein*		
Skeletal muscle	0.80 ±0.04	0.44 ±0.02	0.38 ±0.01	0.51 ±0.04	0.29 ±0.02	0.24 ±0.02	0.30 ±0.02	0.20 ±0.01	0.16 ±0.01
Heart muscle	0.85 ±0.02	0.78 ±0.04		0.54 ±0.04	0.43 ±0.05		0.50 ±0.03	0.47 ±0.02	

* The data represent the averages of data from five separate experiments for skeletal muscle and four experiments for heart muscle±SEM. Studies were performed on iron-treated rats 4 days after iron treatment.

With the demonstration that iron played an important role in normal muscle function, a third study was carried out to see whether an increase in body iron would improve running capacity above normal. Two groups of six animals each with identical running times were studied. One of these groups received parenteral iron whereas the other did not. Mean running time of the control group was 25.2 (±0.60) min during the basal period and averaged 25.0 (±0.85) min and 26.7 (±1.1) min, respectively, during the 1st and 2nd wk of the study. The second group treated with parenteral iron had a basal running time of 25.0 (±0.32) min. During the 1st wk after iron injection, mean running time was 23.2 (±0.63) min and during the 2nd wk was 23.8 (±1.2) min.

Biochemical studies. A number of studies were performed to determine which iron containing cellular components associated with energy metabolism might be depleted by iron deficiency, yet be capable of regenerating within 4 days. The concentrations of all cytochrome pigments were found to be markedly and significantly ($P < 0.01$ for all values) lower in mitochondrial preparations from skeletal muscle but not in preparations from heart muscle of iron-deficient animals as summarized in Tables I and II. However, values remained low and unchanged 4 days after treatment with iron. Measurements of myoglobin content of skeletal muscle as shown in Table III were also significantly ($P < 0.01$) lower during iron deficiency, but again no change in levels of the pigment was noted 4 days after treatment with iron. Finally, studies of oxidative phosphorylation were performed on mitochondrial preparations as shown in Table IV. It was found that the P:O and ADP:O ratios determined with pyruvate-malate, succinate, and α-glycerophosphate as substrates for mitochondria from both skeletal and heart muscle showed little or no change from control values in iron-deficient preparations, but rates of ATP formation and respiratory control indices with all three substrates were markedly and significantly ($P < 0.01$ for all values) decreased only in preparation of mitochondria from iron-deficient skeletal muscle. 4 days after iron treatment of deficient rats, rates of phosphorylation of mitochondrial preparations from skeletal muscle with α-glycerophosphate as a substrate had risen to 80% of control values and were markedly and significantly (P

TABLE II

Effects of Iron Deficiency on Concentrations of Cytochromes b_K and b_T in Skeletal Muscle Mitochondria

Cytochrome b, *nmol/mg protein*					
b_T			b_K		
Control	Iron deficient	Iron treated	Control	Iron deficient	Iron treated
0.23 ±0.02	0.13 ±0.01	0.11 ±0.01	0.28 ±0.02	0.16 ±0.01	0.13 ±0.01

* The data represent the averages of data from five separate experiments±SEM. Studies were performed on iron-treated rats 4 days after iron treatment.

TABLE III

Effects of Iron Deficiency on the Myoglobin Content of Skeletal Muscle

State of animals	Myoglobin	Control
	mg/g tissue	%
Control	0.15±0.007	
Iron deficient	0.07±0.009	47
Iron treated	0.08±0.006	53

* All data represent the averages of data from seven separate experiments±SEM. Studies were performed on iron-treated rats 4 days after iron treatment.

TABLE IV

Effects of Iron Deficiency on Oxidative Phosphorylation in Mitochondria from Skeletal Muscle and Heart Muscle

Tissue	State of animal	Pyruvate-malate*					Succinate*					α-Glycerophosphate*				
		³²P:0	³²P esterified	ADP:0	ADP esterified	RCI	³²P:0	³²P esterified	ADP:0	ADP esterified	RCI	³²P:0	³²P esterified	ADP:0	ADP esterified	RCI
Skeletal muscle	Control	2.9 ±0.2	0.34 ±0.04	2.6 ±0.1	0.30 ±0.02	5.3 ±0.2	1.6 ±0.0	0.25 ±0.03	1.6 ±0.0	0.25 ±0.01	3.0 ±0.1	1.7 ±0.1	0.095 ±0.005	1.4 ±0.0	0.084 ±0.004	1.6 ±0.0
	Iron deficient	2.7 ±0.1	0.16 ±0.02	2.4 ±0.0	0.13 ±0.01	2.8 ±0.2	1.3 ±0.1	0.032 ±0.002	1.3 ±0.1	0.053 ±0.006	1.3 ±0.1	1.5 ±0.1	0.047 ±0.002	1.5 ±0.2	0.055 ±0.006	1.3 ±0.0
	Iron treated‡	2.6 ±0.1	0.14 ±0.02	2.5 ±0.1	0.15 ±0.02	3.4 ±0.2	1.4 ±0.1	0.040 ±0.003	1.3 ±0.1	0.063 ±0.010	1.7 ±0.2	1.7 ±0.1	0.076 ±0.006	1.4 ±0.1	0.077 ±0.008	1.4 ±0.0
Heart muscle	Control	—	—	2.7 ±0.1	0.48 ±0.03	4.8 ±0.2	—	—	1.5 ±0.0	0.38 ±0.02	2.7 ±0.1	0.9 ±0.1	0.005 ±0.0007	—§	—§	1.0§
	Iron deficient	—	—	2.7 ±0.0	0.45 ±0.05	4.1 ±0.2	—	—	1.6 ±0.1	0.35 ±0.03	2.2 ±0.2	—	—	—	—	—

The data represent averages of data from at least nine separate experiments ±SEM, except for data for heart muscle which represents averages of at least four separate experiments.

* Activities given above are defined as follows: ³²P esterified, micromoles of ³²P esterified per minute per milligram of protein; ADP esterified, micromoles of ADP phosphorylated per minute per milligram of protein; RCI (Respiratory Control Index) was calculated as the ratio of State 3 rate of oxidation to State 4 rate of oxidation.

‡ Studies were performed on iron-treated rats 4 days after iron treatment.

§ No stimulation was observed when ADP was added to assays of heart mitochondria containing α-glycerophosphate as substrate.

< 0.01) higher than rates found for preparations from untreated iron-deficient rats although rates of phosphorylation with pyruvate-malate and succinate as substrates remained low and unchanged.

In the experiments outlined above, the rats had been rendered severely anemic by being maintained on the iron-deficient diet for 4 wk. In additional studies, biochemical measurements were performed at 2 wk when the running time was essentially normal and showed no decrease in rates of oxidative phosphorylation with α-glycerophosphate as substrate. However, at 2½ wk when animals were beginning to show impairment in running ability, phosphorylation rates with α-glycerophosphate were moderately decreased; thus, the mean running time was 9.4 (±0.15) and rates of oxidative phosphorylation with α-glycerophosphate averaged 0.076± 0.003 μmol of ³²P esterified/min per mg of protein, a decrease of 20% from control values.

DISCUSSION

Attention in iron deficiency has been primarily directed to anemia for several reasons. Most body iron, over 80% in iron-deficient individuals, is contained within the erythron (25). The recognition of iron deficiency usually begins with a hemoglobin determination, and symptoms in the iron-deficient patient are conveniently ascribed to the anemia. In recent years attention has shifted to more sensitive measurements of iron deficiency, including transferrin saturation with iron as an index of iron availability to tissues (26) and the serum ferritin concentration as a reflection of tissue iron stores (27, 28). It has been demonstrated that about half of individuals who are iron deficient by chemical criteria have no demonstrable anemia (29). The question has been repeatedly raised as to whether iron deficiency without anemia represents any physiological liability.

In the present study the deleterious effect of anemia on work performance was reaffirmed. At a level of 6 g/100 ml of hemoglobin, running ability, while showing some difference in degree, was severely depressed in both control and iron-deficient animals. This presumably reflects the overriding effect of a decreased oxygen supply. However, an impressive difference between the normal and iron-deficient animals became apparent as the hemoglobin concentration was increased. Normal running ability was regained in the normal animal at about 10 g/100 ml of hemoglobin, consistent with other observations in animals and man. On the other hand, the running time of the iron-deficient animal was still markedly impaired at this hemoglobin concentration.

In the second study, a hemoglobin concentration compatible with maximum work performance was produced. Again, iron-deficient animals showed a very limited ability to run. This dysfunction was less severe in animals on an iron-deficient diet for only 3 wk and increased in severity during the 4th wk (group E of Fig. 2). It was evident that muscle dysfunction exists in iron deficiency of sufficient severity but is ordinarily hidden by anemia. Of further interest was the rapid reversibility of this lesion over a period of 3–4 days with iron treatment.

In the two preceding studies, the running ability of normal animals was observed to be somewhat less

than that of animals supplemented with parenteral or oral iron (Figs. 1 and 2). A more controlled study was therefore carried out in which normal animals were compared with animals injected with parenteral iron. No difference was observed, indicating that surplus iron provided no advantage to the otherwise normal animal.

In searching for the muscle abnormality responsible for decreased running ability in iron deficiency, it might be anticipated that considerable difficulty would be encountered in identifying the critical deficiency from among the many iron containing compounds which were depleted. It was reasoned, however, that impaired function might relate to electron transport and oxidative phosphorylation, and therefore a mitochondrial abnormality was suspected. The marked deficiency of cytochrome c as previously described (10) and other cytochrome components was confirmed, but lack of reversibility was felt to exclude these abnormalities as responsible. Similarly, myoglobin in skeletal muscle was decreased but again did not change with treatment as did muscle function. Of particular interest was the decrease in α-glycerophosphate-mediated phosphorylation by the skeletal muscle mitochondria and the increase in this activity with iron therapy over a 4-day period.

Since the mitochondrial α-glycerophosphate dehydrogenase previously isolated from brain is a nonheme iron containing enzyme (30), it would appear probable that the mitochondrial enzyme in skeletal muscle is also an iron metalloprotein. Its role in skeletal muscle metabolism would seem an important one for the following reasons: first, it appears well established that the energy for sustaining skeletal muscle activity is mainly derived from ATP formation during glycolysis (31) and that mitochondrial oxidation of α-glycerophosphate via the α-glycerophosphate oxidase system is an important reaction in the α-glycerophosphate shuttle (32, 33) which is largely responsible for the regeneration of the NAD^+ necessary for the continued operation of the glycolytic cycle; and second, additional ATP is generated from oxidation of α-glycerophosphate by oxidative phosphorylation. Changes in the concentration of this enzyme both during depletion and in response to iron therapy are temporally related to changes in muscle function. Thus among the iron containing enzymes studied, this enzyme appears the only promising candidate to explain the disorder in muscle function.

Biochemical changes similar to those found in skeletal muscle are not found in cardiac muscle. The α-glycerophosphate oxidase system is known to be virtually absent in mitochondrial preparations from normal heart muscle, and this was verified in our study. Levels of cytochrome pigments and rates of oxidative phos-

phorylation mediated by pyruvate-malate and succinate were also found to be unaffected by iron deficiency. Such biochemical differences between cardiac and striate musculature are consistent with the almost completely aerobic metabolism of the heart where glycolysis is used only as an extra source of energy in emergencies (33) and with the previous findings that cardiac function in iron deficiency anemia is unimpaired (4).

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