Exhibit 22

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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Erythropoietin

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Assays for Erythropoietin

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I. INTRODUCTION

A. HORMONE ASSAYS

Assays for hormones have been categorized according to their biological, chemical and immunological methodologies (Loraine and

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Bell, 1966). Biological assay techniques generally are time consuming, cumbersome, expensive, many times require elaborate animal preparations, often show great degrees of variability and for the most part are not usually sensitive enough for the measurement of normal hormonal blood levels. However, such biological assays may be quite specific since they are based on extensions or modifications of the original physiological phenomenon associated with the particular hormone. Also, it must be pointed out that it has been mainly on the basis of such biological assays that the biochemistry of hormones has evolved to such an extent that the biochemist has been able to purify, analyse and finally synthesize certain hormones.

Chemical hormone assays should theoretically provide the ideal final assay; however for the most part, where it has become possible to employ chemical procedures, they have usually required the use of large samples and employed concentration, isolation and purification steps. Chemical assays have been used for thyroid and steroid hormones. However there is a large category of proteinaceous hormones, existing in very low concentrations in blood, that do not have the unique chemical groupings of the steroids, or the iodine of the thyroid hormones on which one can base a chemical assay. These hormones are often made up of lengthy amino acid chains. They have specific amino acid sequences and specific biological effects, but other than this, they are not particularly distinguishable from the overwhelming amount of plasma protein which surrounds them in blood. The exploitation of immunological technology in the development of the radioimmunoassay by Berson and Yalow (1958, 1968) has resulted in a major advance in hormone assay. It is now possible to quantitate many of the protein and polypeptide hormones utilizing the radioimmunoassay technique. Generally, this technique is highly sensitive and very reproducible. It is very economical since such dilute concentrations of reactants are used and most determinations can be done on small samples of plasma or serum. This technique also has the advantages that large numbers of samples can be set up in a single assay and further that many radioimmunoassays utilize native plasma or serum, with no extraction step required. Theoretically it should be possible to apply the radioimmunoassay technique to any material, which can be obtained in pure form, which is or can be made antigenic and which will accept a radioactive label. As a tribute to this technique, some of the hormones, formerly measured by chemical assays, like the steroids and thyroid hormones, are now routinely assayed by

radioimmunoassay. The main thrust of this article is concerned with attempts to apply this radioimmunoassay technique to the hormone, erythropoietin.

B. ERYTHROPOIETIN

The production of red blood cells in the normal individual is subject to a control which responds positively when confronted by a reduction in circulating red blood cell concentration, or, more fundamentally by a deficiency in the delivery of oxygen to the tissues. Also, red cell production is negatively affected in the face of excessive numbers of circulating red blood cells or an over abundant delivery of oxygen relative to the oxygen requirements of the tissues. The result of these controls is to maintain a degree of constancy in the total mass of circulating red cells. That this control is humorally mediated is now generally accepted, and is the subject of several reviews (Grant and Root, 1952; Gordon, 1959; Krantz and Jacobson, 1970).

Carnot and Déflandre (1906) were the first to suggest that a humoral factor was involved in controlling erythropoiesis. Although the extremely rapid results they describe are difficult to accept, in the context of present knowledge of the subject as supportive of a humoral control, they must be given credit for the initiation of this hypothesis. This concept received its greatest indirect support from the experimental work of Reissmann (1950), using parabiotic rats, one breathing normal air and the other air with a reduced oxygen concentration, and the clinical observations by Stohlman *et al.* (1954) in a patient with congenital heart disease which resulted in the delivery of normoxic blood to the upper half and hypoxic blood to the lower half of the body. Red cell production was increased in both studies, not only in the marrow receiving hypoxic blood, but also in the marrow which received blood containing a normal concentration of oxygen. These studies indicated that a receptor in the tissues receiving oxygen deficient blood responds with the elaboration of a humoral mediator which stimulated erythropoiesis in all the marrow tissue present. These observations argued strongly against one of the then prevalent hypotheses, that hypoxia stimulated marrow directly. More direct evidence, demonstrating the presence of a humoral factor in the serum of anemic animals which could specifically stimulate erythropoiesis was provided by the work of Toha *et al.* (1952); Erslev (1953) and Borsook *et al.* (1954).

"Erythropoietin", a term first used by Bonsdorff and Jalavisto (1948), has been accepted as the name for the hormone involved in the control of erythropoiesis.

Erythropoietin can be extracted from the plasma and urine of a variety of animal species, including the human. The two main sources of this hormone have been the plasma of phenylhydrazine anemic sheep and the urine of severely anemic humans. It has been purified to specific activities in the order of 8000-12 000 units per milligram by Espada and Gutnisky (1970), and Dukes *et al.* (1971), using the urine from anemic humans and by Goldwasser and Kung (1971), utilizing the plasma of anemic sheep. Erythropoietin has been characterized as a glycoprotein hormone with an estimated molecular weight of 45 000, made up of approximately 70% protein and 30% carbohydrate of which 10.8% is sialic acid (Goldwasser and Kung, 1971). It is now generally accepted that erythropoietin exerts its controlling influence on erythropoiesis, primarily, by directing the differentiation of a multipotential stem cell into erythroid precursors (Alpen and Cranmore, 1959). The most convincing evidence supports the kidney as the source for this hormone (Jacobson *et al.*, 1957a).

II. THE ERYTHROPOIETIN UNIT

The unit now accepted as the standard for erythropoietin had its origin in the work of Goldwasser *et al.* (1958). One unit of erythropoietin was the amount that would produce a response in the starved male Sprague Dawley rat assay equivalent to that produced by 5 μM cobaltous chloride. The need for a common erythropoietin standard, as expressed by Bangham (1960), lead to the Erythropoietin Standard A which consisted of a sheep plasma extract with a unitage which was related to the effect of cobalt. When this material ran out, it was replaced by Erythropoietin Standard B which was of human urinary origin and had a biological potency in terms of Erythropoietin Standard A. Erythropoietin Standard B was accepted as the first International Reference Preparation of Erythropoietin (Cotes and Bangham, 1966). This has since been replaced by the second International Reference Preparation of Erythropoietin, which is also of human urinary origin and has a unitage which is equivalent to the first International Reference Preparation of Erythropoietin (Annable *et al.*, 1972). The term milliunit (mu) used in this article is,

of course, equivalent to 0.001 unit of the International Reference Preparation of Erythropoietin.

Another unit that appears in the erythropoietin literature is the CS unit (Keighley *et al.*, 1959). The CS unit is equal to approximately 10 Erythropoietin Standard A units (or 10 "cobalt units").

III. BIOASSAYS *IN VIVO* FOR ERYTHROPOIETIN

The bioassay for erythropoietin has been covered in detail in several good reviews (Krantz and Jacobson, 1970; Gordon and Weintraub, 1962; Camiscoli and Gordon, 1970; Cotes, 1971). This discussion will be limited to a summary of highlights of the development of such assays. The earliest assays for erythropoietin consisted of increases in red cell count, circulating reticulocytes, hematocrit and hemoglobin concentration. In the initial work of Carnot and Déflandre (1906), an increase in red cell count in the rabbit was used as the index of erythropoietic stimulation. After the injection of only 9 milliliters of bled donor rabbit serum an incredible increase of greater than 50% was seen in 24 hours with a doubling in 3 days. Krumdieck (1943) using larger doses (20 milliliters of bled serum daily for 3 days) was able to show an increase in circulating reticulocytes in the third to the fifth day in rabbits. Gley (1952), using normal rats as recipients, gave bled horse serum and showed an increase in reticulocyte concentration on the third day. Toha *et al.* (1952) found not only an increase in reticulocyte number, but also an increase in circulating hemoglobin and red cells after giving anemic rabbit serum to normal rabbits. Erslev (1953) also working with rabbits, showed that large doses of anemic serum were required to give significant increases in these parameters. Gordon *et al.* (1954), in addition to increases in reticulocyte numbers, red cell counts, hematocrits and hemoglobin concentrations, showed increases in nucleated erythrocytes in the bone marrow of normal rats injected with extracts of phenylhydrazine anemic rabbit plasma. Borsook *et al.* (1954) produced a polycythemia in rats, using an acidified, heat extraction of phenylhydrazine anemic rabbit plasma. These investigators observed increases in total blood volume, as well as increases in reticulocyte numbers, hemoglobin concentrations and hematocrits, thereby establishing the absolute nature of the

erythropoietic stimulation with the resulting polycythemia. Incidentally, these authors also made the first biochemical observation about erythropoietin, i.e. it is heat stable. Van Dyke *et al.* (1957) were able to produce a significant increase in total red cell mass in normal rats following the injection of untreated urine from an anemic human. Injection of an extract of such urine for 14 days resulted in the production of a polycythemia in normal rats significantly greater than that resulting from the exposure of normal rats to a hypoxic environment equivalent to an altitude of 15 000 feet for the same period of time. In a later study, Garcia and Van Dyke (1959) observed increases in total circulating hemoglobin as well as total red cell mass in normal rats injected with such extracts. Gordon and Weintraub (1962) also demonstrated the polycythemic effect of anemic human urine in normal rats by observing increases in the total number of circulating red cells. Such studies circumvented the possible objection of simultaneous plasma volume alterations. Thus the early work demonstrated that stimulation of erythropoiesis by erythropoietin was possible in normal animals in terms of increases in reticulocytes, red cell and hemoglobin concentrations, and stimulation of bone marrow nucleated erythroid elements, as well as increases in the total circulating red cell mass, red cell number and hemoglobin. These studies also demonstrated that the response could be observed not only when the recipient animal was of the same species, but also when it differed from the donor. Thus erythropoietin appeared not to be species specific.

Huff *et al.* (1950) demonstrated the usefulness of the measurement of radioiron (^{59}Fe) incorporation into newly formed red cells as a means of determining the rate of erythropoiesis. It was the exploitation of this phenomenon that led to the development of bioassays for erythropoietin in their present form. Plzak *et al.* (1955) first demonstrated an increase in the rate of incorporation of radioiron into red cells following the administration of anemic rat plasma into normal rats. From this, a more sensitive bioassay involving hypophysectomized rats as recipients was developed (Fried *et al.*, 1956). Such animals were several times more sensitive to the administration of small amounts of anemic plasma than were normal rats. These same authors (Fried *et al.*, 1957) later demonstrated that rats subjected to an atmosphere of high O_2, starvation or transfusion polycythemia also had a decreased radioiron red cell incorporation and an exaggerated response to the administration of anemic plasma. The development of such assays for erythropoietin were important since they involved only one or two injections of test material and

avoided the lengthy assays with prolonged administration required to observe increases in circulating red blood cells, hemoglobin or hematocrits. The value of such radioiron red cell incorporation assays in rats was substantiated by the high correlation observed with the polycythemia produced after prolonged administration of erythropoietin (Garcia and Van Dyke, 1959). Rats with depressed erythropoiesis following dehydration (Rambach *et al.*, 1961), administration of nitrogen mustards (Korst *et al.*, 1958), and irradiation (Stohlman and Brecher, 1956) have also been used as assay animals, however, the starved rat has been perhaps the most widely used rat preparation. Erythropoiesis in such an assay animal is not completely depressed and still requires the administration of an amount of erythropoietin in the order of one unit to obtain a significant result.

Unlike the situation in the rat, erythropoiesis in the mouse is much more completely depressed as a result of polycythemia. Jacobson *et al.* (1957b) first showed that the number of reticulocytes in the peripheral blood fell essentially to zero in 6 days in mice following the transfusion of homologous red cells. The polycythemic mouse has become, and still remains, the most sensitive bioassay preparation *in vivo* for erythropoietin. A careful study of the ideal time relationships for the administration of the erythropoietin and radioiron and the sampling of the blood was done by DeGowin *et al.* (1962a). They were able to quantitate as little as 0.05 units of erythropoietin given in a single subcutaneous injection into the hypertransfused polycythemic mouse. Most bioassays *in vivo* presently being used are modifications of their assay. Beside the polycythemia produced by the transfusion of red cells, a variety of other schemes have been used to produce the polycythemic mouse. Mice made polycythemic by exposure to hypobaric hypoxia (Cotes and Bangham, 1961; DeGowin *et al.*, 1962b), exposure to carbon monoxide (Fogh, 1966), or hypoxia produced by silicone rubber membrane enclosures (Lange *et al.*, 1966) have all been used satisfactorily as sensitive bioassay preparations *in vivo*.

IV. SPECIFICITY OF BIOASSAYS *IN VIVO* FOR ERYTHROPOIETIN

Unfortunately, the polycythemic mouse bioassay for erythropoietin is not as erythropoietin specific as one would like. A variety of

materials are now known which will influence red cell radioiron incorporation in such animals. Polycythemic mice injected with sheep plasma erythropoietin along with normal rat serum will show a markedly increased effect as compared to that seen when the same amount of erythropoietin is given with saline (Garcia and Schooley, 1965). A similar observation has been made for human urinary erythropoietin by Moores *et al.* (1960). Other materials which will stimulate radioiron incorporation in polycythemic mice include: testosterone (Fried *et al.*, 1964), ACTH and triiodothyronine (Fisher *et al.*, 1967), prolactin (Jepson nd Lowenstein, 1965), vasopressin (Jepson *et al.*, 1968), serotonin (Lowry *et al.*, 1970), cyclic AMP (Schooley and Mahlmann, 1971a) and certain prostaglandins (Schooley and Mahlmann, 1971b). Recently, Rodgers *et al.* (1975) have observed that the prolonged treatment of normal mice with cyclic AMP produced a polycythemia in terms of an increase in total red cell mass as well as hematocrit and hemoglobin concentration. The stimulatory effect of most of these materials is abolished by the use of an anti-erythropoietin antiserum, indicating that their effects are erythropoietin dependent. This is discussed in detail elsewhere in this volume. For the most part these materials are thought to have their effect in causing the release of endogenous erythropoietin; however, Naets and Wittek (1968) feel that the effect of testosterone is one of enhancing the effect of erythropoietin. Human placental lactogen also enhances the effect of erythropoietin in polycythemic mice (Jepson and Friesen, 1968). Orosomucoid has been reported to enhance erythropoietin activity (Goldwasser, 1968). Van Dyke *et al.* (1968) have also reported on a factor which will enhance the biological activity of erythropoietin. In view of the fact that the polycythemic rat (Schooley and Garcia, 1970) and even the more sensitive polycythemic mouse (Schooley and Mahlmann, 1972) still contain a certain amount of endogenous erythropoietin in their circulations, the possibility that this small amount of erythropoietin may be enhanced when plasma or serum is injected must be considered.

The existence of inhibitory factors on erythropoiesis has been demonstrated in the plasma from hypertransfused animals (Krzymowski and Krzymowska, 1962; Whitcomb and Moore, 1965) and in the plasma of high altitude residents after return to sea level (Reynafarje, 1968). Jepson and Lowenstein (1966) found erythropoietic inhibitory activity in the plasma of patients with erythroblastopenia which they postulated to be anti-erythropoietin antibody. Erythropoiesis-inhibiting factors have also been isolated

from the urine of normal and anemic patients (Lewis *et al.*, 1969; Lindemann, 1971).

Thus the bioassay *in vivo* for erythropoietin appears to be sensitive to materials other than erythropoietin, some of which may stimulate endogenous erythropoietin in the assay animal, others may enhance or inhibit the effect of endogenous erythropoietin in the assay animal or exogenous erythropoietin injected along with the test material. The assay of serum, plasma or urine samples with such an assay must be looked upon as representing the effect of a summation of all such materials in the specimen tested, including the erythropoietin molecules present.

V. BIOASSAYS *IN VITRO* FOR ERYTHROPOIETIN

A variety of assay systems *in vitro* for erythropoietin have been developed utilizing cultures of bone marrow, spleen or fetal liver. An increase in the number of erythroblasts in rat marrow cultures incubated with anemic, as compared to normal human plasma, has been reported (Friederici, 1958). Korst *et al.* (1962) found that serum from anemic rabbits increased radioiron incorporation into rat or sheep marrow cells. An erythropoietin dose-response relationship was obtained by Erslev (1962) using radioiron incorporation by rabbit bone marrow cells incubated with varying concentrations of sheep erythropoietin. Krantz *et al.* (1963) were able to stimulate heme synthesis in rat bone marrow cultures with the development of an erythropoietin dose-response curve. Cultures utilizing human bone marrow with sheep plasma erythropoietin (Krantz, 1965) and dog bone marrow with human urinary erythropoietin (Ward, 1967) was able to block this effect by the use of an erythropoietin antiserum. The lower limit of sensitivity of this assay was approximately 0.05 unit per milliliter. Dukes *et al.* (1964) have described an assay system *in vitro* in which a dose-response relationship is obtained between sheep plasma erythropoietin and the incorporation of radiocarbon labeled glucosamine into the stroma of cultured rat bone marrow cells. Assay systems *in vitro* utilizing spleen fragments from polycythemic mice have also been used (Nakao *et al.*, 1966; Miura *et al.*, 1967). In these assays erythropoietin initiated the formation of new erythroblasts which was paralleled by radioiron incorporation into heme. Radioiron incorporation into fetal mouse liver cells has

been utilized as an erythropoietin assay *in vitro* (Wardle *et al.*, 1973). These authors obtained a dose–response relationship for erythropoietin down to a concentration of 0.005 unit per milliliter of incubation medium (10 ml total volume).

Not all reports utilizing marrow culture systems *in vitro* have been positive in terms of responding to erythropoietin (Alpen, 1962). Culture systems *in vitro* for the assay of erythropoietin require extreme care regarding the preparation of all reagents including the concentration of cells in the medium, transferrin and iron concentrations, etc. They are also subject to the effects of non-specific inhibitory factors which are present in plasma, serum and crude erythropoietin preparations. Thus, the interpretation of results utilizing such materials in assays *in vitro* must be accepted with caution. Assays *in vitro* may be more useful in following biochemical purification procedures, however caution must be exercised here also. Dukes *et al.* (1970) have observed great differences in the biological activity of various erythropoietin preparations when assayed by the polycythemic mouse assay system *in vitro* measuring radiocarbon-labeled glucosamine incorporation into rat bone marrow cells, and the system *in vitro* measuring the incorporation of radioiron into heme of rat bone marrow cells. Finally, Goldwasser (1966) has observed that removal of the sialic acid from the erythropoietin molecule does not interfere with its action *in vitro* on hemoglobin synthesis or glucosamine incorporation in cultured marrow cells, although its biological activity in the bioassay *in vivo* is completely destroyed. It must be admitted here that the attempt, so far, to develop a radioimmunoassay for erythropoietin has this same discrepancy with the bioassay *in vivo* for erythropoietin. Both intact and asialoerythropoietin appear to be equally reactive in the radioimmunoassay for human erythropoietin (Garcia, 1972).

VI. IMMUNOLOGICAL ASSAYS FOR ERYTHROPOIETIN

A. GENERAL COMMENTS

Since the first demonstration of the ability to produce antibody, which will neutralize the biological activity of erythropoietin (Schooley and Garcia, 1962), various investigators have been

concerned with the application of immunological techniques to the measurement of erythropoietin. A precipitating rabbit antiserum to rat erythropoietin was produced by Goudsmit *et al.* (1967) and Krugers Dagneaux *et al.* (1968) which cross-reacted with human erythropoietin with the formation of a single precipitin line. These investigators used this antibody and an agar double diffusion technique in an immunological assay for human erythropoietin. They obtained a correlation with biological assays *in vivo*, with high levels of erythropoietin in patients with aplastic anemia, and normal human plasma levels between 0.5 and 2 mu/ml. They also reported that no erythropoietin could be detected using this assay on plasma from a patient with chronic renal insufficiency combined with anemia.

Other investigators (Lange *et al.*, 1969) produced an erythropoietin antibody which caused hemagglutination of tanned red cells sensitized with erythropoietin. They found that this hemagglutination could be inhibited by various urinary erythropoietin extracts and by human serum with a high titer of erythropoietin. Using this technique, they obtained erythropoietin values in normal human serum of 7 to 30 mu/ml. In a later study, using the hemagglutination-inhibition technique, Lange *et al.* (1970) observed high erythropoietin values in the sera of patients with anemia associated with uremia. In addition to human serum, the hemagglutination-inhibition assay for erythropoietin was used to measure erythropoietin in the serum of the rat, rabbit, mouse, sheep and pig (Lange *et al.*, 1972).

Studies utilizing the radioimmunoassay approach to the measurement of erythropoietin in human urine (Fisher *et al.*, 1972) and human plasma or serum (Garcia, 1972, 1974; Lertora *et al.*, 1975) have been reported. Also, Cotes (1973) has developed a radioimmunoassay for sheep plasma erythropoietin. More discussion of these studies concerning the radioimmunoassay of erythropoietin will be presented below. However, basically, the radioimmunoassay depends on the ability of unlabeled hormone to inhibit the binding of labeled hormone by specific antibody. The determination of the concentration of the hormone in a sample is obtained by comparison of the degree to which it inhibits the binding of the labeled hormone by antibody, with the degree of inhibition obtained by a series of standard hormone concentrations. The essential requirements for the development of a radioimmunoassay for a hormone are: (1) the availability of the hormone in a pure form and its ability to accept a radioactive label; (2) the ability to produce specific antibody to that

hormone and (3) a technique for the separation of antibody-bound and non-antibody-bound hormone after a given incubation period. In the next sections I will present his considerations and experiences concerning the development of a radioimmunoassay for human erythropoietin.

B. ANTI-ERYTHROPOIETIN ANTIBODY

The production of antibody varies greatly, both in quantity and quality, from animal to animal even with the same immunization schedule and within the animal itself at different times throughout the immunization. The obtaining of a good antibody for radioimmunoassay purposes, i.e. one with a high avidity for the antigen, may require the immunization of a large number of animals. In practice, one uses the dilution of an antiserum which will result in the binding of 50-70% of a given amount of labeled antigen. Then one chooses the antiserum which results in the greatest amount of inhibition when a small amount of unlabeled antigen is added.

Antibody to erythropoietin was produced in rabbits immunized with erythropoietically active human urinary extracts from severely anemic patients prepared by pressure filtration through a collodoin membrane (Van Dyke *et al.*, 1957). Ten milligrams of such an extract containing approximately 200 units of erythropoietin were dissolved in one milliliter of distilled water and emulsified with one milliliter of complete Freund's adjuvant, and given in 4 subcutaneous sites at weekly intervals for 3-4 weeks. Parenthetically, this investigator has considered the possibility that, as a result of our erythropoietin extraction procedure, small amounts of collodoin may have persisted in our eythropoietin extracts and may have contributed additional adjuvants effects. This may have facilitated our initial attempts at the production of erythropoietin neutralizing antibody (Garcia and Schooley, 1962). This procedure usually resulted in erythropoietin neutralizing antiserum in greater than 50% of the rabbits immunized. Precipitin lines can be demonstrated when such anti-erythropoietin antiserum is allowed to react in Ouchterlony plates with a wide variety of purified human plasma protein fractions. However, if the anti-erythropoietin antiserum is absorbed with these proteins, no reduction in the erythropoietin neutralizing ability of the antiserum is observed (Schooley and Garcia, 1965). Thus, the anti-erythropoietin antiserum contains a mixture of antibodies against a variety of proteins, as well as antibody specifically directed against erythropoietin. The anti-erythropoietin

activity can be assessed by its effect in completely depressing the radioiron incorporation into red cells of normal mice. Also, as a corollary to this, the fact that erythropoiesis in normal mice can be depressed by the administration of anti-erythropoietin antiserum must mean that the normal production of red cells in mice is mediated by erythropoietin. Erythropoietin is not just a hormone brought into action in severely anemic animals, or by exposure to anoxic environments, but plays a role in the maintenance of day-to-day erythropoiesis. This will be covered in more detail in Chapter 6. Erythropoietin antibody can also be assessed by combining it with a standard amount of erythropoietin and assaying in the polycythemic mouse for any remaining erythropoietin. In addition to human erythropoietin, increased erythropoietin levels produced in a variety of animal species, including mice, rats, rabbits and sheep, can also be neutralized with such antiserum (Garcia and Schooley, 1963). These data suggested that portions of the erythropoietin molecule in all these species may be similar. Since rabbit erythropoietin appeared to be neutralized by antiserum produced in rabbits, a study of the hematological state in rabbits being immunized with human erythropoietin was initiated (Garcia, 1972). A transient anemia develops in the rabbits following immunization, and as the hematocrit falls, erythropoietin neutralizing ability is present in the serum. It appears that the presence of anti-erythropoietin in the serum reacts with endogenous erythropoietin in the rabbit. This anemia, coincident with the presence of erythropoietin neutralizing antibody in the serum, further supports, in the rabbit, the hypothesis that normal erythropoiesis is erythropoietin dependent. The presence of anti-erythropoietin ability of the rabbit serum disappears along with the anemia. This is the usual picture we have observed in rabbits immunized with human urinary erythropoietin. However, one rabbit, after an initial immunization with human urinary erythropoietin, was given an intravenous booster with sheep plasma erythropoietin, and after the disappearance of the usual transient anemia, the serum of this rabbit retained the ability to neutralize human erythropoietin for about one year. The incubation *in vitro* of serum from this rabbit with rabbit erythropoietin showed that it no longer could neutralize rabbit erythropoietin (Garcia, 1972). These data suggest that, although there are similarities between human and rabbit erythropoietin giving rise to antibody which will neutralize both, there must also be molecular differences between these two erythropoietins which can give rise to antibody which will neutralize one and not the other.

More complete study of our antisera produced in rabbits immunized with human urinary erythropoietin has revealed other species differences in their ability to neutralize various erythropoietins. Although most of our antisera will neutralize 25 units of human erythropoietin per milliliter, they will usually neutralize only about 2.5 units of rat erythropoietin. Rioux and Erslev (1968) have also observed immunological differences between sheep and mouse erythropoietin.

Species differences in erythropoietin molecules may be highly significant in attempts at radioimmunoassay. So far we have not been able to show competition in our radioimmunoassay competition for human erythropoietin with sheep and rabbit erythropoietin. We consider our antiserum to be a mixture of antibodies against a variety of proteins as well as antibodies specifically directed against erythropoietin. Also, it must be considered as a mixture of antibodies of varying avidities against various antigenic sites in the erythropoietin molecules. Some of these antibodies must be directed against antigenic sites which are common in a variety of species erythropoietins and some must be directed against antigenic sites only present on the particular species erythropoietin molecule used for immunization. It is not surprising that in such a spectrum of antibodies against erythropoietin that the antibody of highest avidity would be that directed against different antigenic sites present in the erythropoietin used for immunization, in this case human erythropoietin. Considering the extreme dilution and time constraints imposed in the development of a radioimmunoassay system, only antibody of the highest avidity would be expected to express its effect. Indeed, the method for selection of a good antiserum for radioimmunoassay discussed above, would favour selection of the antiserum with the highest avidity for the labeled antigen.

The antiserum used in the radioimmunoassay described here was obtained from a single bleeding of one rabbit. This rabbit had previously been immunized with a series of subcutaneous injections of relatively crude human urinary erythropoietin in Freund's adjuvant and had responded, as described above, with a transient anemia coincident with erythropoietin neutralizing serum. It was later given a single booster immunization of 13 micrograms (approximately 100 units) of a highly purified erythropoietin of human urinary origin obtained from Dr Espada. The rabbit was bled 9 days after this immunization and had a very high neutralizing ability for human erythropoietin. One milliliter of this antiserum neutralized more than 300 units of human erythropoietin. Fractionation of this

antiserum on Sephadex G-200 revealed that both the erythropoietin neutralizing ability and the ability to bind labeled erythropoietin resided with the 7S gamma globulins (Garcia, 1972). This antiserum was used in the radioimmunoassay for human erythropoietin at a final incubation dilution of 1 : 1 000 000.

It was not felt necessary to absorb the antiserum with normal human plasma prior to its use in the radioimmunoassay, as has been done by Fisher *et al.* (1971) in their erythropoietin radioimmunoassay. Because of the considerations presented above regarding the necessity of obtaining antibody with the highest avidity for the hormone, it was felt that such a procedure should, in fact, be avoided. The presence of even the small amount of erythropoietin expected in normal human plasma would serve only to bind the most avid erythropoietin antibody present in the antiserum, thereby seriously jeopardizing ones chance of obtaining a sensitive radioimmunoassay for the hormone. Besides, if one considers the radioimmunoassay scheme, the only antigen-antibody reaction which is of consequence is that between labeled antigen and its antibody. The presence of antibody against human serum albumin, for instance, would not affect the radioimmunoassay system for erythropoietin so long as no labeled human serum albumin was present in the incubation, regardless of the amount of unlabeled human serum albumin present. This of course presumes that no cross-reaction exists between human serum albumin and the specific anti-erythropoietin antibody.

It is for these reasons that it was decided not to absorb any of the other antibodies known to be present in the antiserum. Instead, in attempting to achieve a sensitive radioimmunoassay for erythropoietin, it was decided to concentrate on the possibility of obtaining a labeled fraction which consisted only of labeled erythropoietin molecules.

C. LABELED ERYTHROPOIETIN

The human erythropoietin used for labeling was obtained from Dr Espada. It was extracted from the urine of anemic patients and purified by a series of chromatographic steps to a specific activity of approximately 8000 units per milligram protein. This lyophilized material was dissolved in distilled water, and divided into 50-microliter aliquots containing 6.5 micrograms, or 50 units of erythropoietin. The glass ampoules were sealed and kept frozen until

used for labeling. The erythropoietin was labeled with ^{125}I, using the method of Greenwood *et al.* (1963). The radioiodine was obtained from New England Nuclear (NEZ-33L) as carrier free Na ^{125}I, in a concentration of approximately 400 millicuries per milliliter at a pH of 8-10. Usually 2 millicuries ^{125}I and 5-6.5 micrograms of erythropoietin were used in each iodination. One hundred microliters of phosphate buffer (0.5 M, pH 7.5) and 5 microliters of Na ^{125}I were added to the ampoule containing the erythropoietin which was then stoppered with a rubber cap. The other reagents were added through the rubber cap by the use of microsyringe. They were added in the following order with mild agitation: 30 microliters chloramine T (4 mg/ml), 100 microliters sodium metabisulfite (2.4 mg/ml) and 200 microliters potassium iodide (10 mg/ml). These reagents were made up in phosphate buffer (0.05 M, pH 7.5). Approximately 30 seconds were allowed following the addition of the chloramine T, whereas the other reagents were added fairly quickly. The total contents of the ampoule were then immediately transferred to a small Sephadex G-50 column and eluted in approximately one-milliliter aliquots with barbital buffer (0.07 M, pH 8.6). Usually the labeled erythropoietin was eluted in the third or fourth tube and the remaining unreacted radioiodide peaked at approximately the eighth tube. This procedure resulted in preparations of labeled erythropoietin which usually had specific activities greater than 100 microcuries per microgram. In our early studies concerning the production of antibody to human erythropoietin, in addition to immunizing rabbits with erythropoietically active human urinary extracts, we also immunized some rabbits with a similar extract prepared from normal human urine (Schooley and Garcia, 1962). Some of the antiserum prepared against normal human urinary protein was combined with the labeled erythropoietin and submitted to gel filtration on Sephadex G-150. Such a fractionation resulted in two peaks of radioactivity. The first peak, occuring at the void volume, contained any erythropoietin damaged in the labeling process and any labeled protein contaminants to which antibody was present in the antiserum against normal urinary extract. The second peak, at approximately 1.6 times the void volume, contained undamaged labeled erythropoietin molecules freed of some of its labeled contaminants. In our experience biologically active erythropoietin occurs at approximately 1.6 times the void volume on Sephadex G-150 and at 1.8 times the void volume on Sephadex G-200 (Garcia, 1974). The second peak, containing the labeled erythropoietin, at 1.6 times the void volume

was combined with an anti-erythropoietin antiserum and again placed on a Sephadex G-150 column. Again two peaks of radioactivity were eluted. The first peak, at the void volume, consisted of labeled erythropoietin molecules bound to anti-erythropoietin gamma globulin. The second peak consisted of labeled material which was not antigenic with either of the antisera used. Garcia and Schooley (1971) have shown that the biological activity of erythropoietin in an erythropoietin-anti-erythropoietin complex can be recovered by an acidification-heating procedure similar to that used by Borsook *et al.* (1954). The labeled erythropoietin was dissociated from its antibody by acidification to pH 5.5 and heating in a boiling water bath for 5 minutes. The labeled erythropoietin, resulting from such a procedure is thus freed of any damaged labeled erythropoietin, some labeled contaminants and some labeled non-antigenic material. Gel filtration on Sephadex G-200, of such "immunologically purified" labeled erythropoietin, shows a single symmetrical peak of radioactivity coinciding with both biological and immunological erythropoietin activity at approximately 1.8 times the void volume (Garcia, 1974). This "immunologically purified" labeled erythropoietin was also characterized in electrofocusing studies (Garcia, 1972). The major peak of radioactivity occurred at pH 3.5, which is in agreement with Lukowsky and Painter (1972) who observed similar results for sheep plasma erythropoietin.

D. ERYTHROPOIETIN RADIOIMMUNOASSAY

The radioimmunoassay presented here was designed for the measurement of plasma or serum concentrations of human erythropoietin. A double-antibody technique was used for the final separation of the antibody-bound labeled erythropoietin. Goats immunized with rabbit gamma globulin were used as a source of the precipitating second antibody. Initially, the first International Reference Preparation of human erythropoietin was used as the standard. More recently, the second International Reference Preparation has been used, with similar results. The erythropoietin standard was dissolved in a diluent made up of 5% human serum albumin in 0.05 M phosphate buffer at pH 7.5. Halving concentrations of the erythropoietin standard from 100 mu/ml down to 0.78 mu/ml were made using the same 5% human serum albumin phosphate diluent. This was done in an attempt to keep, at least, this protein constituent in the erythropoietin standards similar to that in normal plasma or serum.

One milliliter of the varying erythropoietin standard dilutions was pipetted into a 15-ml test tube. Other test tubes were set up with 1 ml of plasma or serum to be assayed. Two milliliters of the "immunologically purified" labeled erythropoietin, containing 5000-10 000 c.p.m. and approximately 0.1-0.2 mu of erythropoietin were added to each tube. This was followed by the addition of two ml of a 1 : 400 000 dilution of the rabbit anti-erythropoietin. The diluent for both the labeled erythropoietin and the anti-erythropoietin consisted of 0.05 M phosphate buffer at a pH of 7.5 with 0.05% bovine serum albumin added. The tubes were then capped and incubated at 4° for a period of 4-5 days. After the incubation period 1 ml of a 1 : 10 dilution of normal rabbit serum was added as a source of carrier rabbit gamma globulin. This was followed by an amount of goat anti-rabbit gamma globulin serum which had previously been determined would maximally precipitate the rabbit gamma globulin in the test tube and any labeled erythropoietin which was antibody bound. After a two-hour period at 4°, the test tubes were centrifuged at 700 x *g* in a refrigerated centrifuge for 30 minutes, and the supernatant decanted. Using such an excess of rabbit gamma globulin resulted in a readily visible precipitate which held together as a pellet in the bottom of the test tube on decanting. The radioiodine in the precipitates was then counted in a Nuclear Chicago automatic well-type scintillation counter. Curves were plotted using semilogarithmic paper with the standard erythropoietin concentration (i.e., the initial concentration in the 1 ml used) on the logarithmic scale and the percentage of labeled erythropoietin bound to antibody on the linear scale. This allows for a direct reading of the concentration of erythropoietin in the 1 ml of plasma or serum used in the assay. A curve of a typical radioimmunoassay for human erythropoietin is presented in Fig. 1. With the antibody concentration and the incubation period used, the quantity of labeled erythropoietin which is bound is approximately 70% when no unlabeled erythropoietin is added. It appears from Fig. 1 that human erythropoietin in plasma or serum can be assayed when the concentration lies between 1 and 100 mu/ml. The separated 7S gamma globulin of the anti-erythropoietin antiserum can equally be used in the radioimmunoassay, and will result in a similar curve. Removal of the sialic acid from erythropoietin, using the enzyme neuraminidase, does not appear to interfere either with the labeled erythropoietin or the unlabeled erythropoietin used for the development of the standard curve, although this treatment completely destroys the biological activity of erythropoietin. The

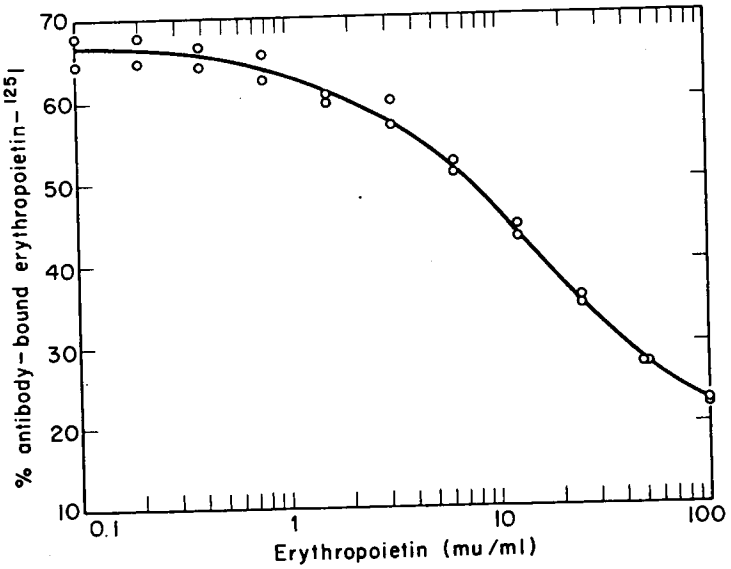

Fig. 1. A typical standard curve for the radioimmunoassay of human plasma erythropoietin.

curve obtained with neuraminidase-treated erythropoietin was identical to that obtained with non-neuraminidase-treated erythropoietin. Although 100 mu of the human erythropoietin standard results in maximal competition, 1000 mu of rabbit or sheep erythropoietin do not compete with the binding of labeled human erythropoietin for the antibody. Thus the radioimmunoassay appears to be specific for human erythropoietin and unfortunately not useful for animal studies.

Dilutions of human sera, which have high erythropoietin values, results in a parallel relationship with the erythropoietin standard, thus supporting the identity of the immunoreactive material in the sera with the erythropoietin standard.

E. DISCUSSION AND COMPARISON OF RESULTS WITH IMMUNOASSAYS

Using the radioimmunoassay for human erythropoietin described here on a large series of samples of serum from normal individuals resulted in an average value of 4.3 mu/ml for females as compared to 4.9 mu/ml for males. This small difference was significant with a P value <0.02. No difference was seen between heparinized plasma and serum taken at the same time. Serum samples taken in the morning from normal subjects all gave higher values than samples taken in the afternoon on the same subjects, suggesting a diurnal pattern.

Although erythropoietin bioassay systems have generally been

insensitive to the erythropoietin (Ep) concentration in plasma or serum of normal individuals, it has been possible to measure the excretion of erythropoietin in concentrates of urine from normal individuals (Finne, 1965; Adamson *et al.*, 1966). Using such urinary concentrates, Alexanian (1966) has observed a greater excretion of erythropoietin in normal men as compared to normal women. Also Adamson *et al.* (1966) have observed diurnal variations in the excretion pattern of erythropoietin in normal individuals.

With the radioimmunoassay for erythropoietin, it was possible to demonstrate an increase in serum erythropoietin following a moderate bleeding (Garcia, 1974), whereas no erythropoietin was demonstrable when these same serum samples were assayed in the polycythemic mouse bioassay. However, as demonstrated by Adamson (1968), by utilizing concentrates of urine, it was possible to show a rise in the excretion of erythropoietin, as measured by increased radioiron incorporation in polycythemic mice, which coincided with the observed rise in serum erythropoietin as measured by radioimmunoassay. Thus results obtained in normal individuals by radioimmunoassay of serum are in a certain amount of agreement with those reported for urine concentrates measured by bioassay.

Generally, using the radioimmunoassay presented here on serum from anemic individuals results in higher erythropoietin values than that observed in normal subjects, although not as high as results obtained by bioassay of the same samples. In one case, a severely anemic patient (Fanconi's anemia) with extremely high bioassayable erythropoietin in serum showed a normal value for serum erythropoietin by the radioimmunoassay. Also, a great discrepancy has been observed utilizing this radioimmunoassay on the serum of severely anemic kidney patients, either anephric, or in patients with kidney disease undergoing hemodialysis. These patients have very high serum erythropoietin levels as determined with the radioimmunoassay, although no erythropoietin was detectable in the serum of anephric patients with the polycythemic mouse assay. The results in the anephric patient have a model in the results obtained with asialoerythropoietin, in that, whereas the erythropoietin immunoreactivity is retained, its biological activity in the polycythemic mouse is completely lost. The possible effect of enhancing or inhibiting factors in the bioassay *in vivo* may account for some of the discrepancies seen with the radioimmunoassay. In the bioassay for erythropoietin such factors could express themselves by modifying the biological activity of the erythropoietin molecules, whereas the radioimmunoassay may be more closely correlated with

the number of erythropoietin molecules present in the serum. In this respect, Wallner *et al.* (1975) concluded on the basis of a bioassay *in vitro* that there is a substance in serum of anemic patients which suppresses heme synthesis and that this "uremic toxin" may be responsible for the clinically severe anemia seen in these patients. The possible existence of various erythropoietin molecules (Dukes *et al.*, 1971) or various forms of one erythropoietin molecule by aggregation or fragmentation also must be considered. As pointed out above, the best evidence is that the kidney is the main source of erythropoietin. More recent evidence further supports this hypothesis (Burlington *et al.*, 1972; Erslev, 1974). However, reports of extrarenal erythropoietin production are also in evidence (Schooley and Mahlmann, 1974).

High immunoreactive serum erythropoietin levels in anemic uremic patients have also been observed by Lange *et al.* (1970) and Lertora *et al.* (1975), although no erythropoietin was detected in the plasma of such a patient by the erythropoietin immunoassay of Krugers Dagneaux *et al.* (1968). The low erythropoietin values obtained in normal serum with the radioimmunoassay presented here are not consistent with those reported by Lertora *et al.* (1975) who observe a range of 52-84 mu/ml in normal subjects. Their values are within the range of the polycythemic mouse assay for erythropoietin although no erythropoietin was detected using such an assay. The results on normal serum presented here are rather more consistent with the much lower results reported by Goudsmit *et al.* (1967) and the lower end of the range (7-30 mu/ml) reported by Lange *et al.* (1969).

Cotes (1973) reported on a radioimmunoassay for sheep plasma erythropoietin which utilized a highly purified sheep plasma erythropoietin obtained from Goldwasser and Kung (1971). Unlike the results presented here regarding generally lower erythropoietin immunoassay levels in human serum relative to the bioassay, the results obtained by use of the sheep radioimmunoassay were generally higher than bioassay results. Bleeding of sheep resulted in a rise in immunoreactive erythropoietin which coincided with a rise in biologically active erythropoietin. The labeled sheep erythropoietin was fractionated on Sephadex G-150 and was eluted at approximately 1.6 times the void volume which was coincident with the elution of biologically active erythropoietin as measured in the polycythemic mouse assay. The labeled erythropoietin was further fractionated by ultracentrifugation which resulted in the formation of two peaks of radioactivity. Only the lighter of the two peaks

coincided with the biological activity and could be bound by anti-erythropoietin antibody. This purified labeled sheep erythropoietin was used in the radioimmunoassay of sheep plasma erythropoietin. A later study (Cotes, 1975) demonstrated the presence of small amounts (less than 3%) of biologically active erythropoietin occurring at the void volume of Sephadex G-150 fractionation of both erythropoietin of human urinary and sheep plasma origin. This small amount of "big erythropoietin" was equally reactive with anti-erythropoietin antibody and it was suggested that it may be formed by an aggregation of the monomeric form of erythropoietin. The radioimmunoassay for human plasma erythropoietin reported by Lertora *et al.* (1975) apparently used this large form of erythropoietin since the labeled erythropoietin used by these investigators was eluted at the void volume of Sephadex G-150 fractionation. In their studies, Lertora *et al.* (1975) observed that this relatively small peak of labeled erythropoietin which occurred at the void volume was immunologically reactive, whereas a much larger peak of radioactivity was not immunologically reactive although it occurred in an area where biologically active erythropoietin is eluted from such a column.

All of the radioimmunoassays discussed here have utilized highly purified erythropoietin fractions of human or sheep origin. The reasons for the differences observed between the various radioimmunoassay systems are not obvious. Whether the answers lie in material damaged in the iodination process, resulting in aggregation or fractionation of the erythropoietin molecule, or whether they lie in the avidity of the various antisera used must await further work.

The hemagglutination-inhibition assay described by Lange *et al.* (1969) appears to have reactants which parallel those used in the radioimmunoassay scheme, i.e. tanned red cells sensitized with erythropoietin corresponding to the labeled erythropoietin, antibody which causes agglutination of the erythropoietin sensitized tanned red cells corresponding to the antibody binding the labeled erythropoietin, and finally inhibition of the agglutination reaction in the presence of added erythropoietin corresponding to the competition in the radioimmunoassay system. Because of this parallelism it would seem that the hemagglutination-inhibition assay system would be subject to the same requirements and limitations as the radioimmunoassay technique. One of the requirements being the need for highly purified erythropoietin used in sensitizing the tanned cells, as highly purified labeled erythropoietin is required in the

radioimmunoassay system. Any contaminant present in the erythropoietin extract used to sensitize the red cells could also react with antibody present in the antiserum specific for the contaminant. Absorption of the antiserum with normal human serum does not sufficiently rule out this possibility. Most would agree that erythropoietin is present in normal human serum and yet the absorption procedure does not absorb out all the anti-erythropoietin antibody. Thus, it cannot be assumed that antibody against all of the unknown contaminants present in the crude immunizing erythropoietin extract have been removed. The erythropoietin fractions used by Lange *et al.* (1969) for immunization and sensitization of the tanned red cells have been relatively impure. Yet, in the carefully controlled conditions in their laboratory, they find a good correlation between biological and immunological activity using their hemagglutination-inhibition assay system. Other investigators who have attempted to use this assay system for erythropoietin have not found it as satisfactory (Kolk-Vegter *et al.*, 1975).

Finally, the only data whereby an immunoassay approach to the measurement of erythropoietin has resulted in complete agreement with bioassay results are those obtained by Krugers Dagneaux *et al.* (1968). The results obtained by these investigators are meager however.

VII. CLOSING COMMENTS

In general, all bioassay systems for erythropoietin, both *in vivo* and *in vitro*, are not sensitive enough to measure erythropoietin concentration in untreated plasma or serum much below 50 mu/ml. The commonly used practice of expressing the sensitivity of a given assay in terms of absolute units can be misleading when applied to serum. Some bioassay systems *in vitro* are very sensitive to small amounts of extracted erythropoietin, but examination of the protocol reveals that a relatively small volume of serum can be used without the risk of non-specific inhibitory effects. If the implication of an assay is that it can be used on unextracted serum, its sensitivity should perhaps be given in terms of the minimal concentration of erythropoietin detectable in serum.

Details of a radioimmunoassay for erythropoietin are given here which allows for the measurement of erythropoietin concentrations

in unextracted human serum down to approximately 1 mu/ml. Using this assay a significant difference has been observed between the normal male and female serum erythropoietin (Garcia, 1974). Moderate bleeding of a normal individual resulted in an increase in the serum concentration of immunoreactive erythropoietin. Conversely, return of the blood resulted in a depression of the immunoreactive serum erythropoietin concentration. No erythropoietin was detectable by bioassay of the serum in this study. However, a correlation with these results was shown to exist when bioassays in the polycythemic mouse assay were performed on concentrates of urine. Bioassay of urinary concentrates has also revealed a difference in the excretion of erythropoietin in humans, between normal males and females. Thus erythropoietin results obtained in the normal individual by radioimmunoassay of serum appear to be supported by results obtained by bioassay of urinary concentrates. However there still are discrepancies with the radioimmunoassay of serum erythropoietin. Most notably are the results seen in anemic patients without kidneys, where the highest serum immunoreactive erythropoietin is observed and yet no erythropoietin is detectable by bioassay of such serum in the polycythemic mouse assay.

Some puzzling results have been obtained in the use of the erythropoietin bioassay *in vivo*. As has been observed by many others, Alexanian (1969) found that erythropoietin was detectable in the plasma of mice exposed to hypoxia only during the first few days of hypoxic exposure. After this time no erythropoietin was detectable in the plasma by bioassay although there was a sustained stimulation of erythropoiesis throughout the prolonged hypoxic exposure. Schooley (J. C. Schooley, personal communication) is able to detect measurable erythropoietin in the serum of male mice during the first few days of a 30-day exposure to a simulated altitude of 14 000 feet. No erythropoietin was detectable, at any time, in the serum of female mice similarly exposed. However both male and female mice accumulated the same net increase in total red cell mass of 30% after the 30-day exposure. These studies emphasize the need for erythropoietin assay systems which are capable of measuring erythropoietin concentrations in serum with a sensitivity of better than 50 mu/ml.

Adamson and Finch (1975), in a recent review, point out that the total oxygen transport system; hemoglobin concentration, hemoglobin saturation with oxygen, hemoglobin affinity for oxygen, as well as other factors, are involved in setting the erythropoietic state

of the marrow. These factors must also be considered then to play a significant role in the regulation of the plasma level of erythropoietin. Consideration of some of these factors may result in more meaningful correlations as new erythropoietin assay systems are developed.

Many hormones, existing in very low concentrations in plasma, are now being routinely measured by the use of radioimmunoassay techniques. For instance, growth hormone has been shown to play an acute regulatory role in intermediary metabolism (Glick *et al.*, 1965). Hypoglycemia, exercise, administration of certain amino acids result in consistent prompt rises in the plasma concentration of this hormone. Such a volatility for growth hormone would not have been predicted before the development of a radioimmunoassay for this hormone.

Generally, there appears to be a barrier at about 50 mu/ml in the ability to assess erythropoietin concentrations in serum or plasma using any of bioassay systems for erythropoietin. An exciting adventure most certainly lies below this barrier. The radioimmunoassay technique offers some hope in this direction.

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