Exhibit 33

to the Declaration of Cullen N. Pendleton in Support of Amgen's Opposition to Roche's Motion for Summary Judgment that Claim 7 of the '349 Patent is Invalid Under 35 USC §112 and is Not Infringed

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An Assay for Erythropoietin *in Vitro* at the Milliunit Level

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ABSTRACT. A method is described for the assay of erythropoietin using primary cultures of adult rat bone marrow cells. Either total labeled iron uptake by the cells or hematin synthesis from labeled iron may be used as the measure of erythropoietin action. The method is useful in the range 0.001 to 0.010 U, where the log response is linear with the log dose, and can be carried out in 2 working days. This method has the disadvantage of detecting asialoerythropoietin which has no activity *in vivo*. (*Endocrinology* 97: 315, 1975)

THE need for a rapid, sensitive, and precise method of assay for erythropoietin has been apparent for a long time. Conventional bioassay methods, based on measurement of increased rates of red cell formation, require either too much erythropoietin, too much time, or both, in addition to having a low degree of precision. In the 11 years since we published data showing that rat bone marrow cells, in primary culture, respond to graded amounts of erythropoietin with a dose-response curve similar to that found *in vivo* (1), there have been several reports (2–5) showing that marrow or fetal liver cells can be used *in vitro* for the quantitative estimation of erythropoietin. In this paper, we describe an *in vitro* method for the routine, rapid determination of erythropoietin in the range from 0.001 to 0.01 U.

Materials and Methods

Cells. Femora and tibiae from male Sprague-Dawley rats (10 to 12 weeks old) are the source of marrow cells. Cells are removed sterilely by flushing out the bones after puncturing one end with a 22 gauge needle and injecting medium into the other end. Several flushings are needed to maximize the yield, which should be 4–5 × 10^8 nucleated cells per rat. The cell clumps are dispersed by repeated gentle ejection from a dropper or pipet. Rat marrow cells tend to aggregate very readily, and repeated dispersal is required to yield reproducible aliquots. Before the final dilution is made, the cell suspension is filtered through a sterile stainless steel screen (100 mesh) to remove bits of bone and connective tissue.

Medium. The basic constituents of the medium are NCTC 109, fetal calf serum, and rat serum. As long as the original NCTC 109 is used, rather than the modification, NCTC 135, the source of supply is immaterial. Tests of additions to the NCTC 109 formulation are described below.

The fetal calf serum is heat inactivated at 56 C for 30 min before use, in order to minimize the loss of erythrocytes during the culture period due to complement-promoted lysis. Each new lot of serum must be tested for adequacy of cellular response to erythropoietin before it can be used routinely. We have found that lots of serum from K. C. Biological Inc., Lenexa, Kansas; International Scientific Industries Inc., Cary, Illinois; Baltimore Biological Laboratories, Cockeysville, Maryland; and Reheis Chemical Company, Kankakee, Illinois, were all suitable.

Rat serum is used as a source of transferrin, both for unlabeled iron, which is required for optimal response by the cells (6), and for labeled iron. Data from this laboratory have shown that rat marrow cells utilize iron from rat transferrin to a considerably greater extent than from transferrins of other species, although human transferrin can also be used (7). As a source of unlabeled iron we use unhemolyzed rat serum to which 73 nmoles of ferric nitrate per ml have been added. Rat transferrin is labeled with radioiron as follows: to 5.0 ml of frozen, unhemolyzed serum are added 4.0 ml of NCTC 109, 0.5 ml of 0.9M $NaHCO_3$, and 0.5 ml

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of ^{59}Fe (supplied as $^{59}Fe\ Cl_3$ in 0.1M HCl, 200 μCi/ml, 13 to 30 mCi/mg of Fe, by Amersham-Searle Company, Arlington Heights, Illinois). The serum is allowed to thaw, is mixed thoroughly, without frothing, and stored at 4 C overnight before use. This quantity is sufficient for 500 samples. If a large number of assays is not being done routinely, it would be better to prepare a smaller quantity rather than to store the labeled serum for an extended time.

Method. The details of how the conditions of the assay method were determined are presented in the Results section of this paper, the final method is described here.

Cells are suspended in sterile medium consisting of 30% fetal calf serum, 5% rat serum with unlabeled iron, and 65% NCTC 109 containing 30 mM morpholinopropane sulfonic acid (MOPS) at pH 6.9. In addition, the medium contains 0.05 mg/ml of Gentamicin (Schering Corporation) as the sole antibiotic. The nucleated cell density is adjusted to 15 million per ml as determined by hemocytometer count.

Two-tenths milliliter aliquots of the cell suspension are pipetted into the wells of "Disposo" trays (Model FB-16-24-TC, Linbro Chemical Company, New Haven, Connecticut) and, where indicated, erythropoietin samples (1 to 10 μl), dissolved in 0.1% bovine serum albumin, 0.15M NaCl, are added to the appropriate wells. We use six replicates per sample, including the control group containing no exogenous erythropoietin. The wells are sealed with the sterile plastic sheet supplied by the manufacturer and incubated at 37 C for 20 h. The use of MOPS as a buffer eliminates the need for gassing with CO_2 as long as the trays are kept sealed. After the 20 h, ^{59}Fe-labeled rat serum is added, 20 μl per well, using a 1.0 ml syringe (Hamilton Model 100) and a repeating dispenser (Hamilton Model PB 600-1). The trays are re-sealed and incubated at 37 C for 5 h, after which the contents of each well are washed over into glass culture tubes (13 × 100 mm) with 2 × 1.0 ml of a slightly hypertonic solution (0.145M NaCl, 0.013M phosphate, pH 7.3). Washing the cells with this solution reduces nonspecific adsorption of iron to the cells (8). The tubes are centrifuged at 500 to 1000 × g for 10 min at 4 C, and the supernatant solution is removed by careful aspiration. The cells are washed once with 1.5 ml of the same solution, then twice with 1.5 ml of cold 5% TCA.

At this stage, the amount of ^{59}Fe incorporated into the cell pellet may be determined if only the total iron uptake is to be measured (8). We also measure the incorporation of ^{59}Fe into hematin by the following procedure: The cell pellet is suspended in 0.5 ml of the phosphate-saline solution used above. After brief mixing with a Vortex mixer, 0.5 ml of Drabkin's solution [12 mM $NaHCO_3$, 0.8 mM KCN, 0.6 mM $K_3Fe(CN_6]$ and 0.10 ml of a solution of carrier hemoglobin (2.6 mg/ml) are added. If no carrier is added, the loss of labeled hematin at the interface during the subsequent extraction step becomes appreciable. At this stage, the total uptake of ^{59}Fe is determined in the same culture tubes by counting in an automatic gamma counter. After counting, 0.1 ml of 1M HCl is added to each tube, the suspension mixed thoroughly, 2 ml of cyclohexanone are added and the contents of the tubes mixed very thoroughly. The tubes are then centrifuged at room temperature to separate the phases; 1.0 ml aliquots of the upper (organic) phase containing hematin are removed with an automatic pipet and delivered to glass tubes for counting.

Materials. Organic buffers were bought from Sigma Chemical Company, St. Louis, Missouri; NCTC 109 from Microbiological Associates, Bethesda, Maryland; cyclohexanone from Eastman Kodak, Rochester, New York, and disposable glass tubes from Becton-Dickenson and Company, Rutherford, New Jersey. Thin layer chromatography was done on silica-coated plastic sheets from Brinkman Instrument Company, Westbury, New York, using a solvent mixture consisting of 25 ml methanol, 10 ml chloroform, and 3 ml pyridine. The developed chromatogram was scanned on a Packard Model 7200 radiochromatogram scanner.

Results

The technique of hematin extraction used originally (1), which was based on the method of Teale (9), had two disadvantages: (a) butanone-2 is appreciably soluble in water and the volume of the heme extract is influenced by the volume of the aqueous phase, making it necessary to remove all of the organic phase for counting, with the danger of contamination by a small amount of the highly labeled water phase; and (b) under some conditions a small, but appreci-

able fraction of the nonhematin radioactivity is extracted into the butanone. Replacement of butanone with cyclohexanone eliminates the first of these problems, since the latter is very slightly soluble in water. With short-term cultures, the second problem also disappears. Thin-layer chromatographic analysis of the cyclohexanone extract shows that only a negligibly small amount of the total radioactivity remains at the origin, where inorganic iron is to be found. the remainder is associated only with hematin (Fig. 1). The amount of nonhematin ^{59}Fe varies with the experiment from about 1% to 6%. We find that, after exposure to trichloracetic acid, there are two iron-porphyrin components resolved by the thin layer method. They have R_f values of 0.55 and 0.65; both contain labeled iron and have hematin absorption properties. We have not identified these compounds since, for the purpose of this paper, it is sufficient to indicate that essentially only hematin ^{59}Fe is present in the cyclohexanone extract. When longer-term cultures (48 h or more) are employed, an appreciable fraction of the radioactivity extracted is not hematin, and an alternative method should be used (10). This is important for the study of erythropoietin action *in vitro*, but not for the purpose of the assay as described here. When we used ethyl acetate as the nonaqueous phase as described by Thunell (11), only about 75% of the labeled hematin was recovered.

Since we have shown previously (12) that essentially all of the heme radioactivity is derived from hemoglobin, it is justifiable to use the cyclohexanone extraction method as a measure of hemoglobin synthesis.

The effects, on both iron uptake and hemoglobin synthesis, of increasing the percentage of fetal calf serum in the medium are shown in Fig. 2. The data indicate that, as the percent of fetal calf serum is raised, there is a greater effect on erythropoietin-stimulated cells than on control cells. There is only a small advantage in using more than 20% fetal calf serum in the medium.

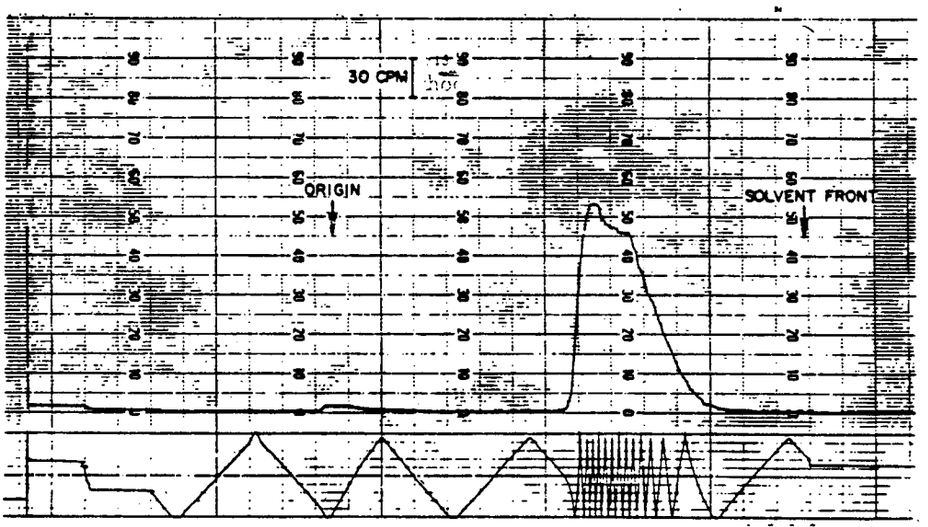

FIG. 1. Thin layer chromatography of cychlohexanone extract. Cyclohexanone extracts from 12 replicate cultures of cells incubated with 10 mU of erythropoietin were pooled and concentrated to dryness with a rotary evaporator at low pressure. The residue was dissolved in a small volume of chloroform, methanol, pyridine, 10:25:3 vol/vol/vol and applied to a silica gel thin layer foil. The chromatogram was developed with the same solvent, dried and scanned for ^{59}Fe on a radiochromatogram scanner at 300 cpm full scale.

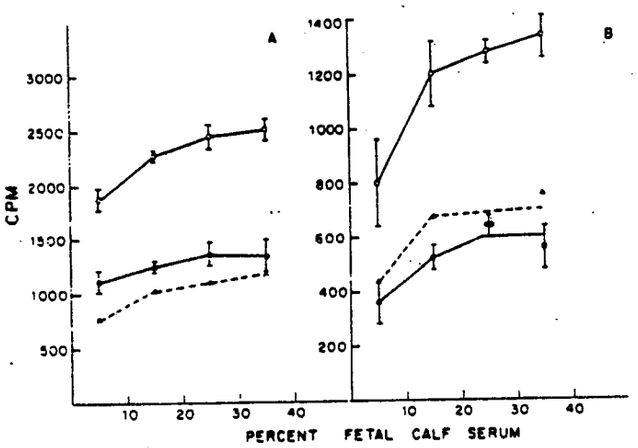

FIG. 2. Effect of fetal calf serum concentration on iron uptake and hematin synthesis. Cells were cultured as described in text at different volume percent of fetal calf serum. Closed circles represent controls, open circles represent erythropoietin treated cells (5 mU), triangles represent Δ cpm due to erythropoietin and vertical bars represent ± one standard deviation. Panel A: total iron uptake. Panel B: hematin synthesis.

Four different supplies of fetal calf serum (at 30%) were tested, with the results shown in Table 1. The differences in controls and stimulated groups are statistically significant, indicating that both baseline iron uptake and the erythropoietin effect on iron uptake can vary by as much as 40%, depending on the source of fetal calf serum.

In an attempt to eliminate the need for gassing the cultures with CO_2 during the incubation, we tested, in different experiments, several alternative buffers, all at pH 7.4 in the presence of 10% CO_2, for their effects on cultured marrow cells (Table 2). Tricine [*N*-tris(hydroxymethyl)methyl glycine] and HEPES [*N*-2-hydroxyethyl piperazine-*N*'-2-ethane sulfonic acid] caused the complete loss of response to erythropoietin, whereas glycylglycine, TES [N-Tris(hydroxymethyl methyl-2-aminoethane sulfonic acid], and MOPS permitted a response. The data in Table 2 also demonstrate that, in a closed system, without gassing with CO_2, the use of MOPS at a final concentration of 20 mM at pH 7.2 allowed a reasonable dose-response relationship.

The effect of pH on the erythropoietin dose-response relationship is appreciable, as demonstrated in Fig. 3. When total iron uptake was measured (Fig. 3A) at pH 6.8, 7.1, and 7.3, the slopes of the ln dose (in milliunits) vs ln response (in cpm over

TABLE 1. Test of different lots of fetal calf serum

	Total iron uptake (cpm ± SD)			
	Lot A	Lot B	Lot C	Lot D
Control	430 ± 30	510 ± 20	520 ± 40	370 ± 40
Erythropoietin (6 mU)	790 ± 40	1,010 ± 40	940 ± 30	840 ± 30
% Increase	84	98	81	127

TABLE 2. Test of different buffers

Buffer	Control (cpm ± SD)	Erythropoietin (6 mU) (cpm ± SD)	% Stimulation
CO_2	630 ± 30	940 ± 40	50
HEPES (10 mM)	420 ± 40	440 ± 20	5
Tricine (15 mM)	730 ± 100	720 ± 30	0
TES (10 mM)	730 ± 50	1,040 ± 40	42
Glycylglycine (10 mM)	730 ± 30	1,000 ± 30	37
MOPS (20 mM)	710 ± 50	1,030 ± 30	45

	Control	3 mU	6 mU	12 mU
	(cpm ± SD)	(cpm ± SD)	(cpm ± SD)	(cpm ± SD)
MOPS (20 mM)	1,180 ± 80	1,680 ± 90	1,890 ± 70	2,060 ± 100

The experiments summarized in the upper part of the table were done with 10% CO_2 in the gas phase. That in the lower part was done in a closed system without added CO_2.

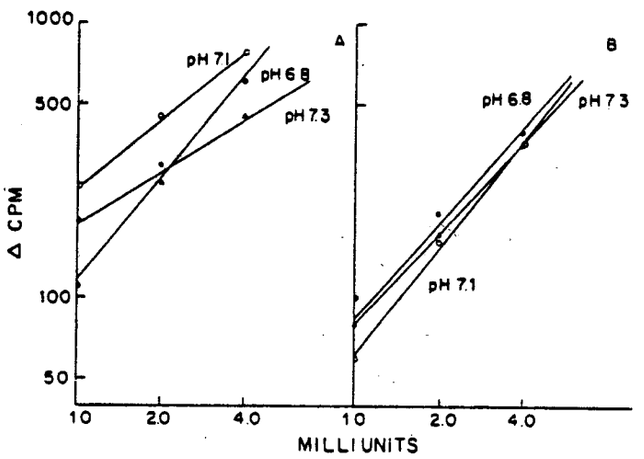

FIG. 3. Effect of pH on dose-response relationships. Cells were cultured as described in text using 20 mM MOPS in medium adjusted to pH 6.8 (●); 7.1 (○) and 7.3 (△). Panel A: total iron uptake. Panel B: hematin synthesis.

control) curves were 1.24, 0.81, and 0.62, respectively. When hemoglobin synthesis was measured (Fig. 3B), the corresponding slopes were 1.16, 1.29, and 1.08. The correlation coefficients of the regression fit for all six curves were 0.99 or greater. We have chosen to use pH 6.9 as the standard condition.

Because of the instability, in solution, of penicillin and streptomycin, the antibiotics commonly used in cell cultures, we tested the stable antibiotic, gentamicin. The data demonstrated no significant difference between the antibiotics in either control or stimulated cells; therefore, the more stable compound may be used.

There is commercially available a modification of NCTC 109, NCTC 135, from which cysteine is absent. When it was tested (Table 3), the cells took up significantly less iron, and the absolute magnitude of the response to erythropoietin was decreased. when NCTC 135 was supplemented with cysteine or with an equimolar amount of reduced glutathione, the response was somewhat greater than that found with the original NCTC 109. This increase may be due to the loss of some cysteine in NCTC 109 due to oxidation during storage.

As the cell density is increased, there is an increase in the magnitude of the response to 4 mU of erythropoietin (Fig. 4), with respect to both iron uptake and hemoglobin synthesis. The maximal percent response was found at 15 to 16 million nucleated cells per ml, and 15 million was chosen as the standard cell density for the assay. A comparison of dose-response relationships with respect to total iron uptake, using 8 and 15 million nucleated cells, is shown in Table 4. At the lower cell density, only the 4 mU response was significantly dif-

TABLE 3. Effect of sulfhydryl compounds on marrow cell cultures

	Control	Erythropoietin (6 mU)	
Medium	(cpm ± SD)	(cpm ± SD)	Δ cpm
NCTC 109	1,000 ± 50	1,710 ± 50	710
NCTC 135	850 ± 30	1,390 ± 50	540
NCTC 109	2,000 ± 50	2,890 ± 70	890
NCTC 135	1,180 ± 20	1,870 ± 60	690
NCTC 135 + cysteine (1.65 mM)	2,280 ± 120	3,570 ± 90	1,290
NCTC 109	950 ± 110	1,660 ± 120	710
NCTC 135	540 ± 60	1,150 ± 20	610
NCTC 135 + GSH* (1.65 mM)	1,280 ± 120	2,220 ± 120	940

* GSH = reduced glutathione.

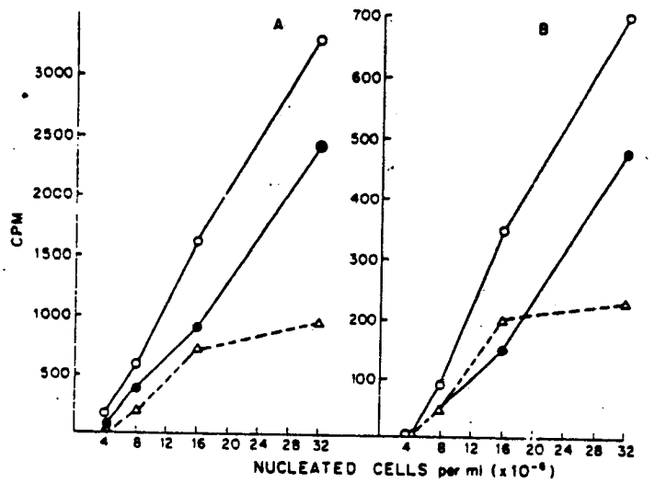

FIG. 4. Effect of cell number on marrow cell cultures. Cells were cultured as described in text at varying cell densities. Closed circles represent controls, open circles represent erythropoietin treated cells (4 mU) and triangles represent Δ cpm due to erythropoietin. Panel A: total iron uptake, Panel B: hematin synthesis.

ferent from the control; at the higher cell density, however, all of the points were significantly above the control level at a value of $P < 0.02$.

When erythropoietin is assayed *in vivo*, increased sensitivity to exogenous hormone is achieved by reducing endogenous erythropoiesis. This can be done by making assay animals plethoric (13) or by starvation (14). We have studied the effect of starving the donor rats on the response of their marrow cells *in vitro*. The results (Fig. 5) show that control cells and cells exposed to 1, 2, or 4 mU of erythropoietin have decreased baseline iron uptake and response to hormone as the period of fasting increases. While they are not shown, the slopes of the ln dose vs ln response curves do not change appreciably with time of starvation.

TABLE 4. Dose-response relationships at different cell densities

	Total iron uptake (cpm ± SD)	
	8×10^6 cells	15×10^6 cells
Control	250 ± 20	430 ± 50
1 mU	250 ± 20	510 ± 50*
2 mU	260 ± 30	580 ± 60*
4 mU	330 ± 60*	760 ± 60*

Values labeled with an asterisk are significantly different from controls at the $P \leq 0.02$ level.

In the standard assay, therefore, marrow cells from unstarved rats are used.

Marrow cells from rats made polycythemic by exposure to low pressure have a dose-response curve very similar to that seen with cells from normal animals (Fig. 6). Since erythropoietin is known to act on both differentiated erythroid and undifferentiated cells in the marrow population; since plethoric animals have few, or none, of the former type of cell; and since the time required between inducing the first steps of erythroid differentiation and increase in iron uptake is unknown, we tested cells from polycythemic donors at both 18 and 42 h of incubation with erythropoietin. The amount of iron taken up in a 5-h pulse at the later time is about 60% less than that taken up in a pulse of the same length at 18 h. The response to erythropoietin is decreased by about 80% at the later time. While it is feasible to use cells from polycythemic rats for assay of erythropoietin *in vitro*, our data indicate that there is no advantage over the use of cells from normal rats.

The method described in this paper may, with caution, be used for the assay of fractions obtained during the purification of both plasma and urinary erythropoietin. The data in Table 5 show the results of

comparative assays *in vivo* and *in vitro* of several fractions derived from anemic sheep plasma or human urine. The human material was from a chromatogrpahic separation on QAE cellulose, whereas the sheep fractions were derived from chromatography on DEAE cellulose. In the case of the human erythropoietin, fractions A and B showed more activity *in vitro* than *in vivo*. The 33% greater activity of fraction A is probably within the limit of error of the *in vivo* assay method, as is the 64% greater activity of fraction B. The larger discrepancy for B is probably due to the fact that the *in vivo* activity was considerably lower than the lowest standard used (1 U) in the fasted rat assay.

Plasma from phenylhydrazine-treated sheep, assayed by this method, has about 37% of the activity found *in vivo*. This low value probably reflects an inhibitory action of some constituent of the hemolyzed sheep plasma. Three of the sheep plasma fractions showed approximately 60% recovery of *in vivo* activity by *in vitro* assay; the fourth showed a 23-fold increase. The decrease could be accounted for by either the error inherent in the rat bioassay or by inhibition due to crude plasma preparations (15), or both. The large increase of *in vitro* activity seen with one sheep plasma fraction may, probably, be explained by our finding that erythropoietin that has been desialated has no activity *in vivo* and increased activity *in vitro* (16). This fraction (number 3) may consist of a small amount of native hormone and a larger amount of the desialated form.

Assay of rat serum from animals made anemic by removal of 5–6 ml of blood eight hours before harvesting yielded dose-response curves with slopes of 0.376 (total iron uptake) and 0.491 (heme synthesis) in contrast to the values of 0.785 and 0.778 found with partially purified sheep plasma erythropoietin. At the lowest level

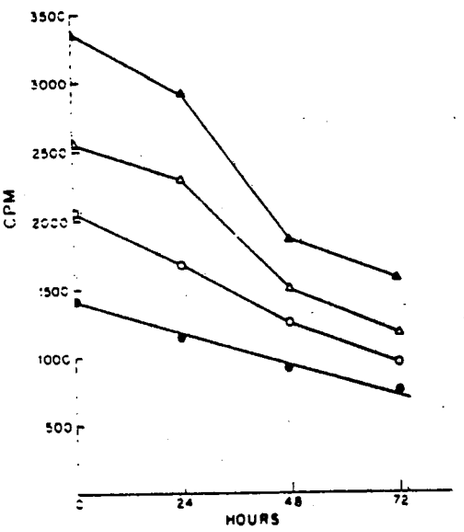

FIG. 5. Effect of starvation of rats on marrow cell iron uptake. Rats were deprived of food for the times indicated, and the marrow cells were cultured as described in text. Controls (●) had no added erythropoietin, open circles (○): 1 mU, open triangles (△) 2 mU and closed triangles (▲) 4 mU.

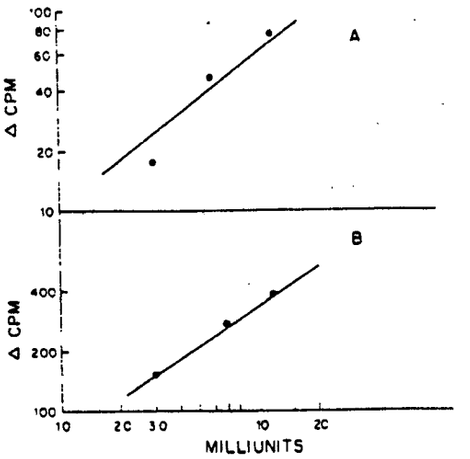

FIG. 6. Dose-response curves of cells from plethoric rats. Animals were kept at 0.5 atmospheres for 3 weeks, allowing 3–5 h per day at normal pressure. Their drinking water contained 0.16 mg/ml of ferrous sulfate. The cells were harvested after the rats had been returned to normal pressure for 6 days when their hematocrits were 68. Culture conditions were as described in the Text. Panel A: hematin synthesis, Panel B: total iron uptake.

TABLE 5. Comparison of assays *in vivo* and *in vitro*

Test material	Assay results (U)	
	in vivo	*in vitro*
Human urine fraction A*	3.6	4.80
Human urine fraction B	0.5	0.82
Human urine fraction C	3.8	3.94
Human urine fraction D	0.0	0.0
Sheep plasma fraction 1**	0.9	0.56
Sheep plasma fraction 2	0.4	0.26
Sheep plasma fraction 3	0.1	2.3
Sheep plasma fraction 4	0.5	0.30

* The fractionation of human urinary erythropoietin was done in this laboratory by Dr. S. Chiba, using the preparation H-1 Talsl collected and concentrated by the Department of Physiology, University of The Northeast, Corrientes, Argentina, further processed by the Hematology Research Laboratory, Children's Hospital of Los Angeles, for distribution by the National Heart and Lung Institute under research grant HE 10880.

** The fractionation of sheep plasma was done by C. Hancher, Oak Ridge National Laboratory, Oak Ridge, Tennessee.

The *in vivo* assay was done with the fasted rat method (14).

of anemic rat serum tested (2 μl per 200 μl) the assay values were 0.5 U/ml (total iron uptake) and 0.37 (heme synthesis); the titer found by the fasted rat method was 0.5 U/ml. At higher amounts (4 and 8 μl) we found lower titers per ml, due to the shallower dose-response curve. At present we cannot account for these differences but suggest, that because of the possibility of inhibitory materials being present, accurate measurement of serum titers can be made only at low levels (approximately 1 mU).

We have used this method to estimate the erythropoietin titer of normal rat serum by comparing cultures containing 30% rat serum in place of fetal calf serum, with cultures containing 30% serum derived from ex-hypoxic rats with hematocrits greater than 65. When total iron uptake was measured, the difference between the two sets of cultures could be accounted for by the presence of 0.01 units of erythropoietin per ml of normal rat serum. While this value is in the expected range, this method does not have a high degree of accuracy.

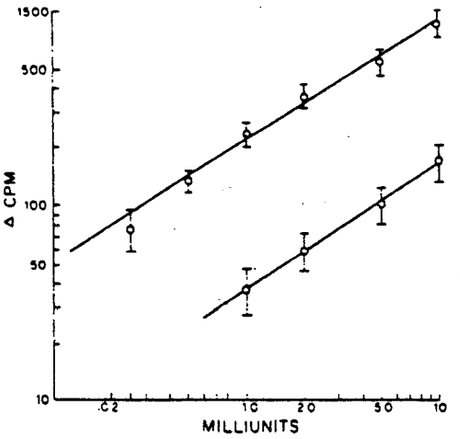

FIG. 7. Erythropoietin Assay: dose-response curve. Cells were cultured as described in the text. The upper curve represents total iron uptake, the lower curve represents hematin synthesis and the vertical bars indicate ± 1 SD.

Typical dose-response curves showing the effects of erythropoietin at doses from 0.5 to 10 mU are shown in Fig. 7. It is clear that the total iron uptake response is capable of greater sensitivity than is the hematin response. In the case of the latter, the apparent responses to 0.25 and 0.5 mU were not significantly different from the control. In the case of the former, although the lowest point (0.25 mU) falls close to the regression line, it, too, is not significantly different from the control. The slopes of the two curves are very similar: 0.631 for total iron uptake and

TABLE 6. Comparison of *in vitro* assay methods

Method	Volume (ml)	Amount of erythropoietin (U)	Sensitivity (U/ml)
Ward (2)	3.0	0.15	0.05
Stephenson and Axelrad (3)	2.0	0.01	0.005
Wardle *et al.* (4)	2.0	0.01	0.005
Bartley and Powsner (5)	0.225	0.007	0.03
Present method	0.20	0.001	0.005

0.647 for hematin. The index of precision of the iron uptake dose-response curve is 0.16 if the 0.25 mU point is included or 0.14 if it is not, whereas the value for the hematin curve, not including the two lowest doses, is 0.056.

In general, the index of precision is small when the lowest dose used is 1 milliunit; the range over 20 experiments is 0.008 to 0.30, with a mean value of 0.08 for the total iron uptake measurement. When hematin was measured (6 experiments), the range was 0.07 to 0.22 with a mean value of 0.10. While it is possible, especially with the use of ^{59}Fe of higher specific activity, to determine amounts of erythropoietin as low as 0.5 mU, we regard 1 mU as the lower level of activity detectable with any precision.

Discussion

The method described in this paper is a fairly rapid and sensitive one for the assay of erythropoietin. It is based on our earlier findings that bone marrow cells respond *in vitro* to added erythropoietin by increased hemoglobin synthesis and iron uptake. These findings have also been used by others as a means of erythropoietin assay using dog bone marrow (2), 13-day fetal mouse liver (3,4), and rat marrow (5). The differences among these methods are not great, and the choice of cell source will depend on individual preferences. A comparison of the sensitivities of the various methods is presented in Table 6. The present method is capable of detecting a smaller absolute amount of added erythropoietin than the others; it also uses adult rat marrow, which is easily available, rather than fetal mouse liver which may be more difficult to obtain.

One major disadvantage of all of the *in vitro* assay methods relates to the response to asialoerythropoietin. Because asialoerythropoietin has no activity *in vivo*, it would be possible, for example, to use the *in vitro* assay to follow the fractionation of a form of the hormone which would then have no action on a whole animal. Another problem lies in the sensitivity of cultured cells to impurities, making it impossible to determine the amount of erythropoietin in crude preparations with any certainty.

Within the limitations imposed by the above considerations, assays for erythropoietin *in vitro* can be useful for a variety of studies. We have, for example, shown differences in stability toward thermal denaturation and toward tryptic digestion between native and asialoerythropoietin using this method of assay (16). After this paper was submitted for publication, Adamson *et al.* (17) showed that dog marrow cells in culture respond to 1–2 mU of erythropoietin, and that the dose was linear with response from 3 to 30 mIU.

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