TABLE II. Leucocyte Counts in Peaks of Volume Spectrum Relative to Standard Differential Count.

	First peak (small lymphocytes) (%)	Second peak (large cells) Saponin lysis (%)	Whole blood (%)
Average of 15 measurements ± S.D.*	102 ± 23	69 ± 20	107 ± 13

* Standard deviation.

saponin lysis method, was less stable, and generally yielded a count lower than the differential. This is consistent with the experience of others(6), where it was observed that lymphocytes almost always displayed a more normal morphological appearance and swelled less readily than polymorphonuclear granulocytes. However, the large peak obtained with whole blood that had merely been diluted yielded a count in good agreement with that obtained by standard hematological procedures. Thus, it appears that some quantitative information on the distribution of cell types can be obtained from the volume spectrum; however, this is no substitute for a standard differential. In order to improve this technique, a more satisfactory leucocyte concentration method is needed; work on this problem is in progress.

Summary. Leucocytes from normal adults have been studied by electronic sizing and separating methods. The volume distribution was found to be bimodal. Three cell groups of small volume dispersion were isolated and subsequently identified by hematological methods. The smaller-volume leucocyte peak was found to contain small lymphocytes and little else. The large-volume peak contained granulocytes in its central portion and monocytes in its large-volume tail. Using these methods, the relative abundance of a specific type of leucocyte can be greatly increased. These methods may also be applied to other cell suspensions.

The authors thank J. M. Hardin, G. A. Drake, P. M. LaBauve, C. F. Bidwell, J. R. Coulter, R. D. Hiebert, R. B. Glascock and J. D. Gallagher for help in carrying out this investigation.

1. Fulwyler, M. J., Science, 1965, v150, 910.
2. Griffiths, J. D., Salsburg, A. J., in Circulating Cancer Cells, Charles C. Thomas, Publ., Springfield, Ill., 1965, pp19-22.
3. Herbeuval, R., Herbeuval, H., Duheille, J., Triangle, 1963, v6, 47.
4. Eriksson, O., Cancer, 1962, v15, 171.
5. Van Dilla, M. A., Spalding, J. F., Nature, 1967, v213, 708.
6. Wasastjerna, C., Scand. J. Clin. Lab. Invest., 1956, v8, 14.

Received February 3, 1967. P.S.E.B.M., 1967, v125.

An *in vitro* Assay of Erythropoietin.* (32094)

H. P. WARD (Introduced by J. K. Aikawa)
Department of Medicine, Veterans Administration Hospital and University of Colorado School of Medicine, Denver, Colo.

The ability of erythropoietin to stimulate heme synthesis in cultures of bone marrow has been demonstrated by many workers (1-3). Recently, Krantz and coworkers(3,4) described a method of bone marrow culture in which heme synthesis, for periods of incubation longer than 12 hours, was dependent upon the presence of erythropoietin in the culture media. They suggested that the ability of erythropoietin to prolong heme synthesis *in vitro* might be used as an assay for erythropoietin.

We(5) recently used the Krantz method of

* Supported by Clinical Investigator Program, Veterans Administration Hospital.

bone marrow culture in order to investigate the effect of chloramphenicol on heme synthesis and RNA synthesis. In the current study, the effect of erythropoietin on heme synthesis in this culture system was investigated. A log-dose response relationship for erythrpoietin was demonstrated that provides a more sensitive and rapid bioassay for serum levels of erythropoietin than the polycythemic mouse assay(6). In addition, the influence of actinomycin D, trypsin, and erythropoietin antibody on the erythropoietin effect was studied.

Materials and methods. The methods of culture and extraction of Fe^{59} heme have been described previously(5). Modifications of the culture technique, in order to accentuate the effect of erythropoietin, will be discussed in *Results.* In brief: bone marrow was washed from the ribs of a dog with NCTC 109 medium. Fetal calf serum and autologous dog serum were added in concentrations listed in *Results.* A volume of 2.6 ml of this suspension was placed in sterile tissue culture dishes and 0.2 ml of $Fe^{59}Cl$ suspended in autologous dog serum was added. The 0.2 ml of $Fe^{59}Cl$-serum had 3 μc of $Fe^{59}Cl$ for a final culture activity of 1 μc per ml. In all experiments, a volume of 0.2 ml of erythropoietin Standard B suspended in pooled AB+ serum was added to 2.8 ml of the bone marrow suspension culture. The erythropoietin rich cultures were compared with control cultures in which pooled AB+ serum or NCTC 109 had been added.

Actinomycin D was obtained from Merck Sharp and Dohme. Trypsin was purchased from Calbiochemicals, Los Angeles, Calif. $Fe^{59}Cl_3$ was obtained from Abbott Laboratories, North Chicago, Ill. Fetal calf serum and NCTC 109 were purchased from Colorado Serum Co., Denver, Col. The erythropoietin Standard B was obtained from the National Institute for Medical Research, Mill Hill, London, England. Erythropoietin antibody was obtained from J. C. Schooley and J. F. Garcia, Donner Laboratory, University of California, Berkeley. The preparation and properties of the immune serum have been described(7).

The *in vivo* assays for erythropoietin were performed by M. Gesink, National Jewish Hospital, Denver, using the hypertransfused mouse preparation(6).

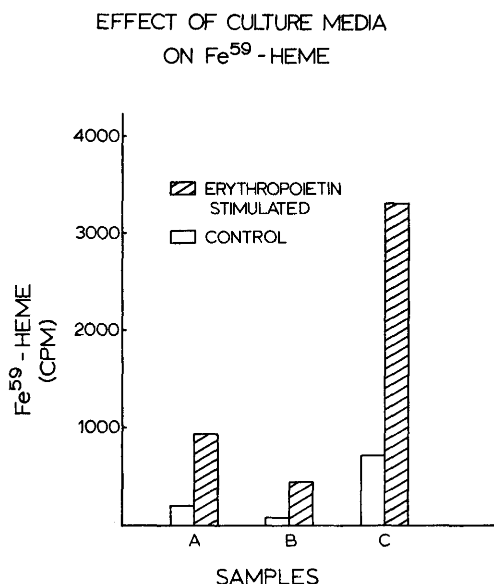

FIG. 1. Effect of the culture medium on Fe^{59} incorporation into heme. $Fe^{59}Cl_3$ suspended in isologous dog serum was added to cultures at 20 hrs of incubation. Cultures were harvested after 6 additional hrs of incubation. A-50% NCTC 109, 38% dog serum, 12% fetal calf serum; B - 88% NCTC 109, 12% fetal calf serum; C - 50% NCTC 109, 50% fetal calf serum.

Results. Development of culture method. Various concentrations of NCTC 109, fetal calf serum, and isologous dog serum were tested to determine which method of culture provided the greatest Fe^{59} incorporation into heme (Fig. 1). Maximum incorporation of Fe^{59} into heme was demonstrated in cultures prepared with 50% fetal calf serum and 50% NCTC 109. These cultures were responsive to erythropoietin-rich serum.

To determine whether the rate of heme synthesis changed during incubation, $Fe^{59}Cl_3$ was added to the cultures after various periods of incubation (Fig. 2). The rate of Fe^{59} incorporation into heme gradually decreased in cultures without erythropoietin. In the presence of erythropoietin, Fe^{59} incorporation into heme remained relatively constant during 20 hours of incubation. At 48 hours, heme synthesis was virtually absent in the control cultures. The erythropoietin stimulated cultures demonstrated Fe^{59}-heme synthesis but at a greatly decreased rate.

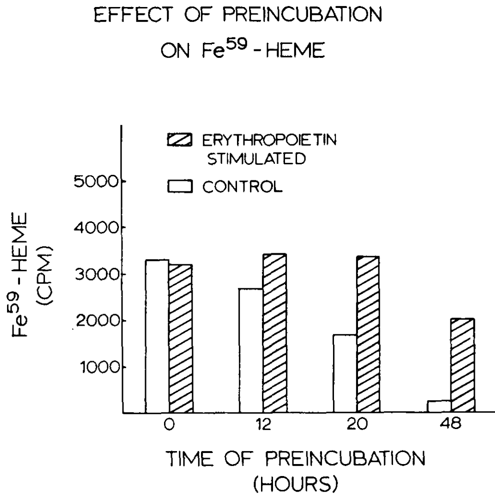

FIG. 2. Effect of preincubation on Fe^{59} incorporation into heme. $Fe^{59}Cl_3$ suspended in isologous dog serum was added into cell cultures at 0, 12, 20 or 48 hrs. Cultures were harvested after 6 additional hrs of incubation.

To assay erythropoietin *in vitro,* the previously described(5) method of bone marrow culture was modified to take advantage of the influence of culture media and prior incubation of the cells on the Fe^{59} incorporation with heme. The bone marrow cells were cultured in 50% NCTC 109 and 50% fetal calf serum. The cells were incubated for 20 hours with erythropoietin prior to the addition of $Fe^{59}Cl$. Fe^{59} incorporation into heme was determined after 6 additional hours of incubation.

Dose response relationship. In a series of experiments, erythropoietin standard B and serum from 2 patients with elevated levels of erythropoietin were assayed, Table I. The covariance analysis showed that the regression slopes among experiments were significantly different ($F = 3.32$, $p<0.005$). Further analysis showed that the source of the heterogeneity was most likely due to a difference in bone marrow samples and not a difference among erythropoietin preparations. The influence of variation in bone marrow samples is demonstrated in Fig. 3, P and P′ (Table I, 2b and 2a). The same erythropoietin-rich serum was assayed in cultures of bone marrow prepared from 2 mongrel dogs. The slopes of the log-dose response line were different although each line demonstrated a relatively good fit by the method of least-squares. However, when the same bone marrow preparation was used to investigate the dose response relationship of different erythropoietin preparations, similar slopes were obtained, Table I: 1b, 1c, and 3a. Standard B suspended in NCTC 109 or in pooled human AB+ serum demonstrated similar slopes, Fig. 3, S and S′.

TABLE I. Slope of the Log-dose Response Regression Line in *in vitro* Assay of Erythropoietin.

Preparation*	Slope of Regression line (±SE)
1. Standard B suspended in:	
a. NCTC 109	62.1 ± 10.9
b. "	54.8 ± 5.0
c. Serum	49.0 ± 6.3
d. "	67.2 ± 10.6
e. "	56.0 ± 10.2
2. Patient Standard #1	
a. Serum	40.1 ± 10.0
b. "	65.8 ± 5.2
c. "	56.8 ± 5.3
3. Patient Standard #2	
a. Serum	52.9 ± 5.3
b. "	36.5 ± 9.5
c. "	57.5 ± 8.2
d. "	19.0 ± 3.5

* In each assay bone marrow from a different dog was used except for 1b, 1c, and 3a in which bone marrow from the same dog was cultured. The slope of the regression line was determined by the least squares method.

In a previous publication(5), we reported that a variation of Fe^{59} incorporation into heme of greater than 5% between duplicate

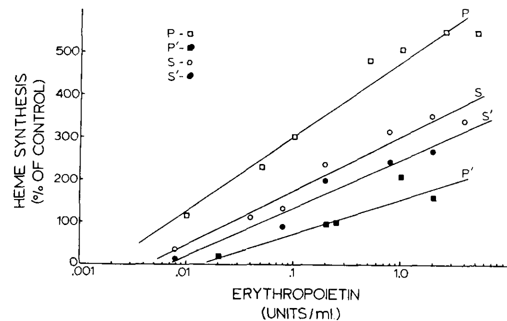

FIG. 3. Log-dose response regession line of *in vitro* ehythropoietin assay. S - Standard B suspended in NCTC 109, S' - Standard B suspended in pooled AB pos. serum, P - erythropietin-rich serum from patient (W.M.) assayed in bone marrow from dog #1, and P' - erythropoietin-rich serum from patient (W. M.) assayed in bone marrow from dog #2.

samples was rarely observed. Additional experience with this technique has confirmed this observation in cultures that demonstrate active Fe^{59}-heme synthesis. However, in cultures with low Fe^{59} incorporation into heme (total count of less than 5 times background) a variation between duplicate samples of greater than 5% was frequently found. Therefore, in order to assay for erythropoietin *in vitro,* the incorporation of Fe^{59} into heme in the control cultures had to be greater than 5 times the background count. By keeping the variation between duplicate samples at 5% or less, a significant difference between control cultures and erythropoietin-stimulated cultures was placed at 25%. An erythropoietin level of approximately 0.05 units/ml consistently demontrated a 25% or greater Fe^{59} incorporation into heme (Fig. 3). The central linear portion of the log-dose response line was 0.05 to 1 unit/ml. Levels of erythropoietin greater than 1 unit/ml failed to demonstrate a log-dose response.

Specificity of erythropoietin. Human serum has an unlimited number of factors that could influence Fe^{59} incorporation into heme. To evaluate the likelihood that erythropoietin was responsible for the stimulation of Fe^{59}-heme, the following tests were performed (Table II). Antierythropoietin blocked the stimulation of Fe^{59} incorporation into heme. Previous incubation of anemic serum with trypsin eliminated the stimulation of Fe^{59} incorporation into heme. Actinomycin D blocked the ability of erythropoietin-rich serum to stimulate Fe^{59}-heme.

Discussion. The important discovery by Krantz and coworkers(3) that erythropoietin prevents the decrease in Fe^{59}-heme synthesis with prolonged incubation was confirmed (Fig. 2). A method for *in vitro* assay of erythropoietin was established by taking maximum advantage of this ability. A preincubation period of 20 hours avoided the lower counts observed frequently with prolonged incubation. Fetal calf serum enhanced Fe^{59}-incorporation into heme without decreasing the responsiveness of the cultures to erythropoietin. What factor(s) in the fetal calf serum was responsible for the stimulation of heme synthesis is not known. The possibility that fetal calf serum was rich in erythropoietin was excluded by the failure of fetal calf serum to stimulate Fe^{59} incorporation into red cells in the hypertransfused mouse assay. The possibility that growth hormone was responsible for the enhanced heme synthesis was investigated by adding purified growth hormone (70 μg/ml)† to the cultures without fetal calf serum. Growth hormone did not stimulate Fe^{59} incorporation into heme. Previous autoradiographic studies (8) demonstrated a direct relationship of Fe^{59} uptake by nucleated red cells and the specific activity of Fe^{59} in the suspending media. In the current study, the serum iron of fetal calf serum was higher than isologous dog serum. Therefore, the specific activity of Fe^{59} in the cultures with fetal calf serum was lower than cultures prepared with dog serum. The cultures with fetal calf consistently demonstrated a greater visual cell layer on the bottom of the culture dish than the cultures without fetal calf serum. This "sticking factor" has been the generally accepted explanation for the effect of fetal calf serum on *in vitro* cultures of cells. Our observations would support this concept.

TABLE II. Effect of Erythropoietin "Inhibitors" on the Erythropoietin Rich Serum Stimulation of Fe^{59} Incorporation into Heme.

Preparation	Fe^{59} Incorporation into heme: cpm	% Greater than control
Control	368	
Erythropoietin	1346	268
a. Erythropoietin + Antibody	379	3
Antibody alone	325	(−12)
b. Erythropoietin + Actinomycin D*	481	30
Actinomycin D* alone	293	(−20)
c. Erythropoietin + Trypsin†	533	45
Trypsin† alone	547	49

* Actinomycin D, 5 μg/ml.
† Trypsin, 500 μg/ml.

The variation in the log-dose response regression slopes is probably due to variation between bone marrow specimens from different dogs. When the same bone marrow was used to assay different erythropoietin samples

† Obtained from H. Elrick and T. Lee, Protein Hormone Bank, V. A. Hospital, Denver, Colo.

a similar slope was obtained. When the same erythropoietin sample was assayed in different bone marrow the slopes were not similar. Therefore, when a serum sample(s) is to be assayed for erythropoietin, bone marrow cultures are prepared from one dog and a dose response relationship of a known (control) erythropoietin preparation is determined. The erythropoietin level of the unknown serum sample is obtained by relating the response of the unknown serum sample to the log-dose response regression of the control specimen.

Variation of the dose response regression has been noted in the *in vivo* assay of erythropoietin(9) and other hormones, including ACTH and gonadotropins(10). Perhaps the variation demonstrated in biological assays represents a limitation of hormone binding on receptor sites of the target tissue and not intrinsic errors of the method itself. Several hormones, including erythropoietin(11), appear to stimulate the production of messenger RNA in specific target tissues. The mechanism of stimulation is unknown, but a popular hypothesis is that the hormone acts as a chemical de-repressor permitting one or more operons to synthesize new messenger RNA. The kinetics of the hormone-repressor substance interaction is speculative. Failure of high doses of erythropoietin to increase Fe^{59} heme synthesis in our studies might represent maximum de-repression of the finite cell population of the *in vitro* culture.

The stimulation of Fe^{59} incorporation into heme most likely represents the effect of erythropoietin in the serum. Erythropoietin antibody obtained from Schooley and Garcia neutralized the effect of erythropoietin rich serum *in vitro*. Extensive investigation by these workers has demonstrated the ability of the erythropoietin antiserum to block erythropoietin *in vivo*. The ability of trypsin to inactivate serum erythropoietin has been demonstrated by several investigators(12). A tryptic digestion of the erythropoietin rich serum blocked the stimulation of Fe^{59} incorporation into heme in our cultures. Krantz and Goldwasser(11) have recently demonstrated that actinomycin D blocks the erythropoietin stimulation of rapidly labeled RNA and of Fe^{59} heme in bone marrow cultures. In agreement with their findings, we observed that actinomycin D prevented the stimulation of Fe^{59} heme synthesis by erythropoietin. However, actinomycin D inhibited synthesis of Fe^{59} heme in the control cultures without erythropoietin (Table II). Therefore, definite conclusions concerning the ability of actinomycin D to specifically inhibit erythropoietin cannot be made.

Summary. The ability of erythropoietin to stimulate Fe^{59} incorporation into heme was confirmed. A method of bone marrow culture was designed in order to assay erythropoietin *in vitro*. A log-dose response relationship was demonstrated with a lower limit of sensitivity of 0.05 units/ml. The effect of erythropoietin *in vitro* was blocked by erythropoietin antibody, trypsin, and actinomycin D.

The author wishes to thank Janet Holman for her capable laboratory assistance, and Dr. Edmond A. Murphy, for assistance in the statistical evaluation of this study.

1. Erslev, A. J., Proc. Soc. Exp. Biol. & Med., 1962, v110, 615.

2. Korst, D. R., Frenkel, E. P., Wilrelm, J. E., in Erythropoiesis, Jacobson, L. O. & Doyle, M., ed., Grune & Stratton, New York, London, 1962, p310.

3. Krantz, S. B., Gallien-Lartigue, O., Goldwasser, E., J. Biol. Chem., 1963, v238, 4085.

4. Gallien-Lartigue, O., Goldwasser, E., Science, 1964, v145, 277.

5. Ward, H. P., J. Lab. Clin. Med., 1966, v68, 400.

6. Fried, W., Plzak, L. F., Jacobson, L. O., Goldwasser, E., Proc. Soc. Exp. Biol. & Med., 1957, v94, 237.

7. Schooley, J. C., Garcia, J. F., Blood, 1965, v25, 204.

8. Ward, H. P., Tauxe, W. N., Kiely, J. M., Brit. J. Haemat., 1966, v12, 99.

9. Keighley, G., Lowry, P. H., Borsook, H., Rambach, W. A., Stohlman, F. Jr., Van Dyke, D. C., Blood, 1960, v16, 1424.

10. Gaddum, J. H., Pharmacol. Rev., 1953, v5, 87.

11. Krantz, S. B., Goldwasser, E., Biochim. Biophys. Acta, 1965, v103, 325.

12. Lowy, P. H., Borsook, H., in Erythropoiesis, Jacobson, L. O., & Doyle, M., ed., Grune & Stratton, New York, London, 1962, p33.

Received February 17, 1967. P.S.E.B.M., 1967, v125.