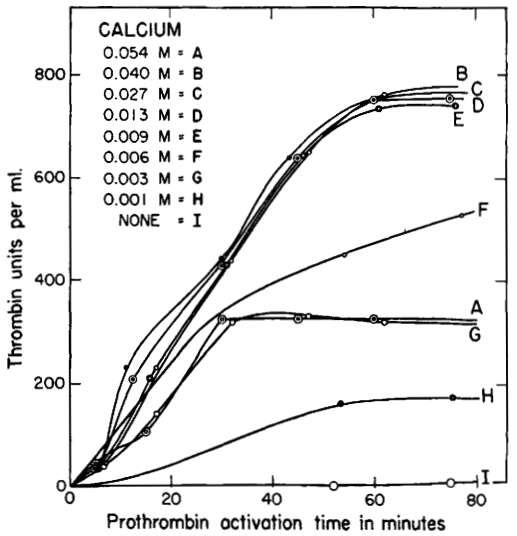

FIG. 1. Activation of purified prothrombin. Only calcium concentration was varied, as indicated for each curve. Molar concentration refers to concentration in reaction mixture described in the text.

activity is thus seen in sharp contrast with the narrow optimum range found with thromboplastin(3) as an activator of prothrombin.

Summary. When threone is involved in the conversion of purified prothrombin to thrombin, the optimum calcium concentration ranges from 0.009 M to 0.04 M. This is a much broader range than found when thromboplastin is used for the activation of prothrombin.

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Attempts to Identify Site of Production of Circulating 'Erythropoietin'. (22556)

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It is becoming rapidly established that humoral mechanisms underlie the erythropoietic effects of anoxia. Thus serum and plasma, or extracts derived therefrom, obtained from animals rendered anemic by bleedings(1-9) or by repeated injections of phenylhydrazine (9-13) exert considerable erythropoietic activity. Attempts to concentrate the active principle(s) from 'anemic' plasma and serum have led some to the conclusion that it is heat-stable and protein-free(9-15). In this connection, boiled filtrates of acidified plasma obtained from blood of phenylhydrazinized rabbits display marked erythropoietic actions in the rat (9-13). Relatively few investigators have attempted to locate the site of formation of the blood erythropoietic factor. Gordon *et al.*(5) detected slight activity in the livers of chronically bled rabbits and other experiments(16) appear to rule out those organs, presumably blood-forming in nature, which are injured by nitrogen mustard.

It seemed important to consider this problem in greater detail by subjecting a variety of organs to the chemical procedure employed

for obtaining 'erythropoietin' from plasma (10,11).

Materials and methods. Donor rabbits. Eight male New Zealand rabbits weighing 4-6 kg were injected daily for 2 days with 1 ml of a 2.5% solution of phenylhydrazine HCl (25 mg) neutralized to pH 7.1. On the 3d day and for the subsequent 2 days, they received 1.5-2.5 ml of this solution (37.5-62.5 mg), graded according to body weight. No treatment was administered on the 6th day because of the death of 2 animals. The remaining 6 animals were intensely anemic on the 7th day, displaying red cell counts of approximately one mill/mm^3 and reticulocyte values ranging from 82 to 96%. At this time, blood obtained from these rabbits by cardiac puncture was heparinized (10 mg/50 ml blood) and centrifuged for 15 minutes. The pooled plasmas (348 ml) were brought to a pH of 5.5 by the addition of 9.9 ml of 1 N HCl and boiled for 10 minutes. Filtration yielded 110 ml of extract. The livers, spleens, thymuses, lungs, brains (excluding medullae and pituitaries), gastrocnemii, bone marrows (femoral, tibial and humeral) and packed blood cells (82-96% reticulocytes) were collected for further preparation. The livers (604 g) were homogenized for 20 minutes in a Waring blendor; 100 ml of acid saline (pH 5.5) were added and the material was acidified to a pH of 5.5 by the addition of 12.1 ml of 1 N HCl. After further homogenization, 300 ml of acid water were added and the mixture was boiled for 10 minutes. Filtration followed the boiling at which time a total volume of 300 ml was collected. One hundred and twenty-five ml of acid saline were added to the splenic substance (25.5 g) during a 20-minute homogenization period. The material was then adjusted to a pH of 5.5 by the addition of 0.8 ml of 1 N HCl. The mixture was boiled for 10 minutes after which it was filtered. A volume of 95 ml of extract was obtained. Thymus material (32.5 g) was also homogenized during which period 125 ml of acid saline were added. The mixture was acidified further to pH 5.5 by addition of 0.21 ml of 1 N HCl. Boiling followed for 10 minutes after which time the material was filtered and 64.5 ml of filtrate collected. One hundred ml of acid saline were added to the lungs (77.5 g) during homogenization. The mixture was then acidified to pH 5.5 by addition of 1.37 ml of 1 N HCl. The material was boiled for 10 minutes and filtration yielded 103.5 ml of extract. The brain substance was homogenized (64.5 g) during which time 125 ml of acid saline were added. Further acidification to pH 5.5 was achieved by the addition of 0.37 ml of 1 N HCl. Boiling for 10 minutes was followed by filtration which yielded 74 ml of extract. One hundred and seventy-five ml of acid saline were added to the muscle tissue (369 g) during homogenization. The homogenate was acidified further to pH 5.5 by the addition of 1.72 ml of 1 N HCl and boiled for 10 minutes following which filtration yielded 188 ml of extract. Bone marrow tissue (60.3 g) was homogenized during which time 150 ml of acid saline were added. Following further homogenization, the mixture was acidified to pH 5.5 by addition of 2.32 ml of 1 N HCl. Boiling followed for 10 minutes after which filtration yielded 173 ml of extract. The packed blood cells (186 ml) were acidified to pH 5.5 by addition of 5.02 ml of 1 N HCl. This mixture was boiled for 10 minutes and filtered; 150 ml of acid saline were added to the residue which was then reboiled for 2 minutes. Filtration followed and both filtrates were combined resulting in a yield of 122 ml. All extracts were stored in a refrigerator at 5°C when not used and brought to room temperature just before injection.

Recipient rats. Nine groups, each consisting of 5 young adult female rats of a modified Long-Evans strain weighing 153-196 g, were employed to assess the hemopoietic activities of the 9 extracts. They were given daily subcutaneous injections for 10 days of the following volumes of extract: plasma—1 ml, equivalent to 2.9 ml of original whole plasma; liver—3 ml, equivalent to 6 g tissue; spleen—1.5 ml, equivalent to 0.39 g tissue, for the 1st 5 days followed by 5 daily 2 ml injections; thymus—1.2 ml, equivalent to 0.6 g tissue; lung—2 ml, equivalent to 1.4 g tissue; brain—1.3 ml, equivalent to 1.04 g tissue; muscle—3 ml, equivalent to 5.7 g tissue; bone marrow—3 ml, equivalent to 1 g

TABLE I. Effects of Boiled Filtrates of Blood and Organs upon Peripheral Red Cell Values in Rats (Means ± Stand. Errors).

	Body wt (g)	RBC (mill/mm³)	Hemoglobin (g/100 ml)	Hematocrit (%)	Reticulocytes (%)
Plasma					
Before	175.4 ± 5.9	7.98 ± .29	17.36 ± .70	50.1 ± 1.54	2.20 ± .23
After	184.2 ± 6.3	10.38 ± .45†	19.03 ± .61	58.0 ± 1.36†	4.40 ± .33†
Liver					
Before	165.2 ± 3.2	7.70 ± .25	16.95 ± .64	53.0 ± 2.28	3.22 ±1.71
After	170.8 ± 3.5	7.42 ± .12	16.83 ± .36	51.3 ± .97	2.30 ± .55
Spleen					
Before	166.8 ± 5.1	8.27 ± .15	16.84 ± .22	51.2 ± .73	2.80 ± .61
After	172.4 ± 5.6	7.74 ± .12*	16.34 ± .20	48.5 ± 1.24	2.80 ± .65
Thymus					
Before	173.6 ± 3.9	7.64 ± .15	16.04 ± .55	49.4 ± 1.58	3.60 ± .50
After	185.2 ± 5.5	6.92 ± .16*	15.59 ± .67	47.9 ± 1.47	2.50 ± .40
Lung					
Before	174.6 ±10.1	7.68 ± .26	16.38 ± .34	50.4 ± 1.39	1.70 ± .66
After	184.0 ± 8.7	7.74 ± .25	16.44 ± .38	47.9 ± 1.29	2.30 ± .26
Brain					
Before	153.2 ± 8.0	8.06 ± .25	16.62 ± .41	51.9 ± .65	2.30 ± .33
After	156.2 ± 6.8	8.07 ± .19	16.56 ± .18	50.7 ± 1.03	2.50 ± .08
Muscle					
Before	169.2 ± 4.8	8.24 ± .25	17.39 ± .42	52.2 ± 1.18	2.40 ± .25
After	172.4 ± 4.7	7.76 ± .08	16.83 ± .65	51.0 ± .98	1.90 ± .19
Bone marrow					
Before	175.6 ± 6.7	8.02 ± .17	17.33 ± .42	53.6 ± .54	1.76 ± .17
After	180.0 ± 7.2	8.54 ± .14*	17.39 ± .30	54.0 ± 1.41	2.04 ± .33
Packed red cells					
Before	179.4 ± 3.6	8.02 ± .27	16.07 ± .29	53.9 ± 1.98	3.20 ± .34
After	179.6 ± 3.1	7.74 ± .16	16.52 ± .70	55.5 ± 1.87	2.30 ± .45

* Indicates probability value, $P<0.05$ for the means 'after' as compared to those 'before' treatment.

† Indicates probability value, $P<0.01$.

tissue and packed cells—2 ml, equivalent to 3 ml of cells. All rats were sacrificed on the 11th day for determinations of the peripheral and bone marrow cellular values. Peripheral cell counts were also taken in all rats before experimental treatment was initiated. Forty untreated rats, studied over the course of a year, which overlapped the period of the present experiments, contributed to the control myelogram values. Freely flowing blood from the tail was employed for the peripheral cell determinations. Red cell counts were made in duplicate and differential counts were determined from smears stained with Wright's. Eosinophil numbers were estimated by the chamber method using Randolph's fluid(17). Reticulocyte counts were made from dry smears of heparinized blood stained with new methylene blue(18); 1000 red cells were counted for each determination and the reticulocytes expressed as a percentage of these. Hemoglobin concentrations were estimated by the acid hematin method with a photoelectric colorimeter. Duplicate hematocrit values from heparinized blood, spun at 11,000 rpm for 5 minutes, were obtained by a capillary micromethod. At termination of the experiments, all recipient rats were anesthetized with ether and exsanguinated by cardiac puncture. The right femur was dissected, split lengthwise and the bone marrow removed and placed in a watch glass containing homologous serum. The marrow was prepared in suspension form by gently drawing it up and down in a glass pipette. Smears were then made, fixed immediately in absolute methanol for 2-5 minutes and treated with May-Grünwald stain. For each animal, a minimum of 1500 nucleated cells was counted and classified according to previously reported procedures(19).

Results. Table I indicates that the acidi-

TABLE II. Effects of Boiled Filtrates of Blood and Organs upon Myelograms of Rats (Mean % ± Stand. Error).

	Nuc. RBC	Lympho-cytes	Neutrophils* Young	Neutrophils* Mature	Eosinophils Young	Eosinophils Mature	Blasts	Misc.
Untreated controls	35.1 ± 1.9	8.9 ± 2.0	9.0 ± .7	36.8 ± 1.0	.2 ± .5	7.6 ± 1.0	.2 ± .8	2.2 ± .8
Plasma	51.8 ± 3.7‡	8.6 ± 3.4	7.7 ± 1.6	25.4 ± 2.6‡	.3 ± .2	4.7 ± 1.0	None	1.5 ± .4
Liver	33.1 ± 3.5	6.0 ± 1.3	15.3 ± 2.4†	35.8 ± 1.5	.8 ± .6	6.6 ± 1.7	.2 ± .2	3.2 ± .9
Spleen	29.9 ± 3.2	6.8 ± 2.7	16.0 ± 1.8‡	31.0 ± 3.6	1.2 ± .8	12.8 ± 2.2†	.3 ± .2	4.0 ± .5
Thymus	35.6 ± 2.4	5.2 ± 1.4	11.6 ± .7	32.4 ± 3.1	.2 ± .2	7.6 ± 1.2	None	5.6 ± 1.0†
Lung	29.6 ± 3.7	7.4 ± 1.7	11.4 ± 1.8	38.4 ± 1.5	1.0 ± .2	9.6 ± 1.3	"	3.2 ± 1.4
Brain	38.4 ± 3.7	5.2 ± 1.8	12.6 ± 2.5	37.0 ± 3.8	.4 ± .4	5.0 ± .9	.4 ± .2	1.0 ± .4
Muscle	33.8 ± 3.3	9.6 ± 2.2	9.4 ± 2.6	38.2 ± 1.2	.4 ± .2	6.0 ± .9	None	2.6 ± .7
Bone marrow	31.0 ± 5.2	6.8 ± 2.4	13.4 ± 2.3	41.4 ± 6.8	None	5.2 ± 1.3	.2 ± .2	2.0 ± .2
Packed red cells	39.0 ± 2.0	5.0 ± 1.6	16.4 ± 2.7†	34.0 ± 2.0	.2 ± .2	3.8 ± 2.0	.2 ± .2	1.4 ± .7

* Young neutrophils include promyelocytes and myelocytes; mature neutrophils include segmented forms.

† Indicates probability values, $P<0.05$ for means compared to controls.

‡ " " " , $P<0.01$.

fied boiled filtrate of plasma obtained from phenylhydrazinized rabbits resulted in highly significant rises in the peripheral red cell, hematocrit and reticulocyte values as well as a trend towards an increase in hemoglobin levels in the recipient rats. The elevation in red cell numbers exceeded, percentagewise, that experienced by the hematocrit and hemoglobin values. Apart from a slight increase in the red cell counts brought about by the marrow extract and slight decreases induced by the spleen and thymus extracts, none of the organ or packed cell filtrates evoked any significant changes in the peripheral red cell parameters. Substantial body weight gains were experienced by most groups except those injected with packed cells, muscle and brain material.

Total white, differential and eosinophilic cell (chamber count) values were not altered significantly by the filtrates of plasma, packed cells or any of the organs employed.

Table II shows that among the different materials tested, only the plasma extract stimulated erythropoiesis within the femoral bone marrow. The highly significant increases in the percentages of nucleated erythrocytes, induced by the plasma filtrate, were accompanied by a drop in the percentages of mature neutrophilic forms. The extracts of liver, spleen and packed cells induced increases in the percentages of young neutrophilic elements while those of spleen and thymus evoked rises in the percentages of mature eosinophilic cells and miscellaneous elements respectively. No other significant actions were exerted by the tissue extracts upon the marrow parameters examined.

Discussion. The present results confirm previous reports(5,9-13) that boiled filtrates of plasma obtained from anemic rabbits contain considerable erythropoietic activity. This is evidenced by the significant elevations in peripheral red cell numbers, hematocrit and reticulocyte percentages and by the increased percentages of marrow nucleated erythrocytes in the rats receiving daily injections of this filtrate. It is of interest that in these experiments, unlike those previously reported (10,13), the percentage increases in peripheral red cell numbers were greater than those displayed by the hematocrit and hemoglobin values, which is indicative of the production, in response to the filtrate, of smaller-size erythrocytes associated with a lowered corpuscular hemoglobin. Similar results have been reported by Linman and Bethell(9). The explanation for the microcytosis is not evident although preliminary experiments(20) tend to indicate that alterations in iron metabolism may be responsible for this phenomenon.

None of the organ or packed cell extracts employed in this study proved to be erythropoietic. This should not be considered as indicating necessarily that the various tissues examined do not participate in the production of the erythropoietic factor. It is conceivable, for example, that an efficient storage

mechanism for this principle does not exist, with the factor being released into the circulation as rapidly as it is produced. The present results indicate that plasma may be the only site from which appreciable amounts of 'erythropoietin' are recovered. Experiments are in progress to determine whether the factor may actually be formed in plasma as the result of an enzymatic action upon substrates provided by the blood-forming organs.

Summary. Acidified boiled filtrates were prepared of plasma, liver, spleen, thymus, lung, brain, skeletal muscle, bone marrow and packed blood cells of rabbits made severely anemic by phenylhydrazine. When tested in normal rats, only the plasma extracts proved to be erythropoietic, as evidenced by significant elevations in peripheral red cell, hematocrit and reticulocyte values as well as by a marked increase in the percentages of marrow nucleated erythrocytes. The site of formation of circulating 'erythropoietin' remains unidentified.

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Canine Intestinal and Liver Weight Changes Induced by *E. coli* Endotoxin. (22557)

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Shock has been observed in the human following transfusion of contaminated blood(1), the injection of purified products of bacteria (endotoxin) to induce hemorrhage in malignant tumors(2), and following the treatment of Gram-negative bacteremia with antibiotics. Irreversible hemorrhagic shock in the dog has also been related to the deleterious effects of bacterial products(3). Previous investigation(4) has shown that the intravenous injection of endotoxin prepared from *Escherichia coli* regularly results in a profound decline in systemic blood pressure with an accompanying elevation of portal vein pressure. This prompt hemodynamic reaction did not occur in the hepatectomized animal. Three possible explanations for this phenomenon are evident: 1) large quantities of blood are trapped in the liver, 2) the liver releases vasodepressor substances or 3) both 1 and 2 occur. Attempts