Humoral Regulation of Erythropoiesis. IV. Relative Heat Stability of Erythropoietine. (23367)

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The heat stability of erythropoietine, a substance in the plasma of anemic animals capable of stimulating erythropoiesis, has been the subject of recent controversy. Borsook(1) originally reported that the plasma of rabbits with phenylhydrazine-induced anemia retained its effectiveness after deproteinization by boiling for 10 minutes at pH 5.5. Subsequently Gordon(2), Linman(3) and Mirand (4) reported similar observations. In Erslev's(5) experience as well as our own(6,7), however, plasma so treated was ineffective. In the latter instances plasma from animals with post-hemorrhagic anemia or those which had been exposed to altitude was used as the source of erythropoietine. Some recent studies have indicated that boiled plasma does not produce a uniform response(8,9,10). Jacobsen(8) first noted this in studies on plasma from animals with phenylhydrazine anemia. She suggested that the erythropoietic activity could be demonstrated in boiled plasma only if the phenylhydrazine intoxication had been severe enough to induce significant liver damage. Prentice and Mirand(11) noted that the plasma from hypoxic animals with liver damage due to carbon tetrachloride was more effective than that of hypoxic animals with normal livers. In many of the above studies the assay used for the demonstration of erythropoietic activity differed as did the method of production of erythropoietine in the donor animals. From the published data it was impossible to decide whether the discrepant reports were due to the use of different bio-assays, a relationship of activity to liver function, a multiplicity of humoral factors(12) or whether erythropoietine was indeed heat labile. The present study was undertaken in an effort to resolve these questions.

Methods and materials. Types of assay. Three different assay technics were utilized: (a) The sublethally irradiated animal previously reported by us(6). (b) The hypophysectomized animal suggested by Fried *et al.* (13) and Mirand and Prentice(4). Male Sprague-Dawley rats weighing 150 g were hypophysectomized* and used either 8-10 days after hypophysectomy as suggested by Fried(13) or 30-40 days as recommended by Mirand and Prentice(4). At the time of assay, the animals weighed from 120-160 g. They were randomly assigned to groups of 5-7 animals. The plasma to be tested was injected intravenously in amounts equivalent to 2% of the recipient's body weight. In one experiment a similar amount of boiled plasma was given subcutaneously daily for 3 days. Forty-eight hours after the initial injection of plasma, Fe^{59} bound to homologous plasma was administered intravenously. Forty-eight hours thereafter the amount of Fe^{59} incorporated into the circulating red cells was determined using a blood volume value of 6% of the body weight. (c) Normal animals were injected subcutaneously daily for 10-12 days with amounts of boiled plasma extract equivalent to 2% of the body weight. Five to 7 days prior to the institution of treatment 0.2-0.3 cc of blood was withdrawn by cardiac puncture for the determination of red cell values. At the time of the last injection of plasma, Fe^{59} tagged homologous plasma was given I.V. and 24 hours thereafter the Fe^{59} incorporation determined. At this time the red cell values were again determined. *Preparation of plasma.* Plasma from 4 sources was utilized; plasma from rats or rabbits made anemic by bleeding(6), plasma from normal rats exposed to a simulated altitude of 23,000 feet for 24 hours(14), plasma from rabbits treated with phenylhydrazine. In the latter instance rabbits weighing 1.5-2.5 kg were given 12.5 mg/kg of phenylhydrazine for 4-5 days and 48 hours after the last injection bled by cardiac puncture. At that time the hemato-

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TABLE I. Effect of Temperature on Erythropoietine.

	Anemic plasma			
	Temperature of incubation			
Control	25° 24 hr	50° 6 hr	60° 0.5 hr	70° 0.5 hr
24 ± 5.2 (7)*	38 ± 5.7 (6)			
23 ± 2.3 (6)		40 ± 2.7 (4)		
26 ± 3.4 (9)			28 ± 3.2 (6)	27 ± 3.5 (4)

* Fe^{59} values given represent % of total inj. dose present in circulating red cells 48 hr after inj. ± a single stand. error. No. of animals in each group is included in parentheses. The sublethally irradiated rat was used for the assay.

crits ranged from 12-20 and reticulocytes from 60-95%. Liver damage was apparent grossly in about 25% of the animals so treated. Microscopic examination was in agreement with gross findings in the first 25 animals and thereafter was omitted. Boiled plasma extract was made as described by Borsook(11). The plasma was adjusted to pH 5.5 and then placed in a 100°C water bath for 10 minutes. The precipitate was separated by centrifugation. An amount of water equivalent to the original volume was added to the precipitate. This was agitated with a mechanical stirrer for 10 minutes and the wash water removed after centrifugation. This step was repeated a second time. The supernatant and wash water were pooled and either evaporated to the desired volume in a rotating evaporator at 50°C or lyophilized and the residue resuspended in ion free water. No differences were noted between the 2 methods of concentration. The pH was then adjusted to 7.0 with 1 N NaOH.

Results. The effectiveness in sublethally irradiated animals of altitude or anemic plasma was determined following incubation at 25°C for 24 hours, 50°C for 6 hours, and 60° or 70°C for 30 minutes. Following exposure at 60° or 70°C an erythropoietic effect could no longer be demonstrated (Table I). Boiled acidified plasma from rabbits with post-hemorrhagic or phenylhydrazine anemia was ineffective when tested in the sublethally irradiated animal. Since a number of investigators had described activity when multiple injections were given to normals, these fractions were tested according to the method described by Linman and Bethell(3). Again activity could not be demonstrated.

Concentrated fractions from homologous as well as heterologous plasma were then tested in both hypophysectomized and sublethally irradiated animals. A 3-fold concentration of boiled plasma was used so that each animal received the boiled extract of an amount of plasma equivalent to 6% of the recipient's body weight. By this increase in dosage significant stimulation of erythropoiesis by boiled anemic or altitude plasma was achieved in each of 4 experiments in which hypophysectomized animals were used (Table II). Stimulation of erythropoiesis was also noted in 4 experiments in the sublethally irradiated animal. However in both hypophysectomized

TABLE II. Effect of Boiling on Erythropoietine.

Source and treatment of plasma	Equivalent amount of native plasma (cc)	Fe^{59} incorporation	
		Irradiated rat assay	Hypophysectomized rat assay
None	—	16 ± 3.3 (7)*	40 ± 3.1 (8)
Normal rat	3	23 ± 7.3 (7)	40 ± 6.4 (7)
Altitude "	3	36 ± 3.7 (7)	65 ± 3.5 (6)
Boiled normal rat	9	21 ± 4.5 (6)	37 ± 2.2 (7)
" altitude "	9	26 ± 2.3 (6)	52 ± 1.69(7)
" normal rabbit	9	22 ± 2.8 (6)	43 ± 5.5 (6)
" phenyl " with liver damage	9	27 ± 2.9 (6)	55 ± 2.2 (7)
Idem, without liver damage	9	32 ± 3.6 (7)	69 ± 2.3 (7)

* Fe^{59} values given represent % of total inj. dose present in circulating red cells 48 hr after inj. ± a single stand. error. No. of animals in each group is included in parentheses.

TABLE III. Effect of Dilution on Erythropoietic Activity of "Altitude" Plasma.

Source of plasma	Amt of plasma given (% of body wt)	% Fe^{59} incorporation
Normal plasma	2 %	20 ± 3.3 (6)*
Altitude "	2	35 ± 3.0 (6)
" "	1	32 ± 2.8 (6)
" "	0.5	26 ± 2.0 (6)

* Fe^{59} values given represent % of total inj. dose present in circulating red cells 48 hr after inj. ± a single stand. error. No. of animals in each group is included in parentheses. The sublethally irradiated rat was used for the assay.

and irradiated animals unconcentrated native altitude plasma produced a greater response ($p<.05$)† than did 3 times the amount of boiled acidified altitude plasma obtained from the same plasma pool. This indicated that more than 70% of the erythropoietic activity had been destroyed on heating. In an effort to quantify further the erythropoietic response a dilution experiment utilizing fresh plasma was conducted (Table III). Here, an amount of plasma equivalent to 0.5% of the body weight gave a response similar to that observed from the extract of 12 times as much boiled plasma. It is not suggested that these are quantitative determinations but merely rough semi-quantitative measures indicating that between 70-90% of the activity is lost on boiling for 10 minutes.

In the experiment presented in Table II the plasma derived from phenylhydrazine treated rabbits with normal livers gave a greater response than did that from animals with liver damage. In the hypophysectomized assay this difference was significant ($p<.02$). In 2 other experiments significant stimulation of erythropoiesis was produced by the injection of concentrated boiled extracts of phenylhydrazine plasma obtained from rabbits with normal livers.

Finally, the stability of acidified phenylhydrazine plasma was determined after prolonged periods of boiling (Table IV). Activity could be demonstrated in unconcentrated plasma which had been boiled for 5 minutes but not after boiling for 30 or 60 minutes (Table IV). It may be recalled that in previous experiments activity could no longer be demonstrated in unconcentrated plasma boiled for 10 minutes. Concentration, however, permitted detection of activity in those aliquots boiled for 30 or 60 minutes, as had been the case previously with plasma boiled for 10-15 minutes. The response to concentrated plasma boiled for 30 minutes was less than that of plasma boiled for 5 minutes, though not significantly. No further decrease was noted after boiling for 60 minutes.

Discussion. These data demonstrate that erythropoietic activity is greatly decreased but not completely destroyed by prolonged boiling. While the assay technics used are semiquantitative it nevertheless was possible to estimate that 70-90% of the activity was destroyed by heating acidified plasma at 100° for 10 minutes. Heating for periods of 30-60 minutes did not produce a further decrease in activity. Thus, it is evident that the reaction is non-linear and likely that the kinetics of this reaction are complex. The assay technic is undoubtedly too insensitive to detect differences of a few percent which might occur at the tail of a simple exponential curve or a higher order reaction. Another possibility is that a portion of the circulating erythropoie-

TABLE IV. Effect of Duration of Boiling on Erythropoietine Activity.

	Control	Normal rabbit plasma, boiled 5 min.	Phenylhydrazine rabbit plasma, Boiled for 5 min.	Boiled for 30 min.	Boiled for 60 min.
Unconcentrated	19 ± 1.44 (6)*	10.8 ± 2.7 (6)	27 ± 2.9 (6)	20 ± 3.1 (6)	20 ± 2.0 (6)
3-fold conc.	19 ± 4.6 (6)	22 ± 3.2 (6)	36 ± 2.8 (6)	32 ± 5 (6)	32 ± 2.9 (6)

* Fe^{59} values given represent % of total inj. dose present in circulating red cells 48 hr after inj. ± a single stand. error. No. of animals in each group is included in parentheses. The sublethally irradiated rat was used for the assay.

† Significance was determined using a one-sided t test.

tine is protein bound and that the bound form is heat stable. The data do not exclude the existence of 2 humoral factors, one heat stable and the other labile(12). Since the observations do not necessitate the assumption of a second heat stable erythropoietine, however, it would seem justifiable for the present to consider that there is a single humoral factor a major portion of which is destroyed by boiling.

These experiments are in concert with the reports of Mirand and Prentice(4) who were able to detect erythropoietic activity in all instances where concentrated boiled plasma extracts were utilized. Recently Erslev(15) has also been able to demonstrate activity in boiled plasma when concentrates were used. The experience of these investigators as well as our own suggest that the inability to demonstrate activity in some lots of anemic plasma whether from human or animal sources is not necessarily due to the lack of erythropoietine in such plasma but may be due to the destruction of a large portion of the material prior to testing.

While no systematic study has been conducted on the role of the liver in relation to the level of erythropoietine, it is clear from these data that the presence of liver damage is not a prerequisite for the demonstration of erythropoietine in phenylhydrazine induced anemia (Table III). Erythropoietic activity was demonstrated on 3 occasions in a concentrated extract of acidified boiled plasma from rabbits with phenylhydrazine-induced anemia but with normal livers. It is possible that in a more extensive study, some correlation between the concentration of erythropoietine and the state of liver function could be established.

Summary and conclusions. Stimulation of erythropoiesis in the sublethally irradiated or hypophysectomized rats was demonstrable following injection of a 3-fold concentrated acidified boiled extract of plasma from either rats exposed to simulated altitude or rabbits with phenylhydrazine-induced anemia. No relationship could be established between activity and coexisting liver disease in phenylhydrazine-treated animals. It was estimated that 70-90% of the erythropoietic activity was destroyed by heating acidified plasma at 100° for 10 minutes. Heating for 60 minutes did not further reduce activity. It is suggested that the small fraction of residual activity present after prolonged boiling does not warrant the designation of erythropoietine as a heat stable material nor does it necessitate the assumption of a separate heat stable component, although it does not entirely exclude this possibility.

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