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EVIDENCE FOR THE DE NOVO SYNTHESIS OF ERYTHROPOIETIN IN HYPOXIC RATS

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ABSTRACT

Significant increases in the serum erythropoietin of male rats occur after the end of a brief hypoxic exposure. These increases in the hormone are almost completely abolished when the kidneys are removed after the hypoxic exposure. Injection of puromycin or cyloheximide after the hypoxic exposure significantly decreases the subsequent increases in serum erythro-poietin titers; whereas injections of actinomycin D at this time have no significant effect on erythropoietin levels. Injections of actinomycin D before the hypoxic exposure prevent the increase in serum erythropoietin that normally occurs. These findings suggest that a brief period of hypoxia initiates a DNA-dependent RNA synthesis that regulates the de novo ribosomal synthesis of protein(s) involved in the biogenesis of erythropoietin, and the kidney is essential for these reactions to occur.

Considerable evidence suggests that the level of erythropoietic activity in mammals is ultimately determined by the ratio to oxygen supply to oxygen need in the tissues producing erythropoietin. The low level of blood erythropoietin present in normal animals is not generally measurable in the plethoric mouse biological assay, but within a short time after altering the oxygen supply to an animal, such as an exposure to a simulated altitude, the blood erythropoietin levels are markedly increased and readily detectable in the biological assay. Evidence will be presented that (a) this increase in circulating erythropoietin is the result of de novo protein synthesis and not simply the release of stored hormone; (b) the synthesis of the hormone, once triggered, continues for some time after termination of the hypoxic conditions; (c) the initial triggering of the synthesis of the hormone probably involves a DNA-dependent RNA synthesis; and (d) the kidney is essential for synthesis of the hormone.

MATERIALS AND METHODS

Male Sprague-Dawley rats weighing about 350 g were used. Nephrectomy and sham operations were performed under methoxyflurane (Metofane, Pitman-Moore) anesthesia as previously described.[1] Rats were conscious within 15-20 min of the indicated time of nephrectomy.

Rats were exposed to simulated altitudes of 22,000 ft (6,706 m, 321 torr) or 28,000 ft (8,534 m, 247 torr) for varying periods of time. In one experiment rats were exposed in a lucite chamber to a gas mixture of 10% O_2, 90% N_2, which flowed through the chamber at a rate of 3 L/min (equivalent to 18,000 ft, 380 torr).

At various times after simulated altitude exposure blood was collected from the abdominal aorta under ether anesthesia, allowed to clot, and the serum

was removed and stored frozen until erythropoietin levels were determined using the 7-day post-CO female LAF_1/JAX mouse.[1] One ml of test sera was injected subcutaneously. Each sample was assayed in 6-8 mice weighing 20-26 g. The hematocrits were greater than 60% at the end of the assay.

Actinomycin D was dissolved in saline and injected intravenously at a dose of 0.5 µg/g body weight. Cycloheximide was dissolved in saline and injected subcutaneously at a dose of 0.3 mg/rat. Puromycin dihydrochloride was dissolved in saline, the pH adjusted to 6.6, and injected intravenously at a dose of 10 mg/100 g body weight. The injection of these antibiotics at the dosages used have been shown by a number of investigators to significantly inhibit protein synthesis in vivo in the rat.[2-4] Sera collected from rats receiving antibiotics were dialyzed against several changes of distilled water for 24 hr. The dialyzed sera were lyophylized and restored to their original volume with saline. These procedures were performed to minimize the effects on the plethoric mice of different antibiotics that might still be in the rat sera used for erythropoietin assay.

The results are expressed as the percent of the injected radioiron incorporated in 72 hr in the calculated blood volume, which was assumed to be 7% of the body weight. Estimations of units of erythropoietin were made from the 72-hr ^{59}Fe uptakes by reference to a standard curve prepared by using the International Reference Preparation (IRP). No attempt to convert percent uptake to units of erythropoietin has been made with ^{59}Fe uptakes < 3.0% because of the log-dose nature of the biological assay. Since plethoric mice injected with normal rat sera and uninjected plethoric mice have a 72-hr ^{59}Fe uptakes of 0.62% ± 0.3 (S.E.), we consider that values > 1.5% with a P < 0.001 compared to these controls indicate the hormone is present in the injected sera. The standard error of the mean is indicated. It is assumed that the

increase in radioiron incorporation in the plethoric assay mice was a direct result of erythropoietin in the injected sera.

RESULTS

The temporal changes in serum erythropoietin levels in male rats during and after a 2-hr exposure to a simulated altitude of 22,000 ft (P_{O_2} 67.1 torr) are shown in Fig. 1. Small but detectable increases in the hormone occur after a 2-hr exposure to this altitude. Peak titers of almost 0.85 IRP units/ml of serum are found 2 and 3 hr after the hypoxic stimulus has been terminated. The titer rapidly decreases between the 3rd and 4th hr. Thereafter, it decreases more slowly, returning to normal by 12 hr after the initial stimulus.

The effects of nephrectomy and ureter ligation on the peak titer of serum erythropoietin are shown in Fig. 2. The time of operation relative to the hypoxic exposure is indicated by arrows. Unless otherwise stated, serum was collected 3 hr after the end of a 2-hr exposure to a simulated altitude of 22,000 ft or 5 hr after the beginning of the hypoxia. Some erythropoietin is present in the serum of rats exposed to this hypoxic stimulus for 1 hr and bled 4 hr later (Group 1). A significant titer of 0.08 IRP units/ml of the hormone is found after 2 hr exposure to hypoxia (Group 2), but 3 hr later (Group 3) the titer has increased to 0.85 IRP units/ml of serum. The titer of hormone is increased to more than 2.0 IRP units/ml if the rats are continuously exposed to hypoxia for 5 hr (Group 4). Sham nephrectomy (Group 5) or unilateral nephrectomy (Group 7), immediately after the 2-hr hypoxic exposure, does not significantly depress the serum erythropoietin levels measured 3 hr later; however, ureter ligation performed at this time (Group 6) does significantly lower ($P < 0.005$) the subsequent increase in the titer of the hormone.

If bilateral nephrectomy is performed just prior to the hypoxic exposure, elevated levels of serum erythropoietin do not occur in rats either immediately after the hypoxic exposure (Group 8) or 3 hr later (Group 10). A significant increase in serum erythropoietin (0.22 IRP units/ml) does occur in bilaterally-nephrectomized rats exposed to the hypoxic conditions continuously for 5 hr (Group 9) immediately after recovery from the operation.

When bilateral nephrectomy was performed immediately after the hypoxic exposure (Group 11), elevated serum erythropoietin titers did not occur in rats 3 hr after a 2-hr hypoxic exposure. In contrast, significant increases in serum erythropoietin titers of 0.10 and 0.22 IRP units/ml respectively were observed 3 hr after the hypoxic stimulus when the kidneys were removed 1 hr (Group 12) or 2 hr (Group 13) after the end of the hypoxic exposure.

The serum erythropoietin levels in male rats exposed to a more severe hypoxic stress of 28,000 ft (P_{O_2} 51.7 torr) for brief periods of time is shown in Fig. 3. About 25% of the rats died within 2 hr at this altitude. In this particular experiment the serum level is not elevated after a 1-hr exposure (Group 1), nor does the level change if the serum is collected 4 hr after the end of hypoxia (Group 2). It must be noted that we have found the response of rats to this altitude is variable, and in some experiments elevated levels are observed within 1 hr after a 1-hr exposure, and the hormone titer is elevated for the next 3 hr. A highly significant increase (0.18 IRP units/ml) in serum levels of the hormone is consistently found after a 2-hr exposure at this altitude. Because of the variability in erythropoietin changes seen at this altitude after a 1-hr exposure, most of our other studies were performed on rats exposed to altitudes of 22,000 ft or less for 2 hr.

The effect of actinomycin D and cycloheximide on the changes in serum erythropoietin titers resulting from a brief hypoxic exposure is shown in

Fig. 4. A highly significant increase in the serum concentration of the hormone is measurable 3 hr after a 2-hr hypoxic exposure to 22,000 ft (Group 1). This increase does not occur if actinomycin D is injected before the hypoxic exposure (Group 2). Injection of the antibiotic after the hypoxic exposure (Group 3) has little if any effect ($0.10 > P < 0.05$) on the erythropoietin titer. In contrast, the high erythropoietin level detected 2 hr after the end of the hypoxic exposure (Group 4) is significantly reduced by the injection of 0.3 mg cycloheximide immediately before or immediately after the 2-hr hypoxia (Groups 5 and 6). The subcutaneous injection of 0.5 mg or 0.6 mg of cycloheximide into rats immediately after this 2-hr hypoxic exposure decreased even more the erythropoietic activity of the serum collected 3 hr later; the 72-hr ^{59}Fe incorporations were 2.5 ± 0.3 and 2.2 ± 0.2 respectively. Similar results were obtained after intravenous injections of cycloheximide.

It is unlikely that the depressed erythropoietic response observed, as a result of injections of serum obtained from rats injected with these different antibiotics, was the result of an action of the antibiotics on the erythropoietic activity of the assay mouse. Additions of the entire dose of these various antibiotics to 10 ml of serum containing erythropoietin did not significantly depress the 72-hr radioiron incorporation if the serum was dialyzed against distilled water for 24 hr. For example, the 72-hr ^{59}Fe incorporation elicited by 1 ml of altitude rat serum after dialysis, lyophylization, and reconstitution with saline was $18.1 \pm 1.0\%$; whereas, the same serum sample containing cycloheximide and similarly treated gave a 72-hr ^{59}Fe incorporation of $20.8 \pm 0.8\%$. Similar results were obtained with additions of the other antibiotics to serum.

The response of male rats to a less severe hypoxic stress produced by an atmosphere containing 10% O_2 (approximately 18,000 ft, P_{O_2} 79 torr) is shown in Fig. 5. Significant elevations are not observed after 2-hr exposure to

hypoxia (Group 1), but 2 hr later the titer of the hormone is significantly
increased to 0.25 IRP units/ml (Group 2). This post hypoxic increase in
hormone titer is significantly depressed to 0.07 IRP units/ml when puromycin
is injected immediately before and during the hypoxic exposure (Group 3),
and to 0.09 IRP units/ml when the antibiotic is injected immediately after
the hypoxic exposure and 1 hr later (Group 4).

DISCUSSION

When male rats are exposed to a simulated altitude of 22,000 ft for
2 hr, the serum erythropoietin levels of their blood, measured in the ple-
thoric mouse, are only slightly elevated upon cessation of the hypoxic stress.
The serum erythropoietin levels markedly increase during the next few hours
even though the rats are no longer exposed to hypoxic conditions, specifically
by the second and third hour about 0.85 IRP units of erythropoietin are
found per ml of serum. The serum erythropoietin then rapidly disappears in
a manner analagous to the pattern of erythropoietin disappearance observed by
Stohlman and Howard[5] after a single injection of exogenous erythropoietin in
the rat. They suggest that the initial rapid disappearance of the hormone
after the peak value is probably due to its distribution into the extravascular
fluid; whereas, the second component of the curve is probably related to its
biological half-life. It should be emphasized that this pattern of appearance
and disappearance of serum erythropoietin after exposure to a simulated al-
titude of 22,000 ft is found in the male and not the female rat. These sex
differences will be presented in a subsequent communication. Interestingly,
the male rabbit responds to this brief hypoxic stimulus like the male rat.
Male Long-Evans rats weighing about 100 g less than the rats used in these ex-
periments are reported[6] to increase their serum erythropoietin levels after
only a 1-hr hypoxic exposure (approximately 23,000 ft), although significant
levels of the hormone were not present immediately after the hypoxic stress.

The data of Altland et al.[7] indicate that the arterial oxygen saturation of rats exposed acutely to an altitude of 22,000 ft is about 60%, compared to the normal value of 91.3%. It is well known that the arterial oxygen saturation rapidly returns to normal when animals are returned from hypoxic to normal conditions. The arterial oxygen saturation of the blood of rabbits exposed to 22,000 ft is normal immediately after the 5-10 min required to return the altitude chamber to atmospheric conditions. It appears reasonable to assume that the oxygen supply to tissues producing or releasing erythropoietin into the circulation approaches normal soon after the termination of the hypoxic stress.

The duration, as well as the severity, of the brief hypoxic exposure are of importance in determining the magnitude and the temporal pattern of serum erythropoietin. Significantly elevated serum erythropoietin levels are not consistently observed in rats either immediately after a 1-hr exposure to a simulated altitude of 22,000 or 28,000 ft or 4 hr after the end of these exposures. Two hours after the exposure of rats to 28,000 ft a highly significant increase in serum erythropoietin occurs in the biological assay; whereas, only a slight increase occurs in rats exposed to 22,000 ft for the same period of time. The serum erythropoietin levels are significantly increased 3 hr after the end of exposure to both of these altitudes. This indicates that the sites of production of erythropoietin must remain hypoxic for at least 1 hr before they are induced to produce highly significant levels of serum erythropoietin. It is extremely likely that increased amounts of the hormone are produced soon after the beginning of the hypoxic exposure. Gurney and co-workers[8] have clearly shown that an exposure of only 15 min to severe hypoxia elicit erythropoietic response in plethoric mice. In our experiments increases in the hormone's concentration must be sufficient to produce an

erythropoietic response in the plethoric mouse, and these increases cannot be detected until the concentration of the hormone in the entire plasma volume of the rat has been elevated.

Removal of the kidneys either immediately before or immediately after a 2-hr exposure to 22,000 ft almost completely stopped the subsequent increases in serum erythropoietin. Sham operation, ureter ligation, or unilateral nephrectomy after the hypoxic exposure did not prevent the increases in serum erythropoietin seen several hours later, although the erythropoietin titer of the serum of ureter-ligated rats was significantly less than that found in control rats ($P < 0.005$). We have consistently observed that ureter-ligated rats exposed continuously to hypoxic conditions for variable periods of time produce significantly less erythropoietin than sham-operated controls, suggesting that either the uremic rat does not become as hypoxic as the controls, possibly because of a shift in the oxygen dissociation curve, and/or because some inhibitor interfers with the initiation of erythropoietin production. The effect of ureter ligation after the brief hypoxic exposure is less likely to be related to these changes, and we suggest that the effect is probably related to an altered blood flow through the kidney. The failure of unilateral nephrectomy to significantly decrease the serum erythropoietin levels suggests that the kidneys' contributions are not the rate-limiting process in the hormone's production after the end of the hypoxic exposure.

The production of erythropoietin by anephric rats has been studied by a number of investigators with conflicting results, since the initial observations of Jacobson and co-workers[9] implicated the kidney in rodent erythropoiesis. We have recently reported[1] that some of these conflicting results are related to the finding that the ability of an anephric male or female rat to respond to a hypoxic stimulus of 22,000 ft with elevated erythropoietin levels pro-

gressively decreases with increasing time after nephrectomy. Sera from rats nephrectomized immediately before a continuous 5-hr exposure to 22,000 ft do contain about 0.22 IRP units/ml. Sera from anephric rats collected 3 hr after a 2-hr exposure to 22,000 ft contain about 0.05 IRP units/ml. One day after nephrectomy rats do not have any erythropoietin in their sera before or after an exposure to 5 hr at 22,000 ft. We conclude from these results that (a) only renally-regulated erythropoietin production is significant in the present experiments, and (b) extrarenal erythropoietin production requires more sustained hypoxic conditions in order to commence.

Any interpretation of the role of the kidney in erythropoietin production, based on the failure of anephric animals to respond to hypoxic stimuli, must be made cautiously. The complex physiological changes in hemodynamics and acid-base balance occurring after nephrectomy may play a more important role in the ability of the anephric rat to detect a hypoxic stimulus than has been appreciated. These problems have certainly been minimized in our experiments since the kidneys were not removed until after completion of the brief hypoxic exposure. We conclude that, although the normal mass of kidney tissue is not essential for normal erythropoietin production, some renal tissue is always necessary for its significant production after the end of these brief hypoxic exposures. The longer renal tissue exists in the rat after the hypoxia the greater the production of erythropoietin. The function of the kidney in the production of the hormone is unknown. We have never been able to demonstrate the presence of the hormone in renal tissue after these brief hypoxic exposures, and feel that if the hormone is made in the kidney it does not accumulate, and therefore must rapidly enter the blood.

Antibiotic inhibitors of ribosomal protein synthesis and of RNA formation are useful tools in in vivo studies of protein synthesis. The complex interrelationships between other hormones and erythropoietin production suggest

that interpretations of the effects of these antibiotics on erythropoietin production in the intact animal as a result of a hypoxic stimulus must be tentative.

Giger[10] found that the serum erythropoietin levels of rats exposed to hypoxia (about 19,500 ft) for 16 hr were significantly depressed by the injection of actinomycin D. He concluded that erythropoietin synthesis was DNA-dependent. We have corroborated this finding and have demonstrated that erythropoietin production was severely depressed in normal and anephric rats exposed to 22,000 ft for 5 hr in the presence of the antibiotic.[1] It has been suggested that this depression could have occurred because of a decreased oxygen demand by the organism.[11] We could not detect with continuous monitoring, any differences in oxygen consumption or CO_2 production between normal or actinomycin D-injected rats in 5 hr, although the possibility that changes in the oxygen demand of tissues involved in the biogenesis of the hormone could not be excluded. In the present experiments the processes involved in the production of erythropoietin after the end of a brief hypoxic exposure are not actinomycin D-sensitive, since injection of the antibiotic had no significant effect on the subsequent production of the hormone. Injection of actinomycin D before the hypoxic exposure almost completely prevented erythropoietin production in these experiments. We tentatively conclude that the initiation of erythropoietin production requires a DNA-dependent-mRNA synthesis. The mRNA must be relatively short-lived since detectable erythropoietin production occurs for only a few hours after the end of the hypoxic stimulus, and the production of erythropoietin after this time is not actinomycin D-sensitive.

The injection of puromycin and cycloheximide before a brief hypoxic exposure almost completely prevents the subsequent elevation in serum erythropoietin levels seen in control rats similarly exposed to hypoxia. The

injection of puromycin or cycloheximide <u>after</u> the brief hypoxic exposure either abolishes or markedly reduces the subsequent elevation in serum erythropoietin. These results are consistent with the concept that ribosomal protein synthesis is important in the biogenesis of erythropoietin, and suggest that the erythropoietin produced after the hypoxia is the result of a <u>de novo</u> protein synthesis.

It is useful to consider our results in terms of the concept set forth by Gordon and associates[12] that the biogenesis of erythropoietin involves the action of an enzyme produced by the kidney upon a serum substrate to produce the active hormone. They believe that the serum substrate normally occurs in the serum, although its concentration may be elevated by a hypoxic exposure. This would suggest that the <u>de novo</u> synthesis occurring in our experiments is primarily involved in the production of the renal enzyme. We have been unable to corroborate the <u>in vitro</u> observations of these workers in the maximally suppressed plethoric mouse in a large number of experiments. Furthermore, we have been unable to demonstrate that injections of renal extracts into rats after these brief hypoxic exposures modify the temporal pattern of erythropoietin production even when the kidneys are removed. Our results would not support the view recently suggested[6] that the primary effect of hypoxia on erythropoietin production is the release of stored renal enzyme into the circulation. If this process occurred during the brief hypoxic exposure, removal of the kidneys after the exposure should not alter the subsequent increase in serum erythropoietin.

We favor the view that the biosynthesis of erythropoietin has at least three components.[1] These <u>in vivo</u> experiments indicate that at least one of these components increases by <u>de novo</u> synthesis after a hypoxic stimulus, although the site of this <u>de novo</u> synthesis is unknown. Although the results with anephric rats indicate that renal tissue is essential for erythropoietin

production to occur in our experiments, we have no direct evidence that de novo protein synthesis occurs in the kidney. We have demonstrated, using immunodiffusion techniques, that different proteins are present in the cytoplasmic proteins of hypoxic kidneys, and these proteins are not present in actinomycin D-injected hypoxic rat kidneys. The relationship of these proteins to the synthesis of erythropoietin is being investigated.

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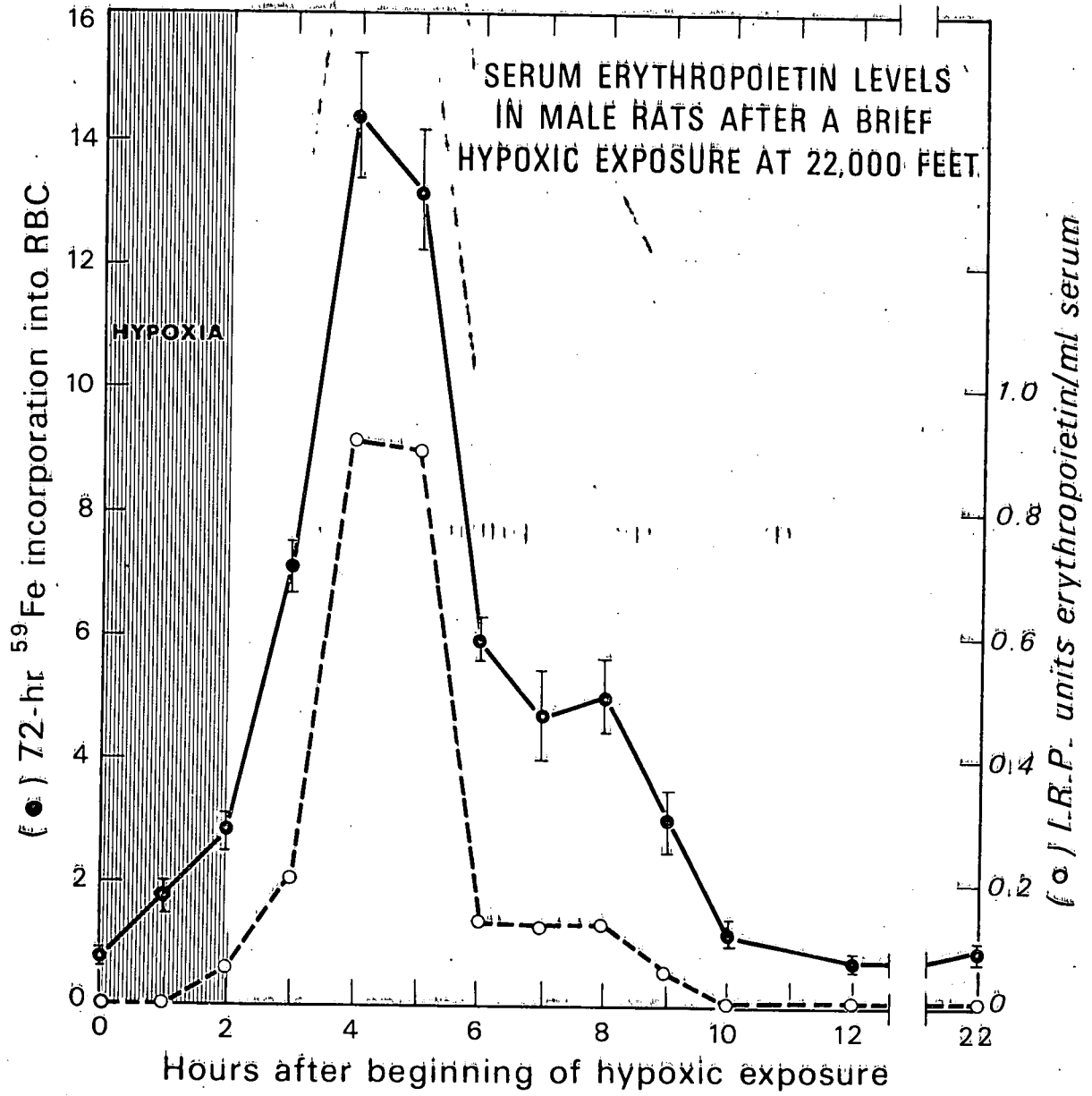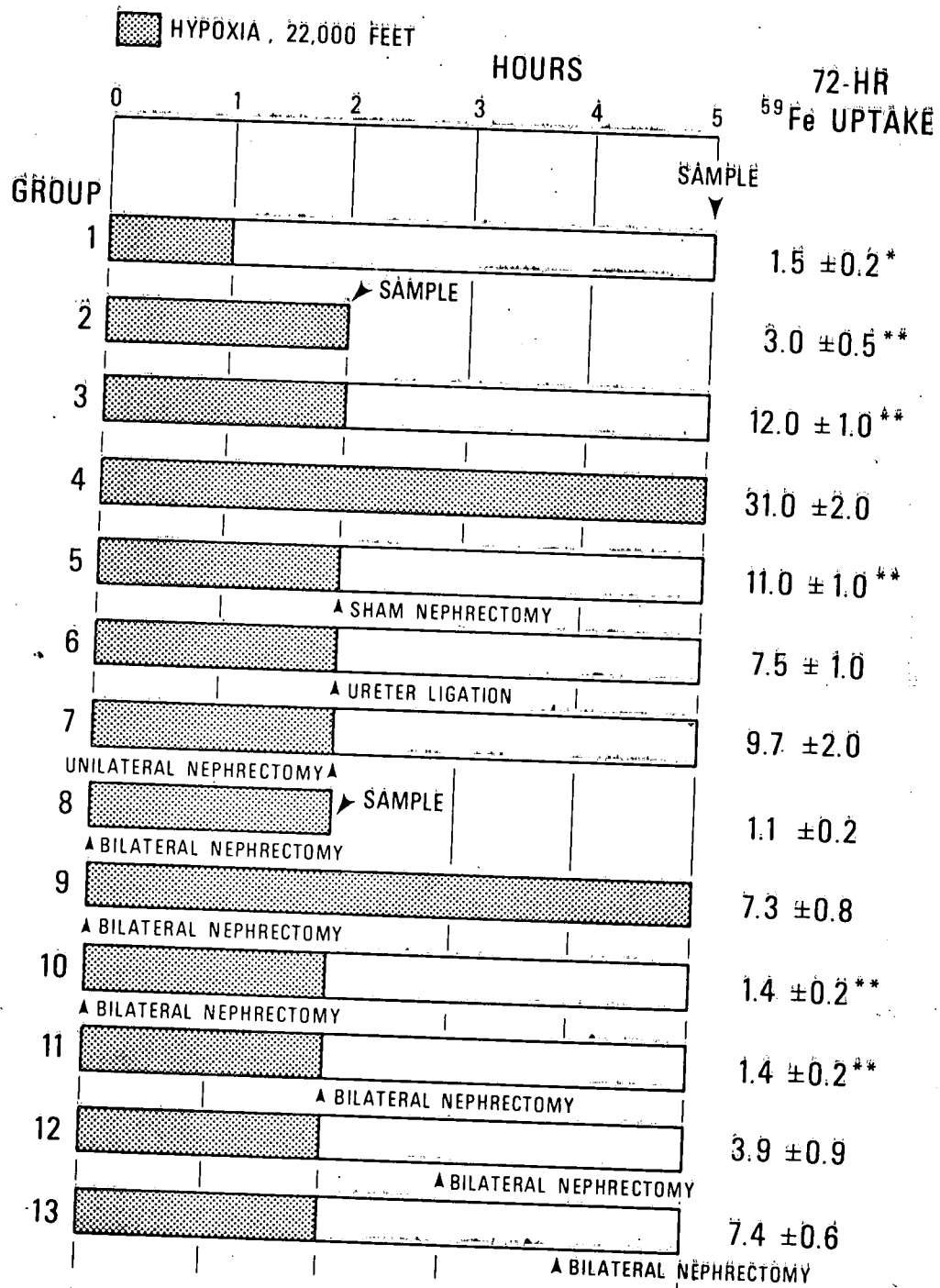
SERUM ERYTHROPOIETIN LEVELS IN MALE RATS AFTER A BRIEF HYPOXIC EXPOSURE AT 22,000 FEET

DBL 718-5949

Fig 1

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"Evidence for the _de novo_ synthesis of
erythropoietin in hypoxic rats" Title of manuscript

HYPOXIA , 22,000 FEET

HOURS

72-HR
^{59}Fe UPTAKE

GROUP		72-HR ^{59}Fe UPTAKE
1	SAMPLE ▼	1.5 ±0.2 *
2	► SAMPLE	3.0 ±0.5 **
3		12.0 ± 1.0 **
4		31.0 ±2.0
5		11.0 ± 1.0 **
6	▲ SHAM NEPHRECTOMY	7.5 ± 1.0
7	▲ URETER LIGATION	9.7 ±2.0
8	UNILATERAL NEPHRECTOMY ▲ ► SAMPLE ▲ BILATERAL NEPHRECTOMY	1.1 ±0.2
9	▲ BILATERAL NEPHRECTOMY	7.3 ±0.8
10	▲ BILATERAL NEPHRECTOMY	1.4 ±0.2 **
11	▲ BILATERAL NEPHRECTOMY	1.4 ±0.2 **
12	▲ BILATERAL NEPHRECTOMY	3.9 ±0.9
13	▲ BILATERAL NEPHRECTOMY ▲ BILATERAL NEPHRECTOMY	7.4 ±0.6

* Standard error of the mean
** Average of two experiments

DBL 7112 6106

Fig. 2
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" Evidence for the <u>de novo</u> synthesis of erythropoietin in hypoxic rats "— title of manuscript.

HYPOXIA, 28,000 FEET

HOURS

72-HR ^{59}Fe UPTAKE

GROUP

1	SAMPLE ▼	0.8 ±0.1*
2	SAMPLE ▼	1.1 ±0.2
3	▼ SAMPLE	5.6 ±0.9
4	▼	9.3 ±0.8
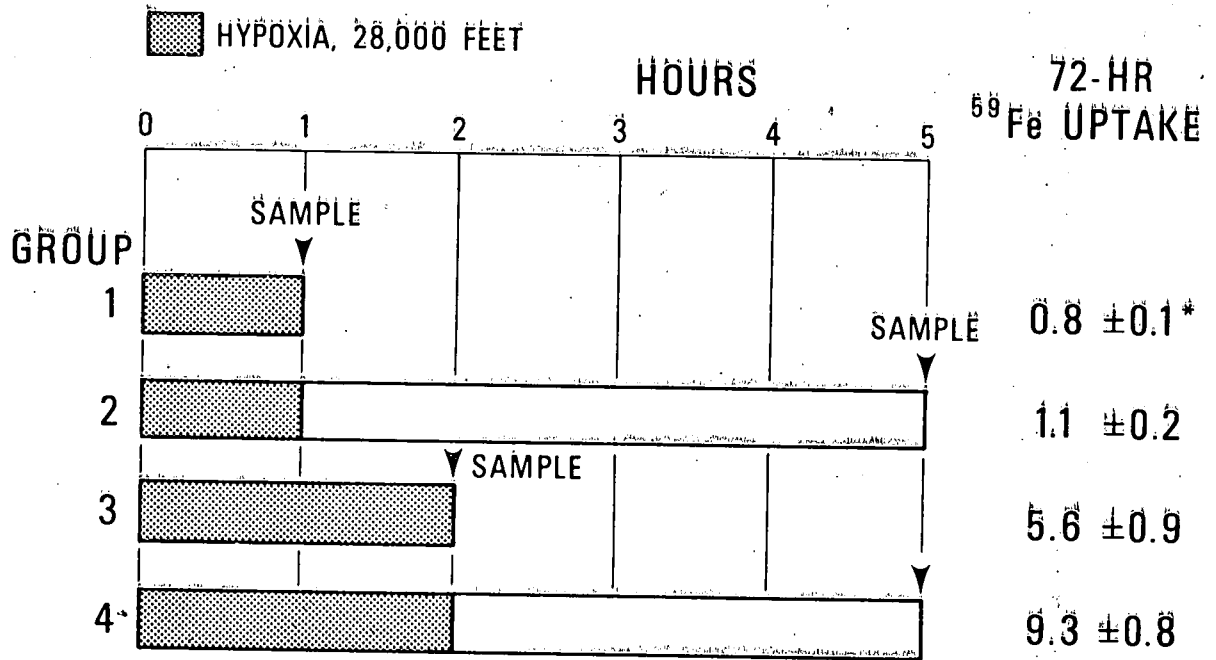
*Standard error of the mean

DBL 721-5126

Fig 3

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"Evidence for the _de novo_ synthesis of erythropoietin in hypoxic rats" - title of manuscript.

HYPOXIA, 22,000 FEET

HOURS

72-HR ^{59}Fe UPTAKE

GROUP		72-HR ^{59}Fe UPTAKE
1	HYPOXIA (0–2)	17.0 ±3.0*
2	HYPOXIA (0–2)	2.9 ±0.2
3	ACTINOMYCIN D	12.0 ±1.0
4	ACTINOMYCIN D	14.0 ±1.0
5	CYCLOHEXIMIDE	4.1 ±0.4
6	CYCLOHEXIMIDE	4.4 ±0.3

SAMPLE

*Standard error of the mean
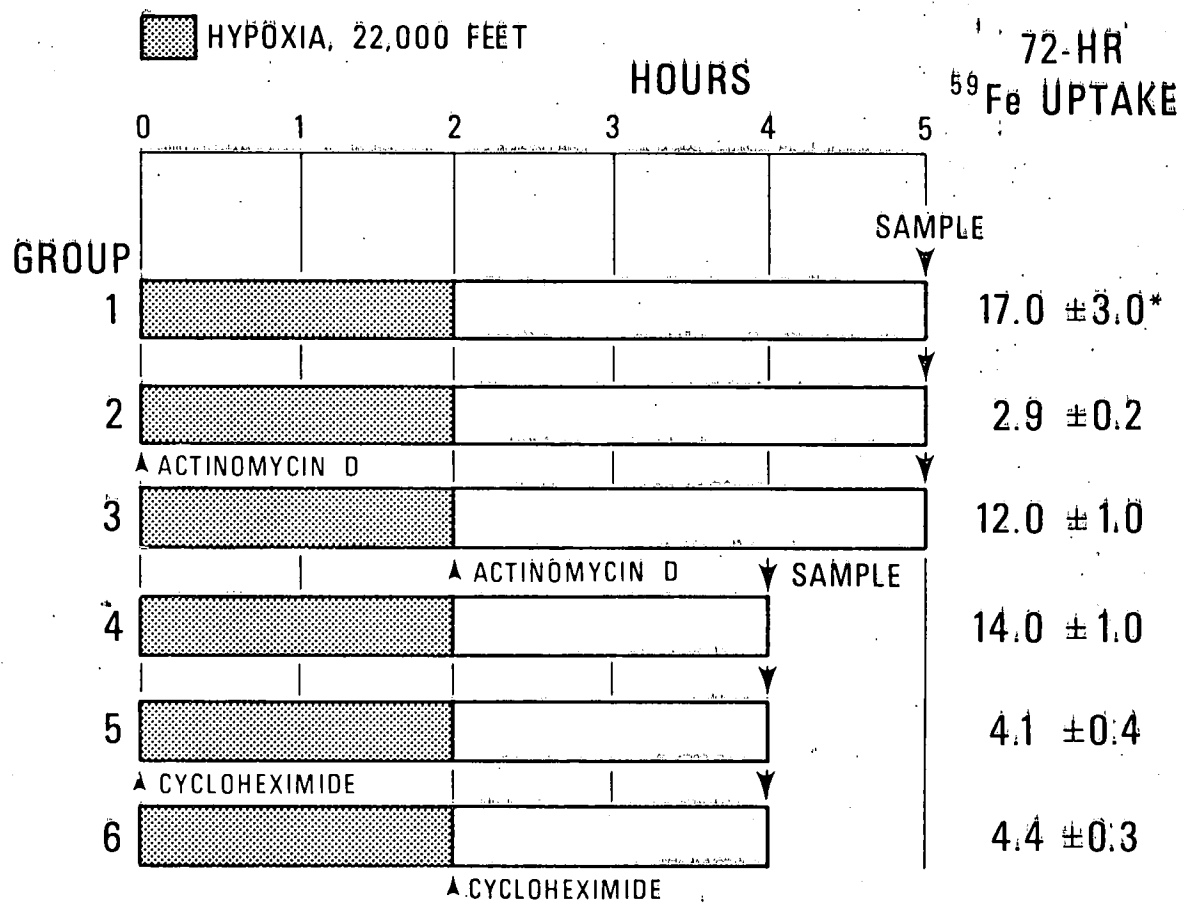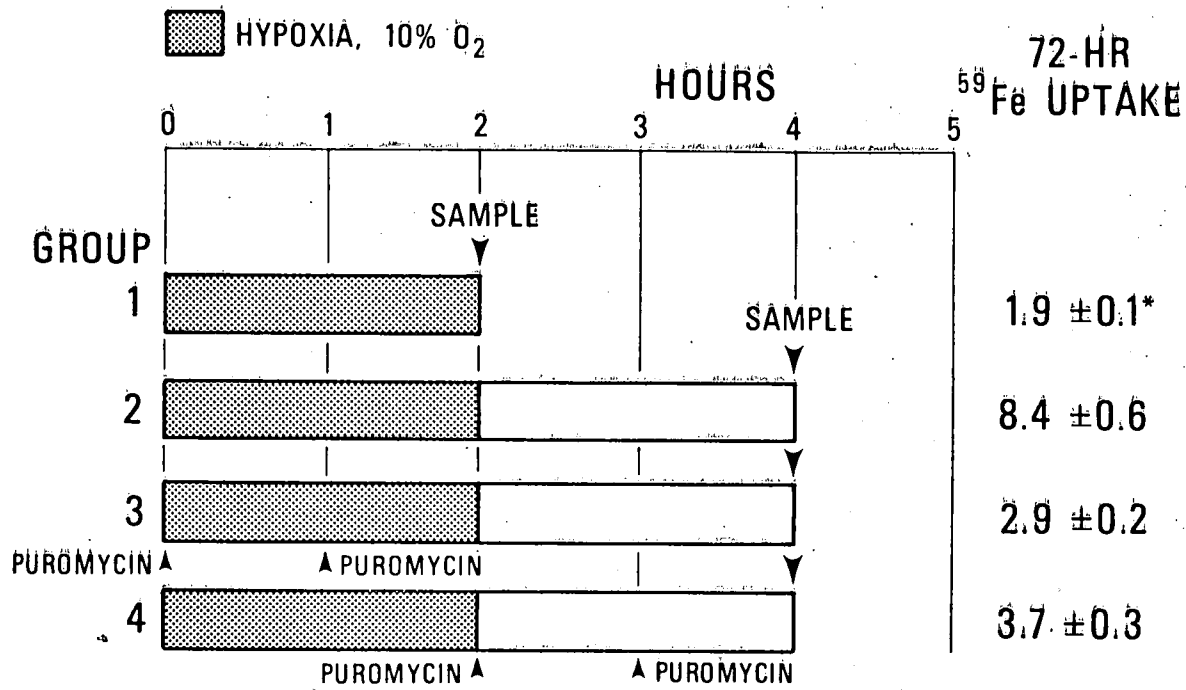
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Fig. 4

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"Evidence for the de novo synthesis of erythropoietin in hypoxic rats" — title of manuscript

HYPOXIA, 10% O_2

HOURS

72-HR
^{59}Fe UPTAKE

0   1   2   3   4   5

GROUP

SAMPLE

1   1.9 ±0.1*

SAMPLE

2   8.4 ±0.6

3   2.9 ±0.2

PUROMYCIN ▲   ▲ PUROMYCIN

4   3.7 ±0.3

PUROMYCIN ▲   ▲ PUROMYCIN

* Standard error of the mean

DBL 721-5125

Fig. 5

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"Evidence for the de novo synthesis of erythropoietin in hypoxic rats" — title of manuscript

Fig. 1 Temporal pattern of serum erythropoietin changes during
and after a 2-hr exposure to 22,000 ft.

Fig. 2 Effect of nephrectomy on erythropoietin production after
a brief hypoxic exposure.

Fig: 3 Effect of a brief hypoxic exposure of 28,000 ft on
erythropoietin production.

Fig. 4   Effect of actinomycin D and cycloheximide on erythropoietin

production after a brief hypoxic exposure.

Fig. 5 Effect of puromycin on erythropoietin production in rats
exposed to a 10% O_2 atmosphere.