Topical Reviews in

Haematology

Volume One

Edited by S. Roath

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EDITED BY

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Bristol
John Wright & Sons Ltd,
1980

Published by John Wright & Sons Ltd., 42–44 Triangle West, Bristol BS8 1EX

British Library Cataloguing in Publication Data

Topical reviews in haematology.
Vol. 1
1. Hematology
I. Roath, S
616.1'5 RB145

ISBN 0 7236 0540 8

Printed by John Wright & Sons Ltd.,
at The Stonebridge Press, Bristol BS4 5NU

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Preface

The object of *Topical Reviews in Haematology* is to provide haematologists, trained and in training, with a series of 'state of the art' papers in haematological areas where this appears to be valuable. In general this would include fields where there has been enough investigation for an authority personally involved in that area to make a statement concerning what he or she thinks are the important data and critically analysing the views of those producing data. There is no obligation on the author's part to produce a consensus viewpoint!

The spread of articles varies from fine structure on the one hand to clinical appreciation of patients on the other. In general the subject matter is confined to human haematology, although where appropriate reference to and experience of animal-based data is of course used.

Fine structure, both of the cell's surface and interior, is now an acceptable part of the descriptive background to cell-based disease in haematology. Preparation times for electron microscopy are now a matter of hours so this technique can readily be applied to the solution of urgent patient-based problems. The work of Dr Newell and Professor Smetana helps in understanding the behaviour of 'abnormal' cells – whatever that may mean – in e.g. leukaemias and of course the function of normal cells and their precursors.

Dr Church and Dr Dunn have provided a timely review of the basophil cell, the multi-system effects and interactions of which are only now being appreciated. Dr Dunn has summed up the present confusing status of erythropoietin assays – warts and all – and allows us to make our own judgement on the value of the techniques involved. Research in this area appears to have reached a technological plateau, so this is an opportune moment for this review.

Dr Brozović's article is certainly topical and gives a sound informational basis in another research area where further developments are in the offing. Perhaps a review in 5 year's time on the same topic would again make interesting reading.

Dr. Lewis has provided a typically thorough analysis of technological developments in haematology laboratories which will allow practising haematologists everywhere to assess the benefits of the automated devices available and the changes they could bring to laboratory practice.

The three reviews in the leukaemias cover aetiological, cellular and management aspects. Dr Moloney and Dr Rosenthal's timely presentation on this recently emphasized aspect of the acute leukaemias, Dr Catovsky's on patterns of cellular enzymology and Dr Powles' intriguing overview of acute leukaemia management all show how this subject has recently produced new data and conclusions.

Topical Reviews will appear again at about 18-month intervals when other haematological topics of current interest will be featured. Comment from haematologists about this and future reviews would be welcomed by the editor.

S. R.

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C. D. R. Dunn and R. D. Lange

1 Erythropoietin: Assay and Characterization

INTRODUCTION

In his opening remarks to a session on erythropoiesis at the ICN–UCLA symposium on Hemopoietic Cell Differentiation, Erslev (1) suggested that the link between atmospheric oxygen pressure and red cell concentration in the peripheral blood was first propounded by Jourdanet (2) – observations which were published in 1863. Jourdanet observed that the blood of his patients from the Mexican highlands was thick and viscous and suggested this may have been related to the low oxygen content of arterial blood secondary to the low atmospheric oxygen concentrations at high altitude. Bert (3), Viault (4) and Miescher (5) extended these observations, with Miescher suggesting that hypoxia increased red cell production by a direct stimulating effect on the bone-marrow. The possibility that the effects of hypoxia on erythropoiesis were indirect and mediated by a hormone is generally considered to have been first discussed in two articles by Carnot and De Flandre in 1906. (6, 7) Nevertheless, it was the 1950s before Reissmann (8), in a study with parabiotic rats, and Stohlman et al. (9), from a series of observations in a patient with regional hypoxia, produced indirect support for the hypothesis of the humoral control of erythropoiesis. Direct evidence was detailed by Erslev (10) when he demonstrated that large volumes of plasma from anaemic rabbits caused a reticulocytosis when injected into normal animals. Jacobson et al. (11), in a series of organ extirpation experiments, produced evidence which implicated the kidney as the major site of production of the hormone now termed 'Erythropoietin' (Ep). Jacobson et al. (12), Alpen and Cranmore (13) and Filmanowicz and Gurney (14) suggested that Ep acted on morphologically unrecognizable ('stem') cells in the bone-marrow. Since then, comprehension of the basic principles of erythropoietic regulation has progressed rapidly in some areas but has been disappointingly slow in others. Although other erythroid stimulating (15) and inhibiting (16, 17, 18, 19) factors have been recognized, there seems little doubt that Ep is the most important (*see*, for example, the reviews by Krantz and Jacobson (20), Metcalf and Moore (21), Fisher (22), Fried (23) and Graber and Krantz. (24)) Usually, elevated urinary and serum levels of the hormone are found in circumstances of increased erythrocyte need and decreased levels of Ep are found in cases of an excess of erythrocytes.

Our understanding of the mechanism of action of Ep, at least at the cellular level, has been aided by improvements in the technology of cell separation. These improvements were a spin-off from the development of a

technique (the spleen colony assay) to functionally quantitate multipotential haematopoietic stem cells. (25) Thus, erythropoietin responsive cells (ERC) can be separated from both spleen-colony-forming-cells (26) and morphologically recognizable erythroid precursors (27), suggesting that ERC represent a distinct entity in erythropoiesis. The ERC population has been further delineated by the introduction of methods for cloning erythroid progenitors in the semi-solid media provided by a plasma clot (28, 29) or methyl cellulose. (30, 31) Principally from the studies of Iscove (32) and Gregory and associates (33, 34, 35) three distinct populations of cells have been defined on the basis of their proliferation rates, size, Ep-sensitivity and characteristics of the colonies they produce. These ERC are developmentally between the multipotential stem cells and the proerythroblasts. As the cells mature through these ERC compartments their Ep sensitivity and proliferation rate increases and their size and proliferative capacity decrease. The hormone may also regulate the population size of the more mature compartments. (32, 36) The initial commitment step from multipotentiality to erythroid differentiation appears independent of Ep; however, the factors regulating this process are currently unknown. (32)

Apparently the principal mechanism of action of Ep is to induce cell division within the ERC compartment (or subcompartments thereof). Maturation and cell division within the morphologically recognizable cells follows as a consequence but independently of the presence of the hormone. (13, 37, 38) An influence of Ep on more mature cells, such as controlling reticulocyte release (39) or cellular haemoglobin concentration (40), has been suggested but the function of these possible roles of Ep in the overall control of erythropoiesis is probably minimal.

In contrast to the relative wealth of information on the site of action of Ep, progress in our understanding of the stimulus for, and the mechanism and sites of production of the hormone has progressed at a much slower pace. It seems clear that the stimulus is tissue hypoxia (41) but the mechanism and site by which hypoxia is sensed and how this is translated into Ep production is still obscure. Although extra-renal sites of generation of the hormone have been recognized since at least 1957 (42) they are of secondary importance compared with the kidney. We still do not know if extra-renal sites of Ep production are active in the presence of intact kidneys. Many potential cellular sites of Ep production in both the kidney cortex and medulla have been suggested but with little unanimity among investigators. (43, 44, 45, 46, 47, 48) The liver has been implicated as the main extra-renal site of production. (23) Many other 'organ sites' (for example, uterus, cerebellar tissue) have been shown to be involved in pathological situations. (49) At least two theories have been advanced to explain the general failure to extract large quantities of Ep from the kidneys even of animals presumed to be engaged in active hormone production (e.g. as a consequence of exposure to short periods of hypoxia).

Much attention has been focused on the hypothesis first proposed by Kuratowska et al. (50) that an Ep-precursor, renal erythropoietic factor or erythrogenin, is formed in response to hypoxia and that this factor is activated by, or activates, a serum factor to produce biologically active Ep. Alternatively, Erslev and Kazal (16) have suggested that Ep co-exists in renal tissue with a potent lipid inhibitor from which it is released by the action of some serum component. Sherwood and Goldwasser (51) have claimed that they were able to extract active Ep directly from the kidney.

Progess has also been slow in other major areas of erythropoietic regulation. We still do not have a suitable rapid, sensitive, and specific assay for Ep nor do we fully know the chemical characterization of the hormone which might eventually lead to its synthesis for clinical use. The remainder of this article is concerned with the present status of these two problem areas.

ERYTHROPOIETIN ASSAY

Historical

Assays for Ep in body fluids, begun in the 1950s, were developed following the confirmation (8, 9, 10) of the original suggestion of Carnot and De Flandre (6, 7) that erythropoiesis was under hormonal control. The earliest assays utilized normal animals. It was soon found, however, that animals with suppressed erythropoiesis were more sensitive to the hormone. The use of specially prepared rats gave way to the use of erythrocythaemic mice. Hypertransfused or exhypoxic mice as an *in vivo* tool have become the 'standard' assay procedure, but this has certain disadvantages, to be detailed later.

In vitro bioassays have also been developed. Bone-marrow cells in suspension culture were used (52) in early tests. When fetal mouse liver cells (FMLC) were found to be sensitive to Ep during the 12th to the 15th day of gestation (53, 54), they were utilized to assay the hormone. (55, 56, 57) In 1971 it was found (28) that erythroid colonies could be grown *in vitro*. The method could potentially be used for assay purposes (58) because the number of colonies that develop in plasma clots or methylcellulose have a dose-response relationship to Ep.

In 1962, two groups (59, 60) described the production of antisera against Ep. The attempts to develop immunoassays for the hormone which followed have been based on four forms.

1. Immunoprecipitation. (61)
2. Cutaneous anaphylaxis. (62)
3. Haemagglutination inhibition. (63)
4. Radioimmunoassay. (64, 65, 66)

In most assay systems the Ep unitage of a particular test preparation is compared with either a dose response curve of the second international reference preparation (IRP) or against a laboratory standard which has been calibrated against the IRP. This second IRP was the result of a

collaborative study in 10 laboratories. (67) Each ampoule of standard contains 10 international units (IU) of Ep. The ampoules contain 2 mg of dried extract from approximately 4 mg of urinary concentrate which contained 72 per cent protein. This second standard replaced the earlier International Reference Standard B (68), first reference standard A (69) and definitions such as C–S (70) and cobalt units. (71) (One unit of Ep was originally defined as the erythroid stimulation produced by 5 μmol of cobaltous chloride.)

Bioassays (*in vivo*)

A number of different parameters have been used to measure the effect of the hormone. The first hormonal effect, seen at the cellular level, is an increase in the number of erythroid precursors in the bone-marrow. This is followed by an increased rate of plasma iron disappearance (72) and the incorporation of iron into haem. The next response is a bone-marrow and peripheral blood reticulocytosis. (14) Finally if enough hormone is given an increase in red cell mass occurs. (73) While all of these parameters have been applied at one time or another, the use of the incorporation of radioiron into newly formed red blood cells (74) has achieved almost universal application.

The first animals to be used for Ep assay were normal rabbits (10) and rats. (75) Early studies showed that a large amount of test material was necessary to demonstrate a response. The variability of responses led to the use of animals with suppressed erythropoiesis. Thus, hypophysectomized rats (76) and animals treated with nitrogen mustard (77) or irradiation (78) were employed. Starvation (79) or dehydration (80) were also effective. Starved rats, in particular, received extensive use because the assay could be completed rapidly with no necessity for special equipment. Although some investigators feel that rats offer better precision than do mice (81) their cost is higher and because of their size, larger volumes of test material are required. As a result mice are usually employed as the Ep assay animals.

In mice, erythroid suppression is generally accomplished by the 'exogenous' or 'endogenous' production of erythrocythaemia. By the normal physiological mechanism discussed earlier, erythropoiesis is thereby suppressed. 'Exogenous' erythrocythaemia is induced by hypertransfusion (11) which results in the immediate suppression of bone-marrow erythropoiesis and after five days the assay may be commenced. 'Endogenous' erythrocythaemia is produced by exposure of the mice to an hypoxic environment. (82, 83) Hypoxia triggers the production of red cells which are in excess of requirements when the animals are subsequently returned to the normal ambient environment. Exposure to hypoxia is accomplished by several methods; among them are variations of the hypobaric chamber technique suggested by Cotes and Bangham (82) as well as DeGowin et al. (83) In the late 1960s, workers in this laboratory (84, 85, 86) developed a

method utilizing silicone rubber membrane enclosures which are semi-permeable to oxygen. This method has been successfully used and adapted in other laboratories. (87) Still other investigators have used gas mixtures containing carbon monoxide (88) or decreased amounts of oxygen. (89) All of these hypoxia methods use fewer mice than does the transfusion method; however, special equipment is required. Sometimes the mice are pretreated with iron dextran to prevent a state of relative iron deficiency limiting the response to hypoxia. (83) Both the degree of suppression and the level of response depend on the degree of erythrocythaemia at the time the mice are removed from the hypoxic environment, and when the assay is started. Some investigators have found an extremely low baseline by the use of hypertransfusion. (90) However, in our laboratory when assays were started seven days after transfusion or the removal of animals from the hypoxic environment, there were no differences in erythropoiesis between the two methods of producing erythrocythaemia although ex-hypoxic mice were somewhat more sensitive. (91)

Various routes have been used for injections of test material and ^{59}Fe. The latter is now almost routinely used in the indirect measurement of Ep activity. Thus subcutaneous, intraperitoneal, and intravenous routes have all been utilized. It has been found (92) that the same route of injection should not be used for both the injection of test materials and ^{59}Fe. Most laboratories prefer the subcutaneous route for injection of test substances because the relatively slow rate of absorption frequently enhances the response. In contrast, the rapid absorption from either the intravenous or intraperitoneal route has led to these methods being used to administer the ^{59}Fe. We have found that mice in which the ^{59}Fe has been administered by intraperitoneal injection give as uniform and satisfactory results as those in which ^{59}Fe was given by the intravenous route so the former is generally used because of its convenience. To limit the availability of the ^{59}Fe a relatively large quantity of 'cold' iron in the form of iron dextran is sometimes injected 4–6 hr after the isotope. (93)

The timing of injections also varies from laboratory to laboratory. If greater sensitivity is desired at the expense of greater variability, the assay may be started before the animals reach their nadirs of iron incorporation post-hypoxia, and by giving multiple injections of the test materials. (88) Most investigators give the ^{59}Fe 48 hr after the first injection of test materials and conclude the assay by collecting blood samples 48–72 hr later. Radioactivity in the blood samples is directly proportional to the rate of erythropoiesis in the assay mice although differences in ferrokinetic status between animals may play a small complicating role. (94) The significance of this effect could be minimized by the injection of a large excess of unlabelled iron 6 hr after the labelled tracer. (93, 95)

There are several variables which result in differences between laboratories assaying the same materials. Different mouse strains behave differently towards hypoxia and in their erythropoietic response to Ep preparations.

The mouse strains which have been found to be satisfactory include: C3H, C57B1/6J, CF1, CBA, B6D2F1, Swiss Webster and St/EL. (86, 96) In spite of the use of inbred strains of mice there is still a wide range in the incorporation of iron. In order to obtain valid assays, therefore, groups of at least five animals, and preferably more, should be used.

There are some sources of difficulties which will result in erroneous Ep values. Seepage of test materials and standards can occur from the injection site but if care is taken this is a minor problem. The test materials can cause haemolysis or haemagglutination; during each assay this possibility can, and should, be checked by determining the haematocrit values at the time of assay and discarding the RBC iron incorporation values for any animals with a haematocrit of less than 0·53–0·55. Another source of error is the possible presence of erythroid regulatory substances, other than Ep, in the test system. Such substances can cause an increase in newly formed RBC iron incorporation either by a direct effect on bone-marrow cells or through their effect on the production of erythropoietin. Among the non-Ep materials which can potentially act in this way are cobalt (71), testosterone and other androgens (97), ACTH and tri-iodothyronine (98), placental lactogen (99), cyclic AMP (100, 101), and prostaglandins. (102) While pressor substances are a potential source of error they seemingly have little effect on the *in vivo* bioassay of Ep. The influence of non-Ep materials in the Ep assay has been thoroughly reviewed by Fisher and Gross. (103)

The indirect measurement is obviously a balance between Ep and possible inhibitor substances. At least five inhibitors have been described but none have been fully characterized. (19, 104, 105, 106, 107) Although 'bone-marrow toxins' can be present they can frequently be removed by dialysis of the test specimen before the assay is started.

The current *in vivo* bioassay in use in our laboratory is summarized in *Table* 1.1. While a standard assay technique could be recommended, it is

Table 1.1 *In vivo* Erythrocythaemic Mouse Assay

Day 0	– Mice placed in a silicone rubber membrane enclosure for 23 hr/day.
Day 11	– Mice returned to normal room atmospheric environment.
Day 18	– Injection of one ml test materials, negative control, and standard solutions, subcutaneously.
Day 20	– Injection of 0·5 μC ^{59}Fe in 0·5 ml saline intraperitoneally.
Day 22	– Conclusion of assay. 0·1 ml retro-orbital blood in 0·9 ml H_2O. Two micro-haematocrits obtained and measured. Radioactivity counted and results computed.

probably not necessary to do so. Annable et al. (67), in a collaborative study of the calibration of the second international reference preparation of Ep, found that although systematic differences may occur between widely differing assay systems such as the starved rat and the hyper-transfused or exhypoxic erythrocythaemic mouse, the results of that study

did not show statistically significant differences between Ep estimates using any of these methods.

Bioassays (*in vitro*)

Three different *in vitro* methods based on cultures of spleen fragments, bone-marrow and fetal liver cells have been used.

Nakao et al. (108) and Miura et al. (109) used spleen fragments from polycythaemic mice as an assay system for Ep but this system has not been widely employed.

The earliest *in vitro* studies involved comparing the number of erythroblasts in bone-marrow exposed to plasma of anaemic patients to those produced by plasma from normal subjects. This method was superseded by utilization of radioiron incorporation into haem and expanded by the work of a number of investigators. (52) More recently Adamson's group has used dog bone-marrow cells and radioactive glycine to label haem. (110) Dose response relationships to Ep extracts have been established but difficulty has arisen when this method has been used to assay serum samples. While the *in vitro* bone-marrow method may add greater precision to the assay of Ep, it is not very much more sensitive than *in vivo* assays. (22, 81) Nevertheless, Goldwasser et al. (111) have described a bone-marrow assay for Ep which is sensitive to the hormone down to the milliunit level. Some workers believe that non-specific materials may be involved in *in vitro* assays. (22, 81) As a result meticulous attention must be given to the number of cells and to the concentration of transferrin and iron. Just as in the *in vivo* assays, inhibitors may interfere with the incorporation of iron and care must be taken in ascribing the responses to Ep. Dukes et al. (112) have shown that units of Ep determined by *in vivo* erythrocythaemic mice may not agree with the value found by ^{59}Fe or ^{14}C-glucosamine in *in vitro* cultures. Goldwasser (113) has also found that *in vitro* cultures respond to desialated-Ep as well as to the native hormone. After Cole and Paul (53) demonstrated that FMLC was exceptionally sensitive to Ep several attempts have been made to develop an Ep assay based on these cells.

During the 12th to the 15th day of gestation FMLC are erythropoietic and incorporate iron. Erythropoietin assays were devised by Stephenson and Axelrad (55) as well as Wardle et al. (56) Their methods were expanded by Dunn et al. (57) who found the system to be quite sensitive to Ep extracts. According to these latter investigators, the system not only provides a quantitative measurement of Ep in normal serum, but shows that Ep concentrations vary predictably in a manner consistent with its function as an *in vivo* regulator of erythropoiesis. Dose-response curves for serum Ep showed highly significant parallelism with those obtained from sheep plasma Ep or with the IRP. A brief outline of the method currently in use is shown in Table 1.2.

The same sources of error are possible as described previously for the *in*

Table 1.2. Summary of the Methodology of the Fetal Mouse Liver Cell Assay for EP

Age of Fetus: 13–14 days optimum (11–15 days useable).
Tissue Culture Medium: TCM199 + 5% heat-inactivated fetal calf serum + 1% heat-inactivated pooled human serum.
Livers disaggregated by gentle aspiration through a Pasteur pipette, counted with the aid of a haemocytometer and diluted to provide 5×10^5 cells/ml culture.
Cultures, in disposable tissue culture tubes, incubated for 24 hr in a water-saturated atmosphere of 5% CO_2 in air with 0, 1·56–25 mU Ep diluted in heat-inactivated serum from patients with primary polycythaemia, or 3·125–50 µl heat-inactivated test sera per culture.
Cultures centrifuged, supernatant fluids discarded and replaced by 1·0 m M199 containing 10% heat-inactivated pooled human serum and 0·5 µCi ^{59}Fe ferrous citrate previously incubated for 24 hr.
Following a further four hours' incubation, haem is extracted into methylethylketone using a minor modification (119) of the method originally described by Teale. (121)

vitro bone-marrow methods. For example de Klerk et al. (114) found that at high concentrations, human serum like the Ep standards (115), demonstrated toxicity in the FMLC bioassay for Ep. They found that this was due to complement-dependent IgM heteroantibodies to FMLC and could be overcome by inactivation of complement by heating the test serum at 56 °C for 30 min as is done in the currently used assay (Table 1.2). The same workers (116) in an abstract, stated that heat-inactivated sera did not always produce dose-response curves parallel to the standard due to the inhibition of increasing serum concentrations related to the presence of transferrin-bound iron. These workers achieved both linearity and parallelism if a correction curve for each serum was utilized. Rokicinski et al. (117) and Radtke et al. (118) have confirmed these observations. It seems well recognized that the ratio of radiolabelled iron to 'cold' iron in the cultures can influence radioiron uptake into haem. (114, 117, 118, 119) Whether this actually influences the measurement of the Ep potency is still not clear. (119) In any case, possible problems of isotope dilution can be overcome by using other isotopes which do not require a carrier protein or by washing the cells before adding the ^{59}Fe pre-bound to transferrin. (120) Several other serum constituents (citrate, testosterone, B_{12}, folic acid) have been tested for possible effects on cultures. (119) Only small effects on haem synthesis were observed. It was concluded therefore, that variations in the level of these factors in the sera of patients would not produce significant alterations in the estimated Ep concentrations.

In addition, we believe that the FMLC technique is a useful method for the measurement of Ep for several reasons.

1. Test sera even from normal subjects, produce log dose-log response

relationships parallel to the IRP of Ep or of standards calibrated against the IRP. This is sound pharmacological evidence for the similarity of standard and test materials. (57, 122)

2. The assay specifically measures stimulation of haemoglobin synthesis. (119)

3. Ep in human serum detected by the FMLC assay elutes in one peak from Sephadex G–100 in the same position as the Ep in the IRP (both *in vivo* and *in vitro* assay) with no evidence of enhancing or inhibiting factors. (123)

4. In a variety of clinical disorders and during the course of therapy for these diseases, the FMLC-detectable Ep behaves as expected of a humoral regulator of erythropoiesis. (122)

5. By its effect on relatively immature erythroid precursors, the mechanism of action of Ep on FMLC is similar to its *in vivo* effect. (124)

6. The coefficient of variation of the assay is 30 per cent and it has an index of precision of 0·05–0·15. (122)

7. At least with human preparations, there is a good correlation between FMLC-detectable material and that detected with a standard *in vivo* bioassay. (125)

In general Ep_{FMLC} levels are higher than Ep levels determined by bioassay or immunological methods (Table 1.3). Further studies may determine

Table 1.3 A Comparison of Serum Erythropoietin Titres in Haematologically Normal Individuals in a Variety of Assay Systems

Method	*Reference*	*mU Ep/ml (Range or Mean + SEM)*
Immunological		
a. Gel Diffusion	Goudsmit et al. (61)	0·5–2·0
b. Haemagglutination Inhibition	Lange et al. (129)	37±22
	Oliver and Gould (130)	7–36
c. Radioimmunoassay	Garcia (64, 65)	3·7–11·0
	Lertora et al. (66)	52–84
	Fisher (131)	24–54
Bioassay		
a. *In vivo* erythrocythaemic mouse	Wagemaker et al. (132)	3·0–20
	Erslev et al. (133)	5–16
b. *In vitro* Fetal liver cell assay	Napier et al. (122)	150±100
	Radtke et al. (118)	136±9
	Goudsmit (134)	♂ 48 ♀ 29
	Napier (135)	Approximately 50
	Kimzey et al. (136)	200±40*
	Dunn et al. (137)	240±42*
	Dunn–unpublished observations	55±5

*Determined using the method as originally described by Dunn et al. (57) which does not include the pooled human serum supplement as an iron carrier.

whether this effect is due to an additional factor stimulating haem synthesis in culture and whether such a factor is present in serum as well as Ep detected with the other systems. The results with the FMLC assay may also be complicated by the above mentioned possible iron and transferrin effects unless special care is taken.

The most recently described possible *in vitro* assay of Ep is the solid-phase erythroid colony technique originally detailed by Stephenson et al. (28) In this assay, ERC respond to Ep in a dose-related manner. The methyl-cellulose modification has been used for the assay of Ep extracts. (58) However, as with other tissue culture systems, cells, species, media and supplements must be meticulously selected. Only one fetal calf serum among many may be suitable. The incubation time needs to be selected for measurement of different classes of colonies such as CFU-e, Day 3 BFU-e and Day 8 BFU-e. Visually counting erythroid colonies for routine assays of many test sera would be a cumbersome method. Technical advances could, of course, overcome this problem.

Immunological techniques

Goudsmit et al. (61) used a gel diffusion method to determine Ep in normal human serum (Table 1.3). Baciu et al. (62) have used the technique of passive cutaneous anaphylaxis as an immunoassay. The latter technique is cumbersome and less sensitive than other immunological techniques and the former depends on the use of completely absorbed antisera. Widespread use has been made of the haemagglutination inhibition (HAI) technique, described by our laboratory. (126, 127, 128) Radioimmunoassays have been under development for some time.

The HAI technique is based on the following principles.

1. Indicator red blood cells sensitized with Ep are agglutinated by *completely absorbed* Ep-antiserum.

2. Prior incubation of test materials or the standard with the Ep-antiserum before the indicator cells are added results in inhibition of the agglutination reaction because the reaction sites on the antisera are occupied by Ep in the test material.

3. Comparison of the degree of inhibition by test materials and the standard allows the calculation of the Ep unitage in the test material.

A number of factors may introduce errors into the HAI assay. The concentration of sensitizing antigen is critical and must be evaluated for the particular extract in use. Since EDTA interferes with the procedure, tests should be carried out with serum. The unitage of the standard must be correctly determined and the antiserum must be completely absorbed of contaminating antibodies.

Some difficulties have arisen with the use of the commercial HAI kit for Ep assay (138), although these problems are not insurmountable. (130) Other investigators have been reluctant to embrace the technique because the antisera have been raised to crude Ep preparations and as a consequence