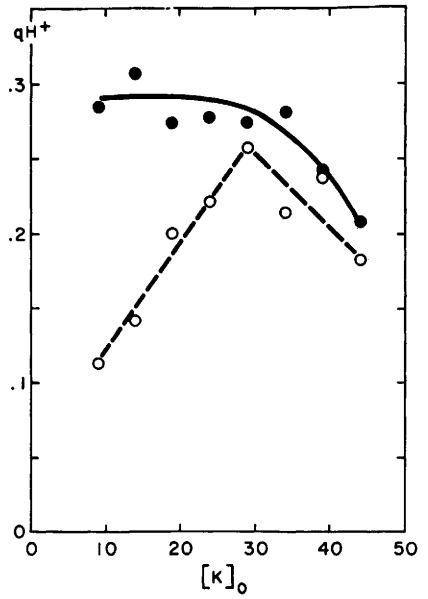

FIG. 3. Ordinates: rates of acid secretion by frog gastric mucosas in μM/mg dry wt/hr at 25°C. Abscissae: K concentrations in mM. Sum of K and Na in all solutions was 129 mM. Each point is the mean of 10 to 25 observations. Solid line is for results without ouabain; interrupted line is for results with 10^{-4} M ouabain.

other effects of the drugs are primary ones or are merely secondary to their alteration of intracellular electrolyte patterns. Burg and Orloff(4) have given a thorough analysis of this problem in the case of kidney tissue, and have demonstrated insofar as is experimentally possible that inhibition by strophanthidin of PAH accumulation by kidney slices is secondary to K depletion. Although they present much other pertinent evidence, their major point is that depression of PAH accumulation caused by strophanthidin can be reversed by increasing the K content of the medium and thereby of the cells. We present similar evidence to explain the inhibition of gastric acid secretion by ouabain, and although other explanations remain possible if not plausible we conclude that at least part of the drug's effect is expressed through alteration of intracellular environment. If we were able to give a valid calculation of intracellular composition of the oxyntic cells under our experimental conditions we could specify the intracellular electrolyte composition required for acid secretion. Until this becomes possible we present our data with the expectation that they will become useful when the basis of such a calculation is at hand.

Summary. Ouabain in concentrations from 10^{-3} to 10^{-6} M inhibits acid secretion by frog gastric mucosas *in vitro* and at 10^{-4} M causes tissue Na to rise and K to fall. Increasing K concentration in the medium from 9 to 29 mM largely abolishes the inhibitory effect of 10^{-4} M ouabain. We conclude that at least some of the inhibitory effect is the result of alteration of intracellular electrolyte pattern.

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Effect of Erythropoietin on the Uptake and Utilization of Iron by Bone Marrow Cells *in vitro*.* (27596)

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During the last decade numerous studies have demonstrated that plasma from severely anemic or severely hypoxic mammals contains an erythropoietic factor, the so-called erythropoietin(1,2,3). This factor is undoubtedly of importance in adjustment of red

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cell production to severe tissue hypoxia. Whether it also controls the normal rate of red cell production is not fully resolved, primarily because the present assay technics are too crude to detect low concentrations of erythropoietin and small changes in its plasma level. Some studies tend to indicate that the accurately tuned regulation of normal red cell production may be controlled by other means (4). However, the simple and very appealing concept that the level of erythropoietin controls rate of red cell production under all physiologic conditions has not been disproved.

Indirect, but quite convincing evidence suggests that erythropoietin *in vivo* acts solely on the erythroid stem cells(5,6). These self perpetuating cells, whether multipotential or unipotential, will feed pronormoblasts into the erythroid tissue and it is believed that erythropoietin is responsible for the differentiation which transforms stem cells into pronormoblasts. The subsequent rates of erythroid multiplication, maturation and release have not convincingly been shown to be controlled by erythropoietin. However, the change in bone marrow structure which takes place after a sudden influx of rapidly-dividing pronormoblasts will undoubtedly influence other erythroid functions, and may lead to the premature release of not fully matured cells.

To gain more insight into the process of stem cell differentiation a number of attempts have been made to study the action of erythropoietin *in vitro*. Early studies of bone marrow suspended in serum revealed that anemic serum induced changes in cellular composition and mitotic index(7,8,9). Isotope studies on the other hand by Thomas *et al.*(10) by Alpen *et al.*(11) and by Erslev and Hughes(12) have shown that uptake and metabolic utilization of iron and glycine is the same in bone marrow suspended in serum from normal animals as in serum from severely anemic animals. Although it was recognized that addition of a few pronormoblasts might not change the overall rate of metabolism in a bone marrow suspension it was disturbing that anemic serum with a pronounced erythropoietic action *in vivo* was inert when studied *in vitro*.

The recent availability of highly concentrated erythropoietic material has made it possible to reevaluate the *in vitro* effect of erythropoietin in a more exact and reproducible manner. Preliminary studies indicating the *in vitro* effectiveness of erythropoietin have been presented by Powsner and Berman (13) and by Erslev(14). The purpose of the present investigation was to define the *in vitro* environment most conducive for *in vitro* action of erythropoietin.

Material and methods. Rabbit bone marrow from the long bones was shelled out into warm rabbit serum, dispersed by means of a stainless steel screen and centrifuged(12). The fatty layer was removed and marrow cells were suspended in a mixture of normal rabbit serum and M.E.M. (Eagle's Minimum Essential Medium with 1% Glutamine). The normal serum used had been brought to a pH of 8.1 and Penicillin 200 units/ml, Streptomycin 200 μg/ml and Mycostatin 20 u/ml had been added. When such serum is exposed to an atmosphere with a pCO_2 of 40 mm, the final pH is about 7.4. Sheep erythropoietin obtained from Nat. Inst. of Health and containing 450 cobalt units per 100 mg material (grade III) was suspended in M.E.M. Radioactive iron as ferric^{59} chloride was added to serum in an amount not exceeding a small fraction of the unbound transferrin. One ml each of bone marrow suspension, erythropoietin and Fe^{59} were added to each of a large number of 25-ml Erlenmeyer flasks and incubated in a shaking incubator for 21 and 45 hr at a tp. of 39°C, a pO_2 of 100 mm and a pCO_2 of 40 mm. At the end of incubation, 0.5 ml of a 700 mg% solution of sodium cyanide was added to inhibit further metabolic activity and the cells were washed 3 times with saline. Cellular uptake of Fe^{59} was finally determined in a well counter and uptake of iron was calculated from the serum iron and total amount of Fe^{59} added. The iron absorbed physically (0 hour value) was determined from aliquot suspensions kept at 4°C. All studies were carried out in triplicate. Serum iron was determined by a modified method of Peterson (15). Fisher's method was used in isolation and recrystallization of heme(16) and paper electrophoresis at pH of 8.6 followed by strip

scanning was used for isolation and measurement of hemoglobin.

Results. 1. *Effect of erythropoietin on cellular iron uptake.* Rabbit bone marrow cells, suspended in a medium made up of one-third normal rabbit serum and two-thirds M.E.M. in a concentration of 2000 nucleated cells per cu mm, were incubated in an atmosphere of 13% O_2 and 5% CO_2. Erythropoietin in various concentrations was added and cellular iron uptake after 21 and 45 hours incubation was determined. Under these conditions (14) erythropoietin was found to increase uptake of iron, an increase which at 45 hours was roughly proportional to the logarithm of the dose (Fig. 1). In 19 different experiments the percentage increase in iron uptake induced by 2.5 units erythropoietin per ml was found after 45 hours incubation to range from 20% to 165% with a mean increase of 68%.

2. *Effect of erythropoietin on heme synthesis.* In previous studies it was found that iron uptake by reticulocytes and nucleated red cells *in vitro* under many but not all conditions (lead and chloramphenicol toxicity) is directly proportional to rate of heme synthesis(17). In the present study, the fractions of absorbed iron utilized in the synthesis of heme varied from 40% to 65%. Table I shows that the erythropoietin-induced increase in cellular iron uptake reflects increased synthesis of heme. However, it appears that cellular iron uptake is increased more than heme synthesis.

3. *Effect of erythropoietin in different media.* Bone marrow cells suspended in serum were found to incorporate more iron at the end of 45 hours incubation than identical cells suspended in serum diluted with M.E.M. However, suspensions with added erythropoietin were not influenced significantly by the composition of the medium. Consequently, the difference between cells with and those without erythropoietin was much more pronounced in the dilute media (Fig. 2). It appears that the erythropoietin either renders the cells more resistant to prolonged incubation or that the cells responsible for the erythropoietin-induced-iron-uptake are hardy and not easily destroyed.

TABLE I. Mean Iron Uptake by Cells, by Recrystallized Heme and by Electrophoretically Separated Hemoglobin from 4 Rabbit Bone Marrows Cultured for 45 Hours in ⅓ Serum + ⅔ M.E.M. at a pO_2 of 100 mm and a pCO_2 of 40 mm. Cellular concentration was 2000 cells/mm^3 and 2.5 units erythropoietin was added/ml of medium.

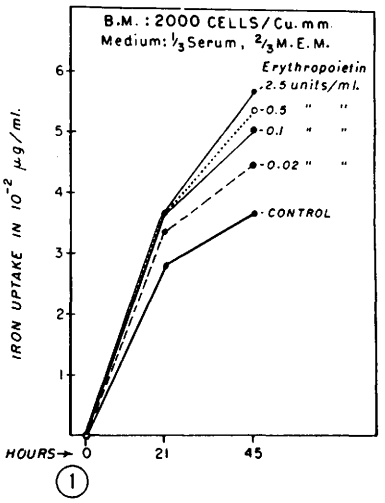
	Cellular uptake of Fe^{59}	Incorporation of Fe^{59} into:	
		Heme	Hemoglobin
	% of control		
Control (4)	100	100	100
Erythropoietin (4)	163	142	148

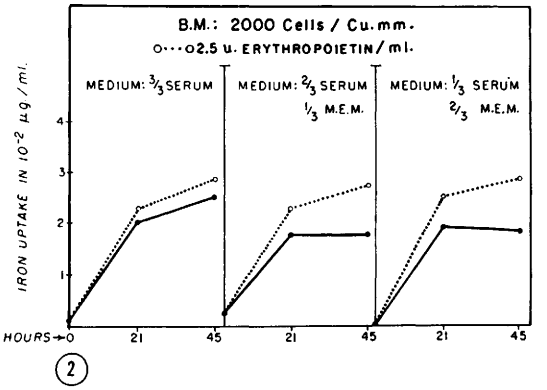
4. *Effect of erythropoietin at different cell concentrations.* A number of unsuccessful experiments led to a study of the influence of cellular concentration on the action of erythropoietin. It was found that at high cell concentrations cellular uptake of iron decreased after 21 hours of incubation indicating cellular destruction. At these concentrations erythropoietin did not elicit a significant increase in cellular uptake. At lower concentrations, cellular destruction was less pronounced and here erythropoietin elicited an increase in cellular uptake. Fig. 3 shows the effect of erythropoietin (2.5 u/ml) on the same bone marrow at 3 different cellular concentrations. In Fig. 4 the percentage increase in iron uptake induced by 2.5 units erythropoietin per ml of bone marrow suspension incubated for 45 hours is charted against cellular concentration. This figure shows that the relative effect of erythropoietin is greatest at low cellular concentrations.

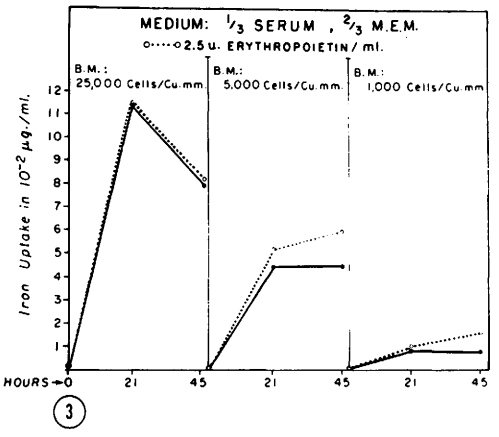
5. *Effect of erythropoietin at different oxygen tensions.* In this laboratory bone marrow studies *in vitro* have been carried out in an atmosphere of 13% oxygen and 5% CO_2 corresponding to the arterial gas tension of 100 mm pO_2 and 40 mm pCO_2(12). Bone marrow suspensions incubated at 95% O_2 and 5% CO_2, 13% O_2 and 5% CO_2 or 5% O_2 and 5% CO_2 incorporated approximately the same amount of iron after 21 hours. After 45 hours the uptake was much lower in samples exposed to the high oxygen tension indicating cellular destruction (Fig. 5). Erythropoietin increased uptake both in the 13% oxygen and the 5% oxygen samples, but did not prevent the cellular destruction in the

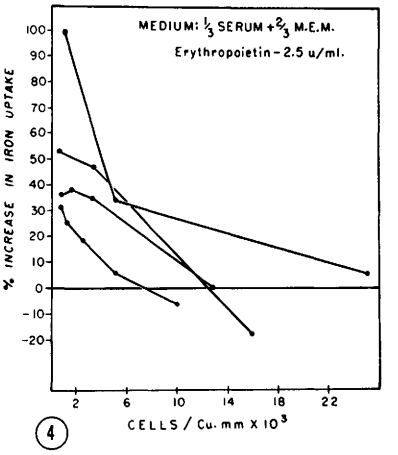
95% oxygen sample. The maximal response to erythropoietin was found in 13% oxygen.

Discussion. Addition of sheep erythropoietin to a suspension of rabbit bone marrow was found to enhance uptake of iron and synthesis of heme. Previous reports(10,11,12) concluding that erythropoietin had no action *in vitro* were based on studies with erythropoietin-containing whole serum and not as in this study or in the study by Powsner and Ber-









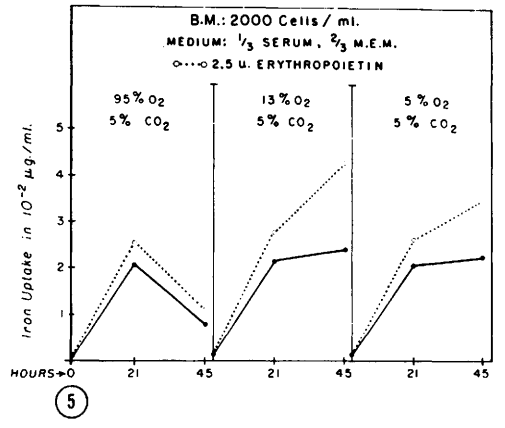


FIG. 1. Effect on iron uptake of various concentrations of erythropoietin.

FIG. 2. Effect on iron uptake of various medias.

FIG. 3. Effect on iron uptake of various cell concentrations.

FIG. 4. Effect of cell concentrations on percentage differences between iron uptake of non-treated bone marrow and of erythropoietin treated bone marrow after 45 hr incubation.

FIG. 5. Effect on iron uptake of various oxygen tensions.

man(13), on highly purified erythropoietin. With such material it is possible to use suspensions in which the only difference between controls and experimental flasks is the presence of a few milligrams of erythropoietic material. The pronounced effect of erythropoietin in the present study is probably also related to the fact that the suspensions were incubated for 45 hours, that a low cell count was employed, that the medium was enriched with an artificial cell culture material and that the incubation was carried out at a physiologic oxygen tension. Furthermore, the use of cellular iron uptake rather than heme synthesis may account for the more striking *in vitro* effect of erythropoietin in this study as compared with the study by Powsner and Berman(13). Heme synthesis was shown not to be accelerated to the same extent as cellular iron uptake.

It has been shown previously that bone marrow suspensions will incorporate iron and synthesize hemoglobin *in vitro* at a steady rate for the first 10 to 14 hours, following which the rate will decline gradually(12). After 21 hours of incubation only small additional amounts of iron will be incorporated and cellular destruction may even lead to a lower uptake of iron after 45 hours than at 21 hours. The enhanced uptake noticed after addition of erythropoietin is relatively much larger after 45 hours incubation than after 21 hours suggesting that erythropoietin either diminishes *in vitro* destruction of erythroid elements or promotes the creation or metabolism of immature erythroid cells capable of *in vitro* multiplication. Since erythropoietin has been shown not to enhance the iron uptake *in vitro* of reticulocytes(14) and to have roughly the same log-dose-response relationship *in vitro* as demonstrated for rats *in vivo*(18), it is possible that the effect observed after 45 hours of incubation represents a physiologically important effect of erythropoietin. The greater difference noticed with low cell counts and with media enriched with M.E.M. appears to be related primarily to an improved survival of cells under these conditions rather than to greater efficiency of the added erythropoietin. This appears also to be the case when different oxygen mixtures are used. The commonly used mixture of 95% oxygen, 5% CO_2 was found to be quite destructive on cell suspensions when the incubation was carried out for longer than 21 hours.

This study has established *in vitro* conditions necessary for demonstration and measurement of the effect of erythropoietin. It is hoped that such systems will clarify the cellular action of erythropoietin *in vitro* and eventually lead to the elucidation of its physiologic role *in vivo*.

Summary. Purified sheep erythropoietin was found to enhance iron uptake and heme synthesis when added to a suspension of rabbit bone marrow. The greatest difference between control suspensions and erythropoietin-treated suspension was found after 45 hours incubation in suspensions with a low cell count, with a medium enriched with tissue culture fluid and with an atmospheric oxygen tension of 13% oxygen.

The author is grateful for the able assistance of Yolanda Rivero.

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Changes in the Free Amino Acid Composition of Cerebrospinal Fluid in Liver Disease. (27597)

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Disturbance in protein metabolism in patients with liver disease has been known since Frerichs(1) established the presence of leucine and tyrosine crystals in urine of patients with acute yellow liver-atrophy. Since then, the amino acid content of serum and urine of such patients has been extensively studied. Far less quantitative data on the free amino acid content of cerebrospinal fluid are available(2,3,4,9).

In the following study, an attempt is made to clarify the following questions which have arisen in the study of cerebrospinal fluid of liver disease patients: (a) Is there any significant difference in the free amino acid composition of cerebrospinal fluid of healthy individuals and patients with liver disease? (b) Is there any relationship between degree of hepatic parenchymal damage and changes in the free amino acid composition of the cerebrospinal fluid? (c) Do these changes play any part in the pathogenesis of hepatic coma?

Materials and methods. This study entailed detailed clinical and laboratory examination of 25 metabolically healthy adults between the ages of 20 to 42 years and of 28 patients with liver disease. Of the latter, 5 were suffering from obstructive jaundice due to malignant tumors and 18 from cirrhosis of the liver. Of the cirrhotics 11 were alcoholics; 1 was associated with cardiac failure; 1 was a result of biliary infection; and 5 followed hepatitis. In 22 of these 28 cases the clinical diagnosis was later confirmed either by peritoneoscopy, biopsy, or autopsy. In most cases clinical diagnosis was also supported by liver function tests such as bromsulphthalein clearance, galactose tolerance, and serum transaminase level as well as by results of serum electrophoretic analysis and by measurement of prothrombin time, serum bilirubin, nonprotein nitrogen, and free alpha-amino-N in plasma. The methods of analysis of the deproteinized cerebrospinal fluid, obtained by lumbar puncture, have been previously described(5). Altogether 35 cerebrospinal fluids from the 28 patients were analyzed. The average cell count ranged between 0-22 cells, and protein content ranged from 22-53 mg %. The free alpha-amino-N (Moore and Stein(6)), indican (Shivaram and Müting(7)), as well as free and combined phenolic bodies (Schmidt(8)) were also measured.

Results. The results are shown in Table I. In 12 patients without hepatic coma, the free alpha-amino-N in cerebrospinal fluid ranged between 0.94-2.07 mg %, the normal being 0.75 mg % ($\pm$ 0.24). Among the free amino acids which most increased were tyrosine, tryptophane, phenylalanine, leucine, isoleucine, and especially methionine, glycine, and glutamine. The increase in 14 amino acids is statistically significant.

In 16 cases with hepatic coma, free alpha-amino-N of the cerebrospinal fluid ranged between 2.45 and 3.90 mg % which is 3 to 4 times the normal value (Table I). Of these patients, 3 with the most severe attack from the clinical point of view also showed the highest values. Breakdown products of methionine such as methionine sulfoxide were demonstrable in 4 cases. In addition to the sulfur containing amino acids, glutamine also showed an increase in cerebrospinal fluid to an extent as high as 10 times normal.