"STUDIES OF ERYTHROPOIETIN"

by

Fiona M. Brooke, B.Sc.

A thesis presented for the Degree of

Doctor of Philosophy

of the University of Edinburgh.

September, 1970.



ACKNOWLEDGEMENTS.

I should like to express my sincere thanks to Dr. Andrew Doig who has shown a great deal of interest in the work and has given generously of his time and effort in the discussion of procedures and in constructive criticism of the results.

It is a pleasure to thank Professor K.W. Donald for his encouragement and the facilities to work in his department.

I am grateful to Professor D.J. Newell, Mrs. D. Weightman and Miss J. Bryan-Jones for their help and advice in the statistical treatment of experimental data.

I should like to thank Dr. I. Riding and Dr. and Mrs. P.C. Das for their invaluable help and advice concerning the immunological aspects of the work.

I am indebted to Mrs. J. Samuel and Mr. J. Davidson for their technical assistance.

My thanks are due to many physicians, nurses and patients of the Royal Infirmary of Edinburgh for their friendly co-operation.

I am extremely grateful to Miss E. Dickie for her skill, efficiency and unfailing patience in typing this thesis.

The work reported herein was undertaken during tenure of a University of Edinburgh Graduate Research Scholarship.

SUMMARY.

1. A review is presented of the literature on the nature and biological role of erythropoietin, the methods used in its estimation and purification and the importance of the hormone in health and disease.

2. The hypoxia-induced polycythaemic mouse assay was used to estimate erythropoietin; the method is described in detail.

3. Two laboratory standards of the hormone were prepared from the urine of patients with aplastic and hypoplastic anaemia. It was established that over a wide dose range both laboratory standards were parallel to the International Reference Preparation of Erythropoietin. The potency of each laboratory standard was estimated relative to the International Reference Preparation.

4. It is established that the assay conditions employed provide a satisfactory, sensitive and precise assay for erythropoietin.

5. Attempts to detect erythropoietic activity in the body fluids of patients with various forms of renal disease are described. Erythropoietin was detected in the renal cyst fluid of one patient but was undetectable in the urine and urine concentrates of all other patients.

6. In two human subjects attempts were made to determine whether evidence of increased erythropoietin secretion could be detected within three to four hours of exposure to a simulated high altitude of approximately 3,000 metres. Erythropoietin could not be detected in the urine or urine concentrates of either subject.

7. Attempts to assay erythropoietin in patients with hypoxia due to severe chronic obstructive airways disease are described. Erythropoietic activity could not be detected in the urine or urine concentrates of any of these patients.

8. Erythropoietin assays on human plasma were unsatisfactory as the plasma was found to lower the haematocrits of the assay animals. The results of experimental investigation of this problem were consistent with damage to mouse erythrocytes by human plasma. These observations cast doubt on the validity of many previously reported values for erythropoietin in human plasma and serum.

9. In rabbits an attempt was made to produce antibodies to erythropoietin extracted from human urine. Immunological investigation of the antisera revealed the presence of precipitating antibodies, chiefly to contaminant albumin which was present in the erythropoietin preparation used for immunisation. In vivo tests for the neutralising ability of the antisera are described.

CONTENTS.

		Page No.
GENERAL INTRODUCTION.		1
	SECTION I. REVIEW OF THE LITERATURE.	
1.	Assay of Erythropoietin.	4
	A. Bioassay of Erythropoietin 'In Vivo'.	4
	B. Bioassay of Erythropoietin 'In Vitro'.	12
	Conclusions.	14
2.	Purification and Chemistry of Erythropoietin.	15
	Purification.	15
	Chemistry.	16
	Conclusions.	25
3.	Site of Production of Erythropoietin.	27
	Evidence for Erythropoietin Production in the Kidney.	27
	Evidence for Extrarenal Production of Erythropoietin.	38
	Conclusions.	39
4.	Erythropoietin and the Control of Erythropoiesis.	41
	Conclusions.	49
5.	Erythropoietin in Health and Disease.	50
	Erythropoietin in Normal Subjects.	50
	Erythropoietin in Disease.	56
	Conclusions.	65

Page No.

SECTION II.
ERYTHROPOIETIN ASSAY.

Introduction. 66

Methods. 67

1. Assay design. 67
2. The Assay Method. 67
3. Collection, Concentration and Storage of Experimental Samples from Human Subjects for Assay. 70
4. Preparation of Laboratory Standards of Erythropoietin ~~Laboratory Standards~~. 71

Statistical Methods. 73

Results and Discussion. 80

1. Degree of Polycythaemia Induced by Exposure to Lowered Atmospheric Pressure and Effectiveness of the Polycythaemia in Depressing Erythrocyte Uptake of Radio-Iron. 80
2. Erythropoietic Activity of Urine Collected from Anaemic Patients for the Preparation of Laboratory Standards of Erythropoietin. 81
3. Log-Dose Response Curves of Erythropoietin Laboratory Standards E.L.S.I and E.L.S.II. 83
4. Log-Dose Response Curve of the International Reference Preparation of Erythropoietin (Standard B). 86
5. Calibration of E.L.S.I and E.L.S.II Against Standard B. 89

SECTION III.
APPLICATION OF THE ASSAY TO CLINICAL AND EXPERIMENTAL HUMAN STUDIES.

A. Erythropoietin in Patients with Renal Disease. 93

Patients Studied. 93

Results. 93

Discussion. 103

Page No.

B. Observations of the Effects of Hypoxia on the Plasma Levels and Urinary Excretion Rates of Erythropoietin. 109

Subjects and Procedures. 110

Results. 111

Discussion. 122

C. Erythropoietin in Patients with Hypoxia due to Severe Chronic Obstructive Airways Disease. 124

Patients Studied. 124

Results. 124

Discussion. 129

Comments on the Estimation of Erythropoietin in Human Plasma Using the Hypoxia-Induced Polycythaemic Mouse Assay. 131

SECTION IV.
PREPARATION OF ERYTHROPOIETIN ANTISERA.

Introduction. 135

Methods. 136

1. Preparation of Antigenic Materials. 136

2. Testing of Antisera. 137

A. Immunological Methods. 137

B. 'In Vivo' Testing of Antisera. 139

Results. 141

1. Immunological Investigation of Erythropoietin Laboratory Standard I. 141

2. Production of Antisera to Erythropoietin in Rabbits. 143

3. Immunological Testing of Rabbit Antisera to Erythropoietin. 147

4. 'In Vivo' Testing of Rabbit Antisera to Erythropoietin. 149

Discussion. 151

Page No.

APPENDIX.

A. Low Pressure Chamber for the Induction of Polycythaemia in Assay Animals. 154

B. Further Details of the Assay Method. 158

C. Materials. 160

D. Determination of the Relative Potency of Two Materials Unknown (U) and Standard (S). A worked example. 163

E. Erythropoietin in Patients with Renal Disease: Patients Studied. 169

F. Methods Used to Confirm that Blood Obtained by Catheter in Patient 16 was from Renal Vein. 172

G. Determination of PAH Concentration in Human Plasma Samples. 174

H. Timetable of Experimental Procedure in Subject 2. Observations on the Effects of Hypoxaemia on the Plasma Levels and Urinary Excretion Rates of Erythropoietin. 176

J. Preparation of Alum-Precipitated Erythropoietin Laboratory Standard I (E.L.S.I). 178

REFERENCES. 179

INTRODUCTION.

It is now generally accepted that the rate of erythrocyte production is related to tissue oxygen tension. For many years it was thought that the state of oxygen tension in the body tissues directly determined the rate of red cell production by the erythroid marrow. A fundamental contribution to haematology was the discovery of a humoral factor, now called "erythropoietin", or the "erythropoietic stimulating factor(s)", which acts as an intermediary in the regulation of erythropoiesis.

Credit for the first description of a humoral stimulant of erythropoiesis is due to the French workers Carnot and Déflandre. More than sixty years ago they observed increased reticulocyte and haemoglobin values in normal rabbits injected with the serum of anaemic rabbits (Carnot and Déflandre, 1906) and postulated the existence of an erythropoietic stimulating factor which they called "hémopoiétine". The term "erythropoietin", introduced in 1949 by Bonsdorff and Jalavisto, is now used in preference to hemopoietine.

The field of the humoral regulation of erythropoiesis lay fallow for nearly fifty years after Carnot and Déflandre's discovery, there being no simple, reproducible assay methods for estimating the stimulating factor. Until the late nineteen fifties, all assays were based on the original method of Carnot and Déflandre; reticulocyte, haemoglobin, haematocrit and red blood cell values were determined before and after administration of the test plasma.

Advances made during the decade 1950-1960 confirmed the postulate of Carnot and Déflandre that erythropoiesis is controlled by a humoral mechanism. Reissman (1950) exposed one member of a

parabiotic pair of rats to a 10% oxygen gas mixture, while the other rat breathed air. Both rats exhibited erythroid hyperplasia, indicating that a humoral substance had passed from one animal to the other. In 1953 Erslev confirmed the results of Carnot and Déflandre by injecting anaemic rabbit serum into normal rabbits and measuring the erythropoietic effect.

In addition to the experimental evidence for humoral control of erythropoiesis, supporting clinical evidence was also forthcoming. Stohlman, et al. (1954) observed in a patient with regional hypoxia and polycythaemia secondary to a patent ductus arteriosus, that both the normally oxygenated and hypoxic marrow areas were hyperplastic, thus nullifying the idea that it is the oxygen content of the bone marrow which regulates erythropoiesis.

During the last ten years the chief site of production of erythropoietin has been localised to the kidney and great efforts have been made to purify and characterize the hormone. A major advance occurred in 1966 when the first International Reference Preparation of Erythropoietin was established (Cotes and Bangham, 1966). Each laboratory performing erythropoietin assays can now calibrate its own standard against the international standard, thus enabling a meaningful comparison of results between laboratories. The use of radioisotope, <u>in vitro</u> and immunological techniques has greatly facilitated work on the physiology of the hormone, and its clinical significance has been established.

This Thesis is presented in four sections. Section I contains a review of the literature pertaining to the nature and biological role of erythropoietin, how it may be purified and estimated, and its importance in health and disease. Section II

describes the assay method employed in the studies reported in the next section, and the methods used to prepare working laboratory standard preparations of erythropoietin. Details of the application of erythropoietin assay to human clinical and experimental investigation are given in Section III. The studies recorded in Section IV represent an attempt to prepare antibodies to erythropoietin with a view to the development of an immunoassay for this hormone.

SECTION I

REVIEW OF THE LITERATURE

SECTION I

REVIEW OF THE LITERATURE

1. ASSAY OF ERYTHROPOIETIN

An essential step towards the understanding of the physiology of any hormone is the development of a sensitive and specific assay. Hormone assay methods may be divided into three main groups: biological, immunological and chemical. Whilst chemical methods are in most instances the least laborious and least expensive, they will not be available for erythropoietin estimation until the chemistry of the factor has been elucidated. Reports of the preparation of erythropoietin antisera (Schooley and Garcia, 1962; Lange, et al., 1964; Fisher, et al., 1967a; Krugers-Dagneaux, et al., 1968) raise hopes for the future development of a satisfactory immunoassay; however, at present this development is being hampered by the impurity of the erythropoietin-containing materials which are being used as antigens.

In the absence of a chemical or immunological assay, biological assay, *in vivo* or *in vitro*, remains the only feasible way of estimating erythropoietin.

Details of the assay techniques used in the last fifteen years are summarised in Tables 1 and 1A.

A. Bioassay of Erythropoietin 'In Vivo'.

1. Choice of Assay Animal Preparation.

As will be seen in Table 1, early studies of erythropoietin employed normal rats, mice, rabbits and guinea pigs

Test Animal				Administration of Assay Material		Metameter of Erythropoietin Activity	Standards Used	Authors
Species	Preparation	Sex	Weight(g)	Injection Route	Number of Injections			
Rat	Normal	-	-	i.v.	3	^{59}Fe incorp. - 12 hr.	-	Plzak, et al., 1955
Rabbit	Normal	M & F	1600-2400	i.p.	4	^{59}Fe incorp. - 24:48 hr	-	Hodgson, et al., 1957
Mouse	Normal	F	-	s.c.	3	Reticulocyte count	-	Lowy, et al., 1959
Rat	Normal	M	110-130	i.p.	1	Reticulocyte count	-	Payne, 1961
Rat	Normal	F	170-230	s.c.	5	Reticulocyte count	Cobalt	Winkert and Gordon, 1960
Rat	Nitrogen mustard treated	-	140-280	s.c. or i.p.	3	^{59}Fe incorp.-18,24,48 hr	-	Korst, et al., 1958
Rat	X-irradiated	F	135-170	i.v.	1	^{59}Fe incorp. - 48 hr.	-	Stohlman and Brecher, 1956
Rat	Starved	M	175	i.v.	3	^{59}Fe incorp. - 16 hr.	-	Fried, et al., 1957
Rat	Starved	M	180-220 250-320	i.v.	4	^{59}Fe incorp. - 20 hr.	-	Hodgson, et al., 1958
Rat	Starved	M	200-235	s.c.	2	^{59}Fe incorp. - 19 hr.	-	Hanson, 1963a
Rat	Starved	M	175-200	i.v.	2	^{59}Fe incorp. - 16 hr.	Cobalt	Schleuter, et al., 1960
Rat	Starved & Hypophys-ectomized	M	100-120	s.c.	2	^{59}Fe incorp. - 16 hr.	-	Gurney and Pan, 1960
Rat	Starved & T.P.	F	180-200	i.v.	2	^{59}Fe incorp. - 18 hr. and Reticulocytes.	Laboratory Standard	Gallagher, et al., 1961a
Rat	Hypophysect.	M	175-200	i.v.	3	^{59}Fe incorp. - 16 hr.	-	Fried, et al., 1956
Rat	Hypophysect.	-	-	i.v. or i.p.	3	^{59}Fe incorp. - 16 hr.	-	Jacobson, et al., 1956
Rat	Hypophysect. and Normal	F	150-200	i.v. s.c. or i.p.	2 or 3	^{59}Fe incorp.-18,24,42 hr	-	Secrest and Bethell, 1958
Rat	T.P.	F	200	i.v.	3	^{59}Fe incorp. - 24 hr.	-	Reichlin and Harrington 1960
Rat	T.P.	M	110-130	i.p.	2	^{59}Fe incorp. - 24 hr.	Laboratory Standard	Penington, 1961
Rat	T.P.	-	Various groups	s.c.	2	^{59}Fe incorp. - 16 hr.	-	Gurney and Pan, 1958
Mouse	T.P.	M & F	22-24 18-20	i.v.	4	Reticulocyte count	-	Jacobson, et al., 1959
Mouse	T.P.	F	20-25	s.c. or i.v.	4	^{59}Fe incorp. -18-20 hr	Cobalt	Jacobson, et al., 1960
Mouse	T.P.	F	20-25	s.c.	1	Reticulocyte count	Cobalt	Filmanowicz & Gurney, 1961
Mouse	T.P.	F	18-22	s.c.	2	^{59}Fe incorp. - 48 hr.	Cobalt & Laboratory Standard	Rosse, et al., 1962
Mouse	T.P.	F	-	s.c.	1	^{59}Fe incorp. - 72 hr.	Cobalt	DeGowin, et al., 1962a
Mouse	H.P.(R.A.P.)	F	16-24	i.p.	2	^{59}Fe incorp. - 20 hr.	-	Cotes and Bangham, 1961
Mouse	H.P. (R.A.P.)	F	20-23	s.c.	1	^{59}Fe incorp. - 72 hr.	Laboratory Standard	DeGowin, et al., 1962b
Mouse G.Pig	H.P.(R.A.P.) T.P.	M -	- 225-275)	s.c.	1	^{59}Fe incorp. - 48 hr.	-	Jepson and Lowenstein, 1964
Mouse	H.P.(CO)	F	20	i.p.	10	^{59}Fe incorp. - 66 hr.	Standard A & I.R.P.S.	Fogh, 1966
Mouse	H.P.(R.A.P.) and Protein depletion	F	-	i.p.	8	^{59}Fe incorp. - 48 hr.	I.R.P.S.	Adamson, et al., 1966
Mouse	H.P.(R.A.P.) and Protein depletion	F	-	i.p.	3	^{59}Fe incorp. - 72 hr.	I.R.P.S.	Alexanian, 1966
Mouse	H.P. Silicone rubber membrane	F	16	i.p.	2	^{59}Fe incorp. - 48 hr.	-	Lange, et al., 1966
Mouse	H.P.(R.A.P.)	F	20-30	i.p.	2	^{59}Fe incorp. - 24 hr.	Standard A & I.R.P.S.	Weintraub, et al., 1963

Abbreviations: i.v. = Intravenous
i.p. = Intraperitoneal
s.c. = Subcutaneous
T.P. = Transfusion Polycythaemia
H.P. = Hypoxic Polycythaemia
^{59}Fe incorp. = Percentage incorporation of ^{59}Fe into erythrocytes during the period stated.
I.R.P.S. = International Reference Preparation of Erythropoietin (Standard B).
R.A.P. = Reduced Atmospheric Pressure
CO = Carbon Monoxide exposure.

TABLE 1A. 'IN VITRO' METHODS OF ERYTHROPOIETIN BIOASSAY.

Tissue Culture System	Metameter of Erythropoietin Activity	Comments	Author
Rat bone marrow.	Mitotic rate of erythroblasts.	Demonstrated stimulation by erythropoietin.	Rosse and Gurney, 1959.
Rabbit bone marrow.	Mitotic rate of erythroblasts.	Demonstrated stimulation by erythropoietin.	Matoth and Ben-Porath, 1959. Matoth and Kaufmann, 1962.
Chicken erythrocytes.	^{59}Fe incorporation into haem.	Demonstrated stimulation by erythropoietin.	Nakao, et al., 1960.
Rat spleen.	^{32}P uptake by DNA.	Demonstrated stimulation by erythropoietin.	Linkenheimer, et al., 1959.
Dog bone marrow.	^{59}Fe incorporation into haem.	Sensitivity = 0.05 I.U. erythropoietin per ml.	Ward, 1967.
Human bone marrow.	^{3}H-thymidine or ^{14}C-formate incorporation into DNA ^{59}Fe incorporation into haem globin.	Failed to demonstrate stimulation by erythropoietin.	Alpen, et al., 1959.
Rabbit bone marrow.	^{59}Fe incorporation.	Failed to demonstrate stimulation by erythropoietin.	Erslev and Hughes, 1960.
Rat bone marrow.	^{59}Fe-incorporation into erythrocytes.	Failed to demonstrate stimulation by erythropoietin.	Thomas, et al., 1960.
Rabbit bone marrow.	^{14}C-glycine incorporation into haem. ^{14}C-formate incorporation into thymine. Oxygen consumption.		

as assay animals. It soon became evident however, that the sensitivity of the assays could be increased by inhibiting the assay animals' endogenous production of erythropoietin. The methods used to achieve this object were starvation, hypophysectomy and the induction of polycythaemia.

The advantage of using hypophysectomised animals is offset by the enhanced erythropoietic stimulating effects of other hormones in this type of preparation. Thus, it has been shown that thyroxine (Evans, et al., 1961) and corticoids (Fisher and Cook, 1962) stimulate erythropoiesis in hypophysectomised animals.

The starved rat preparation is not entirely satisfactory since non-specific nutrients in the sample under assay can stimulate erythropoiesis (Alt, 1938).

Another reported method of reducing endogenous production of erythropoietin in the rat was that of toxic depression of bone marrow activity, using either X-rays (Stohlman and Brecher, 1956) or nitrogen mustard (Korst, et al., 1958). Such preparations were found to be unsatisfactory, presumably as a result of profound disturbance of nuclear metabolism and mitosis. They have not been pursued by other workers.

The most frequently used assay animal at the present time is the mouse made polycythaemic by transfusion or hypoxia. The transfusion-induced polycythaemic (T.P.) rat (Gurney and Pan, 1958) was abandoned by its original proposers in favour of the T.P. mouse, which gave lower baseline values for erythropoiesis and did not exhibit the gastric bleeding which is associated with plethora in the rat (Dahl, et al., 1959). Mice and rats may be transfused

by intraperitoneal injection of washed, homologous red cells suspended in saline. The transfusion of one mouse usually requires the sacrifice of two donor mice, and this makes the method tedious and somewhat costly. On the other hand, assay animals can be prepared in a relatively short period of time.

Cotes and Bangham (1961) introduced an erythropoietin bioassay using mice made polycythaemic by exposure to air at a reduced pressure (0.5 atmospheres for two or three weeks). DeGowin, et al. (1962b) and Weintraub, et al. (1963) devised their own modification of this method, employing discontinuous rather than continuous hypoxia. It was found that lower baselines of erythropoietic activity were obtained using this modification, and mice gained weight despite the severe hypoxia at 0.4 atmospheres. Other methods of producing hypoxia (Table 1) include enclosure in silicone rubber membranes and exposure to carbon monoxide.

Hypoxia solves most of the problems presented by T.P. mice, since sufficient numbers can be maintained in a low pressure chamber and removed at the required intervals. The method eliminates the labour and cost of transfusion. This method of inhibiting endogenous erythropoietin production might be regarded as more physiological than those of starvation and hypophysectomy.

2. Measurement of Erythropoietin Activity.

The variable to be measured in evaluating the erythropoietic activity in any sample is the change which it induces in the red cell production rate of the assay animal. The criteria originally used for this were haemoglobin concentration, red cell counts, haematocrit, or reticulocyte counts. An increase in one or

more of these parameters, after injection of the sample, was regarded as evidence of erythropoietin activity in the administered sample. Since haemoglobin concentration and red cell count are influenced by plasma volume changes, they cannot be regarded as reliable indications of erythropoietic activity. The measurement of the reticulocyte response is time consuming and gives variable results, especially at low dose levels of the hormone (Gallagher, et al., 1961a).

The most satisfactory method at present available for the measurement of red cell production is based on the rate of incorporation of radioactive iron (^{59}Fe) into red blood cells. This rapid and convenient method was first used by Pizak, et al. (1955). For this assay (and that based on reticulocytosis), simultaneous determination of the haematocrit is required to investigate the possible contribution of haemolytic effects of the injected materials, especially when heterologous whole plasma or crude extracts are used. The radioactive iron is removed from the plasma within four to six hours after injection and is incorporated into the newly formed erythrocytes (Hahn, et al., 1940). It does not exchange with the iron already present in erythrocytes (Hahn, et al., 1940), nor is it adsorbed onto the mature erythrocyte (Gibson, et al., 1946). As shown in Tables 1 and 1A, laboratories differ in the time allowed for ^{59}Fe uptake, the range being from 12 to 72 hours.

3. Preparation and Administration of Assay Material.

Tissue extracts from various organs, plasma, serum, urine and other body fluids have all been administered to assay

animals for estimation of their erythropoietic activity. The route of administration of the sample under test does not appear to be critical; it can be given by subcutaneous or intraperitoneal injection. Intravenous administration was found to produce thrombosis in rats (Hansen, 1963a).

Borsook, et al. (1954) devised a method of heat denaturation of plasma proteins, at an acid pH, which decreased the toxicity of rabbit plasma to normal adult rats, while retaining the erythropoietic activity. Other investigators applied the method to assays of human plasma, with positive results (Piliero, et al., 1956; Gurney, et al., 1957). However, three groups of workers all reported that heat denaturation of plasma proteins destroyed a considerable amount of the erythropoietic activity (Stohlman and Brecher, 1957; Gurney and Pan, 1960; Mirand, et al., 1965c). Lowy, et al. (1959) considered that plasma filtrate preparations were more effective than whole plasma because of the removal of antibody-provoking antigens. Garcia and Schooley (1965) observed a greater response to erythropoietin given in serum than in saline and they attributed this to retardation of erythropoietin absorption from the subcutaneous injection site, due to the presence of serum proteins. Neither of these explanations for the stimulatory effects of heterologous whole plasma or serum is satisfactory. A more likely cause would seem to be the haemolysing effect of heteroagglutinins (Landois, 1875; Landsteiner, 1900).

Fractionation of the administered dose of erythropoietin is found to increase the response substantially, though not the sensitivity (Reichlin and Harrington, 1960; Schooley, 1965;

Alexanian, 1966; Fogh, 1966). Whilst the cause of this is uncertain, it is suggested that stem cells in the marrow which do not undergo erythroid differentiation on the first injection become more sensitive to the second injection, and so on (Gurney, et al., 1961; Schooley, 1965). An alternative explanation proposed is that, in the interval between injections, stem cells which were previously channelled into "ineffective erythropoiesis" are now pushed in the direction of "effective erythropoiesis" (Gurney, et al., 1961).

4. International Units of Erythropoietin.

The quantitative estimation of erythropoietin is based on the increase of red cell formation induced by known amounts of a standard preparation, compared with that induced by the sample under assay.

Since 1959, various units of activity have been proposed. The "Cobalt Unit" was introduced by Goldwasser and White (1959) and was based on the response obtained from the injection of 5μ moles of cobalt, using the starved rat assay of Fried, et al. (1957). Such "units" are no longer acceptable, since not only is the mechanism by which cobalt stimulates erythropoiesis unknown, but it is essential that a standard preparation for erythropoietin assay should contain the hormone.

After a co-operative assay organised in the U.S.A., Keighley, et al. (1960) proposed a unit based on the net incorporation into rat red cells of 20% of injected ^{59}Fe, using a "standardised" starved rat assay.

A major advance in standardisation occurred in 1966

when the first International Reference Preparation of Erythropoietin was established as the result of an international collaborative assay (Cotes and Bangham, 1966). Each laboratory performing erythropoietin assays can now obtain a valid international standard against which its own laboratory standard may be calibrated.

5. Accuracy of Assay Methods.

The sensitivity and precision of *in vivo* assay methods depend on many factors, including the design of the assay, animal variability, the numbers of animals used, and the doses of unknown and standard preparations administered.

In clinical work, where samples may contain a very low titre of erythropoietin, the sensitivity of the assay becomes important. A sensitivity of 0.05 I.U. has been achieved with polycythaemic mice (Lange, et al., 1966; Alexanian, 1966) and Fogh (1966) quotes a sensitivity of 0.03 I.U., also using polycythaemic mice. This is an increased sensitivity of 10-20 times that demonstrated in the starved rat assay (DeGowin, et al., 1962c).

B. Bioassay of Erythropoietin 'In Vitro'.

In vitro assays for erythropoietin have only recently been developed, earlier studies of the effects of erythropoietin preparations on *in vitro* systems having produced contradictory results in different laboratories. Thus, Matoth and Ben-Porath (1959) and Rosse and Gurney (1959) demonstrated stimulation of the mitotic rates of erythroblasts in bone marrow culture by erythropoietin preparations, and Nakao, et al. (1960) were able to show stimulation of ^{59}Fe

incorporation into the haem of chicken erythrocytes. On the other hand, Alpen, et al. (1959), Erslev and Hughes (1960) and Thomas, et al.(1960), using radio-isotopic methods, were all unable to confirm that there was stimulation of marrow activity in erythropoietin-containing cultures, compared with controls. Later Matoth and Kaufman (1962) suggested that the negative results obtained by Thomas, et al. (1960) had been due to an unfavourable culture system, and that they had allowed insufficient time to show the effect of erythropoietin on DNA synthesis.

A considerable advance in <u>in vitro</u> assay techniques was made in 1963, when Krantz, et al. described a culture system for rat marrow cells in which haem synthesis, over periods greater than 12 hours, was found to be dependent on the presence of erythropoietin in the culture medium. A log-dose response curve could be obtained, with a central linear plot. It is difficult to compare the sensitivity of the method with <u>in vivo</u> techniques, since the results are standardised in terms of "Cobalt Units". Krantz, et al. (1963) found that the cell cultures were sensitive to inhibitors and toxic materials present in crude urine preparations. The method has been adopted for use with human bone marrow cells (Krantz, 1965).

Ward (1967) applied certain modifications to the Krantz technique and demonstrated a log-dose response relationship which was linear between the doses 0.05 and 1.00 I.U./ml. The sensitivity of Ward's method was 0.05 I.U./ml., which is comparable to that of the polycythaemic mouse techniques. Confirmation of the results of Ward is awaited.

Conclusions.

It would appear from the preliminary experiments of Krugers-Dagneaux (1967), that a sensitive immunoassay for erythropoietin is feasible. A major problem yet to be overcome is the isolation of an erythropoietin preparation which is sufficiently pure to enable the production of specific antisera.

In vitro assay systems are under development (Ward, 1967) and should eventually prove to be rapid and convenient methods for the estimation of erythropoietin. They have the disadvantage of being sensitive to toxic materials which may be present in the sample under test, but this is a characteristic of *in vivo* systems also and therefore not a valid criticism of *in vitro* techniques. As yet, sensitivity has not exceeded that observed for *in vivo* techniques.

Until the development of accurate, reproducible immunological or *in vitro* methods has been completed, ^{59}Fe incorporation in the mouse rendered polycythaemic by hypoxia or transfusion remains the most sensitive and reliable assay method presently available for erythropoietin estimation.

2. PURIFICATION AND CHEMISTRY OF ERYTHROPOIETIN.

The isolation and physico-chemical characterisation of erythropoietin is of great importance. Once the pure hormone has been isolated and its chemical structure determined, many exciting prospects arise. Firstly, a chemical method of estimation of erythropoietin might be feasible; secondly, radioactive labelling of the hormone molecule would be possible, leading to more accurate studies of its physiology; and thirdly, it might be possible to chemically synthesise the hormone and present it in a form suitable for clinical use. At present however, only small amounts of erythropoietin are available in a highly purified form, so that what little is known about its structure has been gleaned from experiments with impure preparations of the hormone.

Purification.

The techniques employed in the purification of erythropoietin are summarised in Table 2. Urine from human patients with severe anaemia is the most frequently used source of the hormone. Erythropoietin has also been extracted from the plasma and urine of rabbits and sheep made anaemic by the administration of phenylhydrazine and from kidney homogenates of hypoxic rats, rabbits, sheep and dogs.

Since the active part of the erythropoietin molecule contains protein, it can be concentrated by protein precipitants such as ammonium sulphate, ethanol, acetone or tannic acid. Dialysis and ultrafiltration are frequently used as initial steps in the recovery of erythropoietin. These preliminary concentration procedures are usually followed by adsorption chromatography using DEAE cellulose or

TABLE 2. METHODS OF ISOLATION OF ERYTHROPOIETIN.

Source	Precipitation Technique	Dialysis	Adsorption Material	Lyophilisation	Other Techniques	Authors
Rabbit plasma	$(NH_4)_2 SO_4$	+	-	+	-	Rambach, et al., 1957
Sheep plasma	$(NH_4)_2 SO_4$	+	DEAE cellulose + Amberlite XE-97 resin Sulphoethyl Sephadex $CaPO_4$ gel.	+	-	Goldwasser and Kung, 1963
Human plasma Human placental serum	$(NH_4)_2 SO_4$	+	DEAE cellulose Amberlite IRC-50 resin Dowex - 50 resin	+	-	Kuratowska, et al., 1962
Rat kidney homogenate	$(NH_4)_2 SO_4$	+	DEAE cellulose Sephadex G-100	-	-	Wong, et al., 1968
Rabbit plasma	Ethanol	-	-	-	-	Grant, et al., 1961
Rabbit plasma) Human urine)	Ethanol	+	-	+	Phenol as preservative	Lowy and Borsook, 1962
Human urine	Ethanol	+	DEAE cellulose	-	Phenol as preservative	Lowy and Keighley, 1961
Human urine	Ethanol	+	Sephadex G-75	-	Phenol as preservative	Winkert, et al., 1962
Human urine	Ethanol	+	-	-	Flash evaporation	Lewis, et al., 1964
Human urine	Ethanol	+	DEAE cellulose + Amberlite CG-50 resin	-	-	Lewis, et al., 1965b
Rabbit urine	Ethanol	+	DEAE cellulose	+	-	(Hodgson, et al., 1960
Human urine	Ethanol/ Acetone	+	Kaolin	+	-	
Human urine Rabbit plasma	Ethanol	+	Polyacrylamide gel or $(NH_4)_2 SO_4$ fractionation	+	-	Lowy and Keighley, 1966
Sheep plasma + kidney extract	Ethanol	+	DEAE Sephadex	+	Precipitation of albumin with polyphosphoric acid.	Hansen, 1963b
Human urine	Tannic acid	-	DEAE Sephadex	-	-	Hansen, 1963c
Human urine	Acetone + $(NH_4)_2 SO_4$	+	DEAE cellulose + $CaPO_4$	+	-	Graham, et al., 1963
Rat kidney homogenates	No precipitation	+	DEAE cellulose	-	Hypotonic buffer extraction	Contrera, et al., 1966
Dog kidney homogenates	No precipitation	-	-	-	Isotonic saline extraction	Naets, 1960a
Rabbit kidney homogenates	No precipitation	+	Dowex - 50 resin	+	-	Kuratowska, et al., 1964
Human urine	No precipitation	-	-	-	Ultrafiltration	Van Dyke, 1960
Rabbit plasma	No precipitation	+	DEAE cellulose	+	-	Rambach, et al., 1958a
Human urine	No precipitation	+	-	-	-	Lewis, et al., 1967
Human urine	No precipitation	+	Kaolin	+	-	Gordon, et al., 1959
Human urine	No precipitation	+	DEAE cellulose	-	-	Lewis, et al., 1965a

Sephadex gels, XE-97 Amberlite or Dowex-50 resins, or kaolin. If the resultant erythropoietically active fraction is not to be used immediately, lyophilisation and storage at temperatures below freezing point are recommended, as for international standard preparations of hormones (Jerne and Perry, 1956). The urine or plasma used as the erythropoietin source is also deep frozen if it is to be kept before use, and dialysis and chromatography are usually performed at low temperatures (e.g. 2-4°C) to ensure maximum recovery of biologically active erythropoietin (Lewis, et al., 1965a). Dilute phenol is added as a preservative to the starting material by some authors (Lowy and Keighley, 1961; Lowy and Borsook, 1962; Winkert, et al., 1962; Hansen, 1963c) as it is thought to exert a protective action on erythropoietin.

One of the most highly purified preparations so far isolated is probably that of Goldwasser and Kung (1968). Starting with anaemic sheep plasma containing 0.007 units/mg. protein a 930,000-fold purification was achieved, but unfortunately the yield was only 2.2%. Goldwasser estimated that, in order to obtain highly purified erythropoietin in an amount sufficient to enable its physical and chemical characterisation (say 10 mg.), the plasma from a flock of 325 sheep would be required.

It seems probable that the only way to achieve significant yields of highly purified erythropoietin will be by the continued use of present techniques, starting with larger initial volumes of sheep plasma or human urine.

Chemistry.

Thermal Stability.

Carnot and Déflandre (1906) observed that their serum factor which stimulated erythropoiesis was unstable when heated to 56°C. Until the mid nineteen-fifties, this was the only information available on the chemical properties of erythropoietin. Borsook, et al. (1954) tested the heat stability of the erythropoietic factor in anaemic rabbit plasma by heating for 15 minutes at 100°C and pH 5.5. Erythropoietic activity appeared to remain. Rambach, et al. (1957) and Lowy, et al. (1959) confirmed Borsook's results, while Linman, et al. (1959) held that there were at least two erythropoietic factors (a) a thermostable factor controlling the division of red cell precursors, and (b) a relatively thermolabile factor governing haemoglobin synthesis. Since these early experiments, evidence has been presented which indicates that plasma erythropoietin is not thermostable, since heating destroys at least part of the erythropoietic activity (Stohlman and Brecher, 1957; Gurney and Pan, 1960; Mirand, et al., 1965c).

Chemical Composition.

Rambach, et al. (1957) and Hodgson, et al. (1960) established that the erythropoietic factor was non-dialysable and therefore probably contained protein. Chemical analysis revealed that the hexoses galactose, mannose and fucose were components of the factor (Rambach, et al., 1958b).

(a) Enzyme Studies.

Mild acid hydrolysis causes biological inactivation of

the hormone with a concomitant appearance of sialic acid. Sialic acid alone does not stimulate erythropoiesis. In vivo experiments with the enzyme sialidase seemed to indicate that sialic acid is a part of the erythropoietin molecule which is essential for its biological activity (Lowy, et al., 1960; Rosse and Waldmann, 1964). Goldwasser (1966) subsequently confirmed that sialidase inactivates erythropoietin in vivo, but found that it had no effect in vitro. He therefore postulated that although sialic acid is an intrinsic part of the erythropoietin molecule, it is not required for interaction with the target cells in the marrow. He further suggested that sialic acid may be required for binding of erythropoietin to a carrier, or to protect it from degeneration in, or excretion by, the assay animal (Goldwasser and Kung, 1968).

The inactivation of a biologically active material by the enzyme trypsin indicates that it is a protein or polypeptide which contains lysine and/or arginine. Slaunwhite, et al. (1957) and Lowy and Borsook (1962) found that trypsin destroyed erythropoietic activity in urine preparations, and this was confirmed by Rosse and Waldmann (1964) for erythropoietin derived from the serum of patients with anaemia or anoxia, and cyst fluid of patients with renal or cerebellar haemangioblastoma cysts.

(b) Substitution Reactions.

Lowy and Borsook (1962) found that partially purified rat plasma erythropoietin was inactivated by reaction with fluorescein isothiocyanate which substitutes on phenolic hydroxyl and free amino groups. This was confirmed by Goldwasser and Kung (1968) who also showed that neither esterification of erythropoietin with

anhydrous formic acid (which reacts with the hydroxyl groups of serine or threonine), nor reaction with p-chloromercuribenzoate (which reacts with sulphydryl groups), caused any loss in activity. Lowy and Keighley (1968) investigated the reaction of human urinary fractions of high erythropoietic activity (100-200 I.U./mg.) with Koshland's tryptophan reagent, and found that 95-100% of the erythropoietic activity was destroyed. L-Tryptophan and its dipeptides had no demonstrable erythropoietic activity. They concluded that one or more intact tryptophan moieties in the erythropoietin molecule are necessary for its biological activity.

Electrophoretic Characteristics.

Rambach and co-workers made significant contributions in 1957, and in the succeeding years, to the elucidation of the chemical components and electrophoretic characteristics of erythropoietin. Their investigations, using continuous flow and paper electrophoresis, indicated that the active principle in heat-denatured plasma had the electrophoretic properties of an α_2 globulin (Rambach, et al., 1957). Using purification techniques they obtained a fraction with high erythropoietic activity which was found to be homogeneous and on paper electrophoresis migrated as a single component, with a mobility between that of α_2 and α_1 globulins (Rambach, et al., 1958b, 1959). Chemical analysis of the active fraction revealed a composition of 69.3% protein, 7.7% hexose, 10% hexosamine and 15.6% sialic acid. Rosse and Waldmann (1964), examining the electrophoretic behaviour of erythropoietin from different sources, found that the biological activity of each material migrated as an α_2 globulin on

zonal electrophoresis. Lewis, et al. (1969) purported to have separated erythropoietic activity into two fractions, one dialysable fraction, and one non-dialysable fraction which they proposed was an erythropoietin-producing enzyme, since it potentiated erythropoietin activity when incubated with normal serum. The most highly purified dialysable fraction contained 11% protein, and on immuno-electrophoresis gave three lines in the α-globulin-albumin region, and one line in the β-globulin region, indicating that their erythropoietin preparation was still impure.

Molecular Weight.

In the absence of pure erythropoietin the only methods of determining the molecular weight of the hormone are those utilising measurements of some aspect of its biological activity. Published estimates of the molecular weight of erythropoietin are shown in Table 3.

Rosse, et al. (1963a), using a crude urine-derived preparation, subjected the erythropoietin molecule to low energy X-ray and high energy electron inactivation. They calculated a molecular weight of 27,000 using the "target theory" computations of Lea (1955) and assuming the material to have the density of most proteins. They also deduced that the erythropoietin molecule is asymmetric, being 10-20 times as long as it is wide.

Molecular weights of 10,000, 40,000 and 40-50,000 were proposed by Rambach, et al. (1958a); White, et al. (1960) and Winkert, et al. (1962). Experiments performed in 1968 and 1969 indicate that the molecular weight is higher than this. Goldwasser and Kung (1968) used sucrose-gradient centrifugation and ultra-

TABLE 3. MOLECULAR WEIGHT OF ERYTHROPOIETIN.

Estimated Molecular Weight	Technique	Source of material	Author
10,000	Ultracentrifugation.	Rabbit plasma.	Rambach, et al., 1958a.
27,000	Low energy X-ray inactivation.	Human urine, renal cyst fluid, cerebellar hemangioblastoma fluid.	Rosse, et al., 1963a.
> 40,000	Gel filtration on Sephadex G-75.	Sheep plasma.	Hansen, 1963b.
39-42,000	Light scattering method.	Sheep plasma.	White, et al., 1960.
52,000	Gel filtration on Sephadex G-100 and ultracentrifugation.	Human urine.	O'Sullivan, et al., 1969.
59,500	(Gel filtration on Sephadex G-150 (columns.	Human urine.	(Lukowsky and Painter, 1968.
61,800		Sheep plasma.	
62,000	Gel Filtration on Sephadex G-200.	Human urine.	Olesen and Fogh, 1968.
66,000	Sucrose-gradient centrifugation and ultracentrifugation.	Sheep plasma.	Goldwasser and Kung, 1968.

centrifugal separation to determine the sedimentation coefficient for highly purified sheep erythropoietin. The value was 5S. They reasoned that if the molecular weight was only 27,000 as proposed by Rosse, et al. (1963a), for a sedimentation coefficient of 5S the frictional ratio would be 0.6, a physical impossibility. A sedimentation coefficient this large is apparently more compatible with a molecular weight of 60-70,000.

Gel filtration in columns of Sephadex (previously calibrated with proteins of known molecular weight) offers the possibility of determining the molecular weight of an impure substance, provided it has measurable biological activity (Andrews, 1965). Since erythropoietin is a protein of high specific activity, this method has been used to determine its molecular weight. Lukowsky and Painter (1968) arrived at a figure of 60,000 for both anaemic sheep plasma and human urinary erythropoietin, using Sephadex G-150 columns. Olesen and Fogh (1968) obtained a value of 62,000 using Sephadex G-200 gel, with human urine as the source of erythropoietin. O'Sullivan, et al. (1969) applied the techniques of gel filtration (Sephadex G-100) and ultracentrifugation to a human urinary preparation and, making certain assumptions about the volume of the molecule, calculated a molecular weight of 52,000 for erythropoietin. In conclusion, the evidence at present available indicates that the molecular weight of the hormone is probably in the range 50-70,000.

Erythropoietin - Protein Complexes.

The concept of erythropoietin existing *in vivo* as a single factor, produced by the kidney and transported in plasma to

its sites of action, may require modification in the light of fairly recent results presented by several workers. Goldwasser, et al. (1962) proposed, on the basis of purification experiments, that erythropoietin exists in plasma in a complex with one or more inert binding proteins, and that the complex can be dissociated under certain conditions. Kuratowska, et al. (1962, 1964) isolated a factor from hypoxic rabbit kidney perfusate which, though erythropoietically inactive alone, became active when pre-incubated with plasma α-globulins. The factor was designated R.E.F.-"renal erythropoietic factor", as distinct from "plasma erythropoietin". Kuratowska proposed that R.E.F. is either an erythropoietin precursor which must be complexed with α-globulins in normal plasma to form "plasma erythropoietin", or that it is an enzyme which activates an erythropoietin precursor present in plasma.

Contrera, et al. (1965, 1966) obtained two major erythropoietically active fractions, by purification of hypoxic kidney extracts from rats, with behaviour similar to the two fractions of Kuratowska. Gardner, et al. (1966, 1967) and Moores, et al. (1966) were also able to substantiate Kuratowska's work, finding that non-neutralising antiserum and normal rabbit, human and mouse sera potentiated the erythropoietic activity of human urinary fractions. By progressive chromatography of urine, Lewis, et al. (1965b) obtained a complex of an α_1-acidic glycoprotein and a smaller basic protein. Erythropoietic activity was demonstrable only as long as the basic polypeptide remained in association with the acidic proteins. Gardner, et al. (1967) and Goldwasser and Kung (1968) later found that orosomucoid (an

α_1-acidic glycoprotein) potentiated the erythropoietic activity of human urinary erythropoietin and sheep plasma erythropoietin respectively. Further investigations are required to explain the mechanism and function of this enhancement of erythropoietin by serum proteins.

Conclusions.

Large scale preparation of erythropoietin is most frequently performed using urine from anaemic humans as the source of active material, though plasma or urine from animals made anaemic experimentally is also employed. The percentage yield is usually small if a high degree of purification is required, necessitating the processing of large amounts of starting material. Though advances have been made in the isolation and purification of the erythropoietic factor from blood, urine and kidney tissue, its chemistry will remain incomplete pending the preparation of pure hormone. It would appear to be a glycoprotein containing sialic acid. Its molecular weight is probably in the range 50-70,000 and it appears to migrate at the rate of α_2 - α_1-globulins on electrophoresis. Phenolic hydroxyl and/or free amino groups may be required for activity, and also the amino acids tryptophan and lysine and/or arginine. Serine and/or threonine hydroxyl groups do not seem to be necessary for biological activity, nor sulphydryl groups. Serum proteins cause some enhancement of erythropoietic activity, particularly in renal perfusates and extracts, giving rise to speculation about erythropoietic factor-protein complexes and their function.

Jacobson's statement in 1960 that, "Despite the

paucity of facts about the properties of pure erythropoietin, we believe that the chemical nature of the hormone will soon be established", seems, nine years later, to have been somewhat wishful thinking.

3. SITE OF PRODUCTION OF ERYTHROPOIETIN.

Evidence suggesting that the kidney plays a role in the control of erythropoiesis arose from clinical observations. Chronic renal failure of moderate to severe degree is always accompanied by a normocytic, normochromic anaemia and polycythaemia occasionally occurs in certain forms of kidney disease; the latter include renal carcinoma (Forssell, 1954; Conley, et al., 1957; Damon, et al., 1958; Frey, 1958; Deweerd and Hagedorn, 1959; Lawrence and Donald, 1959), hydronephrosis (Forssell, 1958; Lawrence and Donald, 1959; Jones, et al., 1960; Jaworski and Wolan, 1963), renal cysts (Forssell, 1958; Jones, et al., 1960; Nixon, et al., 1960; Rosse, et al., 1963b; Kulis, 1964; Vertel, et al., 1967) and renal artery stenosis (Luke, et al., 1965; Tarazi, et al., 1966; Hudgson, et al., 1967). In a number of these reported cases where the kidney disease was unilateral, nephrectomy abolished the polycythaemia, suggesting the possibility of a cause and effect relationship between the renal disorder and the erythrocytosis.

Firmer evidence in support of the production of erythropoietin by the kidney and by extra-renal sites has been obtained from experimental work, and this is supported by more recent clinical observations.

Evidence for Erythropoietin Production in the Kidney.

1. Bilateral Nephrectomy in Animals.

Jacobson, et al. (1957) proposed that erythropoietin is either produced or activated by the kidney, since erythropoietin

could not be detected in the plasma of nephrectomised rats subjected to the erythropoietic stimulus of acute haemorrhage or cobalt treatment. Goldwasser, et al. (1958), Kurtides, et al. (1965) and Mantz, et al. (1967) confirmed these observations and demonstrated that the administration of exogenous erythropoietin prevents the suppression of bone marrow ^{59}Fe uptake which follows bilateral nephrectomy (Kurtides, et al., 1965). In experiments involving total nephrectomy it was shown that the kidney is necessary for normal erythropoiesis in the dog (Naets, 1960b, 1963), and moreover, that it is the sole source of the hormone in this species (Naets and Heuse, 1962a). Naets' results were confirmed by Mirand, et al., (1968b) who found that, while renal allotransplanted dogs responded to bleeding with increased erythropoietin production, bilaterally nephrectomised animals (maintained on peritoneal dialysis) did not.

2. Kidney Perfusion Studies.

Further support for the concept of the renal production of erythropoietin was afforded by the demonstration of the hormone in the venous effluents of isolated and *in situ* kidneys perfused with anoxic blood, and similar results were obtained after cobalt chloride stimulation. These observations were made in the rabbit (Kuratowska, et al., 1961; 1964; Kuratowska and Lewartowski, 1962; Reissman and Nomura, 1962; Reissman, 1963), the dog (Fisher and Birdwell, 1961; Fisher, et al., 1962; Mangheri, et al., 1963; Pavlovic-Kentera, et al., 1965; Abbrecht and Malvin, 1966) and the rat (Mantz, et al., 1967). Erslev, et al. (1965), using a perfused lung-kidney preparation in dogs, were unable to demonstrate erythropoietin after four hours of perfusion with hypoxic or anaemic

blood. They suggested that erythropoietin is released only from injured and disintegrating renal tissue and not from well preserved and active kidneys. This was later refuted by Fisher and Langston (1967) who could find no correlation between erythropoietin production and the glomerular congestion produced in experimentally perfused kidneys. They suggested that the lack of response in the dogs used by Erslev, et al. (1965) may have been due to an insufficient degree of hypoxia (low oxygen gas mixtures of 5% oxygen being used in only two out of twenty experimental animals) or too short a perfusion time. Fisher and Langston (1967) used a 5% oxygen gas mixture in all their hypoxia experiments, and a perfusion time of three or four hours. Pavlovic-Kentera, et al. (1965), perfusing one kidney in situ with mixed venous blood from the right atrium, found that it took five hours to obtain significant increases in plasma erythropoietin content in 50% of their animals and 6-7 hours in all of them.

3. Experimental observations of ischaemic, infarcted and hydronephrotic kidneys.

Experiments on erythropoietin production by kidneys rendered ischaemic from renal artery constriction, or by the injection of plastic microspheres into the renal artery, have produced conflicting results. Details of species used and sample material assayed for erythropoietin are given in Table 4. Takaku, et al. (1962), Hansen (1964b); Fisher, et al. (1965a); Fisher and Samuels (1967) and Fisher, et al. (1967b) all found increased erythropoietin levels on renal artery ligation. There was occasionally the development of a moderate polycythaemia

TABLE 4. EXPERIMENTAL OBSERVATIONS OF ISCHAEMIC, INFARCTED AND HYDRONEPHROTIC KIDNEYS

Authors	Species	Assay Sample	Erythropoietin	Technique
1. Ischaemia and Infarction				
Cooper and Nocenti (1961)	Rat	Central venous plasma.	Not consistently increased.	
Takaku, et al. (1962)	Rat	Central venous plasma.	Increased.	Renal Artery Ligation.
Hansen (1964b)	Rabbit	Kidney extract. Urine.	Increased	
Mitus, et al. (1964)	Rabbit	Central venous plasma.	Not demonstrable.	
Fisher, et al. (1965a)	Rabbit	Arterial plasma.	Increased.	
Fisher, et al. (1967b)) Fisher and Samuels (1967))	Dog	Arterial plasma.	Increased.	
Abbrecht, et al. (1966)) Abbrecht and Malvin (1966))	Dog	Renal venous plasma.	Increased.	Renal Infarction.
2. Hydronephrosis.				
Mitus and Toyama (1964)	Rabbit	- - -	Not measured but erythrocytosis in 8/12.	
Mitus, et al. (1964)	Rabbit	Central venous plasma.	Not consistently increased.	
Melby, et al. (1965)	Rhesus monkey	Peripheral venous plasma.	Not sig. increased.	Ureteral Ligation.
Mirand, et al. (1967)	Dog	Renal arterial and venous plasma.	Frequently undetectable.	
Murphy, et al. (1967a)	Chimpanzee	Peripheral venous plasma.	Not increased.	

(Hansen, 1964a; Fisher, et al., 1965a). Abbrecht and co-workers (1966) produced renal infarction in dogs by the injection of plastic microspheres (30-170 in diameter) into the renal artery and obtained increased erythropoietin levels and a reticulocytosis (Abbrecht and Malvin, 1966; Abbrecht, et al., 1966). On the other hand, Cooper and Nocenti (1961) and Mitus, et al. (1964) failed to show any elevation in the erythropoietin level after renal artery ligation and Cotes and Lowe (1963) found that the constriction of one renal artery did not affect the red cell mass, while constriction of both actually decreased it.

One possible explanation for the varied results obtained in this field is that whilst the renal artery constrictions in the negative experiments may have been sufficient to cause renal ischaemia, they may not have been severe enough to cause renal hypoxia. It has been shown that alterations in renal blood flow over a wide range do not cause significant renal hypoxia (Lassen, 1964). Fisher and Samuels (1967) found that the reduction of renal blood flow to 51-71% of pre-experimental values gave only a slight increase in erythropoietin levels measured at 24 hours, while reduction to 22-35% of pre-experimental values caused a significant increase in plasma erythropoietin at 12 hours. Thus, results from experiments involving constriction of renal arteries to produce a reduction in blood flow must be interpreted with caution.

Hydronephrosis produced experimentally by ureteral ligation frequently does not alter haematocrit or plasma erythropoietin levels in the Rhesus monkey (Welby, et al., 1965),

the chimpanzee (Murphy, et al., 1967a) or the dog (Miraud, et al., 1967), though Mitus and Toyama (1964) obtained an erythrocytosis in rabbits. With this exception, it has not proved possible in the experimental animal to reproduce the polycythaemia which occasionally occurs in human patients with hydronephrosis, and which is sometimes capable of relief by surgical treatment (Ferrell, 1953; Jones, et al., 1960; Jaworski and Wolan, 1963).

4. Demonstration of Erythropoietin in Renal Tissue.

Kidney extracts from various species of normal and anaemic animals have been found to exert an erythropoietic effect (Suki and Grollman, 1960; Rambach, et al., 1961; Piha, 1962; Zangheri, et al., 1962; Penington, 1963; Demopoulos, et al., 1965; Contrera, et al., 1965, 1966). Naets, on the other hand, failed to obtain conclusive evidence of erythropoietin in kidney tissue from anaemic dogs (Naets, 1960a, 1963).

Assessment of results in this field is hampered to some extent by the diversity of the extraction and assay methods used.

5. Cellular Site of Production of Erythropoietin.

Although it is now generally accepted that the main source of erythropoietin is the kidney, the cellular site of production has not been defined. The cells of the kidney medulla (Reissman and Nomura, 1962), cortex (Sokabe and Grollman, 1962; Abbrecht and Malvin, 1966), both medulla and cortex (Gordon, et al., 1967; Zanjani, et al., 1967), tubules (Reissman, et al., 1960; Fisher, et al., 1963; Mantz, et al., 1967), renal vasculature

(Kuratowska, et al., 1960) and juxtaglomerular apparatus (Osnes, 1958; Hirashima and Takaku, 1962; Reeves, et al., 1963; Mitus and Toyama, 1964; Demopoulos, et al., 1965; Jepson and McGarry, 1968) have all been suggested as sites of production. The renal tubules were proposed on the basis of suppression by diuretic drugs or mercuric or uranyl ions of the erythropoietic response to cobalt (Fisher, et al., 1963), anaemia (Reissman, et al., 1960) or anoxia (Mantz, et al., 1967).

The juxtaglomerular (J.G.) cells were proposed as the site of erythropoietin production by Osnes (1958). In rats in which erythropoiesis was experimentally stimulated or depressed, Hirashima and Takaku (1962), showed that erythropoietin production changed in parallel with the granularity of the J.G. cells. Demopoulos, et al. (1965) later devised a method of extracting erythropoietin from the renal cortex and demonstrated a correlation between the amount of extractable erythropoietin and J.G. granularity. There is insufficient clinical evidence in support of J.G. cell production of erythropoietin to draw any definite conclusions, but Jepson and McGarry (1968) reported one case of a child with histologically proven hypertrophy of the J.G. apparatus who was polycythaemic and had increased plasma and urinary erythropoietin levels. Other workers have obtained results which do not favour a role for J.G. cells in erythropoietin production. Goldfarb and Tobian (1962, 1963b) investigated J.G. cell granularity in rats under different conditions and were unable to record any differences between normal, hypoxic and hyperoxic rats. Further attempts were made to correlate J.G. granularity and

erythropoietin production by measuring erythropoietin in saline extracts of kidneys with varying degrees of granularity (Goldfarb and Tobian, 1963a). They could obtain no conclusive evidence that the juxtaglomerular apparatus does or does not secrete erythropoietin. Similar inconclusive results were obtained by Hansen (1964a) using rabbits which developed polycythaemia following unilateral renal ischaemia produced by experimental renal artery constriction.

It is well established that the enzyme renin is secreted by the juxtaglomerular apparatus (Edelman and Hartroft, 1961; Cook and Pickering, 1962) and that increased juxtaglomerular granularity is associated with augmented production of renin. An inter-relationship has been established between renin, angiotensin II and aldosterone in the control of sodium balance and arterial blood pressure (Hartroft and Hartroft, 1953; Tobian, et al., 1958; Slater, et al., 1963). Since J.G. granularity may increase coincidentally with increased erythropoietic activity, it is possible that an erythropoietic effect could be exerted by renin, angiotensin II or aldosterone either directly, or indirectly through erythropoietin. Angiotensin II was found to have an erythropoietic effect in hypophysectomized rats by Fisher and Crook (1962), in dogs and mice by Fisher, et al. (1967b), in hypophysectomized rats and normal rabbits by Nakao, et al. (1967), though not by Bilsel, et al. (1963) using hypophysectomized rats and polycythaemic mice. Nakao, et al. (1967) postulated that the erythropoietic activity of angiotensin II resulted from increased

erythropoietin production which may have been induced by a renal ischaemia through the administration of angiotensin II.

Gallagher and Donati (1968) were unable to demonstrate increased erythropoietic activity in rats after the exogenous administration of renin, angiotensin II or aldosterone, and stimulation of the renin-angiotensin-aldosterone mechanism by salt deprivation in experimental animals failed to produce any increase in red cell mass (Gallagher and Donati, 1968). This group of workers has also shown a dissociation of erythropoietin and renin activity in rats (Donati, et al., 1968). Clinical observations lend further support to the idea of independent secretion of renin and erythropoietin by the kidney. Thus, in most clinical reports of raised plasma renin concentrations associated with renal artery stenosis there is no mention of erythrocytosis. In a study of 19 patients with renovascular hypertension and 11 with essential hypertension Bourgoignie, et al. (1968) reported raised peripheral blood levels of erythropoietin and renin in the majority of patients with renovascular hypertension, whereas in those with essential hypertension the only abnormality detected was an elevation in plasma renin in four of six patients who had malignant hypertension.

Further studies are required to confirm the relationship between the juxtaglomerular apparatus and erythropoietin. Fisher, et al. (1965b) localised a fluorescence-labelled antibody for erythropoietin in the sheep kidney. However, the J.G. area did not appear to fluoresce although there was intense fluorescence

of the capillary walls of the glomerular tuft, suggesting that erythropoietin is produced or stored in the latter. There was complete absence of fluorescent staining of the renal tubules.

6. Clinical Evidence of Renal Erythropoietin Production.

Indirect evidence in support of the production of erythropoietin by the kidney is provided by the following observations in man :-

(a) The plasma of severely anaemic patients is usually rich in erythropoietin, but in the anaemia of renal failure erythropoietin is usually undetectable (Gallagher, et al., 1960; Penington, 1961; Naets and Heuse, 1962b; Brown, 1965; Eschbach, et al., 1967).

(b) Erythropoietin has been detected in renal cyst fluid (Nixon, et al., 1960; Waldmann and Rosse, 1962; Rosse, et al., 1963b; Vertel, et al., 1967; Waldmann, et al., 1968), and renal carcinoma extracts (Hewlett, et al., 1960; Waldmann, et al., 1968).

(c) Normal erythropoiesis may be restored following renal transplantation (Abbrecht and Greene, 1965; McMain, et al., 1965; Nies, et al., 1965; Abbrecht and Greene, 1966; Calne, et al., 1966; Denny, et al., 1966; Hoffman, 1968). In those cases where plasma erythropoietin was measured, it was noted that erythropoietin levels rose somewhat later than the improvement in renal function as estimated by creatinine clearance etc. (Abbrecht and Greene, 1966). Occasionally polycythaemia develops after successful renal transplantation, presumably due to excessive production of erythropoietin (Nies, et al., 1965;

Calne, et al., 1966; Hoffman, 1968). However, in most instances there is a concomitant decrease in plasma erythropoietin as the haematocrit increases and homeostasis is achieved (Abbrecht and Greene, 1965).

These observations of plasma levels of erythropoietin after renal transplantation raise interesting questions about the stimulus to the hormone's production. From the information just presented, it would appear that the pre-operative anaemia is remedied in successful renal grafting when the transplanted kidney has been functioning for a long enough period to secrete erythropoietin and thus restore normal erythropoiesis. In unsuccessful renal transplantation one would expect failure of erythropoietin production or a reduction in output in cases of delayed rejection. Whilst observations have been recorded by Abbrecht and Greene (1965) which are in accord with this concept, those reported by other workers have produced conflicting evidence. Thus, Westerman, et al. (1967) and Mirand, et al. (1968a) have each reported a case of polycythaemia with increased erythropoietin levels in urine and plasma respectively, in patients with transplanted kidneys which were shown to have histological evidence of renal ischaemia and rejection. Westerman postulated that the increased erythropoietin level was due to renal hypoxia, caused by the renal vascular occlusion demonstrated histologically; he concluded that increased urinary excretion of erythropoietin and erythrocytosis are unfavourable portents in renal transplant patients. This view has received support from the experimental work of Abbrecht, et al. (1968) in dogs. These investigators showed that there was a marked increase in plasma

erythropoietin after renal transplantation, the time course of peak hormone values being consistent with the temporal pattern of rejection phenomena. Mirand and co-workers (1965a, 1965b) found that erythropoietin levels rose little in Rhesus monkeys with successful autotransplants, compared with significantly higher levels in animals bearing autotransplants which were ischaemic due to arterial thrombosis. Murphy, et al. (1965) also showed that successful, compatible homotransplants in Rhesus monkeys did not cause an increase in post-operative erythropoietin levels, while, in the presence of arterial occlusion and renal infarction in animals transplanted incompatibly, there were significant increases in erythropoietin. In baboons, erythropoietin levels increased within seven days of arterial thrombosis in grafted kidneys, and the progressive ESF elevations correlated directly with the reduction in renal blood flow due to rejection (Mirand, et al., 1968c).

Evidence for Extrarenal Production of Erythropoietin.

Though Naets and Heuse (1962a) and Mirand, et al. (1968b) have shown that the kidneys are probably the sole source of erythropoietin in the dog, it has been demonstrated in other animals, and in man, that erythropoietin can be produced from extrarenal sources. Bilaterally nephrectomised rats (Mirand and Prentice, 1957; Rosse and Waldman, 1962; Fried, et al., 1969), rabbits (Fischer and Friederici, 1961; Gallagher, et al., 1961b), and tamarins (Mirand, 1963) have been shown to respond to hypoxia or bleeding by the production of endogenous erythropoietin. Erythropoietic activity has also been demonstrated

in anephric man (Erslev, et al., 1967; Naets and Wittek, 1968; Mirand, 1968; Mirand, et al., 1968a, 1969; Murphy, et al., 1968a). Mirand and co-workers (1968a) reported the case of a 10 year old boy who was anephric for 66 days prior to renal transplantation, during which period erythropoietin levels, as measured by ^{59}Fe uptake in the hypoxic mouse assay, were high compared with controls.

Erythropoietin has been detected in abnormal extra-renal tissue, including cerebellar hemangioblastoma cyst fluid (Waldmann, et al., 1961), and tumour extract (Waldmann and Rosse, 1962), and pheochromocytoma extracts (Waldmann and Bradley, 1961). The presence of erythropoietin in tumour tissue or cysts may represent selective concentration of erythropoietin from plasma, rather than production by the tissue or cyst wall itself. In the case of renal cysts it has been suggested (Vertel, et al., 1967) that the cysts themselves are incapable of erythropoietin production by virtue of their fibroid structure (Cohen, 1960; Jones, et al., 1960; Kulis, 1964; Vertel, et al., 1967), but that they compress normal renal parenchyma and result in the production of the hormone from its normal renal site.

Though extrarenal sites of erythropoietin production seem to exist, there is no information implicating any particular organ as being responsible. It is possible that extrarenal sites of production only come into play after nephrectomy, and do not function under normal circumstances.

Conclusions.

From experimental animal work and clinical observations, it is evident that erythropoietin is of renal origin.

In the dog, the kidney appears to be the sole source of the hormone, but in other animals and in man, there is good evidence for extra-renal sites of erythropoietin production.

4. ERYTHROPOIETIN AND THE CONTROL OF ERYTHROPOIESIS.

Under ordinary environmental conditions the rate of erythrocyte production in man averages 2.1×10^{11} cells per day, the mean red cell life-span being 120 days. With this enormous turnover of cells, controlling mechanisms must balance the rate of erythrocyte production against the rate of destruction and elicit appropriate marrow responses to external and internal stimuli.

Erythropoietin and Physiological Erythropoiesis.

In common with many other biological systems, the regulation of erythropoiesis is probably through a humoral mechanism of feedback control. The fundamental stimulus to erythropoiesis is oxygen deficiency, its effects on the bone marrow being mediated by erythropoietin. The following general outline of the control of erythropoiesis was proposed by Fried, et al. (1957) and is supported by a considerable amount of experimental evidence. When the oxygen needs of the tissues exceed their oxygen supply, oxygen-sensitive sensors signal to the renal (and possibly other) sites of erythropoietin production and the hormone is then released into the circulation. The direct effect of hypoxia on isolated kidneys, causing the release of erythropoietin, was shown by Kuratowska, et al. (1960, 1961). The action of erythropoietin on the bone marrow is to increase the production of erythrocytes. This effect ultimately leads to a higher pO_2 in the tissues and at this stage the output of erythropoietin is reduced.

Whilst it has been demonstrated that erythropoietin

produced in response to anaemia and anoxic stress will stimulate erythropoiesis, it has not been fully established that this hormone is involved in the regulation of erythropoiesis under normal conditions, as first suggested by Erslev (1955) and Alpen and Cranmore (1959). However, several lines of evidence may be advanced in support of erythropoietin having a physiological role in the control of erythropoiesis :-

(a) In mice made plethoric by transfusion of red cells, the rate of erythropoiesis falls to a very low level and virtually ceases in the bone marrow (Jacobson, et al., 1957). Administration of exogenous erythropoietin to such plethoric animals causes a wave of erythropoiesis, as evidenced by an increase in haemoglobin concentration and a rise in the reticulocyte count (Filmanowicz and Garney, 1961).

(b) Investigation of the erythropoietin/haematocrit relationship in normal man shows that relatively small changes in the haematocrit, achieved by phlebotomy or transfusion, lead to predictable changes in the daily urinary excretion of erythropoietin. The latter bears a logarithmic relationship to the haematocrit (Adamson, 1968).

(c) Rabbit anti-human urinary erythropoietin antiserum injected into normal mice causes a depression of erythropoiesis (Schooley and Garcia, 1962).

(d) Erythropoietin has been demonstrated in concentrates of normal human urine (Finne, 1965; Adamson, et al., 1966; Alexanian, 1966).

Other observations cast some doubt on the thesis of regulation of physiological erythropoiesis by erythropoietin.

Firstly, the hormone has not been conclusively demonstrated in normal plasma, though several authors claim to have done so (Gurney, et al., 1957; Reichlin and Harrington, 1960; Payne, 1961; Miraud, et al., 1965c). Since none of these workers employed a standard preparation of erythropoietin in their assays, the possibility cannot be ruled out that their results were attributable to non-specific erythropoietic stimulation. Gallagher, et al. (1959), Penington (1961), Maets and Heuse (1962b) and Brown (1965) all failed to demonstrate erythropoietin in normal human plasma. Secondly, it has been reported that cells produced after stimulation of erythropoiesis by erythropoietin are morphologically abnormal (Stohlman, 1961a; Borcook, et al., 1962). Using transfusion-induced polycythaemic rats, Stohlman (1961a) found that the red cells formed in response to severe anaemia or injected erythropoietin were macrocytes with a shortened life-span. On the other hand, Sephton-Smith (1963) could detect no abnormality of size, life-span or fragility in ^{59}Fe-labelled red cells of polycythaemic mice receiving purified erythropoietin or plasma or urine from a patient with aplastic anaemia. It should be noted that the dose of erythropoietin given by Smith was just sufficient to initiate a wave of erythropoiesis at a rate not exceeding that found in the normal mouse. Smith has suggested that Stohlman's findings resulted from excessive stimulation of erythropoiesis by large doses of erythropoietin (Sephton-Smith, 1963).

Site of Action of Erythropoietin.

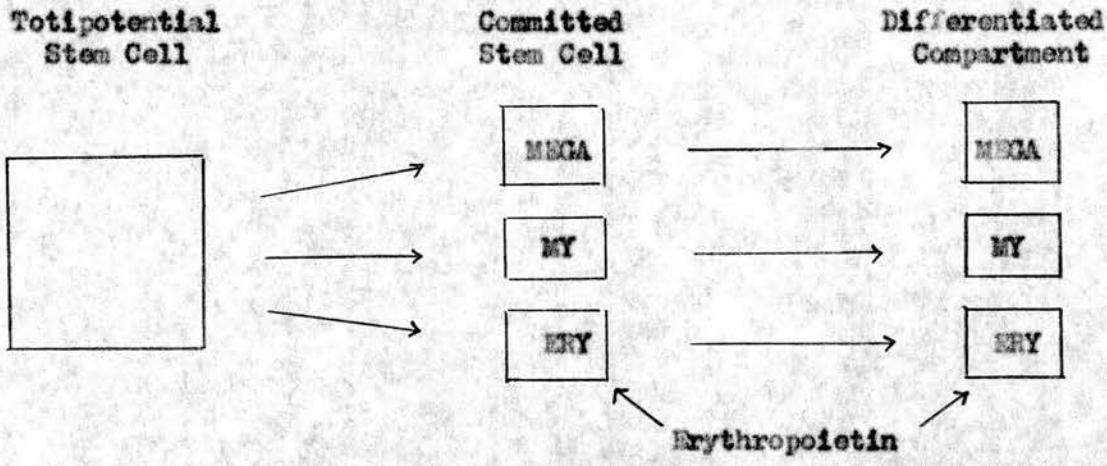
Three cell populations are involved in medullary

erythropoiesis :-

1. The haematopoietic stem cells, a self-maintaining population capable of differentiation.
2. The dividing normoblast series, a transit population in which proliferation and differentiation occurs.
3. The non-dividing population of late polychromatic normoblasts and marrow reticulocytes, which compose the compartment undergoing maturation.

Since nucleated red cells proceed along a pathway of differentiation and are not self-maintaining, the stem cells fulfil the dual function of a precursor population which also maintains its own numbers. The morphological and biochemical characteristics of the stem cells are completely unknown.

It is now established that erythropoietin causes the differentiation of stem cells into identifiable erythroid precursors (Lajtha and Oliver, 1960; Filmanowicz and Gurney, 1961; Stohlman, 1961b; Erslev, 1964; Fujioka, 1966). More recent evidence suggests that two levels of stem cell exist, one being a primitive totipotential stem cell and the other a more differentiated precursor cell for each of the blood cell lines, these latter being the erythropoietin-sensitive cells in the case of the erythroid series (Morse and Stohlman, 1966; Schooley, et al., 1967; O'Grady, et al., 1968; Stohlman, et al., 1968). This concept and the point at which erythropoietin is considered to act is depicted over :-



MEGA - megakaryocytes; MY - myeloid; ERY - erythroid

(Adapted from Stohlman, et al., 1968).

Useful information has been obtained from the study of colony forming units. In the lethally irradiated mouse intravenously injected bone marrow cells seed in the spleen and form colonies of proliferating cells called "clones" (Till and McCulloch, 1961). These colonies appear as gross nodules and are easily counted. Since the identity of the cells giving rise to the colonies is uncertain, they are referred to as colony forming units - C.F.U. Chromosome abnormalities (induced by irradiation) were used by Becker, et al. (1963) as cell markers, to show that all the cells in one colony were derived from a single cell. In X-irradiated mice made polycythaemic, the erythroid nodili are completely suppressed and only granuloid and megakaryocytic clones develop. The injection of erythropoietin elicits the re-formation of erythroid clones (Bleiberg, et al., 1965). There is some controversy as to whether C.F.U. and stem cells are one and the same, and on erythropoietin's role in the kinetics of erythroid

development. Bruce and McCulloch (1964) postulated two kinds of stem cell, the C.F.U. capable of forming spleen colonies and re-establishing erythropoiesis in the lethally irradiated host, and erythropoietin-sensitive cells (E.R.C.) derived from C.F.U. This postulate is compatible with the concept illustrated above, assuming the totipotential stem cell to be the C.F.U., and the committed erythroid stem cell the E.R.C. Porteous and Lajtha (1966) do not consider that it is necessary to postulate two kinds of stem cell, and Weisman, et al. (1967) obtained experimental results which led them to suggest that E.R.C. and C.F.U. are closely related if not identical lines of cells.

Action of Erythropoietin on Differentiated Cells.

There has been some speculation on the possible action of erythropoietin on differentiated cells which are still capable of RNA synthesis. These comprise cells up to and including the early polychromatophilic normoblasts (Stohlman, et al., 1968). In preparations of isolated, perfused femurs from rats it has been found that the administration of erythropoietin causes a prompt release of reticulocytes from the marrow (Gordon, et al., 1962). Fruhman and Fisher (1962) obtained similar results and concluded that erythropoietin acts on nucleated erythrocytes in the marrow well beyond the stem cell stage. Using tissue cultures of mouse foetal liver, Paul (1969) measured ^{59}Fe incorporation into haemoglobin and postulated from his results that erythropoietin acts on erythropoietin-responsive cells beyond the stem cell, including reticulocytes.

Biochemical Actions of Erythropoietin.

In various species *in vivo* studies have shown that erythropoietin causes an increase in DNA and RNA synthesis (Rambach, et al., 1957; Linkenheimer, et al., 1959; Pieber-Perretta, et al., 1965; Fujioka, 1966; Hodgson, 1967; Rudolph and Perretta, 1967; Perretta and Tirapegui, 1968), and in DNA and RNA polymerases, the enzymes involved in nucleic acid synthesis (Fischer, 1962). The exact nature of the RNA formed in response to erythropoietin remains to be determined, though Gross and Goldwasser (1969) did examine the nature of the RNA formed in response to the erythropoietin stimulation of rat marrow cells *in vitro*. Within a few minutes of exposure of the cells to erythropoietin a very large RNA type was formed which could not be detected in unstimulated cells. Smaller RNA components were later formed. It is thought that erythropoietin may act as a gene derepressor, causing the transcription of genetic information from nuclear DNA into messenger RNA (Karlson, 1963), since mRNA-like material was observed by Krantz and Goldwasser (1965) after adding erythropoietin to bone marrow cultures.

In vitro systems employing marrow cell cultures and spleen have been developed to facilitate interpretation of data obtained from the whole animal. Various indices of mitotic activity have been shown to increase on the addition of erythropoietin to culture systems. These include the incorporation of radioisotope-labelled formate, thymidine and uridine into nucleic acids (Perretta and Thomson, 1961; Pieber-Perretta, et al., 1965; Powsner and Berman, 1967), and ^{14}C-glucosamine into cell stroma (Krantz and Goldwasser, 1965; Dukes, 1967) and marrow cells

(Dukes, et al., 1964; Dukes and Goldwasser, 1966; Dukes, 1968). Haem synthesis is also increased (Erslev, 1962; Korst, et al., 1962; Krantz, et al., 1963). In both *in vitro* and *in vivo* studies, Peets and Gordon (1968) found that erythropoietin stimulated bone marrow cell glucose oxidation via the hexose-monophosphate pathway; they suggested that this was a primary effect of erythropoietin to provide the precursors necessary for increased nucleic acid and protein synthesis in differentiating cells.

Use of Mitotic Inhibitors.

Much information about the mode of action of erythropoietin, and erythrokinetics in general, has been obtained by the use of mitotic inhibitors. Actinomycin D, which selectively inhibits DNA-dependent RNA synthesis (Reich, et al., 1961; Reich and Goldberg, 1964), has been shown to impair the haematopoietic response to erythropoietin in mice (Gurney and Hofstra, 1963) and the erythropoietin-induced stimulation of haemoglobin synthesis in spleen and bone marrow cultures (Goldwasser, et al., 1964; Gallien-Lartigue and Goldwasser, 1965; Miura, et al., 1968). When erythropoietin is added to the marrow culture twenty-four hours before the actinomycin D, there is no longer any sensitivity to the inhibitor. This would seem to indicate that the early stages of erythropoietin action are dependent on new DNA-dependent RNA synthesis, while the later stages are not (Goldwasser, et al., 1964). Erythropoietin-dependent glucosamine incorporation is sensitive to actinomycin and puromycin (Dukes and Goldwasser, 1966) implicating both RNA and protein synthesis. The mitotic inhibitor colchicine

has been found to have no effect on erythropoietin-stimulated glucosamine incorporation (Dukes and Goldwasser, 1966), but it decreased erythropoietin-stimulated haemoglobin synthesis (Gallien-Lartigue and Goldwasser, 1965).

Conclusions.

Erythropoietin is intricately involved in the regulation of physiological erythropoiesis and in the erythropoietic response to anaemic or anoxic stress imposed by disease or experiment. The use of *in vivo* techniques and cell culture systems in which spleen or bone marrow is shown to respond to erythropoietin has produced evidence which strongly suggests that the chief action of erythropoietin is on the stem cells of the bone marrow, which are probably one generation removed from the totipotential stem cell common to all three haematopoietic cell lines. Whilst erythropoietin may also influence differentiated cell maturation and the release of erythrocytes from the marrow, these actions have not been fully established.

5. ERYTHROPOIETIN IN HEALTH AND DISEASE

Erythropoietin in Normal Subjects.

Despite the relative insensitivity of currently used bioassay techniques, it is possible to estimate erythropoietin levels in the body fluids of healthy human subjects. The fluid under study is usually concentrated by freeze-drying or by membrane dialysis against a polyethylene glycol compound. Erythropoietin has been demonstrated in urine concentrates (Finne, 1965; Adamson, et al., 1966; Alexanian, 1966; Van Dyke, et al., 1966), concentrated extracts of plasma (Gurney, et al., 1957; Mirand, et al., 1965c), though not convincingly, and amniotic fluid (Finne, 1964). Alexanian (1966) obtained figures for the mean daily urinary excretion of erythropoietin of 2.8 I.U. (Range 1.5 - 5.2) in men, 0.9 I.U. (Range 0.5 - 1.8) in women, and 1.0 I.U. (Range 0.6 - 1.2) in prepubertal boys. The results of Adamson, et al. (1966) indicate that there is diurnal variation in excretion of the hormone, the highest output occurring in the six hour period between 7 p.m. and 1 a.m. Also, there are wide variations in day to day erythropoietin excretion by individual subjects (Adamson, et al., 1966).

Erythropoietin in Pregnancy and the Newborn.

Experimental data obtained in recent years suggest that erythropoietin is a major controlling factor of erythropoiesis in pregnancy. In mice, plasma erythropoietin activity increases from the fifth day of pregnancy, and by the tenth day there is a reticulocytosis followed by an increase in red cell mass (Jepson

and Lowenstein, 1968a). In the human, plasma erythropoietin levels are increased throughout pregnancy, the peak occurring during the second trimester (Manasc and Jepson, 1969). Human placental lactogen, present in large amounts in the plasma of pregnant women, is found to have some erythropoietic activity when injected into mice; it also causes an increase in the animal's plasma volume (Jepson and Lowenstein, 1968a). Further experiments, using anti-human placental lactogen and anti-sheep erythropoietin in mice, indicated that the main action of human placental lactogen is augmentation of the erythropoietic effect of erythropoietin (Jepson and Friesen, 1968).

Ovarian hormones (oestradiol and hydroxyprogesterone) injected into mice are erythropoietically ineffective and oestrogen appears to inhibit the activity of placental lactogen (Jepson and Lowenstein, 1968a). It is suggested that placental lactogen and erythropoietin may be jointly responsible for the increase in red cell mass during pregnancy, and that their stimulatory effect is balanced by the inhibitory effect of increased oestrogens (Jepson and Lowenstein, 1968a).

Erythropoietin has been detected in the foetus of pregnant mice and available evidence suggests that it is foetal rather than maternal in origin. Thus Finne (1964, 1967) showed a lack of correlation between erythropoietin levels in maternal blood and foetal blood, and maternal blood and amniotic fluid, in mice. Matoth and Zaizov (1969) exposed rats in their third week of pregnancy to erythropoietic stimuli (including intermittent hypoxia and anaemia induced by bleeding), and found that, whilst

EDINBURGEN EX BIBL UNIV.

red blood cell ^{59}Fe-incorporation increased in the mother, this did not occur in the foetus. Since the arterial blood gas oxygen tension in the foetus must have decreased in the hypoxic group, it would appear that foetal regulation of erythropoiesis in the rodent is independent of maternal regulation. Reported studies on the possible transplacental passage of erythropoietin are inconclusive (Finne, 1967) but erythropoietin may be transferred across the placenta in the manner of other plasma proteins (Gelfand, et al., 1960; Freda, 1962).

In the human, greatly increased levels of erythropoietin are found in cord blood and amniotic fluid at 32 weeks' gestation (Finne, 1966a). In a study of erythropoietin levels in cord blood from normal full-term infants, infants born more than two weeks after term, premature infants and infants of pre-eclamptic, diabetic and Rh-immunised mothers, Finne (1966a) found that the greatest frequency of increased erythropoietin levels was in the pre-eclamptic group. Normal infants had higher erythropoietin levels than the premature group. High erythropoietin levels in the amniotic fluid of pregnancies with severe anaemic erythroblastosis occur abruptly when the haemoglobin concentration falls to less than 11 or 10 g. per 100 ml. (Finne, 1966b). The high erythropoietin levels in amniotic fluid in these pregnancies correlate with those in cord blood and foetal urine (Halvorsen and Finne, 1963; Finne, 1964). The value of erythropoietin levels in cord blood and amniotic fluid is as an indication of intrauterine hypoxia. Following birth there is a decrease in erythropoiesis and erythropoietin is not detectable, except in

hypoxic newborn infants (Halvorsen, 1963). Thus it has been concluded (Halvorsen, 1963; Finne, 1966a) that erythropoiesis in the human foetus and infant is regulated by erythropoietin in the latter part of life *in utero*, and in the neonatal period.

In the rodent however, experimental evidence seems to indicate that erythropoiesis in the foetus and newborn animal is governed by a different mechanism than in the adult. Nephrectomy does not greatly affect erythropoiesis in the first twenty days of extra-uterine life, whereas it substantially decreases erythropoiesis in the adult rat (Lucarelli, et al., 1964). Presumably, any erythropoietin which is produced at this stage is extra-renal in origin (Lucarelli, et al., 1964, 1968; Carmena, et al., 1966, 1968a, 1968b). It is possible that the control of erythropoiesis undergoes a serial transition from foetal erythropoiesis, which is independent of erythropoietin, through a newborn stage, partly dependent on erythropoietin produced outwith the kidney, to the adult state of erythropoiesis controlled by erythropoietin of renal origin.

High Altitude Studies.

It is well recognised that sojourn at high altitude induces a true polycythaemia and that polycythaemia will develop in animals exposed to conditions of simulated high altitude where the oxygen tension of the inspired air is low. Many studies on the physiological effects of altitude have been reported from the United States laboratory in Morococha, a village in the Peruvian Andes situated 4,540 metres (14,900 feet) above sea level. Reynafarje, et al. (1964) studied plasma erythropoietin levels (1) in natives of Morococha and (2) in newcomers to the village, 24 hours, 72 hours

and 8 days after their arrival from low altitudes. Increased levels were found only in the group of newcomers after 24 hours. Two other groups of workers (Van Dyke, et al., 1966; Carmena, et al., 1967) recorded increased daily urinary excretion of erythropoietin in sea-level residents taken up to altitudes of 5,180 metres and 4,540 metres respectively. Van Dyke found a high mean urinary excretion rate of erythropoietin of 9.1 I.U. per day (range 1.1 - 22.4) in five male subjects who had been living at 5,180 metres for periods varying from 3 to 13 years. (In 10 normal males at sea-level the mean daily urinary excretion of erythropoietin was approximately 1.0 I.U.). Carmena, et al. (1967) reported an early increase in daily urinary erythropoietin excretion in five subjects, reaching a peak on the second or third day of exposure and subsequently falling, the decrease being attributed to increased utilisation of the hormone by an active bone marrow.

Few studies of erythropoietin during experimental hypoxia have been made in human subjects using low pressure chambers or low oxygen gas mixtures to simulate high altitude. Siri, et al. (1966) reported a single experiment measuring serum and urinary erythropoietin in a man exposed to an atmospheric pressure of 405.6 mm.Hg (equivalent to an altitude of 5,000 metres) for four days. Serum erythropoietin was detected after 12 hours, reached a peak on the third day and declined thereafter. During acclimatization the rise and fall of serum erythropoietin concentration correlated with changes in cardiac and pulmonary function, while there was no significant change in red cell mass or plasma volume. It would seem that acute exposure to hypoxia produces a sudden erythropoietin

response, with further exposure resulting in a gradual decrease in levels until they become undetectable. This may be accounted for by a reduction in functional hypoxia as acclimatization continues, the increase in erythropoiesis resulting in improved tissue oxygenation with a consequent reduction in erythropoietin production. Stohlman and Brecher (1959) suggested that the low erythropoietin levels on chronic exposure to hypoxia were due to a high rate of utilisation of the hormone by the bone marrow, in parallel with a high rate of production. Their conclusions were based on experiments in rats in which they found higher erythropoietin levels in animals made hypoplastic by marrow-irradiation and subjected to hypoxia than in those which were not irradiated but subjected to the same degree of hypoxia.

Several animal studies have been performed in high altitude conditions, usually in low-pressure chambers since this is one of the most frequently used methods of preparing mice for erythropoietin bioassay (See Review, Chapter 1 - "Assay of Erythropoietin"). Two groups have made interesting observations of animals native to high altitudes (Bullard, et al., 1966; Scaro and Aggio, 1966). Bullard found that high altitude mammals are not polycythaemic and in fact have lower haemoglobin concentrations, haematocrits and red cell counts than animals living at lower altitudes. In a study using the llama, vicuna and chinchilla, which normally live at high altitudes, Scaro and Aggio reported an increase in their plasma erythropoietic activity when they were exposed to acute hypoxia in a chamber, but the increase was less in the llama than in the other two species; the erythropoietin titres

were also less than those of lower altitude species (mouse, rat, rabbit, sheep) subjected to the same hypoxia. The high oxygen affinity of the blood of the llama was postulated as an explanation for the poor response in this species (Scaro and Aggio, 1966).

Erythropoietin in Disease.

Studies of erythropoietin in disease have largely been concerned with estimations of plasma concentrations and urinary excretion rates of the hormone. A major difficulty in these studies has arisen from the limitations of current bioassay techniques in measuring the amounts of erythropoietin normally present in plasma and urine and even in samples where the concentration of the hormone is moderately elevated. Whilst erythropoietin has been demonstrated in the plasma and urine of patients with various types of anaemia and certain forms of polycythaemia, difficulties are encountered in the interpretation of these findings. Plasma levels of erythropoietin are determined by several factors including (a) the rate of hormone production by the kidneys and other tissues, (b) the rate of utilisation by the bone marrow, (c) the rate of hormone inactivation and breakdown, and (d) the amount excreted in the urine. The same factors influence the urinary excretion rates of erythropoietin, the amount excreted also being influenced by alterations in renal function and changes in urinary pH.

Anaemias.

Since reduced tissue oxygen tension is the stimulus to erythropoietin production, increased plasma and urine levels of the hormone are often found in patients with anaemic hypoxia. In

some cases of unexplained anaemia it has been postulated that there is a congenital or acquired deficiency of erythropoietin. This possibility has been considered in relation to the red cell aplasia found in association with thymoma (Schmid, et al., 1965); an alternative explanation of this rare anaemia is an autoimmune process with the production of antibodies against either erythroblasts or erythropoietin (Andersen and Ladefoged, 1963). However, a high plasma titre of erythropoietin has been reported in a single case of pure red cell aplasia (River, 1966); it is therefore necessary to await the results of further assays in similar cases before continuing this discussion.

Hypoplastic and Aplastic Anaemia.

When bone marrow failure occurs, plasma and urine levels of erythropoietin tend to be high (Van Dyke, 1960; Lowy and Borsook, 1962; Lewis, et al., 1964; Okcuoglu and Jones, 1966a); thus the urine of patients with aplastic anaemia is often used as a source of erythropoietin in the preparation of laboratory standards. Erythropoietin titres are measurable in the plasma and urine of patients with aplastic anaemia when the haemoglobin levels are less than 8g. per 100 ml. (Van Dyke, et al., 1961). Above this haemoglobin concentration there would appear to be no direct correlation between the severity of the anaemia and the erythropoietin titre. The high titres of erythropoietin in aplastic anaemia may be explained by a negative feed-back mechanism where there is end-organ failure (in this case, the bone marrow) and the hormone, erythropoietin, is produced in excess.

Haemolytic Anaemias and Post-Haemorrhagic Anaemias.

Increased amounts of erythropoietin can often be detected in the plasma and urine of patients with haemolytic and post-haemorrhagic anaemias, although the titres are lower than in those with aplastic anaemia (Hammond, et al., 1962). Okcuoglu and Jones (1966a) were unable to detect erythropoietin in the plasma of four children with haemolytic anaemia whose haemoglobin values were 6.7 to 10.7 g. per 100 ml. Jones and Klingberg (1960) showed erythropoietin to be present in 27 out of 46 cases of erythroblastosis foetalis and 12 out of 16 sickle cell anaemia cases studied; however, there was no correlation between erythropoietin titre and haemoglobin concentration. It is postulated that erythropoietin levels are lower in these anaemias with intense erythroblastic normoblastic activity since increased utilisation parallels increased production.

Chronic Iron Deficiency Anaemia.

In this common anaemia elevated erythropoietin levels can sometimes be detected in plasma and urine. Early investigators found little or no correlation between plasma or urine erythropoietin titre and haemoglobin concentration (Van Dyke, 1960; Gallagher, et al., 1960; Van Dyke, et al., 1961). This lack of sensitivity may have been due to the insensitivity of the starved rat assay which was used. More recent reports indicate that there is a significant relationship between plasma and urine erythropoietin level and haemoglobin concentration (Moores, et al., 1963; Movassaghi, et al., 1967). Hammond, et al. (1968) found that plasma erythropoietin levels in children with iron deficiency anaemia were raised at haemoglobin concentrations between 2.4 and

9.5 g. per 100 ml. and that plasma erythropoietin titre was inversely proportional to haemoglobin concentration when the latter lay between 2.4 and 9.5 g. per 100 ml.; a similar correlation was obtained between urinary erythropoietin concentration and the degree of anaemia when the haemoglobin was less than 6 g. per 100 ml.

Anaemia Associated with Renal Failure and Chronic Inflammatory Disease.

It is probable that a major aetiological factor in the anaemias associated with renal failure, rheumatoid arthritis and chronic infection is a relative or absolute deficiency in the production of erythropoietin, since it is unusual to find raised levels of erythropoietin in response to the anaemic, hypoxic stimulus (Naets and Heuse, 1962b; Eschbach, et al., 1967; Ward, et al., 1969). This is one instance where the difficulty in measuring normal and low erythropoietin titres is a grave disadvantage. Several attempts have been made to detect erythropoietin in plasma and urine in chronic renal disease, but these have proved unsuccessful (Gallagher, et al., 1960; Naets and Heuse, 1962b; Finne, 1968). It is in this group of diseases that erythropoietin therapy is most likely to find application when a non-antigenic preparation of the hormone becomes available.

Polycythaemias.

Investigations into the role of erythropoietin in the control of erythropoiesis have enabled a greater appreciation of the pathogenesis of certain forms of polycythaemia. Strictly speaking, polycythaemia denotes an increase in all three formed elements in the blood, though it is now generally used to refer to an abnormal increase in red blood cell mass.

Primary Polycythaemia (polycythaemia vera).

In the classical case of primary polycythaemia there is hyperplasia of all marrow elements, with leucocytosis and thrombocytosis in addition to erythrocytosis. Although there is general agreement that plasma and urinary erythropoietin are not increased in polycythaemia vera, the exact role of the hormone is difficult to evaluate, mainly on account of the problems involved in the measurement of low concentrations of erythropoietin. Van Dyke, et al. (1966) and Adamson and Finch (1968) were unable to detect urinary erythropoietin and Murphy and co-workers (1968b) had similar negative results with plasma from primary polycythaemic patients. Noyes, et al. (1962) reported that plasma erythropoietin was within the normal range in a group of seven patients with polycythaemia vera and that the level of the hormone did not rise above normal limits after phlebotomy had been undertaken to reduce the red cell mass. However, after similar therapeutic reductions in the haematocrit by venesection, Adamson and Finch (1968) observed that erythropoietin reappeared in the urine and rose to normal levels.

Thus the erythrocytosis of primary polycythaemia does not appear to result from overstimulation of the bone marrow by excessive amounts of circulating erythropoietin. This view receives further support from in vitro marrow studies which have demonstrated that marrow from patients with polycythaemia vera shows poor haem synthesis in response to added erythropoietin, in comparison to that obtained with normal human marrow, or marrow obtained from patients with secondary polycythaemia (Krantz, 1967, 1968). It has been postulated (Gurney, 1965; Krantz, 1968) that

polycythaemia vera results from the mutational development of an abnormal totipotential stem cell which does not require erythropoietin for its differentiation. This concept is consistent with current theories on the kinetics of erythropoiesis (See Review, Chapter 4 "Erythropoietin and the Control of Erythropoiesis") and would explain the increase in all three formed elements of the blood in primary polycythaemia.

Secondary Polycythaemias.

Since oxygen lack is the physiological stimulus to erythropoiesis and its effect is mediated by erythropoietin, it is appropriate to find raised levels of erythropoietin and erythrocytosis in those states where there is tissue anoxia. However, the occurrence of erythrocytosis and raised levels of erythropoietin in plasma and urine has also been reported in many cases without hypoxia; these have been found in patients with neoplasms and various types of renal lesion. This latter category would seem to be one of inappropriate erythropoietin production and erythrocytosis.

Polycythaemia Secondary to Hypoxia.

Several haemoglobin disorders interfere with oxygen uptake or release and include such genetically determined abnormalities of haemoglobin structure as haemoglobin Chesapeake (Charache, et al., 1966), haemoglobin Yakima (Jones, et al., 1967) and haemoglobin Ranier (Adamson and Stamatoyannopoulos, 1967; Stamatoyannopoulos, et al., 1968). Since the normal erythropoietic response to hypoxia prevails, erythrocytosis occurs in patients with these abnormalities. Adamson and Stamatoyannopoulos (1967) studied the members of two families with

erythrocytosis due to haemoglobin Yakima and haemoglobin Hanier. Urinary erythropoietin excretion was within normal limits, but reduction of the haematocrit to normal levels resulted in increased erythropoietin excretion and increased reticulocyte production. Thus their erythrocytosis is appropriate to their tissue oxygen deficiency and a state of balance is achieved.

In patients with severe chronic obstructive airways disease one would expect increased erythropoietin production and polycythaemia secondary to low tissue oxygen tension. However, polycythaemia does not develop in all patients with chronic obstructive airways disease, even in those with arterial oxygen saturation values as low as 80% (Shaw and Simpson, 1961; Freedman and Penington, 1963; Vanier, et al., 1963). Where erythropoietin assays have been performed, the hormone levels are usually elevated but they are sometimes normal (Freedman and Penington, 1963; Vanier, et al., 1963; Adamson and Finch, 1968). This poor or absent polycythaemic response to hypoxaemia found in some patients with chronic airways obstruction is evidently not due to failure of erythropoietin production.

In the hypoxia of congenital or acquired cardiac disease one would again expect evidence of increased erythropoietin production and secondary polycythaemia. Okeuoglu and Jones (1966b) failed to find elevated plasma erythropoietin activity in 15 out of 18 children with cyanotic congenital heart disease and polycythaemia, using the starved rat assay. Their negative results may have been due to the insensitivity of the assay method used, or possibly the rate of utilisation of the hormone paralleled its production. Since Okeuoglu and Jones (1966b) appear to be the only workers who have measured

erythropoietin in the hypoxia of congenital or acquired heart disease, there is a need for further studies in this field.

Polycythaemia Secondary to Tumours and Cysts.

There have been many reported cases of polycythaemia occurring in association with tumours and cysts of various organs, in which removal of the lesion has produced remission of the polycythaemia. Erythropoietin derived from the tumour or cysts is believed to be the cause of this secondary polycythaemia. Erythropoietic activity has been demonstrated in renal cyst fluid (Nixon, et al., 1960; Rosse, et al., 1963b; Vertel, et al., 1967; Murphy, et al., 1968b), renal cyst wall extract (Nixon, et al., 1960) and the urine and plasma of patients with polycythaemia prior to the removal of renal cysts (Jones, et al., 1960; Vertel, et al., 1967; Murphy, et al., 1968b). Polycythaemia is occasionally found in association with hydronephrosis (Forssell, 1958; Lawrence and Donald, 1959; Jones, et al., 1960; Jaworski and Wolan, 1963). Patients with carcinoma of the kidney may have increased erythropoietin plasma titres and erythropoietin present in extracts of the tumour tissue (Korst, et al., 1959; Hewlett, et al., 1960; Lange and Gallagher, 1962; Donati, et al., 1963; Penington, 1965; Murphy, et al., 1968b; Waldmann, et al., 1968). In eight cases of nephroblastoma (Wilms' tumour) Murphy, et al. (1967b) found no evidence of erythrocytosis, but five patients had elevated plasma erythropoietin levels preoperatively. However, polycythaemia has been reported in cases of nephroblastoma by Thurman, et al. (1966) and Shalet, et al. (1967) in association with high erythropoietin levels in plasma and in extracts of the tumour.

In the erythrocytosis which is occasionally found in association with hepatoma, increased plasma erythropoietin has

been reported in some patients, but not in extracts of the tumour (Donati, et al., 1963; Cannon and Penington, 1967; McFadzean, et al., 1967).

In one case of adrenal adenoma and associated erythrocytosis, Mann, et al. (1967) found increased plasma erythropoietin levels which returned to normal after surgical removal of the lesion, and a similar result was observed by Fried, et al. (1968) in a case of leiomyoma of the oesophagus. Erythropoietin was not detected in extracts of the leiomyoma.

Erythropoietin has been demonstrated in the cyst fluid from a cerebellar hemangioblastoma (Waldmann, et al., 1961) and in tumour extracts and serum of a patient with a phaeochromocytoma (Waldmann and Bradley, 1961).

Though polycythaemia is seen in association with uterine fibromyomata, erythropoietin has not yet been detected in plasma, urine concentrate or tumour extract of such patients (Payne, et al., 1969).

Polycythaemia in Association with Renal Artery Stenosis.

There have been few reported cases of polycythaemia in association with renal artery stenosis in the human (Luke, et al., 1965; Tarazi, et al., 1966; Hudgson, et al., 1967). Plasma erythropoietic activity was measured in one of the patients and described as being "within the normal range" though few details were given of the assay procedure (Hudgson, et al., 1967). In two patients (Luke, et al., 1965; Hudgson, et al., 1967) removal of the affected kidney resulted in a reduction of blood pressure and red cell mass toward normal values. It would therefore seem that there is a cause-effect relationship between the severe reduction of renal

blood flow in renal artery stenosis, and the hypertension and polycythaemia. Further studies are required in this field, particularly with regard to the role of erythropoietin in the production of the renal polycythaemia.

Conclusions.

Data are available for the daily urinary excretion of erythropoietin by healthy human males and females. Studies of the hormone in pregnancy have shed light on the control of erythropoiesis before birth and erythropoietin levels in cord blood and amniotic fluid may prove useful diagnostically as indicators of intrauterine hypoxia. Since the normal regulation of erythropoietin production (and therefore of erythropoiesis) is based on tissue oxygen supply, the various anaemias and polycythaemias may be considered in relation to the erythropoietin status. While the diagnostic uses of erythropoietin titres are limited, they are sometimes of value in distinguishing between primary and secondary polycythaemia; a high erythropoietin titre in the presence of a normal arterial oxygen saturation raises the possibility of underlying renal disease or ectopic hormone production by a tumour. When a pure erythropoietin becomes available for clinical trial, it will probably find a place in the treatment of anaemia due to underproduction of the hormone.

SECTION II

ERYTHROPOIETIN ASSAY

SECTION II.

ERYTHROPOIETIN ASSAY

Introduction

Since the chemical structure of erythropoietin has not been fully elucidated, present methods of estimation depend on bioassay techniques. These are based on the ability of the hormone to stimulate red cell production when injected into normal animals (Plzak, et al., 1955). The index of red cell production most commonly used is the incorporation of radioactive iron (^{59}Fe) into newly formed erythrocytes, Degowin, et al. (1962a) having demonstrated that this is a function of the amount of erythropoietin administered over a wide dose range. In the studies described in this thesis, radio-iron incorporation into mouse red cells was used as the measure of erythropoietic activity.

The sensitivity of the bioassay procedure is increased by suppressing endogenous erythropoietin production in the animals used for assay. Suppression may be achieved in several ways including starvation, transfusion-induced polycythaemia, or hypoxia-induced polycythaemia (See Review - Chapter 1 - "Assay of Erythropoietin"). In the studies reported in this thesis the latter method was employed by exposure of mice to air at reduced pressure (Cotes and Bangham, 1961).

METHODS.

1. Assay Design.

A four point assay design was used, as first described by Gaddum (1933). Apart from the group of control mice receiving saline, four groups of mice were used, two groups receiving a standard preparation of the hormone (S), and two receiving the substance under test (U). Results were plotted as $\log_{10}$-dose response curves and from them the potency of U in terms of S was calculated. The slopes of the $\log_{10}$-dose response curves of S and U were tested for parallelism, since a lack of parallelism indicates that there are qualitative differences between S and U and that the assay is invalid (Finney, 1952). Details of statistical methods are described in Section II, page 73.

The standard preparation of erythropoietin used in routine assays was a laboratory standard prepared as described below. Two laboratory standards were prepared and calibrated against Standard B, the World Health Organisation International Reference Preparation of erythropoietin (Cotes and Bangham, 1966).

2. The Assay Method.

Virgin female mice of the albino Schofield strain were used, within a weight range of 18 to 26g. Before exposure to low atmospheric pressure each animal was given a single intraperitoneal injection of iron dextran (2 mg.) to prevent iron deficiency during the development of polycythaemia.

The mice were kept in a low pressure chamber, the details of which are given in Appendix A. The pressure was

maintained at approximately 380 mm.Hg. The mice had free access to food and water; the pressure was restored to atmospheric for approximately one hour each day during which time cage cleaning was performed and fresh food and water given.

After 14 days at 380 mm.Hg the mice were returned to atmospheric pressure. The schedule of the test procedure is shown in Table 5 and is that recommended by Cotes and Bangham (1961).

TABLE 5

PROTOCOL FOR PREPARATION OF POLYCYTHAEMIC MICE AND THE ASSAY OF ERYTHROPOIETIN.

Day	Procedure
0 - 14	Mice maintained at 380 mm.Hg pressure.
14	Mice returned to atmospheric pressure.
15	First injection of test material/saline, intraperitoneally (68 hours before killing).
16	Second injection of test material/saline, intraperitoneally (44 hours before killing).
17	Injection of 0.1μc ^{59}Fe-chloride intravenously (20 hours before killing).
18	Mice anaesthetised, weighed and exsanguinated. Haematocrit measured and radioactivity in blood sample determined.

Mice were randomised into groups of five or six and further randomised for the dose to be given. Saline, standard or test material was given in two divided doses on days 15 and 16, since a greater erythropoietic response is obtained with divided doses in comparison with that obtained when the total dose is given

as a single injection (Reichlin and Harrington, 1960; Schooley, 1965; Alexanian, 1966; Fogh, 1966). Each injection was made up to 0.5 ml. with saline and a control group, receiving two doses of 0.5 ml. physiological saline, was included in each assay. Thus each assay animal received a total volume of 1 ml. by intraperitoneal injection.

The radio-iron incorporation into mouse red cells* was measured 20 hours after the intravenous injection of 0.1 μc $^{59}FeCl_3$. Haematocrits were performed in duplicate using a microhaematocrit method and results from mice with haematocrits of less than 50% were discarded. The radioactivity of the blood samples was determined using a well type scintillation counter, along with three standards of the ^{59}Fe injected and three tubes containing 5 ml. water for background counts.

The percentage uptake of ^{59}Fe was calculated using the following formula :-

$$^{59}Fe \text{ uptake } \% = \frac{\text{Counts per sec./ml. sample volume x blood volume x 100}}{\text{Total counts per second injected.}}$$

At the beginning of the studies the blood volume, as a percentage of body weight, was measured for a batch of polycythaemic mice prepared as already described (See page 67). The method used (^{51}Cr-labelled red cells) was based on that described by Lockner, et al. (1963)**. The blood volume of polycythaemic mice was found to be approximately 7.5% of body weight (mean value for 37 mice was

* See Appendix B(i) for further details.

** See Appendix B(ii) for further details.

7.46 ± 0.12 standard error). This is in good agreement with the results of other workers (Cotes and Bangham, 1961; Weintraub, et al., 1963).

3. Collection, Concentration and Storage of Experimental Samples from Human Subjects for Assay.

A. Urine.

Urine was collected in plastic bottles for the period stated in each case, each specimen being placed in a refrigerator (+4°C) immediately after micturition. On completion of the collection the urine bottle was then transferred to a deep freeze (-20°C) where it was stored until required for assay. For use in an assay the urine was thawed at room temperature and the appropriate dose dilutions made in 0.9% saline. The second half of the dose was deep frozen overnight and thawed for use on the following day.

Concentration of urine by freeze-drying.

Measured volumes of urine were placed in 500 ml. round-bottomed flasks and shell-frozen by immersion in an acetone/solid carbon dioxide mixture. The contents of the flasks were concentrated on an Edwards High Vacuum freeze-drier for approximately 4 hours. The final volume of the concentrated urine was measured (after thawing at +4°C) and the urine stored in polythene bottles in a deep freeze (-20°C) until required for assay.

B. Plasma.

Blood was collected by venepuncture of a forearm vein into a polythene disposable syringe and the blood transferred to a heparinised universal container. The blood was centrifuged at 3,000 r.p.m. for 20 minutes in a refrigerated centrifuge

(+2 to +4°C) and the plasma was separated into a clean container. It was stored in a deep freeze (-20°C) until required. On the first day of the assay the plasma was thawed at room temperature and the appropriate dilutions made in 0.9% saline. The second half of the dose was deep frozen overnight for use the following day.

C. Renal Cyst Fluid.

Renal cyst fluid, obtained at operation or by aspiration under local anaesthesia, was collected in a universal container and stored in a deep-freeze (-20°C) until required for assay. It was thawed, diluted and stored overnight as for urine and plasma.

4. Preparation of Laboratory Standards of Erythropoietin.

Laboratory Standard I. (L.L.S.I).

Refrigerated urine collections were made from two hospital in-patients (Patients 1 and 2), with aplastic anaemia and haemoglobin concentrations of 4.4 g and 6.1 g. per 100 ml. respectively.

Eleven litres of urine were collected and stored as described above. In preparing the standards the urine was thawed at +4°C, filtered (Whatman No. 1 filter paper) and dialysed against distilled water in a cold room at +4°C. Regenerated cellulose dialysis tubing of 2" inflated diameter was used. Dialysis was continued for three days, with a change of distilled water daily.

DEAE cellulose chromatography was carried out as described by Lewis, et al. (1965a). Passage of urine through the chromatography column took five days. The first eluate was kept in the deep freeze and used only in the event of finding no activity in

the second eluate. The latter (600 ml.) was pipetted automatically into 2 ml. glass ampoules, the ampoules flame-sealed and stored at -20°C.

Laboratory Standard II. (L.S.II).

Urine collections were made from two hospital in-patients (Patients 3 and 4) with aplastic and hypoplastic anaemia (haemoglobin values 6.2 g. and 4.8 g. per 100 ml. respectively). These urines were first assayed individually, without special preparation, and shown to give a high percentage uptake of ^{59}Fe compared with the response to saline in a control group, as shown in Table 6.

TABLE 6.

ASSAY OF URINE OF PATIENTS 3 AND 4 FOR L.S.II.

Dose	R.B.C. ^{59}Fe Uptake % Mean ± Standard Error of Mean	Number of Mice
1 ml. urine Patient 3	28.76 ± 2.57	6
1 ml. saline	7.20 ± 0.69	5
1 ml. urine Patient 4	33.55 ± 1.19	5
1 ml. saline	4.38 ± 0.64	5

The thawed urines were pooled, filtered and concentrated in a freeze-drying apparatus (as described above) to approximately one-third of their initial volume (1 ml. urine concentrate ≡ 3.5 ml. of original urine). The urine concentrate was pipetted automatically into 5 ml. glass ampoules which were flame-sealed and stored at -20°C.

STATISTICAL METHODS.

Standard Deviation (S.D.) and Standard Error (S.E.)

The standard deviation and standard error of groups of observations were determined from the following formulae:-

n represents the number of observations in a sample; x the individual observations within a sample, and $\bar{x}$ the mean for n observations.

$$\text{S.D.} = \sqrt{\text{variance}}$$

$$\text{Variance} = \frac{\Sigma(x - \bar{x})^2}{n - 1}$$

$$\text{S.E.} = \frac{\text{S.D.}}{\sqrt{n}}$$

Correlation between two Variables.

Correlation between two variables (x and y) was assessed by calculating the correlation coefficient (r) and determining the probability level (p) of correlation from the appropriate table in Fisher and Yates (1948), with (n - 2) degrees of freedom.

The correlation coefficient was calculated as follows :-

$$r = \frac{\Sigma(x - \bar{x})(y - \bar{y})}{(\text{S.D.}x)(\text{S.D.}y)}$$

The Slope of the Regression Line Relating Two Variables (b).

The slope (b) of the regression line relating two variables (x and y) was calculated as follows :-

$$b = \frac{\Sigma xy - \frac{(\Sigma x)(\Sigma y)}{n}}{\Sigma x^2 - \frac{(\Sigma x)^2}{n}}$$

Significant Difference between Two Means.

Significant difference between means was tested according to Snedecor (1950). Firstly the variance ratio (F) was calculated :-

$$F = \frac{\text{greater estimate of variance of population}}{\text{lesser estimate of variance of population}}$$

If F with (n - 1) degrees of freedom was less than the corresponding value in the 5% table, the variances were not regarded as significantly different and Student's t-test was applied :-

$$t = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{\frac{\Sigma(x_1 - \bar{x}_1)^2 + \Sigma(x_2 - \bar{x}_2)^2}{(n_1 + n_2 - 2)} \times \frac{n_1 + n_2}{n_1 \cdot n_2}}}$$

The probability level (p) was determined from the (t) value using the appropriate table (Fisher and Yates, 1948), with $(n_1 + n_2 - 2)$ degrees of freedom.

If F with (n - 1) degrees of freedom was greater than the corresponding value in the 5% table, the variances was regarded as significantly different and Cochran and Cox's modification of the t-test was applied as follows :-

The variance of each mean was calculated separately :-

$$\text{Variance of mean} = \frac{\Sigma(x - \bar{x})^2}{n - 1} / n$$

The variance of the mean difference was the sum of these two variances, and the standard error of the difference =

$$\sqrt{\text{sum of variances for each mean.}}$$

The ratio $\dfrac{\bar{x}_1 - \bar{x}_2}{\sqrt{\text{sum of variance for each mean}}}$ = t, was calculated.

An approximation to the 5% probability level of this ratio was computed as the weighted means of two values of t. These values were taken from the t-table and were the values corresponding to t (5%) for $(n_1 - 1)$ and $(n_2 - 1)$ degree of freedom. The weights were the two variances $\left(\dfrac{\Sigma(x - \bar{x})^2}{n - 1} / n \text{ for each mean}\right)$.

$$5\% \text{ level} = \frac{t_1 \times \text{variance}_1 + t_2 \times \text{variance}_2}{\text{variance}_1 + \text{variance}_2}$$

If t was greater than the Cochran 5% level, the difference between means was regarded as significantly different.

Test for a Significant Difference between the Slopes of Two Lines, $b_{(1)}$ and $b_{(2)}$.

The following calculations were made for each line :-

(1) Total sum of squares $= \Sigma y^2 - \frac{(\Sigma y)^2}{n}$

(2) Sum of squares due to regression $= \frac{\left[\Sigma xy - \frac{\Sigma x \Sigma y}{n}\right]^2}{\Sigma x^2 - \frac{(\Sigma x)^2}{n}}$

(3) Residual sum of squares $=$ (1) - (2).

The (residual) variance ratio F was calculated :-

$$F = \frac{\text{greater estimate of residual variance}}{\text{lesser estimate of residual variance.}}$$

If F with (n - 2) degrees of freedom was less than the corresponding value in the 2.5% table, the variances were not regarded as significantly different and Student's t-test was applied :-

From the residual sum of squares of both lines the pooled variance was calculated as follows :-

$$\text{Pooled variance} = \frac{\text{Residual sum of squares(1)} + \text{Residual sum of squares(2)}}{(n_{(1)} - 2) + (n_{(2)} - 2)}$$

$$\text{Slope of each line} = \frac{\Sigma xy - \frac{\Sigma x \Sigma y}{n}}{\Sigma x^2 - \frac{(\Sigma x)^2}{n}}$$

S.E. of difference between the slopes =

$$\sqrt{\frac{\text{Pooled variance}}{\Sigma x^2 - \frac{(\Sigma x)^2}{n}_{(1)}} + \frac{\text{Pooled variance}}{\Sigma x^2 - \frac{(\Sigma x)^2}{n}_{(2)}}}$$

$$t = \frac{b_{(1)} - b_{(2)}}{\text{S.E. of difference between the slopes}}$$

The probability level (p) was then determined from the t value using the appropriate table from Fisher and Yates (1948), with $(n_{(1)} - 2 + n_{(2)} - 2)$ degrees of freedom.

Method of Determining Whether one Material Behaved as a Dilution of Another.

In order to determine whether one material behaved as a dilution of another, the procedure according to Finney (1952) was adopted. Log_{10}-dose was plotted against assay response for each material (e.g. erythropoietin standard preparation). The linear portions of the two curves were compared to determine whether there was a significant difference between the slopes of the lines (as above). In the case of four point assays of experimental samples against a standard, the slopes of the regression lines for standard and unknown were compared (as above).

Determination of the Relative Potency of Two Materials.

Once it was established that there was no significant difference between the slopes of the lines, two parallel lines were fitted to the two sets of data (Quenouille, 1950). The two parallel lines were given by the following formulae :-

$$y_{(1)} = \bar{y}_{(1)} + b\,(x_{(1)} - \bar{x}_{(1)})$$

$$y_{(2)} = \bar{y}_{(2)} + b\,(x_{(2)} - \bar{x}_{(2)})$$

where b was the joint slope.

An analysis of variance was completed determining joint sum of squares due to regression, due to deviations from parallelism, due to differences between preparations, due to differences between doses, joint total sum of squares and joint residual sum of squares. The mean square and F (variance ratio) value was determined for each component. The probability level (p) of F with the appropriate number of degrees of freedom was determined from Fisher and Yates (1948) F tables, and the p values examined to confirm the validity of the assay.

The relative potency, R, is a function of the horizontal distance between the two parallel lines (calculated as above). The distance (M) between the two lines on a log scale was given by :-

$$M = (\bar{x}_{(1)} - \bar{x}_{(2)}) - \frac{(\bar{y}_{(1)} - \bar{y}_{(2)})}{b}$$

and Antilog M = R (Relative Potency).

Determination of 95% Fiducial Limits of R.

The accuracy of R was determined by calculating the 95% fiducial limits to M using the general formula below and adding $(\bar{x}_{(1)} - \bar{x}_{(2)})$ to the results :-

$$M \quad \frac{ts}{b} \left\{ (1 - g)\left(\frac{1}{n_1} + \frac{1}{n_2}\right) + \frac{(M - \bar{x}_1 + \bar{x}_2)^2}{\sum x_1^2 - \frac{(\sum x_1)^2}{n_1} + \sum x_2^2 - \frac{(\sum x_2)^2}{n_2}} \right\}^{\frac{1}{2}}$$

$$(1 - g)$$

where s^2 was joint residual mean square

b was joint slope

t was value for 5% probability and joint residual degrees of freedom from tables.

and $$g = \frac{t^2 \quad s^2}{b^2 \left(\sum x_1^2 - \frac{(\sum x_1)^2}{n_1} + \sum x_2^2 - \frac{(\sum x_2)^2}{n_2}\right)}$$

The 95% Fiducial limits for R were given by the antilogs of the limits of M.

Index of Precision (λ).

The index of precision for the $\log_{10}$-dose response relationships was calculated according to Gaddum (1953) :-

$$\lambda = \frac{\text{residual variance}}{\text{slope of regression line.}}$$

RESULTS AND DISCUSSION.

1. Degree of Polycythaemia Induced by Exposure to Lowered Atmospheric Pressure and Effectiveness of the Polycythaemia in Depressing Erythrocyte Uptake of Radio-Iron.

The polycythaemia induced by exposure of mice to an atmospheric pressure of 380 mm.Hg. for a 14-day period was effective in depressing the rate of erythropoiesis, as shown in Table 7.

TABLE 7.

HAEMATOCRIT AND PERCENTAGE ERYTHROCYTE UPTAKE OF RADIO-IRON IN MICE EXPOSED TO AN ATMOSPHERIC PRESSURE OF 380 mm.Hg. FOR 14 DAYS AND IN MICE LIVING AT NORMAL ATMOSPHERIC PRESSURE

	Haematocrit % Mean ± Standard Error of Mean	R.B.C. ^{59}Fe Uptake % Mean ± Standard Error of Mean
Mice exposed to atmospheric pressure of 380 mm.Hg for 14 days (100 animals)	54.7 ± 0.5	9.48 ± 0.58
Mice living at normal atmospheric pressure (34 animals)	49.9 ± 0.5	54.20 ± 3.02

The range of haematocrit in the experimental mice was large, from 36 - 69%. The difference in haematocrit between the groups was significant and only a few mice per assay had to be rejected on the grounds of an haematocrit below 50%. There is a marked difference in percentage red cell ^{59}Fe uptake between the experimental animals and control animals kept at normal atmospheric pressure. Although the ^{59}Fe uptake figures were not as low and the haematocrits not as

high in these hypoxic mice as those recorded in some laboratories using transfusion-induced polycythaemic mice (Filmanowicz and Gurney, 1961; Degowin, et al., 1962a), they are in good agreement with the results of others who use the hypoxia-induced polycythaemic mouse as the assay animal (Cotes and Bangham, 1961; Weintraub, et al., 1963), and were regarded as satisfactory evidence that erythropoiesis was sufficiently reduced for erythropoietin assay by this method.

To assess the effects of hypoxia on body weight and feeding activity, each animal in a batch of 60 mice was numbered and weighed when put into the pressure chamber and further recordings of weight were made at intervals during the two week exposure period. It was observed that the mice became lethargic when the pressure decreased towards 380 mm.Hg and that they ate very little for the first three days of exposure. During this period 55 of the 60 mice (92%) lost weight, the mean weight loss being 13.1 ± 0.6% (standard error of the mean) of their initial weight; of these 55 animals which had lost weight, 37 (67%) then gained weight over the remainder of the two week period of exposure, 9 (16%) remained at the same weight and 9 continued to lose weight. Thus, acclimatisation appeared to commence from about the fourth day of exposure to 380 mm.Hg, and by the beginning of the assay most mice were steadily increasing in weight.

2. Erythropoietic Activity of Urine Collected from Anaemic Patients for the Preparation of Laboratory Standards of Erythropoietin.

In order to collect sufficient urine suitable for the preparation of a laboratory standard of erythropoietin, the urine of patients with anaemia was screened for erythropoietic activity.

TABLE 8

ERYTHROPOIETIC ACTIVITY OF URINE OF ANAEMIC PATIENTS

Patient	Diagnosis	Haemoglobin g./100 ml.	Haematocrit %	Mean R.B.C. ^{59}Fe Uptake % Increase over Saline Controls	Comments
*1	Aplastic anaemia	4.4	15	36.32	Urines combined for assay.
*2	Aplastic anaemia	6.1	19		
**3	Aplastic anaemia	6.2	-	21.56	
**4	Hypoplastic anaemia	4.8	16	29.17	Later diagnosed as acute leukaemia.
5	Aplastic anaemia	4.7	14	5.45	
6	Post-haemorrhagic anaemia	4.6	16	-2.50	Urines combined for assay
7	Chronic iron-deficiency anaemia	4.8	20		
8	Aplastic anaemia	7.0	21		
9	Acute myeloid leukaemia	5.7	20	-3.29	
10	Chronic iron-deficiency anaemia	6.6	26	5.31	
11	Pernicious anaemia	9.1	31	-4.08	
12	Hypoplastic anaemia	10.4	-	-0.92	

* Urine of Patients 1 and 2 used for preparation of Erythropoietin Laboratory Standard I.

** Urine of patients 3 and 4 used for the preparation of Erythropoietin Laboratory Standard II.

Collections were made from patients with aplastic or hypoplastic anaemia and other forms of anaemia unassociated with renal disease. The percentage red cell ^{59}Fe uptake induced by a dose of 1 ml. of urine was compared with that induced in control mice given 1 ml. saline and the results, expressed as the difference between the two groups, are given in Table 8. Taking all the values into consideration there was no significant correlation between the mean percentage red cell ^{59}Fe uptake and the patients' haemoglobin concentration (correlation coefficient = -0.54). Several laboratories have demonstrated a direct relationship between urinary erythropoietin concentration and haemoglobin concentration in patients with aplastic, hypoplastic and chronic haemolytic anaemia (Hammond, et al., 1962) and iron deficiency anaemia (Movassaghi, et al., 1967; Hammond, et al., 1968). However, in this study there was an insufficient number of patients for analysis of the results in terms of different types of anaemia. Those urines which gave a high mean percentage ^{59}Fe uptake over controls were incorporated into laboratory standards E.L.S.I and E.L.S.II as previously described (p.71) and their dose-response curves were plotted and compared with that of the International Reference Preparation of Erythropoietin.

3. Log-Dose Response Curves of Erythropoietin Laboratory Standards E.L.S.I and E.L.S.II.

The percentage erythrocyte ^{59}Fe incorporation in response to several doses of Erythropoietin Standards I and II is plotted in Figures I and II. The points and vertical bars show the mean ($\pm$ standard error of the mean) ^{59}Fe incorporation at each dose, with the number of mice injected with each dose in parentheses.

FIGURE I.

LOG-DOSE RESPONSE CURVE OF ERYTHROPOIETIN

LABORATORY STANDARD I.

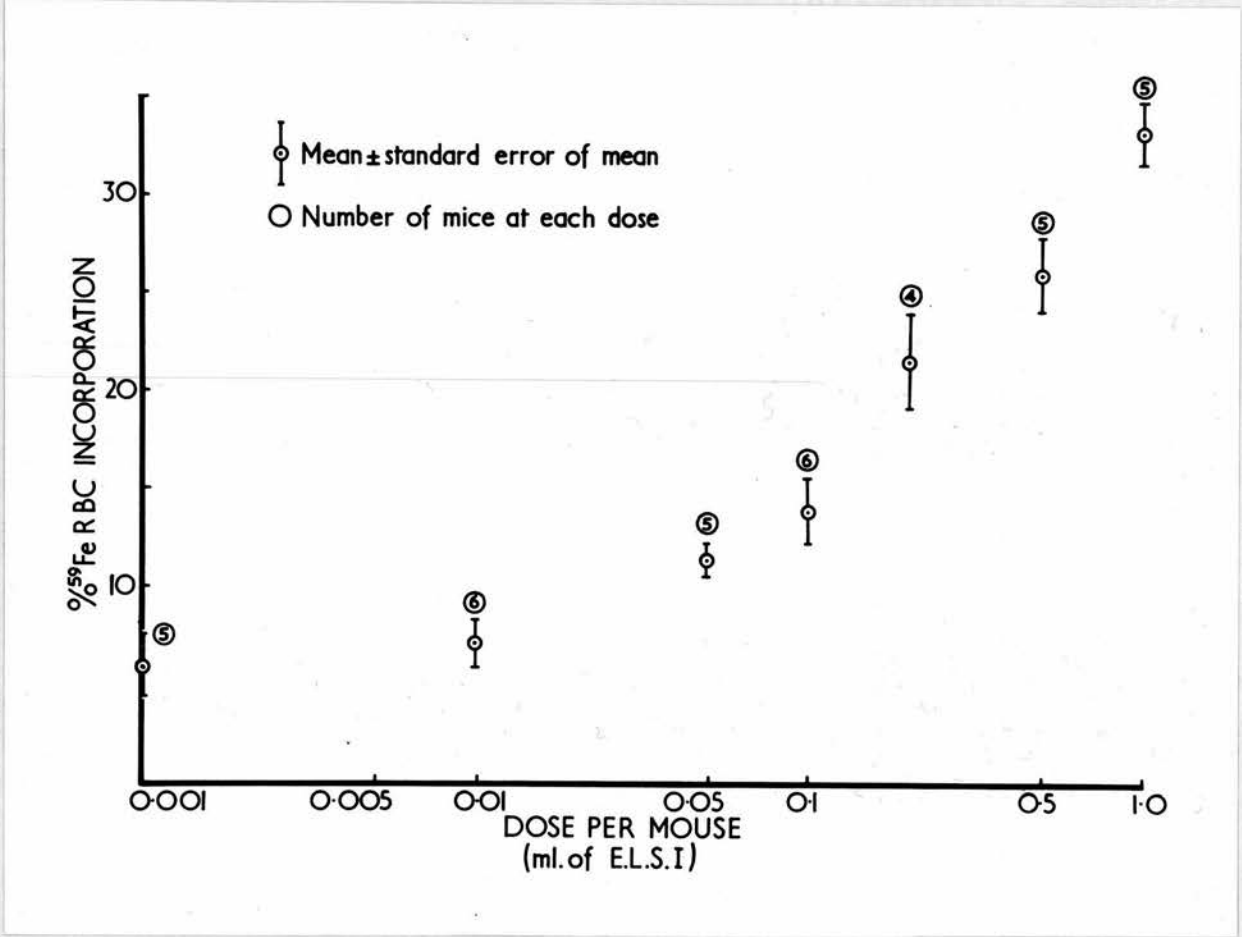
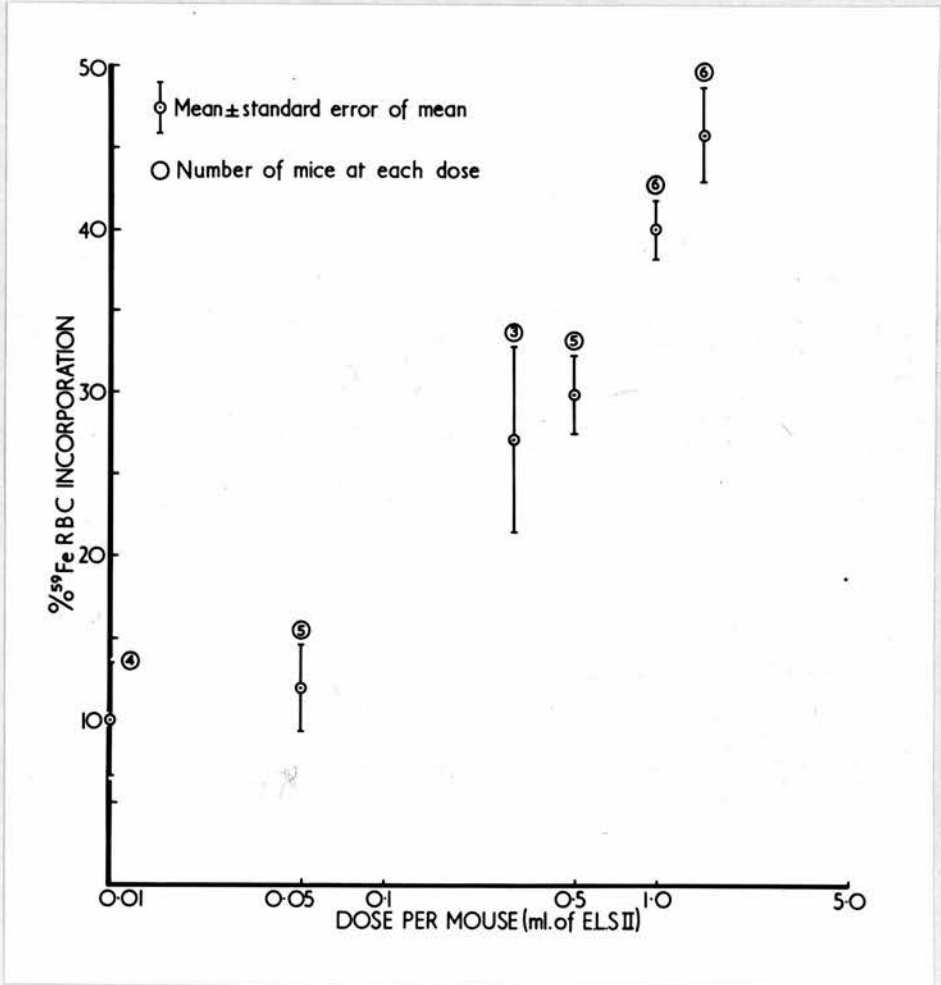


FIGURE II.

LOG-DOSE RESPONSE CURVE OF ERYTHROPOIETIN

LABORATORY STANDARD II.



In Figure I the characteristic shape of a hormone log-dose response curve may be seen over a dose range of E.L.S.I of 1000:1. Doses greater than 1.00-1.50 ml. were regarded as unnecessary and wasteful, so that the position of the plateau of the curve was not determined. The $\log_{10}$-dose response regression line of E.L.S.I did not differ significantly from linearity between doses of 0.05 ml. and 1.00 ml.; its slope (b) was 16.92 and the index of precision (λ) was 0.22. Figure II shows a $\log_{10}$-dose response curve of E.L.S.II over a dose range of more than 100:1. The upper limit of response was not reached at a dose of 1.50 ml. of E.L.S.II and the $\log_{10}$-dose response regression line did not differ significantly from linearity between doses of 0.05 ml. and 1.50 ml.; its slope (b) was 22.35 and the index of precision (λ) was 0.28.

Regression coefficients were calculated and regression lines drawn (Figure III) for E.L.S.I and E.L.S.II over the linear range of their $\log_{10}$-dose response curves. Two regression lines were drawn for each standard, approximately one month elapsing between the two experiments on each standard. It can be seen in Figure III that there is a "shift to the right", i.e. a decrease in activity in each standard with time. The index of precision (λ) and slope (b) of each regression line is marked on the graph.

4. Log-dose Response Curve of the International Reference Preparation of Erythropoietin (Standard B).

Figure IV shows a $\log_{10}$-dose response curve obtained using Standard B* at six dose levels and over a dose range of 1000:1

*Standard B kindly supplied through Dr. D.R. Bangham, Department of Biological Standards, National Institute for Medical Research, London, N.W. 11.

FIGURE III.

LOG-DOSE RESPONSE REGRESSION LINES FOR E.L.S.I AND E.L.S.II.

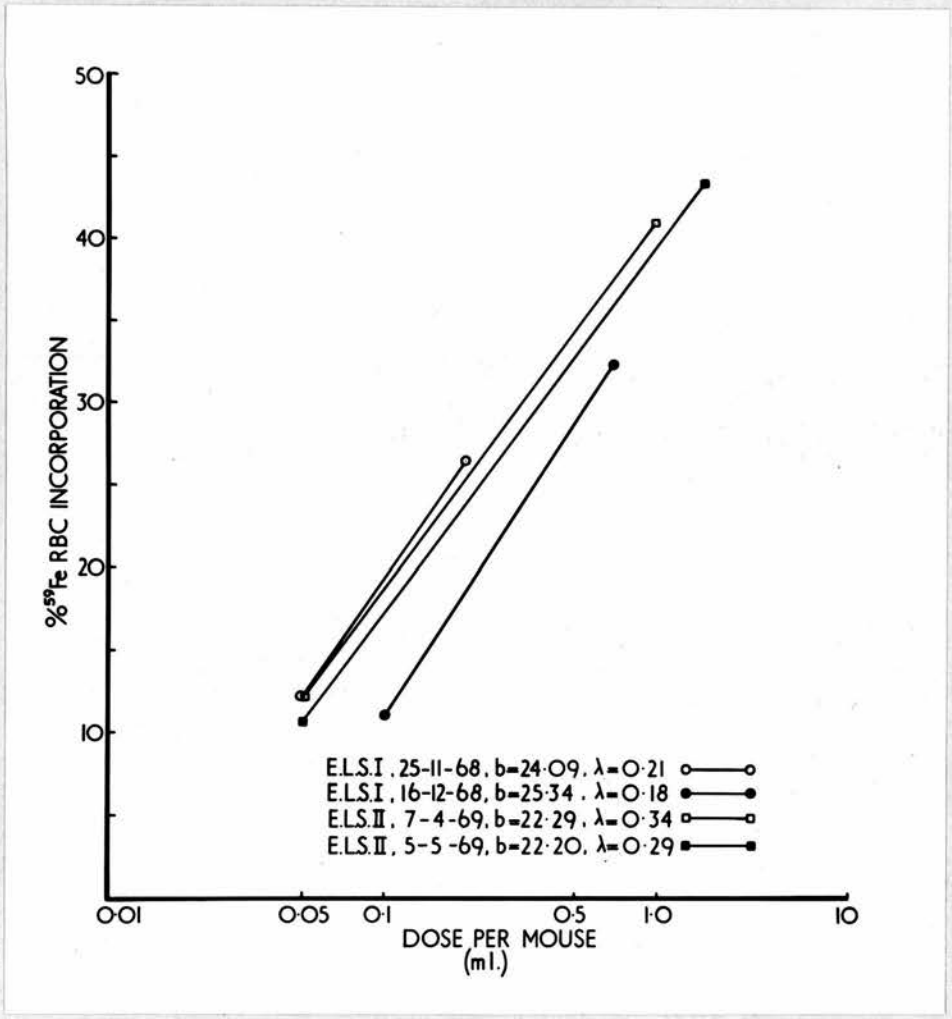
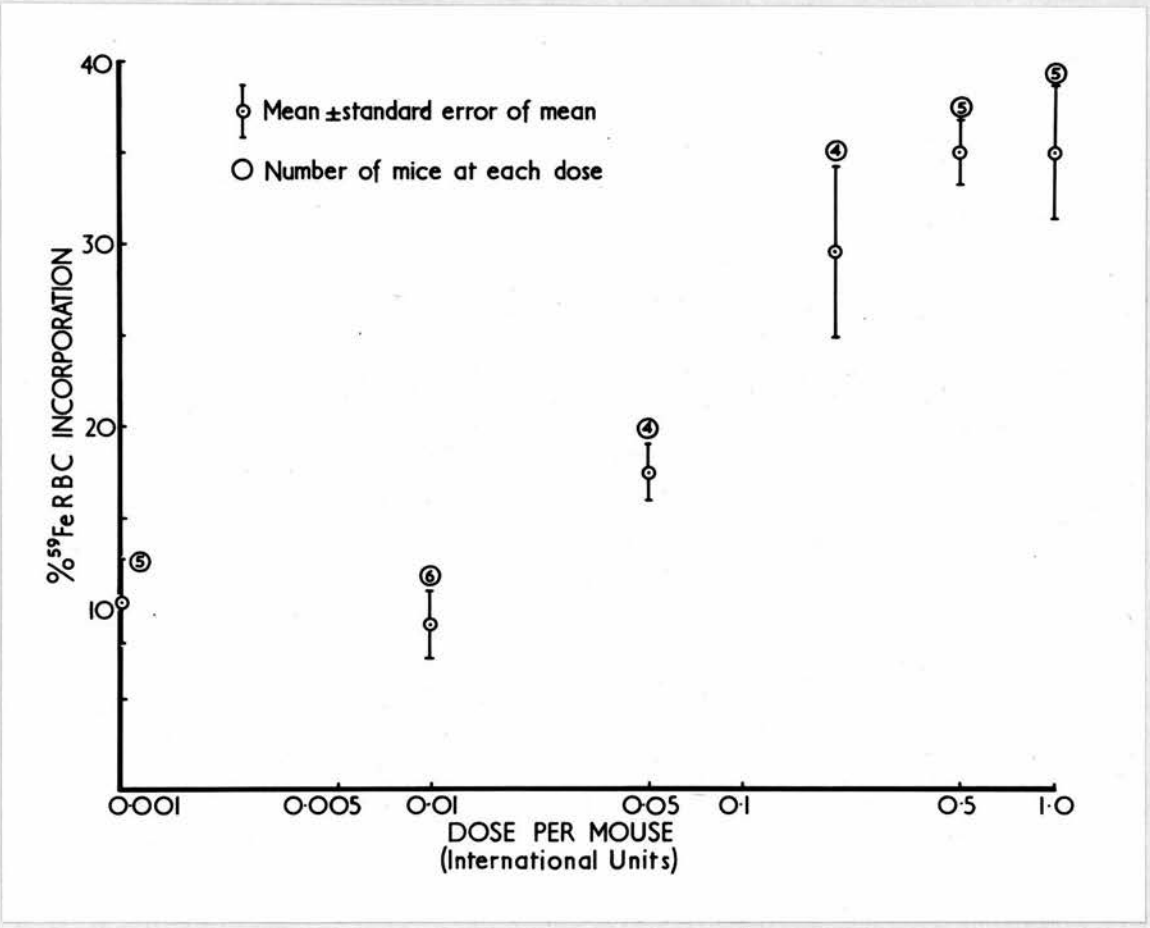


FIGURE IV.

LOG-DOSE RESPONSE CURVE OF INTERNATIONAL REFERENCE PREPARATION OF ERYTHROPOIETIN (STANDARD B).



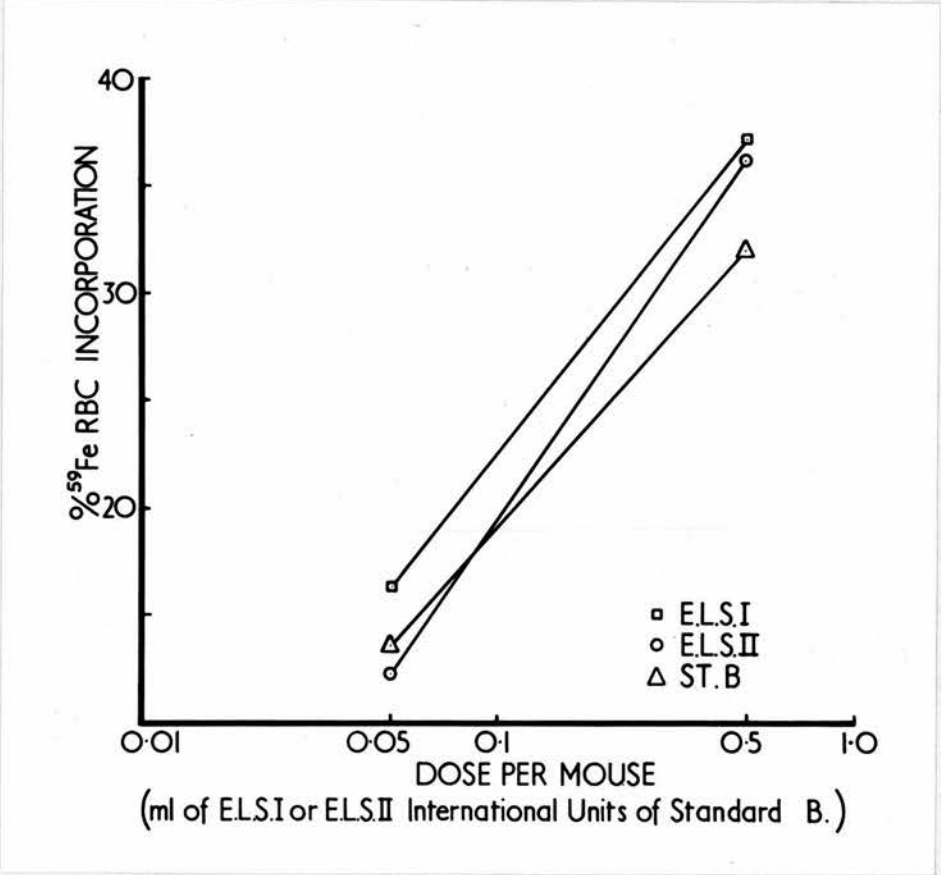
The curve shows a characteristic S-shaped plot. The $\log_{10}$-dose response regression line did not differ significantly from linearity between doses 0.01 and 0.50 International Units of erythropoietin; its slope was 15.51 and the index of precision (λ) was 0.35. These figures compare well with those obtained by other laboratories for the assay of the International Standard; Camiscoli, et al. (1968) obtained values of b = 29.8; λ = 0.27, and Dukes, et al. (1969), assaying the International Standard on three separate occasions, gave values of b from 10.5 to 20.4 and 0.03 to 0.45 for λ.

5. Calibration of E.L.S.I and E.L.S.II Against Standard B.

Since preliminary log-dose response curves of E.L.S.I, E.L.S.II and Standard B had established that doses of 0.05 and 0.50 ml. of E.L.S.I and E.L.S.II and 0.05 and 0.50 International Units of Standard B lay on the linear part of their $\log_{10}$-dose response lines, a bilateral four point assay was undertaken using these doses for each standard. The $\log_{10}$-dose response lines are shown in Figure V. Comparison of the slopes of the $\log_{10}$-dose response regression lines of E.L.S.I and E.L.S.II with that of Standard B showed that the slope of each laboratory standard was not significantly different from the slope of the International Standard. An analysis of variance was completed and the components of the analysis for both assays were examined in detail (See Statistical Methods, p.73, and Appendix D for a worked example). The data was very satisfactory for both assays, the "linear regression" and "between doses" components being highly significant ($p<0.001$) and "deviations from parallelism" and "between preparations" components being not significant for both assays. The estimate of potency of each

FIGURE V.

CALIBRATION OF E.L.S.I AND E.L.S.II AGAINST INTERNATIONAL REFERENCE PREPARATION OF ERYTHROPOIETIN (STANDARD B).



laboratory standard relative to Standard B, and the 95% fiducial limits of this estimate are shown in Table 9.

TABLE 9. RESULTS OF ASSAY TO ESTIMATE POTENCY OF E.L.S.I AND E.L.S.II

Preparation assayed	Slope of $\log_{10}$-dose response regression line	Relative potency L.S. : St.B.	Fiducial limits p = 0.95	λ
E.L.S.I	20.77	1.64 : 1	0.82 - 3.44	0.29
Standard B	18.41			0.18
E.L.S.II	23.72	1.25 : 1	0.71 - 2.21	0.17

It will be seen from the Table that the potency of E.L.S.I relative to Standard B was 1.64:1, and that of E.L.S.II 1.25:1. Ascribing an arbitrary unitage of 1 ml. of laboratory standard ≡ 1 laboratory standard unit,

1 unit E.L.S.I ≡ 1.64 International Units of erythropoietin,

1 unit E.L.S.II ≡ 1.25 International Units of erythropoietin.

Thus, in assays of patients' samples against one of the laboratory standards, multiplication by the appropriate factor above converted the potency of the unknown to International Units.

The indices of precision for the assay of Standard B and E.L.S.II placed these assays in the category defined by Loraine and Bell (1966) as "very precise and well suited to clinical studies", while the assay of E.L.S.I was "less precise but can still be used with reasonable confidence".

It was concluded that the strain of mouse used,

the preparation of the mice using the low-pressure chamber, the assay design selected and the laboratory standards of erythropoietin prepared were all satisfactory and provided a fairly sensitive and precise assay for erythropoietin in clinical specimens.

SECTION III.

APPLICATION OF THE ASSAY TO CLINICAL AND EXPERIMENTAL HUMAN STUDIES.

SECTION III

APPLICATION OF THE ASSAY TO CLINICAL AND EXPERIMENTAL HUMAN STUDIES.

A. ERYTHROPOIETIN IN PATIENTS WITH RENAL DISEASE.

The concentration of erythropoietin was assayed in the body fluids of six patients with various renal disorders, three of whom had polycythaemia.

Patients Studied.

A summary of patients studied with their haematological findings and body fluids assayed is given in Table 10. Further details of these cases are presented in Appendix E.

Results.

(a) Erythropoietin in Patients with Renal Cysts.

The assay of renal cyst fluid from Patient 13 against E.L.S.I was the only positive assay in this group of patients (see Table 11). The mean response at each dose of renal cyst fluid and E.L.S.I is plotted in Figure VI. Comparison of the slopes of their $\log_{10}$-dose response regression lines showed that there was no significant deviation from parallelism ($t = 0.24$, $p>0.80$) and the analysis of variance indicated a valid and satisfactory assay.

The potency of the renal cyst fluid was 0.68 of that of E.L.S.I, equivalent to a potency of 1.11 I.U. erythropoietin per ml.

Plasma from this patient was assayed against E.L.S.I but comparison of the slopes of the $\log_{10}$-dose response regression lines for plasma and standard showed that there was a significant

TABLE 10. ERYTHROPOIETIN ASSAYS IN RENAL DISEASE - PATIENTS STUDIED.

Patient	Age	Sex	Diagnosis	Plasma urea (mg/100 ml)	Hb. (g/100 ml)	P.C.V. (%)	Haematological data		Red Cell Mass (ml/Kg.body wt)	Fluid sample assayed for erythropoietin
							W.B.C. (per cu.mm)	Platelets (per cu.mm)		
13	66	F	Solitary renal cyst	44	15.0	42	9,000	-	-	Renal cyst fluid Peripheral venous plasma
14	52	F	Solitary renal cyst	36	13.8	40	5,100	145,000	-	Renal cyst fluid
15	71	M	Polycystic renal disease and polycythaemia rubra vera	40	22.6	68	20,400	145,000	67.0	Renal cyst fluid Urine
16	46	M	Chronic unilateral pyelonephritis and erythrocytosis	32	16.6	53	11,800	270,000	42.3	Renal venous plasma* Peripheral venous plasma
17	41	M	Hypernephroma and erythrocytosis	28	20.6	66	7,100	160,000	-	Peripheral venous plasma Urine
18	25	F	Renal homotransplant and suspected erythrocytosis	45	16.0	49	8,300	120,000	29.2	Urine

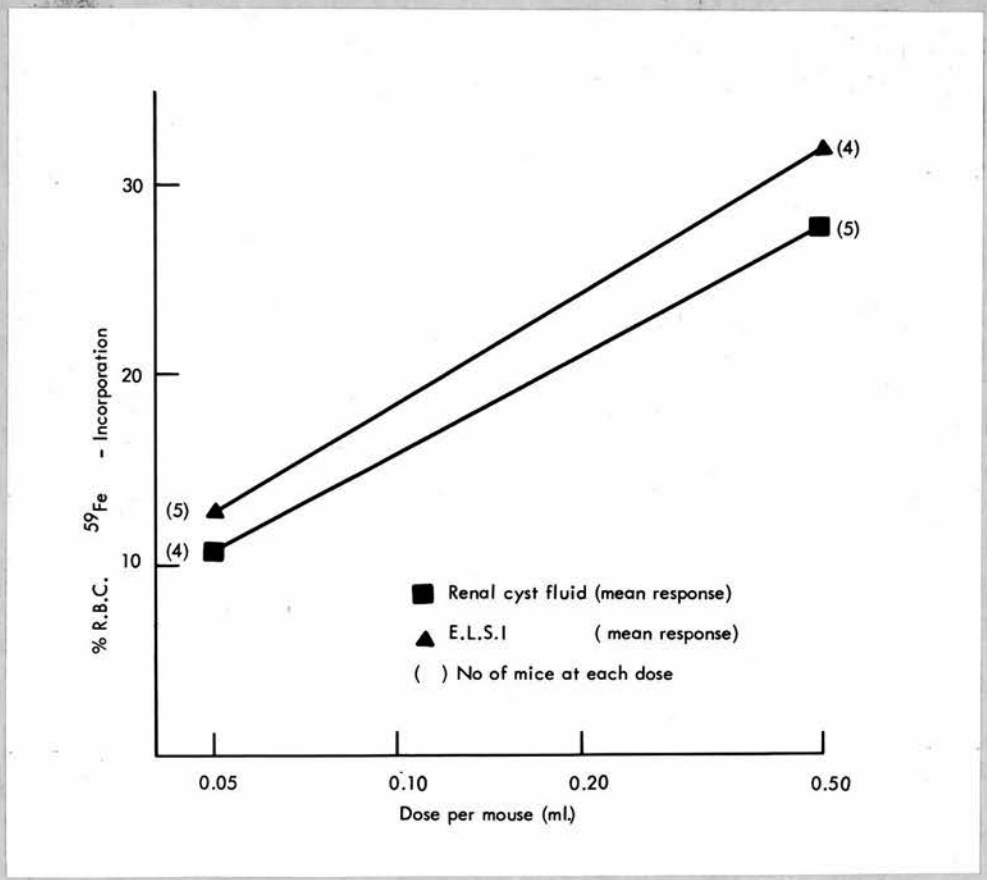
*Confirmation of source of blood from renal vein was obtained by estimation of oxygen saturation and renal extraction ratio of sodium para-aminohippurate (see Appendix F).

TABLE 11. RESULTS OF ASSAY OF RENAL CYST FLUID, PLASMA AND URINE OF THREE PATIENTS WITH RENAL CYSTS

Patient	Material Assayed	Doses used (ml.)	Slope of $\log_{10}$-dose response regression line	Relative potency Unknown: Standard	Fiducial limits p = 0.95	Validity and Comments
13	Renal cyst fluid	0.05 : 0.50	16.71	0.68 : 1	0.18 - 3.39	Valid
	E.L.S.I	0.05 : 0.50	19.04			
	Plasma	0.10 : 0.50	8.34	-	-	Invalid - deviations from parallelism significant
	E.L.S.II	0.10 : 0.50	30.36			
14	Renal cyst fluid	0.10 : 0.50	25.20	-	-	Invalid - deviations from parallelism significant
	E.L.S.I	0.05 : 0.50	11.31			
15	Renal cyst fluid	0.05 : 0.10	-	-	-	Response not significantly different from that of saline control group
	Urine	0.50 : 1.00	-	-	-	

FIGURE VI.

LOG-DOSE RESPONSE LINES FOR THE ASSAY OF RENAL CYST FLUID FROM PATIENT 13.



difference between them ($t = 2.33$, $0.05>p>0.025$) and the assay therefore had to be rejected on the grounds of invalidity due to non-parallelism.

The assay of renal cyst fluid from Patient 14 against E.L.S.I was also invalid due to non-parallelism of the regression lines of unknown and standard ($t = 2.37$, $0.05>p>0.025$). The slope of the $\log_{10}$-dose response regression line of E.L.S.I in this particular assay was much lower than its usual value, which was often in the range of 18.00 to 20.00.

The responses to both renal cyst fluid and urine from Patient 15 were not significantly different from the responses to saline in the control group of mice; renal cyst fluid, 0.10 ml. dose - $t = 0.33$, $p>0.70$: urine, 1.00 ml. dose - $t = 1.27$, $p>0.20$. That is, erythropoietin was not detectable in the renal cyst fluid or urine of this patient.

(b) Erythropoietic Activity in a Patient with Chronic Unilateral Pyelonephritis and Erythrocytosis (Patient 16).

The results of the assay of peripheral venous plasma and renal venous plasma from this patient are given in Table 12. Comparison of the slopes of the $\log_{10}$-dose response regression lines of E.L.S.I and peripheral venous plasma showed that deviations from parallelism were not significant ($t = 0.84$, $0.50>p>0.40$) and similarly for E.L.S.I and renal venous plasma ($t = 0.97$, $0.40>p>0.30$).

The potency of peripheral venous plasma was calculated to be 0.12 E.L.S.I units = 0.19 I.U. erythropoietin per ml.

Study of the analysis of variance on the data of these assays indicated that although the assays were valid on three

TABLE 12. RESULTS OF ASSAY OF PERIPHERAL VENOUS PLASMA AND RENAL VENOUS PLASMA IN PATIENT 16. (CHRONIC UNILATERAL PYELONEPHRITIS AND ERYTHROCYTOSIS).

Material Assayed	Doses used (ml.)	Slope of $\log_{10}$-dose response regression line	Relative potency Unknown: Standard	Fiducial limits p = 0.95	Validity and Comments
Peripheral venous plasma	0.25 : 0.50	4.37	0.12	0.01 - 0.31	Unsatisfactory assay - see text
E.L.S.I	0.10 : 0.50	19.69			
Renal venous plasma	0.25 : 0.50	38.04	0.22	0.04 - 0.48	Unsatisfactory assay - see text

counts, on a fourth they were both unsatisfactory. The analysis of variance for each assay is given in Table 13 and Table 14. In both assays the variance ratios for the regression and between doses components were highly significant and that of the component based on deviations from parallelism not significant, thus indicating the probable validity of the assay in each case. However, the variance ratio for the between preparations component was highly significant for each assay ($p<0.01$) and on this count it was necessary to regard the assays as unsatisfactory (Finney, 1952). The reason for the highly significant variance ratio for the between preparations component is not known, but was possibly due to the imbalance between the dose ratio of standard and unknown in each assay.

(c) Erythropoietic Activity in a Patient with Hypernephroma and Erythrocytosis (Patient 17).

Urine and plasma from this patient were assayed against E.L.S.II. The responses to the highest dose of urine (1.00 ml.) were not significantly different from the responses to 1.00 ml. of saline in the control group of mice ($t = 1.18$, $p>0.20$). A volume of 100 ml. of urine was therefore concentrated in a freeze drying apparatus as previously described (Section II, p.70), such that 1.00 ml. concentrate = 2.38 ml. of original urine. The urine concentrate was then assayed at doses of 0.50 ml. and 1.00 ml., but the response was again not significantly different from the response in the control group. It was concluded that the urine of this patient contained no detectable erythropoietin.

Plasma from this patient was assayed at doses of 0.20 ml. and 0.50 ml. against E.L.S.II. None of the responses in

TABLE 13. ANALYSIS OF VARIANCE FOR ASSAY OF PERIPHERAL VENOUS PLASMA. PATIENT 16.

Nature of variation	Degrees of freedom	Sum of squares	Mean square	F	p
Between doses	3	1137.6244	379.2081	11.4916	<0.01
Linear regression	1	452.2458	452.2458	13.7049	<0.01
Parallelism	1	23.4546	23.4546	0.7107	n.s.
Between preparations	1	661.9240	661.9240	20.0591	<0.001
Residual (Error)	11	362.9856	32.9986		
Total	14	1500.6100			

$$F = \frac{\text{Mean square}}{\text{Residual mean square}}$$

n.s. = Not significant.

TABLE 14. ANALYSIS OF VARIANCE FOR ASSAY OF RENAL VENOUS PLASMA

PATIENT 16.

Nature of variation	Degrees of freedom	Sum of squares	Mean square	F	p
Between doses	3	1003.6368	334.5456	9.5504	<0.01
Linear regression	1	597.4211	597.4211	17.0548	<0.01
Parallelism	1	33.6602	33.6602	0.9609	n.s.
Between preparations	1	372.5555	372.5555	10.6355	<0.01
Residual (Error)	11	385.3230	35.0293		
Total	14	1388.9585			

$$F = \frac{\text{Mean square}}{\text{Residual mean square}}$$

n.s. = Not significant.

TABLE 15. ASSAY OF URINE FROM A PATIENT WITH A RENAL HOMOTRANSPLANT AND SUSPECTED ERYTHROCYTOSIS. PATIENT 18.

Material assayed	Dose (ml.)	R.B.C. ^{59}Fe Uptake % Mean ± Standard Error of Mean	Number of mice	t	p
Urine	0.50	12.40 ± 2.50	6		
Urine	1.00	10.76 ± 1.19	5	0.84	>0.40
Saline	1.00	9.11 ± 1.48	6		
Urine Concentrate	0.20	11.98 ± 2.45	6		
Urine Concentrate	0.50	11.75 ± 2.74	5	0.65	>0.50
Saline	1.00	10.07 ± 0.63	6		

the two groups of mice given plasma could be used to calculate its potency since the haematocrit of every mouse in these groups was less than 50%, the level below which results were discarded because of the possibility of haemolysis of mouse red cells by the test material.

(d) Erythropoietic Activity in a Renal Transplant Patient with Suspected Erythrocytosis. (Patient 18).

A six hour urine collection between 9.00 hours and 15.00 hours yielded a volume of 580 ml. (urine flow = 1.61 ml./min.) from this patient. The urine was assayed at doses of 0.50 ml. and 1.00 ml. against a control group given saline, in a pilot assay. The responses to 1.00 ml. of urine were not significantly different from the responses to 1.00 ml. saline (see Table 15). The urine was therefore concentrated in a freeze-drying apparatus such that 1.00 ml. of urine concentrate = 5.95 ml. of original urine. Assay of the urine was repeated against E.L.S.I, but the responses to 0.50 ml. of urine were again not significantly different from the responses in the saline control group (Table 15). It was concluded that the urine of this patient contained no detectable erythropoietin.

Discussion.

Assay of erythropoietin has been attempted in patients with a variety of renal diseases some of which were associated with polycythaemia.

Renal Cysts (Patients 13-15).

Erythropoietin was detected in the renal cyst fluid of Patient 13 at a concentration of 1.11 I.U. per ml., while the

increased percentage ^{59}Fe uptake results obtained by assay of renal cyst fluid from Patient 14 unfortunately could not be used to estimate its potency as the assay was found to be invalid when statistically tested. The haematological findings in both of these patients were normal.

There are at least 11 other reported cases of increased erythropoietic activity in the renal cyst fluid of patients with normal haemoglobin and haematocrit values (Rosse, et al., 1963b; Murphy, et al., 1968b). However, none of these assays incorporated the simultaneous assay of any kind of standard preparation of erythropoietin. The erythropoietic activity of renal cyst fluid has been measured more commonly in patients with renal cysts and an associated polycythaemia and has frequently been found to be high (Nixon, et al., 1960; Rosse, et al., 1963b; Vertel, et al., 1967; Gallagher and Donati, 1968; Murphy, et al., 1968b; Waldmann, et al., 1968), though again erythropoietic activity is not quoted in International Units of erythropoietin in any of these studies.

Patient 15 of this study had polycythaemia which was probably unrelated to the polycystic disease of the kidneys as there was a leucocytosis and hepatosplenomegaly. Renal cyst fluid and urine from this patient contained no detectable erythropoietin, though satisfactory assay of the renal cyst fluid was hampered by the small volume available for assay. There is one reported case in the literature of estimation of erythropoietic activity in a patient with a renal cyst and primary polycythaemia (Rosse, et al., 1963b); in this case there was no remission in the polycythaemia after the cyst had been excised. The cyst fluid contained erythropoietic

activity, while assays of an extract of the cyst wall and serum were negative.

No conclusions could be drawn from the assay of plasma from Patient 13 since deviations from parallelism between plasma and the laboratory standard rendered the assay invalid. Murphy, et al. (1968b) assayed plasma erythropoietic activity in patients with renal cysts who did not have erythrocytosis and obtained positive results in only three out of seventeen patients. Reports in the literature of assays of plasma or serum of patients with renal cysts and an associated erythrocytosis indicate that of 15 assays, 9 showed increased erythropoietic stimulating activity (Jones, et al., 1960; Lange and Gallagher, 1962; Rosse, et al., 1963b; Vertel, et al., 1967; Gallagher and Donati, 1968; Murphy, et al., 1968b).

The mechanism responsible for the presence of erythropoietin in renal cyst fluid is unknown. If the erythropoietic activity in the cyst fluid is selectively concentrated from plasma, one would not expect to observe such remissions of an associated erythrocytosis after removal of the cyst as have been reported by Nixon, et al. (1960), Rosse, et al. (1963b) and Vertel, et al. (1967). Since Nixon, et al. (1960) demonstrated erythropoietic stimulating activity in a saline extract of a renal cyst wall, attention has been focussed on the cyst wall as a possible site of formation of this excess erythropoietin. Later however, two groups of workers obtained negative results on assaying cyst wall extract for erythropoietic activity (Rosse, et al., 1963b; Murphy, et al., 1968b), and Vertel, et al. (1967) reported that the tissue culture media in which they had incubated cyst wall contained no measurable activity.

The structural composition of the wall of single unilocular or multiple cysts militates against the possibility of hormone production, being generally lined by nondescript flattened epithelium and the covering capsule consisting of fibrous tissue. The histology of the simple unilocular cyst of Patient 14 in this study showed a wall of laminated fibrous tissue containing an occasional atrophic tubule and the absence of an epithelial lining. Reports in the literature of the structure of the renal cyst walls of patients with an associated erythrocytosis show that the wall is often composed of fibrous tissue (Jones, et al., 1960; Kulis, 1964) or lined by a single layer of cuboidal cells resembling tubular epithelium (Nixon, et al., 1960).

In whatever way the erythropoietic activity in renal cyst fluid is formed, it is possible that an associated polycythaemia may be caused by the presence of a supply of erythropoietin in renal cyst fluid additional to the normal output by renal tissue; under such circumstances excision of the renal cyst would reduce the abnormal source of erythropoietin and induce remission of the polycythaemia. In those patients without polycythaemia who have erythropoietic activity in their renal cyst fluid, it is possible that the production of erythropoietin by normal kidney tissue decreases and that a balance is achieved in such a way that normal erythropoiesis is unaffected.

Chronic Pyelonephritis and Erythrocytosis (Patient 16).

The PAH extraction ratio in the diseased left kidney of Patient 16 was low (0.63) compared with the average value of 0.91 in healthy kidneys (Bergström, et al., 1959), confirming

renal function impairment. Although the erythropoietin concentration appeared higher in the renal venous plasma sample than in the peripheral venous plasma sample, the assay results in this case must be accepted with reservation for the reasons which have already been given. The figure of 0.19 I.U./ml. for erythropoietin concentration in the peripheral venous plasma is probably higher than that in normal individuals since most physiological concentrations appear to be too low for measurement by present bioassay techniques. The possible role of erythropoietin in the causation of this patient's polycythaemia is unresolved.

Hypernephroma and Erythrocytosis (Patient 17).

The high haemoglobin and haematocrit values present in this patient prior to nephrectomy for hypernéphroma had decreased to normal levels two months after operation. It is therefore highly probable that the polycythaemia was caused by the renal tumour. The reported incidence of polycythaemia in association with hypernephroma is about two to three per cent (Conley, et al., 1957; Hewlett, et al., 1960) and in Patient 17, as in most recorded cases, the erythroid elements alone were affected.

It has been suggested that excessive production of erythropoietin by the kidney tumour is the cause of the erythrocytosis. Erythropoietic activity has been detected in renal tumour extracts by Korst, et al. (1959), Hewlett, et al. (1960), Murphy, et al. (1968b) and Waldmann, et. al. (1968) and in the plasma of such patients by Korst, et al. (1959), Lange and Gallagher (1962), Donati, et al. (1963), Murphy, et al. (1968b) and Waldmann, et al. (1968). Murphy, et al. (1968b) also detected a high concentration

of erythropoietin in the urine of a patient with a carcinoma of the kidney, the concentration falling post-operatively. Erythropoietin was not detectable in the urine of Patient 17 prior to nephrectomy.

Suspected Erythrocytosis in a Renal Transplant Patient. (Patient 18)

Erythropoietin excretion was estimated in this patient to determine whether the high normal value for red cell mass was due to excessive production of erythropoietin. Despite concentration of the urine by a factor of 5.95 times, erythropoietin was not detectable. It was therefore considered improbable that she was producing excessive amounts of erythropoietin. It has been postulated that polycythaemia and increased plasma erythropoietin concentration or urinary excretion are unfavourable features in patients with renal transplants, being thought to indicate renal hypoxia due to chronic rejection of the transplant (Nies, et al., 1965; Abbrecht and Greene, 1966; Westerman, et al., 1967). This view has also received support from experimental work in animals (see Review - Section I, Chapter 3).

B. OBSERVATIONS OF THE EFFECTS OF HYPOXIA ON THE PLASMA LEVELS AND URINARY EXCRETION RATES OF ERYTHROPOIETIN

Introduction.

Nearly a century ago it was demonstrated by Viault (1890) that the hypoxia of high altitude leads to the production of increased numbers of erythrocytes. It has since been established that erythropoietin is secreted in response to the hypoxia and that this hormone stimulates red cell formation by the bone marrow. High levels of erythropoietin have been recorded in the plasma and urine of men taken from sea-level to high altitudes (Reynafarje, et al., 1964; Van Dyke, et al., 1966; Carmena, et al., 1967). In some subjects these high levels were transient and disappeared on continued exposure to high altitude (Reynafarje, et al., 1964; Carmena, et al., 1967).

Several studies of erythropoietin have been performed in animals during experimental hypoxia using low pressure chambers, but few studies have been made in man. Siri, et al., (1966) reported a single experiment in which they measured serum and urinary erythropoietin in a male subject who was exposed to an atmospheric pressure of 405.6 mm.Hg for four days, by means of a low pressure chamber.

In the study reported here, the effects of hypoxia on plasma concentration and urinary excretion rate of erythropoietin were examined, together with renal and respiratory responses, in two human subjects. Hypoxia was induced by the breathing of a low oxygen in nitrogen gas mixture and the studies were extended over a period of approximately four hours.

Subjects and Procedures.

Subjects.

Both subjects were healthy male volunteers aged 44 years (Subject 1) and 24 years (Subject 2).

Procedures.

Each subject had a light breakfast of tea and toast two hours before the commencement of the investigation. An oral water load (500 ml.) was given 4-10 minutes prior to the period of low oxygen breathing. During the investigation hydration was maintained by the intravenous administration of 5 per cent dextrose at 7 ml./min.

An arterial catheter was inserted into the left radial artery to enable repeated blood sampling for gas, pH and chemical analyses and a cannula was inserted into an antecubital vein in the right arm for the infusion of 5 per cent dextrose containing para-amino-hippurate (PAH). The subject wore an "Air-Med P" mask through which he breathed the low oxygen in nitrogen gas mixture; a microphone system incorporated into the mask enabled the subject to communicate with those around him. The oxygen concentration in inspired air was measured with a paramagnetic oxygen analyser* and that of carbon dioxide with an infra-red carbon dioxide analyser**. Respiratory gas volumes were measured with a Tissot spirometer.

Arterial blood gas and pH analyses were performed using an I.L.† pH and gas analyser. Plasma was separated from

* Type - Servomex D.C. 101.

** Type - Uras 1, Hartmann and Braun.

† Instrumentation Laboratory, Boston, U.S.A.

arterial blood samples and deep frozen for erythropoietin assay and chemical estimation of PAH.

Effective renal plasma flow was estimated by determining the clearance of para-aminohippurate (PAH). Plasma and urine specimens were taken before the experiment as blanks. A priming injection of PAH was given intravenously, followed by a sustaining solution of PAH in 5 per cent dextrose administered at a rate of 7 ml./min. Blood samples were taken near the mid point of each urine collection and PAH was analysed by the Bratton and Marshall (1939) diazotisation method (see Appendix G).

Details of the time-table of the experimental procedure in Subject 2 are given in Appendix H.

Results.

1. General Observations.

Subject 1 tolerated an inspired oxygen concentration of between 12.4 and 13.2% for almost an hour. He then showed skin pallor and experienced nausea; the oxygen concentration was subsequently increased to between 14.8 and 15.4% and he became symptom-free. Subject 2 tolerated an inspired oxygen concentration of 12.3 to 13.6% without distress. Both subjects said that they felt lethargic and slightly drowsy during the period of hypoxia.

2. Respiratory Function Measurements.

The variations in arterial oxygen tension (Pa_{O_2}), arterial carbon dioxide tension (Pa_{CO_2}) and blood pH with the percentage of oxygen in the inspired gas mixture are shown for each subject in Figures VII and VIII.

FIGURE VII.

VARIATIONS IN ARTERIAL Pa_{O_2}, Pa_{CO_2} AND pH WITH PERCENTAGE OXYGEN IN INSPIRED AIR. SUBJECT 1.

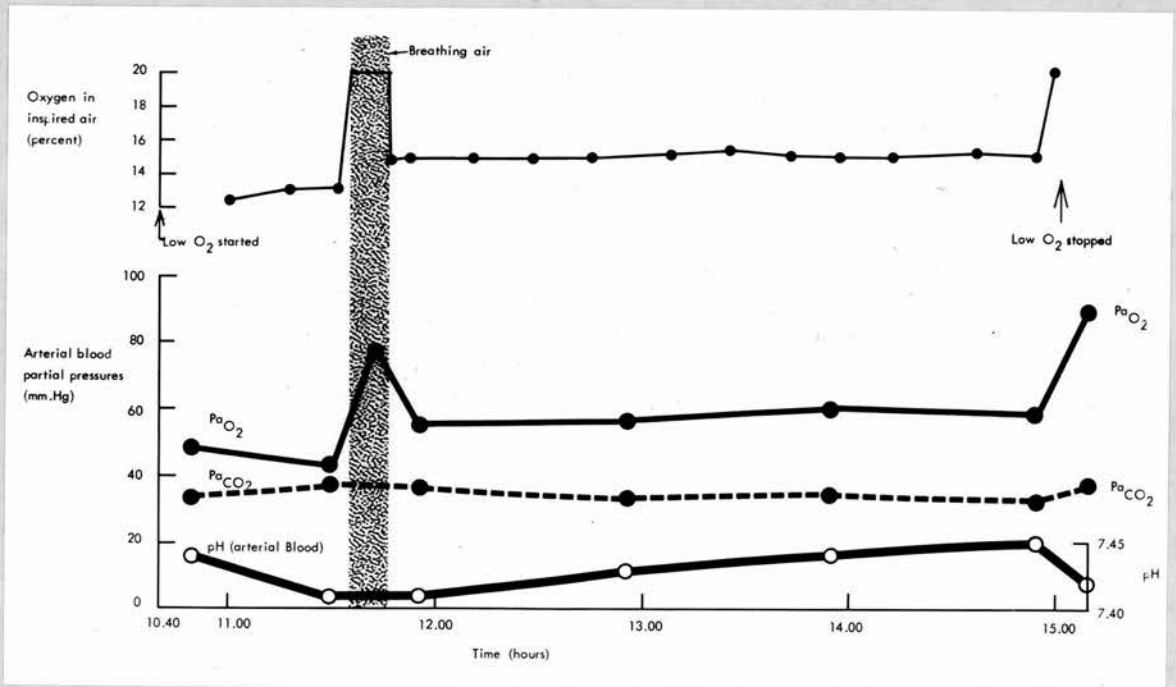
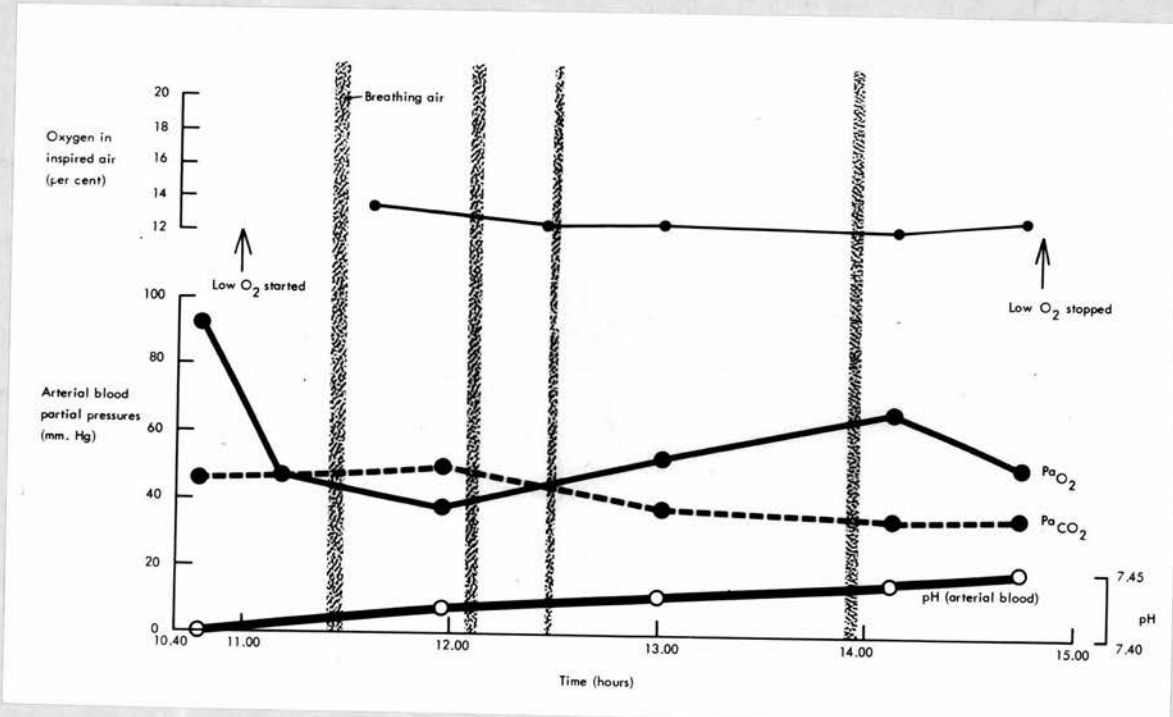


FIGURE VIII.

VARIATIONS IN ARTERIAL Pa_{O_2}, Pa_{CO_2} AND pH WITH PERCENTAGE OXYGEN IN INSPIRED AIR. SUBJECT 2.



In Subject 1 the percentage oxygen breathed was maintained at approximately 15% for just over three hours. The partial pressure of oxygen in the inspired gas mixture (PI_{O_2}) is given by the equation :-

$$PI_{O_2} = (B - 47) \cdot FI_{O_2}.$$

where B is the prevailing barometric pressure and FI_{O_2} is the dry fraction of oxygen in inspired air. For this experiment,

$$PI_{O_2} = (743 - 47) \cdot \frac{15}{100} = 104 \text{ mm.Hg.}$$

As can be seen from Table 16, this is equivalent to the partial pressure of oxygen encountered at an altitude of 2,400 to 3,000 metres. With an FI_{O_2} of 13% as at the beginning of the experiment, the equivalent altitude is 3,500 to 4,000 metres. The arterial oxygen tension achieved with an FI_{O_2} of approximately 15% was in the region of 55 to 60 mm.Hg in this subject.

TABLE 16. ALTITUDE-PRESSURE TABLE.

PI_{O_2} (mm.Hg)	Altitude (metres)	Altitude (feet)
108	2,440	8,000
100	3,050	10,000
91	3,660	12,000
83	4,270	14,000

From "Handbook of Physiology" Section 3
Fenn and Rahn, 1965.

The Pa_{CO_2} remained relatively constant between 30 and 35 mm.Hg while, after an initial decrease, the pH showed a steady rise towards 7.45 (see Figure VII).

In Subject 2 the percentage oxygen in the inspired gas mixture was maintained between 12.3 and 13.6% for more than three hours, with some short breaks to allow the subject to void urine. The resultant arterial oxygen and carbon dioxide tensions are shown in Figure VIII. As in Subject 1, the arterial pH rose steadily to a value of 7.45 before the end of the hypoxic period.

3. Measurement of Renal Function.

Subject 1. Despite oral and intravenous water loading, this subject had an antidiuresis during the period of breathing the low oxygen mixture; this was followed by a diuresis occurring after the cessation of the experiment (see Table 17). Effective renal plasma flow was within normal limits during low oxygen breathing, the para-aminohippurate clearance being 557 ml./minute.

Subject 2 had an initial diuresis which decreased as the experiment continued (see Table 18). Urine flow was highest just after the lowest reading of arterial oxygen tension (see Figure IX). The clearancesof PAH are plotted relative to Pa_{O_2} in Figure X. Effective renal plasma flow (PAH clearance) fell below normal approximately one-and-a-half hours after the start of breathing the low O_2 in N_2 mixture, but rose again during the final hour of hypoxia.

4. Erythropoietin Assay.

Erythropoietin assay was undertaken on two urine samples from Subject 1; U1, obtained at the end of the period of hypoxia and U4, obtained four hours later. The results of the

TABLE 17. URINE FLOW AND EFFECTIVE RENAL PLASMA FLOW IN STUDY OF EXPERIMENTAL HYPOXIA - SUBJECT 1.

Time (hours)	Urine flow (ml./min.)	PAH clearance (ml./min.)
10.53 - 15.15	0.95	557
(Periods of low O_2 breathing		
10.40 - 11.35		
11.47 - 15.02)		
15.02	Experimental hypoxia terminated	
15.15 - 16.20	2.35	-
16.20 - 17.25	12.12	-
17.25 - 19.20	3.13	-
19.20 - 23.45	0.50	-

TABLE 18. URINE FLOW AND EFFECTIVE RENAL PLASMA FLOW IN STUDY OF EXPERIMENTAL HYPOXIA - SUBJECT 2.

Time (hours)	Urine flow (ml./min.)	PAH clearance (ml./min.)
11.29 - 12.06	13.24	557
12.06 - 12.38	16.87	628
12.38 - 13.55	4.10	321
13.55 - 14.56	8.22	723
14.56	Experimental hypoxia terminated	
14.56 - 19.00	0.08	-
19.00 - 21.00	1.00	-

FIGURE IX.

VARIATION IN URINE FLOW WITH ARTERIAL Pa_{O_2}.

SUBJECT 2.

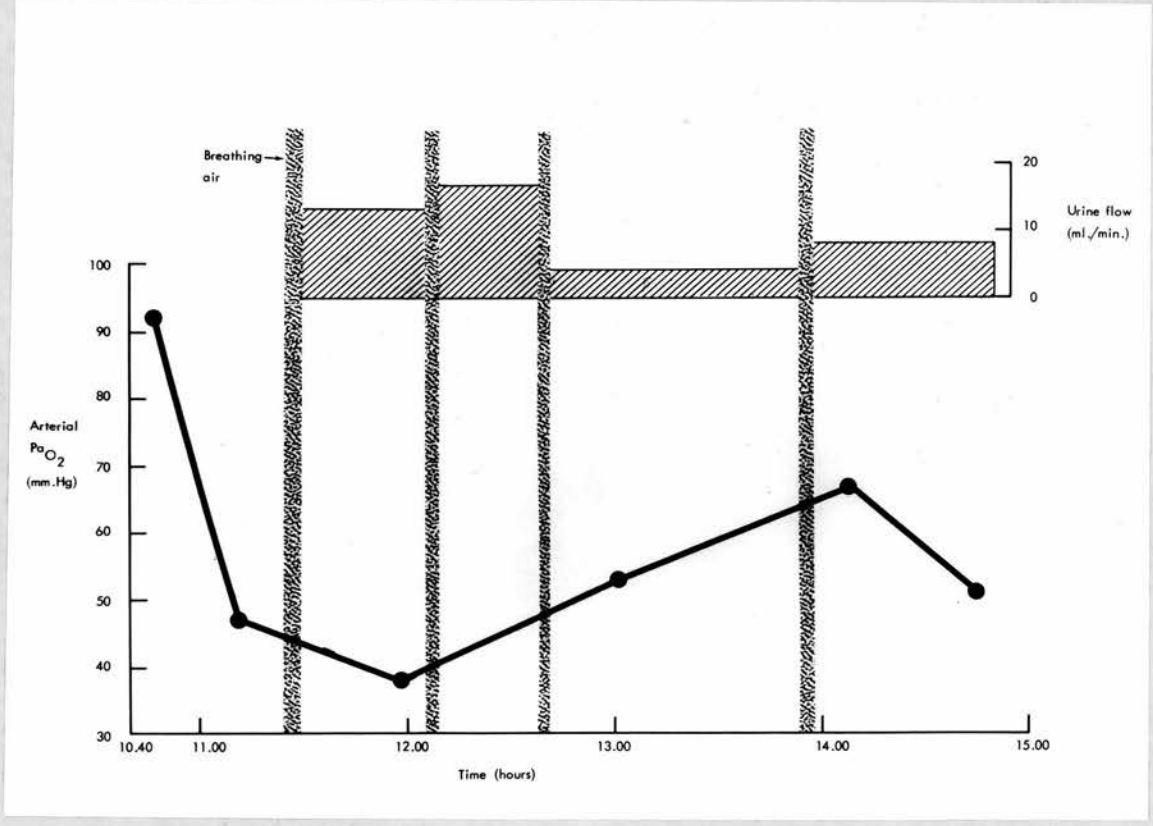
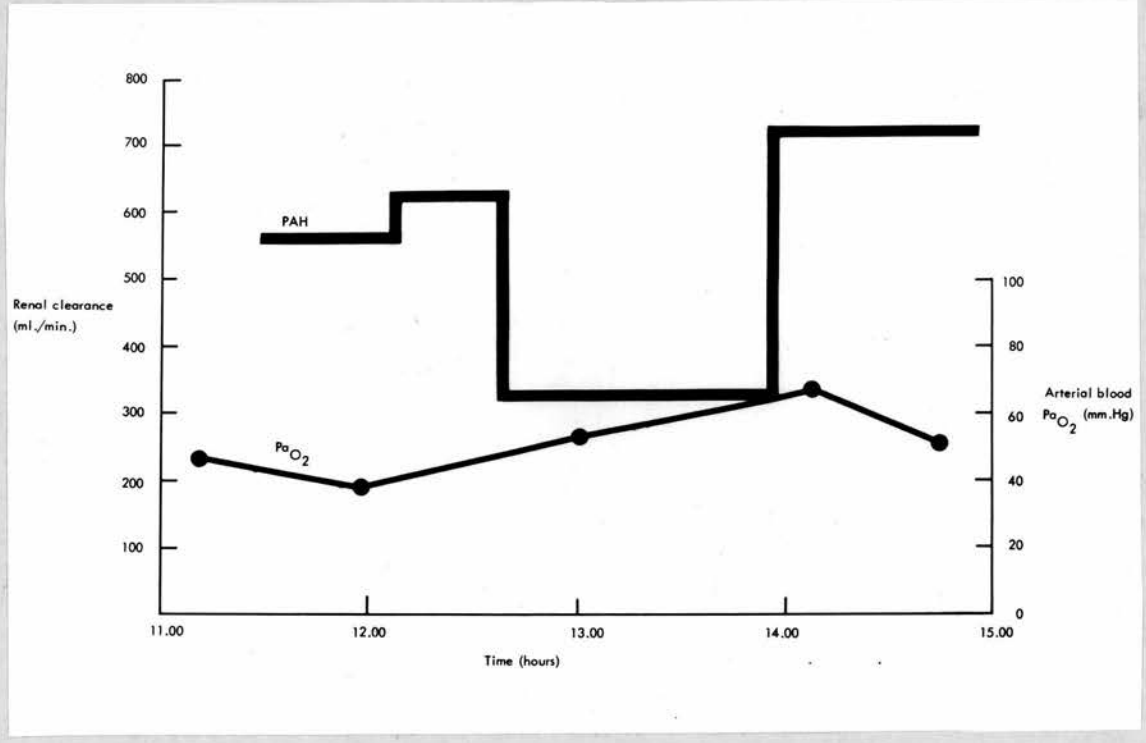


FIGURE X.

CLEARANCE OF PAH RELATIVE TO ARTERIAL Pa_{O_2}.

SUBJECT 2.



assays are presented in Table 19. For U1 and U4 the mean response to 1.00 ml. urine was not significantly different from the mean response to 1.00 ml. saline (U1 - 1.00 ml. dose, $t = 0.08$, $p>0.90$: U4 - 1.00 ml. dose, $t = 0.27$, $p>0.70$). Since there appeared to be no detectable erythropoietin in these two specimens intermediate specimens were not assayed.

In view of the lack of detectable erythropoietic activity in the two urine specimens from Subjct 1, the urine specimens from the second subject were concentrated in a freeze-drying apparatus before assay. The concentration factor varied between 2.40 and 3.03, the value for each being given in Table 20. The results of the assays on urine concentrates of U1, U2, U4 and U6 are presented in Table 21. The mean responses to 1.00 ml. U1, 1.00 ml. U2, 0.50 ml. U4 and 0.50 ml. U6 were not significantly different from the mean responses to saline, as will be seen from the 't' test probability levels. Intermediate specimens were therefore not assayed.

Plasma specimen P1 from Subject 1, withdrawn immediately before inhalation of the low O_2 in N_2 mixture was discontinued, was assayed at doses of 0.50 ml. and 1.00 ml. against E.L.S.I. The responses in the two dose groups could not be used to calculate the potency of the plasma since five out of six mouse haematocrits at the lower dose were less than 50%, whilst five out of five at the higher dose were less than 50%. Similarly, when plasma specimens P3, P4c and P5 (see Appendix H) from Subject 2 were assayed, at lower doses than P1 of Subject 1, haematocrits were less than 50% at the end of the assay and several mice died. Haematocrits in the saline control mice were all above 50%.

TABLE 19. ERYTHROPOIETIN ASSAY ON URINE FROM SUBJECT 1.

EXPERIMENTAL HYPOXIA STUDY.

Urine sample	Dose (ml.)	R.B.C. ^{59}Fe Uptake % Mean ± S.E. of Mean	Number of mice
U1	0.25	10.14 ± 0.51	6
	0.50	10.27 ± 1.70	4
	1.00	10.34 ± 1.46	6
Saline controls	1.00	10.20 ± 0.78	6
U4	1.00	5.80 ± 0.76	5
Saline controls	1.00	5.47 ± 0.86	6

TABLE 20. CONCENTRATION OF URINE SPECIMENS PRIOR TO ASSAY

EXPERIMENTAL HYPOXIA STUDY - SUBJECT 2.

Urine Specimen	Concentration Factor
U1	3.03
U2	2.50
U3	3.03
U4	3.03
U5	2.59
U6	2.40

TABLE 21. ASSAYS OF URINE CONCENTRATES FROM SUBJECT 2.

EXPERIMENTAL HYPOXIA STUDY.

Urine Sample (concentrate)	Dose (ml.)	R.B.C. ^{59}Fe Uptake % Mean ± S.E. of Mean	Number of mice	Probability
U1	0.50	12.09 ± 2.05	5	
	1.00	7.41 ± 2.50	4	>0.60
U2	0.50	8.19 ± 1.30	4	
	1.00	9.25 ± 1.96	5	>0.80
Saline controls	1.00	8.78 ± 1.60	3	
U4	0.50	16.70 ± 3.19	6	>0.40
U5	0.50	17.77 ± 2.17	6	>0.10
Saline controls	1.00	13.50 ± 2.09	6	

Discussion.

The principal aim of the study on these two subjects was to determine whether evidence of increased erythropoietin secretion could be detected within three to four hours of exposure to a simulated high altitude of approximately 3,000 metres. It has been shown by Weil, et al. (1968) that the red cell mass is significantly higher in healthy men who permanently reside at this altitude than in those living at sea level. At Pa_{O_2} levels below 67 mm.Hg these workers also demonstrated a linear relationship between Pa_{O_2} and the red cell mass. In the experiment reported by Siri, et al. (1966) where the subject was exposed to simulated high altitude of 5,000 metres for four days, erythropoietin activity was not detected in urinary concentrates of specimens collected before 24 hours, but results from collections that followed were positive; serum erythropoietin became detectable at 12 hours and reached a maximum on the third day. The Pa_{O_2} of the subject is not quoted, but the partial pressure of oxygen in the inspired air was approximately 85 mm.Hg. Carmena, et al. (1967) reported increased erythropoietin in the 24-hour urine concentrates of five subjects taken from sea level to 4,540 metres after the first day at high altitude.

Although the degree of acute hypoxia hypoxia in the two subjects of the present study was close to the tolerable maximum, erythropoietin could not be detected in the urine after three to four hours of hypoxia; unfortunately the levels of the hormone in plasma could not be assayed as the plasma of both subjects caused a reduction in the haematocrit of the assay animals. The renal clearances of PAH were within normal limits except during the second hour of hypoxia in

Subject 2. This low value probably resulted from incomplete bladder emptying since the value obtained for the next clearance period was high. Previous clearance studies in experimental hypoxia have shown that renal plasma flow either remains unaltered or slightly increases when human subjects breathe gas mixtures containing similar concentrations of oxygen to those used in this study (Berger, et al., 1949; Caldwell, et al., 1949). The failure of the diuretic response to water loading in Subject 1 has previously been described in subjects in whom hypoxia causes distressing symptoms such as nausea, this phenomenon being attributed to the release of antidiuretic hormone by the neurohypophysis (Ullmann, 1961).

These limited observations in two human subjects and in the subject reported by Siri, et al. (1966) suggest that there is probably insufficient erythropoietin secreted during the first few hours of exposure to an altitude of 3,000 - 4,500 metres to enable detection of the hormone in urine. There is a paucity of reports of similar studies on the effects of hypoxia on erythropoietin in man and much work remains to be done to elucidate the kinetics of erythropoietin production in response to varying degrees of hypoxia.

C. ERYTHROPOIETIN IN PATIENTS WITH HYPOXIA DUE TO SEVERE CHRONIC OBSTRUCTIVE AIRWAYS DISEASE.

The urinary concentration of erythropoietin was assayed in three hypoxic patients with chronic bronchitis, two of whom had secondary polycythaemia.

Patients Studied.

Details of the patients, together with their haematological and arterial blood gas data, are summarised in Table 22.

Patient 19 was receiving assisted ventilation during the greater part of each day, this being given by means of a Cape respirator. When the patient was off the machine for several hours he became severely hypoxic (see Table 22). Urine and blood samples were collected for erythropoietin assay (as shown in Table 23) during periods of normal and severely reduced Pa_{O_2} levels.

Patient 20 and Patient 21 were suffering from milder degrees of chronic respiratory failure and did not require assisted ventilation; both had secondary polycythaemia (Table 22). A 24-hour urine collection was obtained from each patient and 100 ml. aliquots were concentrated in a freeze-drier (Concentration factors were - Patient 20, 2.50; Patient 21, 2.63).

Results.

Patient 19. The results of erythropoietin assay on urine samples U1 and U2 are presented in Table 24. For both samples the mean response was not significantly different from the mean response to saline in the control group of mice (U1 - $t = 1.08$, $p>0.30$: U2 - $t = 1.24$, $p>0.20$). 100 ml. aliquots of each specimen were therefore

TABLE 22. ERYTHROPOIETIN ASSAYS IN HYPOXIA DUE TO SEVERE CHRONIC OBSTRUCTIVE AIRWAYS DISEASE - PATIENTS STUDIED.

Patient	Age (yrs.)	Sex	Diagnosis	Haematological data				Arterial blood analyses (breathing air without ventilatory assistance)			
				Hgb. (g/100 ml)	P.C.V. (%)	W.B.C. (per cu.mm)	R.C.M.* (ml/Kg.body wt)	Pa_{O_2} (mm.Hg)	O_2 satn. (%)	Pa_{CO_2} (mm.Hg)	pH
19	55	M	Chronic bronchitis.	11.6	35	6,600	-	26.7	35.5	44	7.39
20	68	M	Chronic bronchitis. Cor pulmonale. Polycythaemia.	18.0	58	10,400	38.1	66.0	92.9	52	7.42
21	65	M	Chronic bronchitis. Cor pulmonale. Polycythaemia.	18.4	56	5,900	42.0	55.0	-	83	7.28

* Red Cell Mass measured by ^{51}Cr-labelled red cell method.

TABLE 23. ERYTHROPOIETIN ASSAY IN A PATIENT WITH RESPIRATORY FAILURE RECEIVING INTERMITTENT PERIODS OF ASSISTED VENTILATION (PATIENT 19).

Time (hours)	Arterial Blood Gases			Blood	Sample collection for erythropoietin assay	
	pO_2 (mm.Hg)	pCO_2 (mm.Hg)	O_2 saturation (%)	pH	Plasma	Urine
09.45	146.0	50.0	97.0	7.39	P1	
10.00	Respirator Off					U1 began
11.00	31.0	38.0	53.0	7.49		
13.00	33.4	37.1	59.0	7.46		
15.00	26.7	44.0	35.5	7.39		
15.30					P2	U1 ended U2 began
15.45	Respirator On					
21.30					P3	U2 ended

TABLE 24. ERYTHROPOIETIN ASSAY OF URINE FROM PATIENT 19 WHEN ON AND OFF RESPIRATOR.

Sample assayed	Dose (ml.)	R.B.C. ^{59}Fe Uptake % Mean ± S.E. of mean	Number of mice
U1 (off respirator)	1.00	7.37 ± 2.00	4
Saline	1.00	9.95 ± 1.41	5
U2 (on respirator)	0.50	8.43 ± 1.17	6
Saline	1.00	10.07 ± 0.63	6

TABLE 25. ERYTHROPOIETIN ASSAY OF URINE CONCENTRATES IN CHRONIC HYPOXIA - PATIENTS 20 AND 21.

Sample assayed	Dose (ml.)	R.B.C. ^{59}Fe Uptake % Mean ± S.E. of mean.	Number of mice
Urine (Pt. 20)	1.00	10.94 ± 6.73	3
Urine (Pt. 21)	1.00	3.97 ± 0.40	5
Saline	1.00	12.24 ± 3.96	4

concentrated in a freeze-drier (U1 by a factor of 2.70, U2 by 2.56), and the urine concentrates assayed. The mean response to each urine concentrate was again not significantly different from the mean response in the saline controls (U1 - t = 0.35, p>0.70: U2 - t = 0.15, p>0.80).

Assay of plasma specimens P1 and P2 was not possible since the haematocrits of four out of six mice given 0.50 ml. P1, and five out of five mice given 0.50 ml. P2 were less than 50%.

Patients 20 and 21. The results of the assay of erythropoietin in the urine concentrates of these patients are shown in Table 25. The mean response to 1.00 ml. of urine concentrate was actually less than the mean response to saline in each case. Thus, erythropoietin could not be detected in the urine concentrates of either of these patients.

Discussion.

Assay of erythropoietin has been attempted in three patients with chronic hypoxia due to obstructive airways disease, two of whom had secondary polycythaemia. While the chronic hypoxia of advanced obstructive airways disease may stimulate erythropoiesis, it is well recognised that many patients fail to develop polycythaemia. This has led to speculation concerning the role of erythropoietin in the genesis of such forms of secondary polycythaemia. From the few assays of the hormone which have been reported in chronic respiratory failure, there is no clear-cut evidence for or against a lack of erythropoietin production in cases with a poor or absent polycythaemic response; Freedman and Penington (1963) reported that plasma erythropoietic activity was increased in all but one of four non-polycythaemic patients with chronic bronchitis; on the other hand,

Vanier, et al. (1963) obtained a positive result for urinary erythropoietic activity in only one out of seven patients with emphysema. In the present study erythropoietic activity could not be detected in the urine or urine concentrate of the non-polycythaemic patient with chronic hypoxia (Patient 19), but there was also no detectable activity in the urine concentrates of the two patients who had secondary polycythaemia (Patients 20 and 21). It is clear that further assays of erythropoietin are required in patients with chronic respiratory failure to elucidate the part played by this hormone in the production of secondary polycythaemia.

COMMENTS ON THE ESTIMATION OF ERYTHROPOIETIN IN HUMAN PLASMA USING THE HYPOXIA-INDUCED POLYCYTHAEMIC MOUSE ASSAY.

In the studies reported in this thesis, difficulty was repeatedly encountered in the estimation of erythropoietin in human plasma samples. In many instances, estimation of the potency of erythropoietin in these samples was impossible because the haematocrits of mice given plasma as test material were less than 50% at the end of the assay. In those assays in which this occurred it was noted that the higher the dose of plasma given, the lower was the mean haematocrit. An example of this phenomenon is shown in Table 26 where the effect of mouse haematocrits of plasma specimen P1 from Subject 1 of the experimental hypoxia study is recorded. When plasma was injected into non-polycythaemic mice it also had the same effect in lowering the haematocrit. The following experiment was therefore set up to investigate this phenomenon *in vitro* :-

The Effect of Human Serum on Mouse Erythrocytes.

Methods.

Blood from three normal mice was collected in sodium citrate solution (3.8%) and, after washing three times in physiological saline, was used to make a 2-5% red cell suspension in saline. Blood from group A, B, O and AB individuals was collected in plain glass tubes and the serum was separated after overnight clotting at 4°C.

To round-bottomed glass precipitin tubes (50 mm. x 5.5 mm) one drop of serum and one drop of cell suspension were added. Control tubes containing one drop of saline and one drop of cell suspension were incorporated for each batch of mouse cells. The

TABLE 26. ASSAY OF ERYTHROPOIETIN IN HUMAN PLASMA - EFFECT OF PLASMA ON MOUSE HAEMATOCRITS.

Test Material	Dose (ml.)	Haematocrit % Mean ± S.E. of mean	Number of mice.
Saline	1.00	55.9 ± 1.3	6
Plasma	0.50	43.4 ± 3.2	6
Plasma	1.00	39.5 ± 2.4	5

Note: The difference between means of plasma-injected and saline-injected groups was significant at both the 0.50 ml. ($p<0.05$) and the 1.00 ml. ($p<0.001$) doses of plasma.

tubes were shaken and left at room temperature for 90 minutes. Each tube was then agitated by tapping with a finger. If the contents showed complete agglutination, obvious to the naked eye, a positive result (+) was recorded, the result being confirmed by examination under the low-power objective of a microscope. Absence of agglutination was scored as negative (-).

Results.

Table 27 shows that red blood cells from the three mice were all completely agglutinated by the sera of normal human subjects of blood groups A, B, O and AB.

TABLE 27. AGGLUTINATION REACTIONS BETWEEN MOUSE ERYTHROCYTES AND NORMAL HUMAN SERUM.

Mouse sample number	Gp.A	Gp.B	Gp.O	Gp.AB	Saline Controls
1	+	+	+	+	-
2	+	+	+	+	-
3	+	+	+	+	-

Discussion.

Serum factors which agglutinate the erythrocytes of a different species (heteroagglutinins) were first discovered by Landois (1875). He found that erythrocytes of the sheep, rabbit, guinea pig, dog and cat were lysed or clumped by human serum.

It would appear that the injection of human serum into polycythaemic assay mice in volumes up to 1.00 ml. caused a serological reaction resulting in red-cell destruction and a decrease in the mouse haematocrits below those of control mice

receiving the same volume of saline. There are many reports in the literature of erythropoietin assays in serum or plasma, but few mention any problems of this kind. However, Borsook, et al. (1954) devised a method of heat denaturation of plasma proteins which decreased the "toxicity" of rabbit plasma to normal adult rats, while retaining the erythropoietic activity; the method was later applied to assays of human plasma (Piliero, et al., 1956; Gurney, et al., 1957). Garcia and Schooley (1965) observed a greater response to erythropoietin given in serum than in saline, though they did not attribute this to the effects of hetero-agglutinins. One possible reason why this problem has been overlooked is that the haematocrits of assay animals in studies reported in the literature appear to be somewhat higher than those in this study; thus, if they were in the region of 70-80% prior to injection of a plasma sample, they could be reduced by 20-30% without a haemolytic effect being suspected.

Since human plasma decreased the haematocrits of many of the polycythaemic mice prepared for erythropoietin assay, the majority of assays on plasma had to be rejected as it was impossible to distinguish between mice whose haematocrit was low due to a poor reaction to hypoxia in the low pressure chamber and mice in which the plasma produced a haemolytic effect and stimulated erythropoiesis. This problem was unexpected in view of the lack of published reports of such an occurrence and its recognition casts doubts on the validity of many previously reported values for erythropoietin in plasma and serum.

SECTION IV.

PREPARATION OF ERYTHROPOIETIN ANTISERA.

SECTION IV.

PREPARATION OF ERYTHROPOIETIN ANTISERA.

Introduction.

The estimation of a hormone by bioassay is a relatively crude procedure and is usually costly in terms of animals and skilled labour. Immunoassays are more rapidly performed and are generally more sensitive. As in other fields of hormone research, it is recognised that the introduction of a satisfactory immunoassay procedure for erythropoietin would represent a significant advance.

Erythropoietin is a relatively weak antigen and as it has not yet been isolated in pure form, any erythropoietin preparation used for immunisation purposes is likely to result in the production of antibodies against contaminant proteins present in the injected preparations. Despite these difficulties, several workers have successfully produced antisera which neutralise the biological activity of erythropoietin from various species, including man (Schooley and Garcia, 1962; Fisher, et al., 1965b; Goudsmit, et al., 1967; Krugers-Dagneaux, 1967; Rioux and Erslev, 1968; Lange, et al., 1968; Zanjani, et al., 1968). The neutralising ability of anti-erythropoietin sera appears to be associated with the γ-globulin fractions (Schooley, et al., 1967; Zanjani, et al., 1968; Lange, et al., 1969).

In this study an attempt was made to prepare antisera to human urinary erythropoietin in rabbits, with a view to the later development of an erythropoietin immunoassay.

METHODS.

1. Preparation of Antigenic Materials.

Erythropoietin laboratory standard I (E.L.S.I) prepared from human urine (see Section II) was used as the starting material. Since Schooley, et al. (1967a) had found that their immune sera with the highest titres of antibody were generally obtained after immunisation with alum-precipitated human urinary erythropoietin, an alum-precipitate of E.L.S.I was prepared using the method of Proom* (1943).

Immunisation injection schedule.

The immunisation procedure was carried out in New Zealand White rabbits which were approximately 12 weeks old at the beginning of the experiment. Following initial injection of 5 ml. of alum-precipitated E.L.S.I intraperitoneally and 2 ml. of non-precipitated E.L.S.I intravenously, intramuscular injections of E.L.S.I (1 ml.) were given at weekly intervals. Disposable syringes and needles were used in all injection procedures. Intravenous injections were made into a marginal ear vein, and intramuscular injections into a hind leg muscle.

Bleeding of rabbits.

The first harvesting of a serum was at five weeks after the initial injection (Sample 1). A second bleeding was performed two weeks later (Sample 2). After three further weekly intramuscular injections of 1 ml. amounts of E.L.S.I, a final bleeding was made (Sample 3).

* See Appendix J for further details.

Blood samples were obtained from a small incision into an ear vein, the blood being allowed to drip directly into a sterile 20 ml. glass universal container. The blood was allowed to clot at room temperature and the serum was separated after centrifugation at 2,000 r.p.m. for 20 minutes. The serum was stored in sterile Bijou bottles at -20°C.

2. Testing of Antisera.

The rabbit antisera were tested by immunological precipitin techniques *in vitro*, and for their biological activity by the *in vivo* erythropoietin assay method described in Section II.

A. Immunological Methods.

(a) Double diffusion in one dimension.

A rapid agar-diffusion method was used as a screening test for the presence of antibodies in the first sampled sera from Rabbits A and B (Sample 1 from each). In this method, based on that of Preer (1956), antibody and antigen diffuse towards each other in a layer of agar :-

Capillary tubing of 1.8 to 2.0 mm inside diameter was internally coated with 0.1% agar in saline-merthiolate (1:10,000) by drawing the agar through the warmed tubing by means of a suction pump. The tubing was cut into 5 cm. lengths. Using a finely drawn Pasteur pipette, 0.6% agar in saline-merthiolate was run into the centre of each 5 cm. tube to give a column 7-8 mm. in height. Doubling dilutions of the injected antigen, E.L.S.I, were made in saline from 1:1 to 1:32,768. Equal column lengths of diluted E.L.S.I and undiluted antiserum were introduced into opposite ends of the tube, care being taken to avoid trapping air bubbles between

the agar and liquid columns. The ends were immediately sealed with paraffin wax and the tubes left on a level surface for two or three days at room temperature until precipitin lines developed in the middle column of agar. The tubes were examined before a bright light for the presence of precipitin lines and their appearance noted by drawing, since this is not a permanent preparation.

(b) Double diffusion in two dimensions.

The method used was that described by Ouchterlony (1958) :-

20 ml. of buffered 1% agar (in phosphate buffer pH 7.0 containing 1% sodium azide), at about 65°C, were pipetted into each disposable Petri dish (8-9 cm. diameter). The agar was allowed to set. Wells for antigen and antisera were cut out using a die and the plates left overnight to enable the agar to adhere to the plate. Next day the appropriate antisera and antigen were placed in the wells and the plates were left for two days at room temperature to allow the development of precipitin arcs. Unprecipitated protein was removed by the addition of saline-azide to each plate, leaving them for two days and changing the wash daily. After two days the agar was carefully lifted out of the Petri dish and placed on a 3½" square glass plate, taking care to avoid trapping air bubbles. It was covered with distilled water-dampened filter paper and dried in a moderate oven (45-60°C). The precipitin arcs were stained by immersing the plates in Ponceau S stain (0.2% in 6% sulphosalicylic acid) for ten minutes. Excess stain was then removed by washing with 2% acetic acid and the plates were left to dry in air.

(c) Immunoelectrophoresis.

The method was based on that of Scheidegger (1955) as modified by Riding (1963) :-

A 1.5% solution of agar in barbitone acetate buffer (pH 8.6, μ = 0.05) was prepared by heating in a water bath until the solution was clear. Microscope slides were placed in series of three in a standard L.K.B.* slat and 10 ml. of the prepared agar solution, at a temperature of about 65°C, was poured over the slides and end pieces. The slides were left under cover to allow the agar to set. Troughs and wells were cut in the agar using a die and the slides were left overnight in a moist chamber. The next day the antigen samples were placed in the wells and subjected to electrophoresis for two hours at room temperature (tank buffer- barbitone acetate buffer pH 8.6, μ = 0.1; fixed current of 7.5 m/amps/ slat of three slides). The appropriate antisera were then introduced into the troughs and the slides were left for two days at room temperature in a moist, enclosed chamber to allow the development of precipitin arcs. Unprecipitated protein was removed and drying and staining completed as described for Ouchterlony plates.

B. 'In Vivo' Testing of Antisera.

The neutralising ability of sera obtained from rabbit A immunised with E.L.S.I was tested *in vivo* using the erythropoietin bioassay described in Section II :-

Known amounts of E.L.S.I and each antiserum were mixed and incubated together for one hour at room temperature. The

* L.K.B. Instruments Ltd.

mixtures were then refrigerated overnight at 4°C. The erythropoietic activity of E.L.S.I, and E.L.S.I with each antiserum, was estimated in polycythaemic mice as described in the assay method. The solutions were administered to the mice in two divided doses as for a normal erythropoietin assay, and the radio-iron uptake by mouse red cells determined.

RESULTS.

1. Immunological Investigation of Erythropoietin Laboratory Standard I.

The Ouchterlony technique of double diffusion in two dimensions (Ouchterlony, 1958) and immunoelectrophoresis were used to investigate the antigens present in Erythropoietin Laboratory Standard I (E.L.S.I).

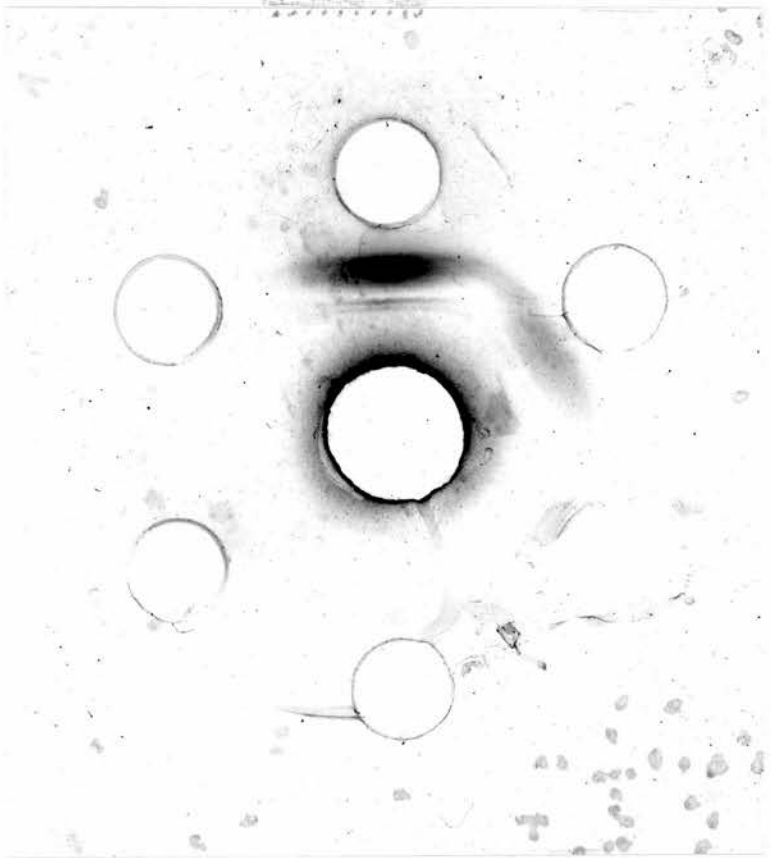
Double Diffusion.

The appearance of precipitin lines obtained by the diffusion of E.L.S.I against whole human antiserum produced in goats (W.H.A.S.) is shown in Figures XI and XII. In Figure XI several precipitin lines are visible from dilution 1:1 to 1:8 of E.L.S.I, but the "fuzz" round the central well of W.H.A.S. indicated that the W.H.A.S. was too concentrated for the E.L.S.I dilutions used. A second plate was therefore set up of undiluted E.L.S.I against double dilutions of W.H.A.S. Two precipitin lines were obtained (Figure XII), one continuous line present at all dilutions of W.H.A.S. from 1:1 to 1:32, and a second line which faded out beyond the 1:2 dilution. Further studies of E.L.S.I were then undertaken using immunoelectrophoresis, in an attempt to identify the precipitating antigens present in E.L.S.I.

Immunoelectrophoresis

Six slides were set up using the Scheidegger technique as previously described (p.139). Normal human serum was placed in the two outer wells of each slide and E.L.S.I in the central well. After electrophoresis W.H.A.S. was added to the channels of each slide using doubling dilutions from slide 1 (1:1) to slide 6 (1:32).

FIGURE XI

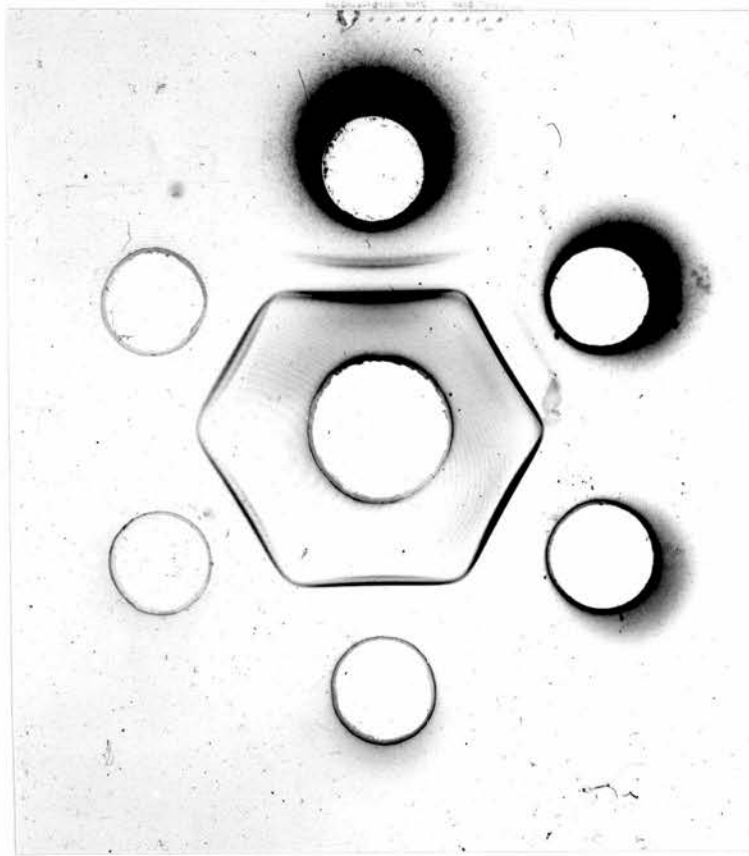


Central Well - Whole human antiserum, undiluted.

Peripheral Wells - Erythropoietin Laboratory Standard I.

1:1 at top and double dilutions to 1:32, moving clockwise.

FIGURE XII.



Central Well - Erythropoietin Laboratory Standard I - Undiluted.

Peripheral Wells - Whole human antiserum

1:1 at top and double dilutions to 1:32, moving clockwise.

The optimum dilution of W.H.A.S. was 1:8; the appearance of the slide with this dilution of W.H.A.S. is shown in Figure XIII. The precipitin lines just visible in the central part of the slide seemed to indicate that albumin was a predominant component of E.L.S.I. To verify this interpretation immunoelectrophoresis of E.L.S.I was repeated against anti-albumin instead of W.H.A.S. The result confirmed the presence of albumin in E.L.S.I.

It was concluded that the continuous precipitin line obtained when E.L.S.I diffused against W.H.A.S. in Ouchterlony plates was an albumin/anti-albumin precipitin line and that the line which faded out beyond a 1:2 dilution of W.H.A.S. (Figure XII) was possibly an erythropoietin/anti-erythropoietin precipitin line.

2. Production of Antisera to Erythropoietin in Rabbits.

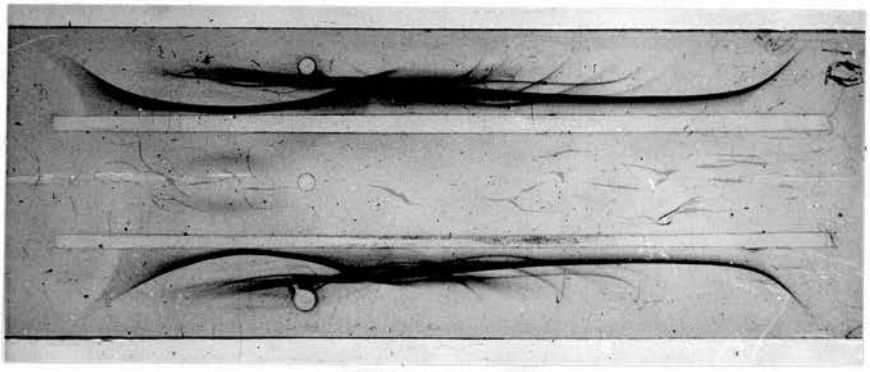
The immunisation protocol and the variation in body weight and haematocrit of Rabbits A and B over the immunisation period is shown in Figures XIV and XV respectively. Following a decrease in weight after the initial injection of alum-precipitated and non-precipitated E.L.S.I, the weight of both rabbits increased steadily over the injection period, as would be expected for healthy young animals. Rabbit B appeared to be anaemic at the beginning of the immunisation, having an haematocrit of 28%. During the injection period there was a gradual rise in haematocrit and it was still rising at the seventh week. Rabbit A, on the other hand, showed a decrease in haematocrit after the start of immunisation which continued for three weeks; thereafter the haematocrit rose.

Blood samples were taken at weeks five, seven and twelve as indicated in Figures XIV and XV. The techniques used to

FIGURE XIII.

IMMUNOELECTROPHORESIS OF ERYTHROPOIETIN

LABORATORY STANDARD I.



Central Well - Erythropoietin Laboratory Standard I diluted 1:8.

Outer Wells - Normal human serum.

Channels - Whole human antiserum.

FIGURE XIV.

PRODUCTION OF ANTISERUM TO ERYTHROPOIETIN.

IMMUNISATION PROTOCOL AND VARIATION OF RABBIT BODY WEIGHT AND HAEMATOCRIT DURING IMMUNISATION PERIOD.

RABBIT A.

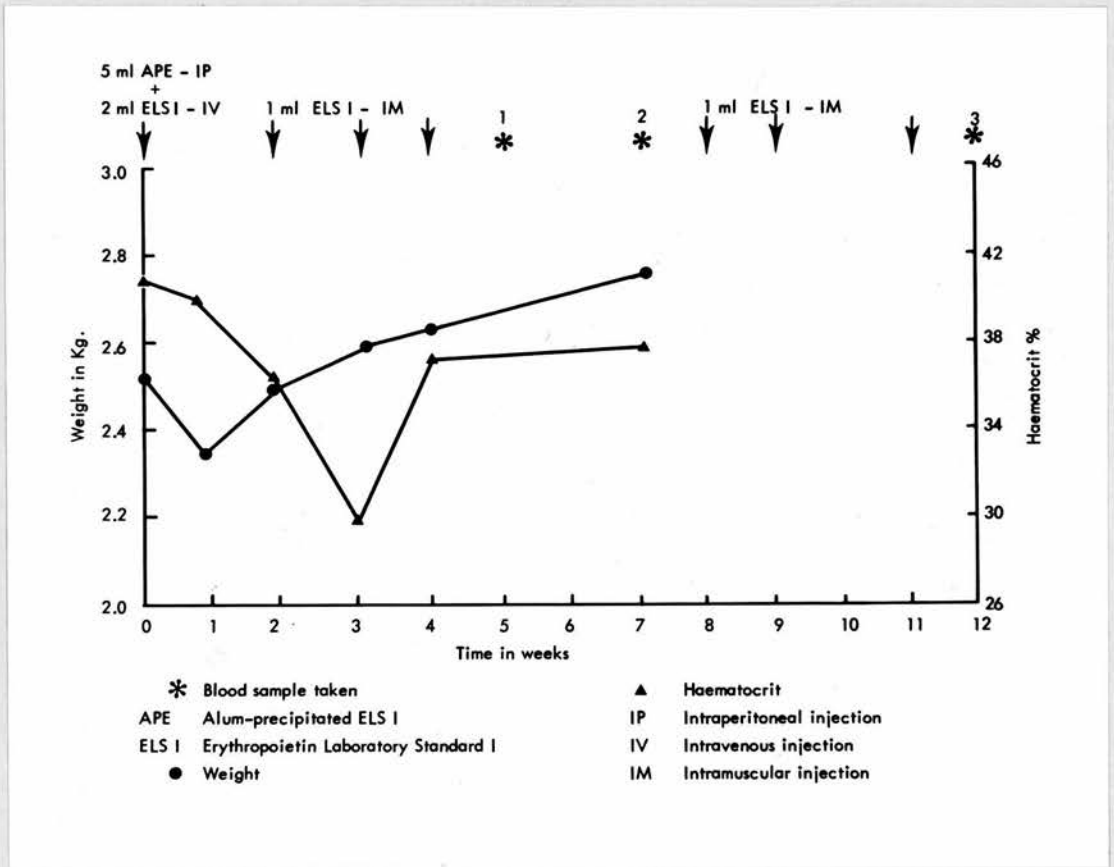
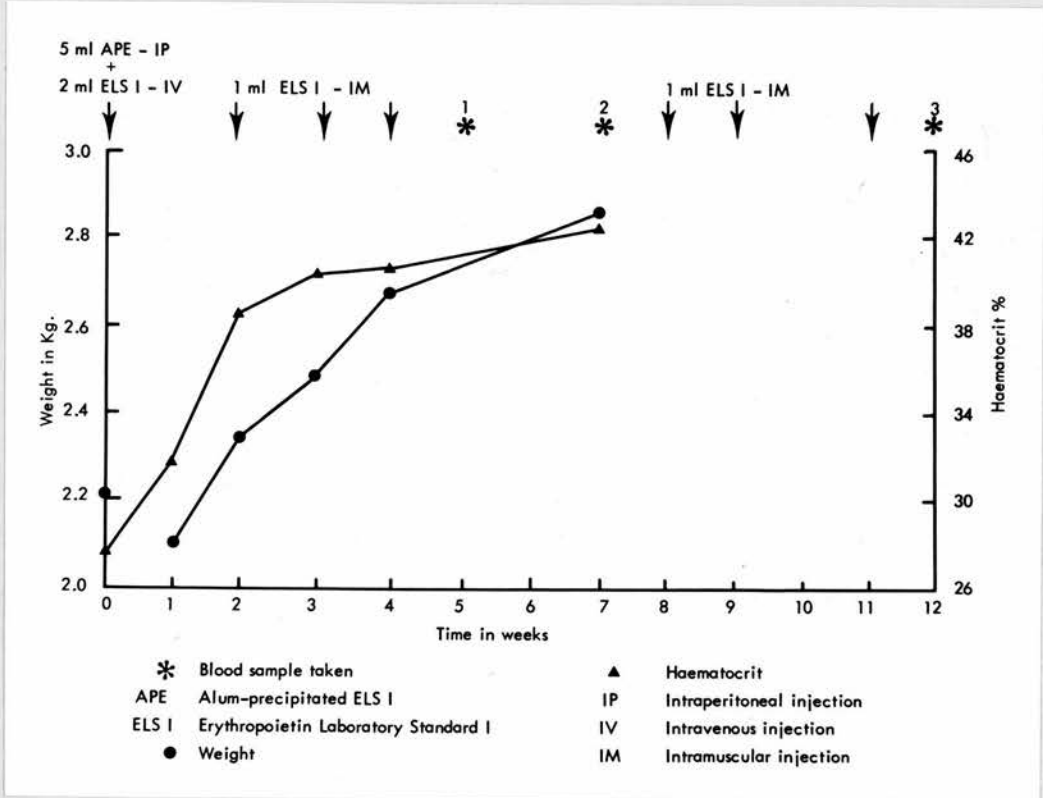


FIGURE XV.

PRODUCTION OF ANTISERUM TO ERYTHROPOIETIN.

IMMUNISATION PROTOCOL AND VARIATION OF RABBIT BODY WEIGHT AND HAEMATOCRIT DURING IMMUNISATION PERIOD.

RABBIT B.



investigate the antisera have already been described (pp.137-140).

3. Immunological Testing of Rabbit Antisera to Erythropoietin.

(a) Double diffusion in one dimension.

Each first sample of antiserum from Rabbits A and B was used undiluted and allowed to diffuse against double dilutions of E.L.S.I from 1:2 to 1:32,768. Rabbit A antiserum produced a precipitin line at dilutions up to 1:8. Rabbit B antiserum did not produce any visible precipitin lines.

(b) Double diffusion in two dimensions.

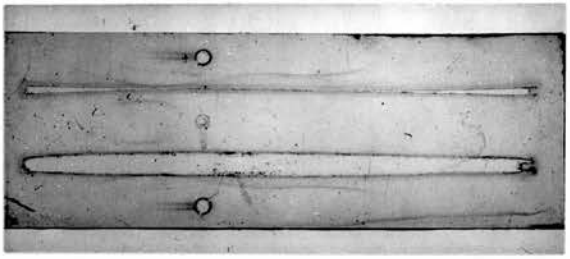
The results on Ouchterlony plates confirmed the initial results of the single diffusion test, precipitating antibodies being detected in antiserum 1 from Rabbit A but not in the antiserum of Rabbit B. When E.L.S.I was placed in the central well of an Ouchterlony plate surrounded by antiserum 1 of Rabbit A diluted 1:1 to 1:32, a single continuous precipitin line was seen to dilution 1:4. Antiserum 1 from Rabbit B produced no precipitin lines when run against E.L.S.I as for Rabbit A. Samples 2 and 3 from both rabbits were not tested by this method.

(c) Immunoelectrophoresis.

The results of immunoelectrophoresis of antiserum 1 from Rabbit A against E.L.S.I are shown in Figure XVI. Precipitin lines are visible in the central part of the slide and seemed to correspond to the position of anti-albumin. When antiserum 1 (Rabbit A) was subjected to immunoelectrophoresis against human albumin an albumin/anti-albumin precipitin line was obtained (Figure XVII). Antiserum 1 from Rabbit B was similarly tested but

FIGURE XVI.

IMMUNOELECTROPHORESIS OF ANTISERUM 1. RABBIT A.



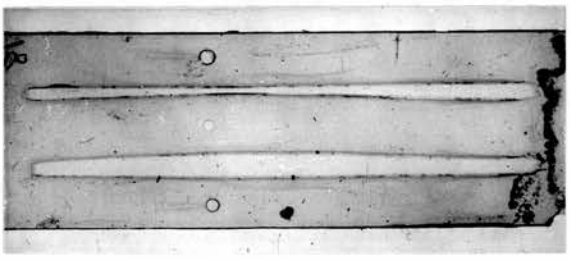
Central Well - Erythropoietin Laboratory Standard I.

Outer Wells - Normal human serum.

Channels - Antiserum 1, Rabbit A.

FIGURE XVII.

IMMUNOELECTROPHORESIS OF ANTISERUM 1. RABBIT B.



Central Well - Albumin

Outer Wells - Normal human serum.

Channels - Antiserum 1, Rabbit A.

there were no apparent precipitin lines against E.L.S.I

Antiserum sample 2, taken from each rabbit 16 days after the first sample, gave negative results when tested against E.L.S.I and against albumin.

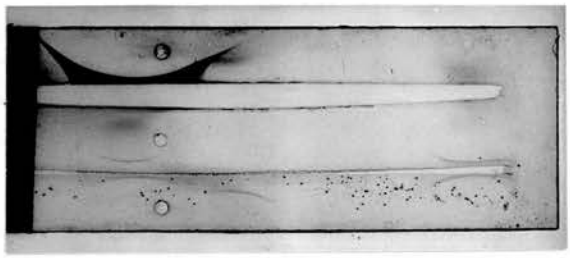
Immunoelectrophoresis of the third sample from Rabbits A and B gave the results shown in Figures XVIII and XIX. The nature of the precipitin lines visible in the E.L.S.I/antiserum 3 region on both slides is difficult to interpret. While it is possible that this is an albumin/anti-albumin line, as obtained with antiserum 1 of Rabbit A (Figure XVII) there are no clear cut precipitin lines of E.L.S.I against anti-albumin in Figures XVIII and XIX.

4. "In Vivo" Testing of Rabbit Antisera to Erythropoietin.

Antiserum 1 from Rabbit A alone was tested in vivo for its ability to neutralise the erythropoietic activity of E.L.S.I as measured by the assay described previously (Section II, p.67). Equal amounts of antiserum sample 1 and E.L.S.I were incubated together and injected into the polycythaemic mice (see p.139). The results of the assay were bizarre. The range of values for the percentage ^{59}Fe uptake in the mice within both control and experimental groups was large. For example, the mean and standard deviation figures for percentage ^{59}Fe uptake in four control mice were 34.41 ± 22.71 s.d. A figure as large as 34% for ^{59}Fe uptake by polycythaemic mice is in itself absurd. The cause of these anomalous results was eventually traced to radioactive contamination of sample tubes. Unfortunately, time did not permit repetition of this in vivo experiment, nor testing of the other antisera by the same method.

FIGURE XVIII.

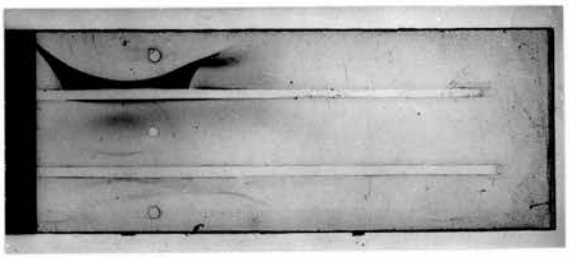
IMMUNOELECTROPHORESIS OF ANTISERUM 3, RABBIT A.



Central Well - Erythropoietin Laboratory Standard I.
Outer Wells - Normal human serum.
Upper channel - Anti-albumin.
Lower channel - Antiserum 3, Rabbit A.

FIGURE XIX.

IMMUNOELECTROPHORESIS OF ANTISERUM 3, RABBIT B.



Central Well - Erythropoietin Laboratory Standard I.
Outer Wells - Normal human serum.
Upper channel - Anti-albumin.
Lower channel - Antiserum 3, Rabbit B.

DISCUSSION.

Erythropoietin has a low level of antigenicity and the production of an antiserum to this hormone is associated with many problems. Because of time limitations, only two rabbits were used for the production of antisera in this study. Possibly a greater degree of success would have been achieved had more animals been used. Lange, et al. (1964) and Schooley and Garcia (1965) found that a suitable antiserum was produced in only 30-40% of the rabbits that they immunised.

Following the initial injections of antigenic material (which immunological investigation revealed as containing several precipitating antigens, including albumin), the haematocrit of Rabbit A decreased and continued to fall until the third week. Rabbit B was anaemic before the experiment but its haematocrit gradually rose during the injection period, in contrast to that of Rabbit A. Other workers have found that anaemia sometimes occurs in immunised animals during the development of neutralising antibodies to erythropoietin (Garcia and Schooley, 1963; Goudsmit, et al., 1967; Krugers-Dagneaux, 1967). This is taken as a sign of an auto-immune state in the rabbit, the production of antibodies against the injected erythropoietin causing neutralisation of endogenous erythropoietin. Schooley and Garcia (1965) reported a correlation between the erythropoietic neutralising ability and haematocrit, the rabbits with highest neutralising titres having the lowest haematocrits. In the present experiments the first serum sample from Rabbit A (whose haematocrit decreased) contained precipitating antibodies whereas these could not be detected in the serum of Rabbit B. However, it was not

known whether either serum had erythropoietic neutralising ability. If the maximum production of erythropoietin antibodies does coincide with the period of lowest haematocrit, then it is important to harvest serum at this time.

Because of the lack of a pure erythropoietin a partially purified extract (Erythropoietin Laboratory Standard I) was used for immunisation. It was therefore expected that the resulting antisera would contain contaminating antibodies to other proteins in E.L.S.I. Immunoelectrophoresis results showed anti-albumin to be the main precipitating contaminant of the antisera from Rabbits A and B. Had additional time been available for this study, such precipitating contaminants as albumin would have been removed by absorption with the appropriate protein. However, it should be noted that this would still have left non-precipitating contaminants in the sera. Opinion is divided as to whether erythropoietin produces precipitating or non-precipitating antibodies. Lange, et al. (1968) reported that the multiple precipitin lines which they observed on immunoelectrophoresis or gel diffusion of antisera produced against plasma or urinary erythropoietin preparations disappeared, or became a single line, when the antisera were absorbed with normal human serum; the absorbed sera retained their erythropoietic neutralising ability. Schooley and Garcia (1965) also found that sera with high neutralising ability showed no evidence of precipitating antibodies. On the other hand Goudsmit, et al. (1967) have reported that erythropoietin antibodies have precipitating properties.

Since the nature of erythropoietin antibodies is at

present ambiguous, *in vivo* tests for the erythropoietic neutralising ability of erythropoietin are of considerable importance. Unfortunately, the results of the assay in these studies were technically unsatisfactory and no conclusions could be drawn. Methods for the detection of erythropoietic neutralising ability vary among laboratories; they generally involve the effect of antiserum on ^{59}Fe erythrocyte uptake in normal mice or in polycythaemic mice in which erythropoiesis is stimulated by the injection of exogenous erythropoietin. The antiserum may be injected into the animal either directly (Garcia and Schooley, 1963; Rioux and Erslev, 1968) or after *in vitro* incubation with erythropoietin (Schooley and Garcia, 1965; Lange, et al., 1968; Rioux and Erslev, 1968). The latter method was selected in these experiments.

APPENDIX.

APPENDIX

A. LOW PRESSURE CHAMBER FOR THE INDUCTION OF POLYCYTHAEMIA IN ASSAY ANIMALS.

A hospital autoclave was adapted for use as a low pressure chamber. The general appearance of the apparatus is shown diagrammatically in Figure XX and in the photograph (Figure XXI).

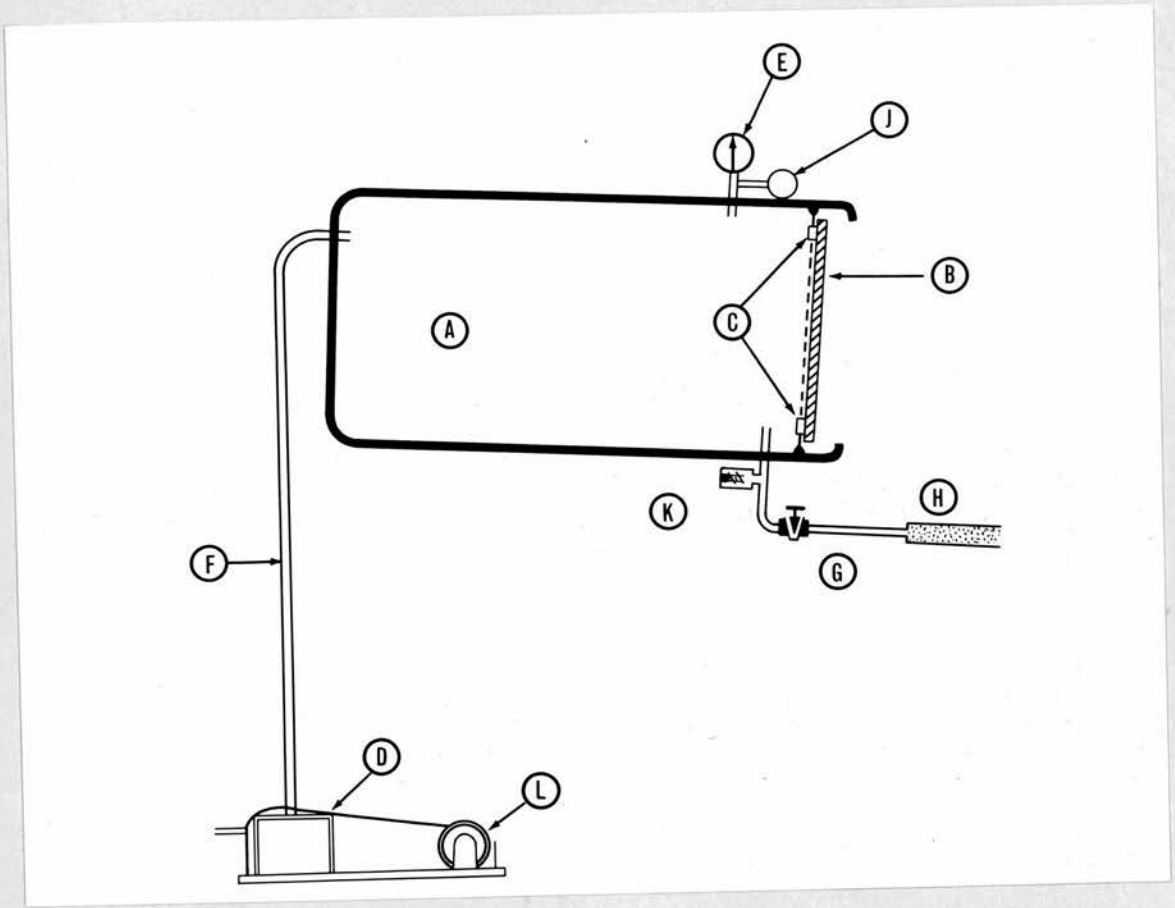
The heavy metal lid of the chamber (A) was replaced by a circular perspex disc 12.5 mm. thick (B). The sealing gasket was made of soft rubber (C). The chamber was permanently tilted forward to ensure that the perspex disc would be instantly released should the pressure in the chamber rise to atmospheric; this situation could arise from an interruption in the electrical power supply to the motor or a mechanical failure in the vacuum pump unit (D).

The pressure in the chamber was lowered by starting the vacuum pump motor after the perspex disc (B) had been clamped on by metal pressure pads. Decreasing pressure within the chamber was registered on the vacuum pressure gauge (E) and it was possible to remove the pressure pads once a partial vacuum had been established. The rate of fall of pressure was controlled by an orifice (F) which was inserted in the suction pipe between the chamber and the vacuum pump unit (D). A diameter of orifice was chosen which would allow the pressure to decrease gradually to 380 mm.Hg (the required working pressure) over a period of half to one hour, while air was admitted to the chamber through a fine control needle valve (G).

Atmospheric air was admitted to the chamber continuously via a simple cotton wool filter (H); the inflow was controlled by

FIGURE XX.

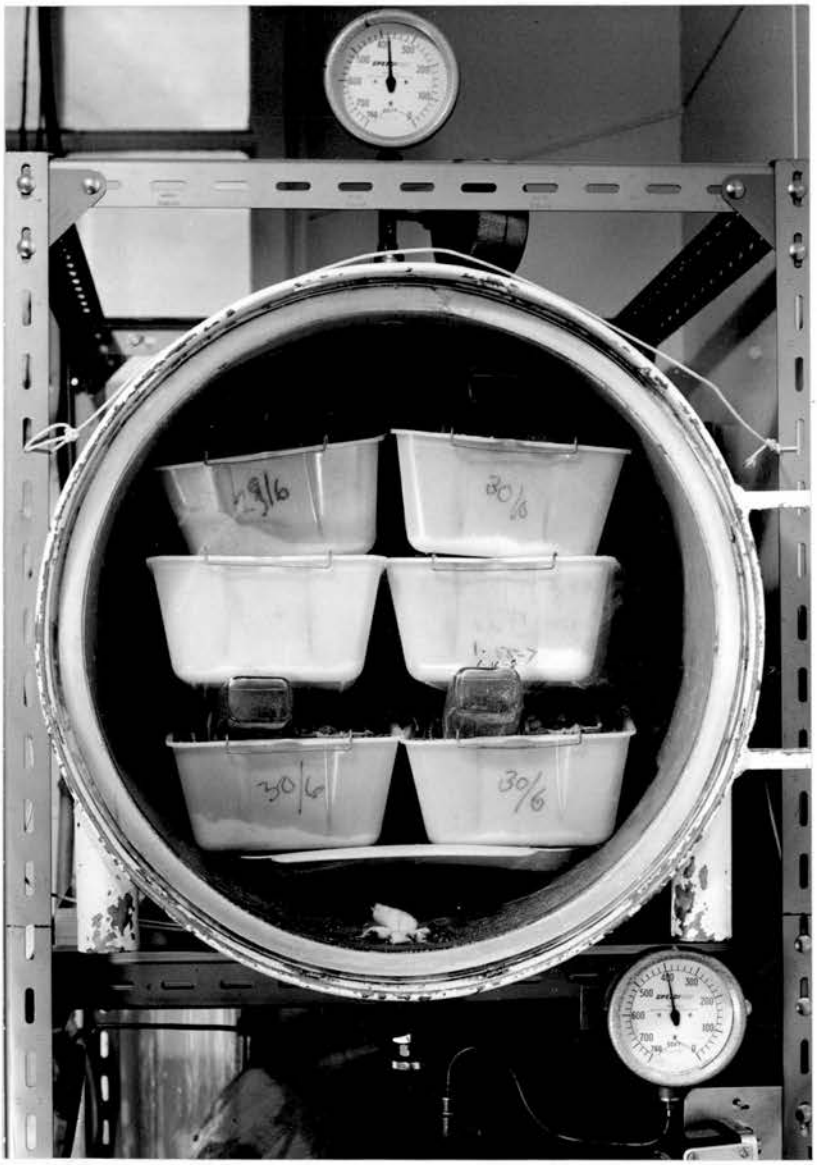
LOW PRESSURE CHAMBER : DIAGRAMMATIC REPRESENTATION



A.	Vacuum Chamber	F.	Orifice
B.	Perspex disc	G.	Needle valve
C.	Sealing gasket	H.	Cotton wool filter
D.	Vacuum pump (Edwards High Vacuum - Model ES75)	J.	Vacuum switch
E.	Dial gauge (Speedivac - Model CG.3)	K.	Pressure relief valve
		L.	Electric motor

FIGURE XXI.

LOW PRESSURE CHAMBER



The pressure gauge of the second chamber can be seen at the bottom of the photograph.

the needle valve (G), the setting of which was checked periodically. The working pressure of 380 mm.Hg was maintained by accurate setting of the needle valve (G) and the vacuum pressure microswitch (J).

The vacuum pressure microswitch was installed as a safety mechanism, its function being to cut off the electricity supply to the vacuum pump motor whenever the chamber pressure dropped below a preset level. As the air pressure in the chamber increased again the electricity supply became re-connected and re-started the vacuum pump motor. As a further safeguard, a pressure relief valve (K) was installed between the needle valve (G) and the chamber, and was set to open to the atmosphere in the event of a pressure switch fault causing the working pressure to fall below 370 mm.Hg.

Several mouse cages could be placed in the chamber, sufficient to accommodate 60 mice. A second chamber was constructed from a larger autoclave and accommodated 72 mice.

Self-indicating silica gel was used as a water vapour absorbent and was spread on trays along the bottom of the chamber.

The apparatus gave satisfactory service over a two-year period of almost continuous operation. Only two breakdowns occurred and both appeared to have resulted from the combined failure of the vacuum pressure microswitch and the second safety valve.

B. FURTHER DETAILS OF THE ASSAY METHOD.

(i) Measurement of Radio-Iron Incorporation.

The intravenous injection of 0.1 μc $^{59}Fe\ Cl_3$ was made into a lateral tail vein after the tail had been warmed in a small, thermostatically controlled water bath. The ferric chloride was dispensed at a concentration of 1 μc ^{59}Fe/ml. in physiological saline. Injection of 0.1 ml. volumes (containing 0.1 μc ^{59}Fe) was achieved using a micrometer syringe outfit ("Agla" syringe, Wellcome Reagents Ltd.). Accuracy of syringe -0.1 ml. ± 0.0014 s.d.

Mice were anaesthetized using ether and weighed (Ohaus animal balance). The chest wall was opened and blood was aspirated from the heart, using a 1 ml. plastic syringe, and transferred to a 1 ml. sequestrene-coated tube. Deep rather than light anaesthesia was found to facilitate the withdrawal of the maximum amount of blood from the heart. The blood sample was pipetted into a plain, disposable 10 ml. sample tube, haemolysed and made up to 5 ml. with water. The samples were counted in a well type scintillation counter and the radio-iron uptake calculated as described in Section II, p.69.

(ii) Blood Volume Estimation in Polycythaemic Mice.

The following method was used, based on that described by Lockner, et al. (1963) :-

Approximately 5 ml. of blood was aspirated from ether anaesthetized donor mice into a heparinised container and centrifuged for 10 minutes at 2,000 r.p.m. The plasma was separated off and retained. The erythrocytes were washed with acid citrate dextrose solution and incubated with sodium chromate $Na_2{}^{51}CrO_4$ (5 - 10 μc)

for one hour at room temperature. The cells were washed three times with cooled saline, centrifuging between washing. The cells were resuspended in their own plasma and 0.1 ml. of the suspension injected into a lateral tail vein using a 1 ml. disposable syringe (accuracy 0.1 ml. ± 0.0024 s.d.). An aliquot of the ^{51}Cr-labelled cell suspension was taken for the preparation of standards. The mice were killed during ether anaesthesia by exsanguination from the heart, and the blood prepared for counting as described in the assay method for ^{59}Fe. Initially, blood volume determinations were made in mice in which ^{59}Fe uptake was also measured and the radio-isotope emissions from ^{51}Cr and ^{59}Fe were then distinguished by pulse height discrimination techniques in the counting apparatus.

C. MATERIALS.

Antisera.

Anti-human sera. Hyland Laboratories, Los Angelos, U.S.A.

Apparatus.

Gel cutting dies. Shandon Scientific Co. Ltd., London.

Immunoelectrophoretic equipment. L.K.B. Instruments Ltd., London.

Microhaematocrit Centrifuge. Hawksley & Sons Ltd., Lancing, Sussex.

pH Meter. Radiometer, Copenhagen.

Refrigerated Centrifuge "Major". M.S.E. Ltd., London.

Radioactivity Counting Apparatus.

Counter - Tracerlab automatic sample changer, type "Gammaguard" No. GG.5334 (100 samples).

Counting Range - Generally 500 - 1500 keV (for ^{59}Fe).

Crystal - NaI (Thallium activated) 3" x 3" well type.

Chemicals.

Alum crystal B.P. Martindale Samoor Ltd., London.

Iron dextran injection B.P. "Imferon". Fisons Pharmaceuticals Ltd., Loughborough, Leics.

Anaesthetic Ether. Macfarlan Smith., Ltd., Edinburgh.

Acid Citrate Dextrose. Edinburgh and South-East Scotland Blood Transfusion Service.

Motor oil (vacuum pump) No. 17 grade. Edwards High Vacuum Ltd., Crawley, England.

Silica gel-coarse self indicating. B.D.H. Ltd., Poole, England.

Physiological Saline. Pharmacy Department, Edinburgh Royal Infirmary.

Saline merthiolate. Pharmacy Department, Edinburgh Royal Infirmary.

DEAE cellulose. Eastman Organic Chemicals, Rochester, New York.

Dialysis Tubing.

2" inflated diameter regenerated cellulose tubing. Viskase, Ltd., Glasgow.

Mouse Food.

'Oxoid' mouse cube diet 41B. Oxo, Ltd., London, E.C. 4.

Needles and Syringes.

"Plastipak" disposable. Becton Dickinson & Co. Ltd., Ireland.

Petri Dishes.

Disposable Petri dishes (9 cm. diameter). Sterilin Ltd., Richmond, Surrey.

Radioactive Material.

^{59}Fe-labelled ferric chloride in dil. HCl. Radiochemical Centre, Amersham, Bucks.

^{51}Cr-labelled sodium chromate. Radiochemical Centre, Amersham, Bucks.

Sample Tubes.

1 ml. sequestrene-coated paediatric tubes. Teklab (Medical Labs.) Ltd., Hockley, Essex.

10 ml. disposable tubes. Staynes Laboratories Ltd., High Wycombe, Bucks.

Microhaematocrit capillary tubes. Hawksley & Sons Ltd., Lancing, Sussex.

Slides.

Microscope slides (76 x 25 mm.). Arnold B. Horwell Ltd., London.

Slide cover glasses (3¼" square). Kodak Ltd., London.

D. DETERMINATION OF THE RELATIVE POTENCY OF TWO MATERIALS UNKNOWN (U) and STANDARD (S). A WORKED EXAMPLE.

In order to facilitate calculations, the original doses were linearly transformed :-

	Unknown		Standard	
Original doses (ml.)	0.05	0.50	0.05	0.50
x 200	10	100	10	100
$\log_{10}$	1	2	1	2

In the calculations which follow these figures 1, 2 : 1, 2 are used as the dose metameters 'x' which produce the response 'y'.

Unknown (U)					Standard (S)				
x	y	x^2	y^2	xy	x	y	x^2	y^2	xy
1	7.71	1	59.44	7.71	1	9.55	1	91.20	9.55
1	12.88	1	165.89	12.88	1	13.21	1	174.50	13.21
1	12.91	1	166.66	12.91	1	17.89	1	320.05	17.89
1	15.83	1	250.58	15.83					
2	41.12	4	1690.85	82.24	2	29.42	4	865.53	58.84
2	37.86	4	1433.37	75.72	2	33.95	4	1152.60	67.90
2	35.48	4	1258.83	70.96	2	33.18	4	1100.91	66.36
2	31.99	4	1023.36	63.98	2	34.57	4	1195.08	69.14
2	39.50	4	1560.25	79.00	2	27.04	4	731.16	54.08
2	30.37	4	922.33	60.74	2	33.60	4	1128.96	67.20
$\Sigma = 16$	$\Sigma = 265.65$	$\Sigma = 28$	$\Sigma = 8531.56$	$\Sigma = 481.97$	$\Sigma = 15$	$\Sigma = 232.41$	$\Sigma = 27$	$\Sigma = 6759.99$	$\Sigma = 424.17$
$(\Sigma x)^2 = 256$	$(\Sigma y)^2 = 70569.92$				$(\Sigma x)^2 = 225$	$(\Sigma y)^2 = 54014.40$			
$\bar{x} = 1.60$	$\bar{y} = 26.56$				$\bar{x} = 1.66$	$\bar{y} = 25.82$			

Analysis of Variance.

Unknown

Sum squares due to regression $= \dfrac{(\Sigma xy - \frac{\Sigma x \Sigma y}{n})^2}{\Sigma x^2 - \frac{(\Sigma x)^2}{n}} = \dfrac{56.93^2}{2.40} = 1350.42$

Total sum squares $= \Sigma y^2 - \dfrac{(\Sigma y)^2}{n} = 1474.57$

Residual sum squares $= 1674.57 - 1350.42 = 124.15$

Standard

Sum squares due to regression $= \dfrac{36.82^2}{2.00} = 677.85$

Total sum squares $= 758.39$

Residual sum squares $= 758.39 - 677.85 = 80.54$

Unknown				Standard			
Component	Sum of Squares	Degrees of freedom	Variance	Component	Sum of Squares	Degrees of freedom	Variance
Regression	1350.42	1	1350.42	Regression	677.85	1	677.85
Residual	124.15	8	15.51	Residual	80.54	7	11.50
Total	1474.57	9		Total	758.39	8	

Comparison of residual variance $= \dfrac{15.51}{11.50} = 1.34$

$F_{0.25}$ (8,7,df), from tables = 4.90 > 1.34

∴ variances not significantly different and 't' test can be applied :-

Slopes - $b_u = \frac{56.93}{2.40} = 23.72$ $\quad b_s = \frac{36.82}{2.00} = 18.41$

$$\text{Pooled slope } b = \frac{56.93 + 36.82}{2.40 + 2.00} = 21.30$$

$$\text{Pooled variance} = \frac{124.15 + 80.54}{(10 - 2) + (9 - 2)} = 13.64$$

$$\text{Standard error of difference between b's} = \sqrt{\frac{13.64}{2.40} + \frac{13.64}{2.00}} = 3.53$$

$$t = \frac{23.72 - 18.41}{3.53} = 1.50$$

∴ probability (p) for (10 - 2) + (9 - 2) degrees of freedom = $p > 0.10$

∴ slopes not significantly different.

Analysis of Variance - Pooled data

Correction factor for following calculation = $\frac{G^2}{n}$

Where G = sum of <u>all</u> y values

n = total number of observations

∴ Correction Factor = $\frac{(232.41 + 265.65)^2}{19} = 13055.9875$

Component — Sum of Squares

1. Linear Regression $= \dfrac{\left[\Sigma xy - \frac{\Sigma x \Sigma y}{n_{(u)}} + \Sigma xy - \frac{\Sigma x \Sigma y}{n_{(s)}}\right]^2}{\Sigma x^2 - \frac{(\Sigma x)^2}{n_{(u)}} + \Sigma x^2 - \frac{(\Sigma x)^2}{n_{(s)}}} = \underline{1997.5142}$

2. Deviation from parallelism = (4) minus (1) + (3)

$= 2030.8888 - (1997.5142 + 2.6045)$

$= \underline{30.7701}$

3. Between preparations

$= \dfrac{(\Sigma y_u)^2}{n_u} + \dfrac{(\Sigma y_s)^2}{n_s} - C.F. = \dfrac{70569.92}{10} + \dfrac{54014.40}{9} - 13055.9875$

$= \underline{2.6045}$

4. Between doses

$= \dfrac{(\Sigma y_u)^2}{n_u} + \dfrac{(\Sigma y_u)^2}{n_u} + \dfrac{(\Sigma y_s)^2}{n_s} + \dfrac{(\Sigma y_s)^2}{n_s} - C.F.$

Σy = sum of responses to each dose of unknown/standard

n = number of observations contributing to each dose of unknown/standard).

$= \dfrac{49.33^2}{4} + \dfrac{216.32^2}{6} + \dfrac{40.65^2}{3} + \dfrac{191.76^2}{6} - 13055.9875$

$= \underline{2030.8888}$

5. Residual (Error) = (6) minus (4) $= \underline{204.6737}$

6. Total = Σy^2 - C.F. = 6759.99 + 8531.56 - 13055.9875

$= \underline{2235.5625}$

Component	Sum Squares	Degrees Freedom	Mean Square	$F = \frac{\text{Mean Sq.}}{\text{Resid. M. Sq.}}$	p
Between doses	2030.8888	3	676.9629	49.6128	<0.001
Linear Regression	1997.5142	1	1997.5142	146.3927	<0.001
Deviations from Parallelism	30.7701	1	30.7701	2.2550	>0.10
Between Preparations	2.6045	1	2.6045	0.1908	>0.20
Residual	204.6737	15	13.6449		
Total	2235.5625	18			

Linear regression, Between doses } components both highly significant.

Between preparations differences, Deviations from parallelism } not significant

Assay very satisfactory and valid.

Estimate of potency.

$$\text{Log}_{10} \text{ potency, } M = \bar{x}_s - \bar{x}_u - \frac{(\bar{y}_s - \bar{y}_u)}{(\text{pooled } b)}$$

$$= 1.66 - 1.60 - \left(\frac{25.82 - 26.56}{21.3068}\right) = 0.0949$$

$$\therefore R = \text{Antilog } 0.0949 = 1.245$$

Potency of unknown:standard

= 1.245:1.

E. ERYTHROPOIETIN IN PATIENTS WITH RENAL DISEASE:

PATIENTS STUDIED.

(a) Renal Cystic Disease.

Patient 13 (Mrs. H.H., aged 66 years). This patient was found to have a large, well-defined mass arising from the lower pole of the left kidney; an intravenous pyelogram disclosed that the lower calyces were distorted by a cyst. The contents of the cyst were aspirated by needle under local anaesthesia and 240 ml. of straw-coloured fluid was obtained; an aliquot was taken for erythropoietin assay. Approximately 20 ml. of peripheral venous blood was taken at the same time and the plasma obtained after centrifugation was also stored for assay. Routine haematological data showed no abnormality.

Patient 14 (Mrs. A.M., aged 52 years). In this patient an intravenous pyelogram revealed a mass in the right kidney. Renal angiography disclosed that it was a large cyst in the upper, outer portion of the kidney. Cyst fluid was aspirated at an operation to excise the cyst and this was stored for erythropoietin assay. Pathological examination of the excised lesion showed that it was a simple unilocular cyst with a wall of fibrous tissue containing an occasional atrophic tubule; there was no epithelial lining. The peripheral blood findings were normal before operation and showed no significant change after removal of the cyst.

Patient 15 (Mr. M.C., aged 71 years). This patient was considered to have two separate diseases, bilateral polycystic renal disease and polycythaemia rubra vera. In addition to an increase in red cell mass there was a leucocytosis and hepatosplenomegaly..

A ten-hour urine collection was obtained for assay and the following day a small amount of renal cyst fluid was obtained by needle aspiration under local anaesthesia.

(b) Chronic Unilateral Pyelonephritis.

Patient 16 (Mr. W.K., aged 46 years). This man was admitted for the investigation of diastolic hypertension and was found to have erythrocytosis. Renal angiography revealed an hypertrophied right kidney and the left was small, contracted and irregular in outline; there was calyceal dilation in relation to the cortical scarring. The peripheral blood findings suggested erythrocytosis and this was confirmed by an increased red cell mass. It was considered possible that the erythrocytosis resulted from excessive production of erythropoietin by the diseased left kidney and an attempt was made to obtain blood from both renal veins for erythropoietin assay. Under fluoroscopic control a catheter was passed into the left renal vein via the right femoral vein and the sampling of renal venous blood was confirmed by estimating oxygen saturation and the extraction ratio of para-aminohippurate (Appendix F). Unfortunately it was technically impossible to catheterise the right renal vein and erythropoietin assays were therefore restricted to blood samples obtained from the left renal vein and a left antecubital vein.

(c) Hypernephroma and Associated Erythrocytosis.

Patient 17 (Mr. W.S., aged 41 years). As a result of radiological and haematological investigation this patient was found to have a carcinoma of the right kidney and erythrocytosis.

A peripheral venous plasma sample was obtained and a urine collection made for erythropoietin assay two days prior to nephrectomy. Histological examination of the tumour showed that it was a clear-cell carcinoma. Two months after operation the patient's erythrocytosis had remitted (Table 28).

TABLE 28. REMISSION OF ERYTHROCYTOSIS IN PATIENT 17 FOLLOWING NEPHRECTOMY.

Haematological data	Pre-nephrectomy	Post-nephrectomy 1 day	2 months
Haemoglobin (g./100 ml.)	20.6	20.2	14.1
P.C.V. (%)	66	65	43
M.C.H.C. (g./100 ml. red cells)	32	32	33
W.B.C. (per cu.mm.)	7,100	16,000	6,200

(d) Renal Homotransplant and Suspected Mild Erythrocytosis.

Patient 18 (Mrs. M.R., aged 25 years). This young woman was suspected of having a mild erythrocytosis three years after a successful renal transplant operation for chronic renal failure. Although her red cell mass (29.2 ml. per Kg. of body weight) was at the upper limit of the normal range, there was a slight chance that this value indicated mild polycythaemia. A six hour urine collection was obtained for erythropoietin assay.

F. METHODS USED TO CONFIRM THAT BLOOD OBTAINED BY CATHETER IN PATIENT 16 WAS FROM RENAL VEIN.

1. Determination of Oxygen Saturation.

The oxygen saturation of blood obtained by catheter was measured by the Kipp reflection oximeter technique. A high value of 92.1 per cent was obtained and provided confirmatory evidence of renal venous sampling.

2. Estimation of Renal Extraction Ratio of Sodium Para-aminohippurate.

The extraction ratio of para-aminohippurate (PAH) is the fraction of this substance removed from the plasma during a single passage through the kidney and is given by $(A - V)/A$ where A and V are, respectively, the concentrations in the arterial and renal venous plasma. Since there is no significant difference between the concentration of PAH in renal arterial blood and systemic venous blood, the extraction ratio of PAH is calculated from the levels of PAH obtained in samples of blood taken simultaneously from a renal vein and a peripheral vein. In Patient 16 blood sampling was performed whilst the plasma concentrations of PAH were maintained at a relatively constant level, this being achieved by the administration of a priming injection of PAH, followed by an intravenous sustaining infusion (Smith, et al., 1945). Blood was sampled simultaneously from the renal vein of the diseased left kidney and a left antecubital vein, the PAH being infused into a right antecubital vein. The PAH was estimated colorimetrically by the diazotisation method of Bratton and Marshall (1939) after precipitation of plasma proteins by cadmium sulphate (Fujita and Iwatake (1931) (see Appendix C).

The concentrations of PAH in the peripheral venous plasma and renal venous plasma were 2.73 and 0.99 mg. per 100 ml. respectively, the extraction ratio being 0.63. This extraction ratio is consistent with values previously reported in diseased kidneys (Bergström, et al., 1959) and provided additional evidence of renal venous sampling.

C. DETERMINATION OF PAH CONCENTRATION IN HUMAN PLASMA SAMPLES.

(a) Precipitation of Plasma Proteins by Cadmium Sulphate (Fujita and Iwatake, 1931).

A cadmium sulphate plasma filtrate was prepared as follows :-

34.67 g. $3CdSO_4.8H_2O$ plus 169 ml. 1.0N H_2SO_4 were made up to 1 litre with distilled water and filtered.

A 1.0N NaOH solution was prepared in distilled water.

The following were added in sequence to an Erlenmeyer Flask :-

20 ml. of water

6 ml. of cadmium sulphate solution

2 ml. of plasma: mixed well and added dropwise with constant agitation.

2 ml. of 1.0N NaOH.

The flask was stoppered, shaken well and allowed to stand for 10 minutes, shaking at least once. The solution was centrifuged for 10 minutes at 3,500 r.p.m. and filtered. This procedure yielded at least 24 ml. of filtrate of 1/15 dilution.

(b) Estimation of PAH by Diazotisation Method (Bratton and Marshall, 1939).

The following were pipetted into an Erlenmeyer Flask :-

10 ml. of $CdSO_4$ plasma filtrate, distilled water or PAH standard solution, each in duplicate.

2 ml. of 1.2N HCl

1 ml. of 0.10% $NaNO_2$

These solutions were mixed and not before 3 minutes and not after 5 minutes was added :-

1 ml. of 0.5% ammonium sulphate

The solution was mixed and not before 3 minutes and not after 5 minutes was added :-

1 ml. of 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride.

The solution was mixed and after 15 minutes was read in a photoelectric colorimeter* at a wavelength of 540 mμ against the reagent blank prepared with 10 ml. distilled water. The optical density of five standard solutions of PAH was plotted against concentration and the concentration of the experimental plasmas read from the graph.

* E.E.L. Spectra colorimeter.

H. TIMETABLE OF EXPERIMENTAL PROCEDURE IN SUBJECT 2.

OBSERVATIONS ON THE EFFECTS OF HYPOXAEMIA ON THE PLASMA LEVELS AND URINARY EXCRETION RATES OF ERYTHROPOIETIN.

Time	Procedures	Sample Code No.
09.57	Catheter inserted into left radial artery.	
09.58	Arterial blood sample taken (blood gas analysis).	I
10.08	Cannula inserted into right antecubital vein.	
10.47	Arterial blood sample taken (blood gas analysis).	II
10.48	Arterial blood sample taken (PAH blank).	Po
10.53	Bladder emptied (PAH blank).	Uo
10.54	Oral hydration with 500 ml. water.	
10.56	Intravenous injection of priming dose of PAH.	
10.57	Sustaining infusion of PAH given in 5% dextrose into right antecubital vein at 7 ml./min.	
10.58	Subject started to breathe O_2-poor gas mixture.	
11.11	Arterial blood sample taken (blood gas analysis).	III
11.25	Subject changed to breathing room air. Bladder emptied (urine discarded).	
11.29	Subject returned to breathing O_2-poor gas mixture.	
11.45	Arterial blood sample taken (PAH)	P1
11.58	Arterial blood sample taken (blood gas analysis).	IV
12.05	Subject changed to breathing room air. Bladder emptied (PAH and erythropoietin assay).	U1
12.08	Subject returned to breathing O_2-poor gas mixture.	
12.25	Arterial blood sample taken (PAH)	P2
12.37	Subject changed to breathing room air. Bladder emptied (PAH and erythropoietin assay).	U2
12.39	Subject returned to breathing O_2-poor gas mixture.	
12.54	Arterial blood sample taken (PAH and erythropoietin assay).	P3
13.01	Arterial blood sample taken (blood gas analysis).	V

Time	Procedures	Sample Code No.
	Continued	
13.54	Subject changed to breathing room air. Bladder emptied (PAH and erythropoietin assay).	U3
13.57	Subject returned to breathing O_2-poor gas mixture.	
14.08	Arterial blood sample taken (blood gas analysis).	VI
14.26	Arterial blood sample taken (PAH).	P4a
14.45	Arterial blood sample taken (blood gas analysis).	VII
14.45	Arterial blood sample taken (PAH).	P4b
14.50	Subject returned to breathing room air.	
14.51	Arterial blood sample taken (erythropoietin assay).	P4c
14.56	Bladder emptied (PAH and erythropoietin assay).	U4
18.55	Venous blood sample taken (erythropoietin assay).	P5
19.00	Bladder emptied (erythropoietin assay).	U5
21.00	Bladder emptied (erythropoietin assay).	U6

J. PREPARATION OF ALUM-PRECIPITATED ERYTHROPOIETIN LABORATORY STANDARD I (E.L.S.I).

The method of Proom (1943) was used :-

11 ml. E.L.S.I and 37 ml. distilled water were mixed together, and to this was added 41 ml. of a 10% solution of potassium alum in distilled water. The pH was adjusted to 6.5 by the dropwise addition of 2.5N sodium hydroxide, at which point the precipitate appeared. After centrifuging the suspension, the precipitate was washed twice with 200 ml. of 1:10,000 saline merthiolate, and the final precipitate made up to 9 ml. with saline merthiolate.

REFERENCES.

REFERENCES.

Abbrecht, P.H. and Greene, J.A.
1965 J. Lab. clin. Med. 66 : 849.
"Erythropoiesis After Renal Homotransplantation."

Abbrecht, P.H. and Greene, J.A.
1966 Ann. intern. Med. 65 : 908.
"Serum Erythropoietin After Renal Homotransplantation."

Abbrecht, P.H. and Malvin, R.L.
1966 Amer. J. Physiol. 210 : 237.
"Renal Production of Erythropoietin in the Dog."

Abbrecht, P.H., Malvin, R.L. and Vander, A.J.
1966 Nature 211 : 1318.
"Renal Production of Erythropoietin and Renin after Experimental Kidney Infarction."

Abbrecht, P.H., Turcotte, J.G. and Vander, A.J.
1968 J. Lab. clin. Med. 71 : 766.
"Plasma Erythropoietin and Renin Activity After Canine Renal Allotransplantation."

Adamson, J.W.
1968 Blood 32 : 597.
"The Erythropoietin-Hematocrit Relationship in Normal and Polycythemic Man. Implications of Marrow Regulation."

Adamson, J.W. and Finch, C.A.
1968 Ann. N.Y. Acad. Sci. 149 : 560.
"Erythropoietin and the Polycythemias."

Adamson J.W. and Stamatoyannopoulos, G.
1967 Blood 30 : 848.
"Erythrocytosis Associated with Abnormal Hemoglobins: Aspects of Marrow Regulation."

Adamson, J.W., Alexanian, R., Martinez, C. and Finch, C.A.
1966 Blood 28 : 354.
"Erythropoietin Excretion in Normal Man."

Alexanian, R.
1966 Blood 28 : 344.
"Urinary Excretion of Erythropoietin in Normal Men and Women."

Alpen, E.L. and Cranmore, D.
1959 "The Kinetics of Cellular Proliferation." 1st Edition. Edited by Stohlman, F. Published Grune and Stratton, New York. p.290. "Observations on the Regulation of Erythropoiesis and on Cellular Dynamics by Fe^{59} Auto-Radiography."

Alpen, E.L., Lajtha, L.G. and Van Dyke, D.C.
1959 Nature 184 : 1228.
"Lack of Direct Effect of Erythropoietin on Human Erythroid Cells *in vitro*."

Alt, H.L.
1938 J. Nutr. 16 : 597.
"Relation of Growth and Nutrition to Reticulocyte Level in the Young Rat."

Andersen, S.B. and Ladefoged, J.
1963 Acta Haematol. 30 : 319.
"Pure Red Cell Anaemia and Thymoma."

Andrews, P.
1965 Biochem. J. 96 : 595.
"The Gel-Filtration Behaviour of Proteins Related to Their Molecular Weights over a Wide Range."

Becker, A.J., McCulloch, E.A. and Till, J.E.
1963 Nature 197 : 452.
"Cytological Demonstration of the Clonal Nature of Spleen Colonies Derived from Transplanted Mouse Marrow Cells."

Berger, E.Y., Gladston, M. and Horowitz, S.
1949 J. clin. Invest. 28 : 648.
"The Effect of Anoxic Anoxia on the Human Kidney."

Bergström, J., Bucht, H., Ek, J., Josephson, B., Sundell, H. and Werkö, L.
1959 Scand. J. clin. Lab. Invest. 11 : 361.
"The Renal Extraction of Para-Aminohippurate in Normal Persons and in Patients with Diseased Kidneys."

Bilsel, Y.C., Wood, J.E. and Lange, R.D.
1963 Proc. Soc. exp. Biol. Med. 114 : 475.
"Angiotensin II and Erythropoiesis."

Bleiberg, I., Liron, M. and Feldman, M.
1965 Transplantation 3 : 706.
"Reversion by Erythropoietin of the Suppression of Erythroid Clones Caused by Transfusion-Induced Polycythemia."

Bonsdorff, E. and Jalavisto, E.
1949 Acta Physiol. Scand. 16 : 150.
"A Humoral Mechanism in Anoxic Erythrocytosis."

Borsook, H., Graybiel, A., Keighley, G. and Windsor, E.
1954 Blood 9 : 734.
"Polycythemic Response in Normal Adult Rats to a Non-Protein Plasma Extract from Anemic Rabbits."

Borsook, H., Lingrel, J.B., Scaro, J.L. and Millette, R.L.
1962 Nature 196 : 347.
"Synthesis of Haemoglobin in Relation to the Maturation of Erythroid Cells."

Bourgoignie, J.J., Gallagher, N.I., Perry, H.M., Kurz, L., Warnecke, M.A. and Donati, R.M.
1968 J. Lab. clin. Med. 71 : 523.
"Renin and Erythropoietin in Normotensive and in Hypertensive Patients."

Bratton, A.C. and Marshall, E.K.
1939 J. biol. Chem. 128 : 537.
"A New Coupling Component for Sulfanilamide Determination."

Brown, R.
1965 Brit. med. J. II : 1036.
"Plasma Erythropoietin in Chronic Uraemia."

Bruce, W.R. and McCulloch, E.A.
1964 Blood 23 : 216.
"The Effect of Erythropoietic Stimulation on the Hemopoietic Colony-Forming Cells of Mice."

Bullard, R.W., Broumand, C. and Meyer, F.R.
1966 J. appl. Physiol. 21 : 994.
"Blood Characteristics and Volume in Two Rodents Native to High Altitude."

Caldwell, F.T., Rolf, D. and White, H.L.
1949 J. appl. Physiol. 1 : 597.
"Effects of Acute Hypoxia on Renal Circulation in Man."

Calne, R.Y., Loughridge, L., MacGillivray, J.B. and Swales, J.D.
1966 Brit. med. J. II : 1345.
"Further Observations on Renal Transplants in Man from Cadaveric Donors."

Camiscoli, J.F., Weintraub, A.H. and Gordon, A.S.
1968 Ann. N.Y. Acad. Sci. 149 : 40.
"Comparative Assay of Erythropoietin Standards."

Cannon, P. and Penington, D.G.
1967 Lancet II : 1276.
"Erythropoietin and Hepatoma."

Carmena, A., Lucarelli, G., Carnevali, C. and Stohlman, F.
1966 Proc. Soc. exp. Biol. Med. 121 : 652.
"Regulation of Erythropoiesis. XIX. Effect of Hypoxia on Erythropoiesis in the Newborn Animal."

Carmena, A.O., Garcia de Testa, N. and Frias, F.L.
1967 Proc. Soc. exp. Biol. Med. 125 : 441.
"Urinary Erythropoietin in Men Subjected to Acute Hypoxia."

Carmena, A.O., Howard, D. and Stohlman, F.
1968a Abst. XIIth Cong. Int. Soc. Haemat.
"Erythropoiesis in the Newborn Rat."

Carmena, A.O., Howard, D. and Stohlman, F.
1968b Blood 32 : 376.
"Regulation of Erythropoiesis. XXII. Erythropoietin Production in the Newborn Animal."

Carnot, P. and Déflandre, C.
1906 C.R. Acad. Sci. 143 : 384.
"Sur l'Activité Hemopoiétique du Sérum au Cours de la Régénération du Sang."

Charache, S., Wetherall, D.J. and Clegg, J.B.
1966 J. clin. Invest. 45 : 813.
"Polycythemia Associated with a Hemoglobinopathy."

Cohen, H.N.
1960 Arch. intern. Med. 105 : 301.
"Polycythemia Associated with Bilateral Unilocular Renal Cysts."

Conley, C.L., Kowal, J. and D'Antonio, J.
1957 Bull. Johns Hopk. Hosp. 101 : 63.
"Polycythemia Associated with Renal Tumors."

Contrera, J.F., Camiscoli, J.F., Weintraub, A.H. and Gordon, A.S.
1965 Blood 25 : 809.
"Extraction of Erythropoietin from Kidneys of Hypoxic and Phenylhydrazine-Treated Rats."

Contrera, J.F., Gordon, A.S. and Weintraub, A.H.
1966 Blood 28 : 330.
"Extraction of an Erythropoietin-Producing Factor from a Particulate Fraction of Rat Kidney."

Cook, W.F. and Pickering, G.W.
1962 Biochem. Pharm. 2 : 165.
"The Location of Renin in the Kidney.

Cooper, G.W. and Nocenti, M.R.
1961 Proc. Soc. exp. Biol. Med. 108 : 546.
"Unilateral Renal Ischemia and Erythropoietin."

Cotes, P.M. and Bangham, D.R.
1961 Nature 191 : 1065.
"Bio-Assay of Erythropoietin in Mice Made Polycythaemic by Exposure to Air at a Reduced Pressure."

Cotes, P.M. and Bangham, D.R.
1966 Bull. Wld. Hlth. Org. 35 : 751.
"The International Reference Preparation of Erythropoietin."

Cotes, P.M. and Lowe, R.D.
1963 Mem. Soc. Endocrin. 13 : 187.
"The Influence of Renal Ischaemia on Red Cell Formation."

Dahl, J.D., Blaisdell, R.K. and Beutler, E.
1959 Proc. Soc. exp. Biol. Med. 101 : 622.
"Gastric Ulceration in Rats with Experimentally-Induced Polycythemia."

Damon, A., Holub, D.A., Melicow, M.M. and Uson, A.C.
1958 Amer. J. Med. 25 : 182.
"Polycythemia and Renal Carcinoma."

DeGowin, R.L., Hofstra, D. and Gurney, C.W.
1962a Proc. Soc. exp. Biol. Med. 110 : 48.
"A Comparison of Erythropoietin Bio-Assays."

DeGowin, R.L., Hofstra, D. and Gurney, C.W.
1962b J. Lab. clin. Med. 60 : 846.
"The Mouse with Hypoxia-Induced Erythremia, an Erythropoietin Bio-Assay Animal."

DeGowin, R., Hofstra, D. and Gurney, C.W.
1962c Fed. Proc. 21 : 71.
"Comparison of Sensitivities of In Vivo Systems for Bio-Assay of Erythropoietin."

Demopoulos, H.B., Higuman, B., Altland, P.D., Gerving, M.A. and Kaley, G.
1965 Amer. J. Path. 46 : 497.
"Effects of High Altitude on Granular Juxtaglomerular Cells and Their Possible Role in Erythropoietin Production."

Denny, W.F., Flanigan, W.J. and Zukoski, C.F.
1966 J. Lab. clin. Med. 67 : 386.
"Serial Erythropoietin Studies in Patients Undergoing Renal Homotransplantation."

DeWeerd, J.H. and Hagedorn, A.B.
1959 J. Urol. 82 : 29.
"Hypernephroma Associated with Polycythemia."

Donati, R.M., McCarthy, J.M., Lange, R.D. and Gallagher, N.I.
1963 Ann. intern. Med. 58 : 47.
"Erythrocythemia and Neoplastic Tumors."

Donati, R.M., Bourgoignie, J.J., Kuhn, C., Gallagher, N.I. and Perry, H.M.
1968 Circulat. Res. 22 : 91.
"Dissociation of Circulating Renin and Erythropoietin in Rats."

Dukes, P.P.
1967 Proc. 19th Mosbach Col. Soc. Phys. Chem. 5 : 197.
"Erythropoietin - Its Nature and Mechanism of Action."

Dukes, P.P.
1968 Biochim. Biophys. Res. Com. 31 : 345.
"Erythropoietin-Stimulated Incorporation of 1-^{14}C-Glucosamine into Glycolipids of Bone Marrow Cells in Culture."

Dukes, P.P. and Goldwasser, E.
1966 Biochim. biophys. Acta 108 : 447.
"On the Mechanism of Erythropoietin-Induced Differentiation. 3. The Nature of Erythropoietin Action on ^{14}C-Glucosamine Incorporation by Marrow Cells in Culture."

Dukes, P.P., Takaku, F. and Goldwasser, E.
1964 Endocrinology 74 : 960.
"In vitro Studies on the Effect of Erythropoietin on Glucosamine-1-^{14}C Incorporation into Rat Bone Marrow Cells."

Dukes, P.P., Hammond, D. and Shore, N.A.
1969 J. Lab. clin. Med. 74 : 250.
"Comparison of Erythropoietin Preparations Yielding Different Dose-Response Slopes in the Exhypoxic Polycythemic Mouse Assay."

Edelman, R. and Hartroft, P.M.
1961 Circul. Res. 9 : 1069.
"Localisation of Renin in Juxtaglomerular Cells of Rabbit and Dog Through the Use of the Fluorescent-Antibody Technique."

Erslev, A.J.
1953 Blood 8 : 349.
"Humoral Regulation of Red Cell Production."

Erslev, A.J.
1955 Blood 10 : 954.
"Physiological Control of Red Cell Production."

Erslev, A.J.
1962 Proc. Soc. exp. Biol. Med. 110 : 615.
"Effect of Erythropoietin on the Uptake and Utilisation of Iron by Bone Marrow Cells In Vitro."

Erslev, A.J.
1964 Blood 24 : 331.
"Erythropoietin In Vitro. II. Effect on 'Stem Cells'."

Erslev, A.J. and Hughes, J.R.
1960 Brit. J. Haemat. 6 : 414.
"The Influence of Environment on Iron Incorporation and Mitotic Division in a Suspension of Normal Bone Marrow."

Erslev, A.J., Solit, R.W., Camishion, R.C., Amsel, S., Ilda, J. and Ballinger, W.F.
1965 Amer. J. Physiol. 208 : 1153.
"Erythropoietin In Vitro. II. Perfusion of a Lung-Kidney Preparation."

Erslev, A.J., McKenna, P.J., Capelli, J.P., Hamburger, R.J. Cohn, H.E. and Clark, J.E.
1967 Clin. Res. 15 : 275.
"Erythropoiesis in Nephrectomized Patients."

Eschbach, J.W., Funk, D., Adamson, J., Kuhn, I., Scribner, B.H. and Finch, C.A.
1967 New Engl. J. Med. 276 : 653.
"Erythropoiesis in Patients with Renal Failure Undergoing Chronic Dialysis."

Evans, E.S., Rosenberg, L.L. and Simpson, M.E.
1961 Endocrinology 68 : 517.
"Erythropoietic Response to Calorigenic Hormones."

Fenn, W.O. and Rahn, H.
1965 Handbook of Physiology. Section III : Vol. II. "Respiration". Published - American Physiological Society. p.1101.

Filmanowicz, E. and Gurney, C.W.
1961 J. Lab. clin. Med. 57 : 65.
"Studies of Erythropoiesis. XVI. Response to a Single Dose of Erythropoietin in the Polycythemic Mouse."

Finne, P.H.
1964 Acta paediat. 53 : 269.
"Erythropoietin Levels in the Amniotic Fluid, Particularly in Rh-Immunized Pregnancies."

Finne, P.H.
1965 Brit. med. J. I : 697.
"Erythropoietin in Concentrates of Urine from Healthy Persons."

Finne, P.H.
1966a Acta paediat. 55 : 478.
"Erythropoietin Levels in Cord Blood as an Indicator of Intra-Uterine Hypoxia."

Finne, P.H.
1966b Acta paediat. 55 : 609.
"Ante-Natal Diagnosis of the Anemia in Erythroblastosis."

Finne, P.H.
1967 Acta paediat. 56 : 233.
"On Placental Transfer of Erythropoietin."

Finne, P.H.
1968 Ann. N.Y. Acad. Sci. 149 : 497.
"Erythropoietin Production in Fetal Hypoxia and in Anemic Uremic Patients."

Finney, D.J.
1952 "Statistical Method in Biological Assay." 1st Edition. Published - Charles Griffin & Co. Ltd., London.

Fischer, S.
1962 "Erythropoiesis". Edited Jacobson, L.O. and Doyle, M. 1st Edition. Published - Grune & Stratton, New York. p.204. "Studies on the Mechanism and Site of Action of Erythropoietin."

Fischer, J. and Friederici, L.
1961 Experientia 17 : 318.
"Erythropoese bei Bilateral Nephrektomierten Kaninchen."

Fisher, J.W. and Birdwell, B.J.
1961 Acta haemat. 26 : 224.
"The Production of an Erythropoietic Factor by the *in situ* Perfused Kidney."

Fisher, J.W. and Crook, J.J.
1962 Blood 19 : 557.
"Influence of Several Hormones on Erythropoiesis and Oxygen Consumption in the Hypophysectomized Rat."

Fisher, J.W. and Langston, J.W.
1967 Blood 29 : 114.
"The Influence of Hypoxemia and Cobalt on Erythropoietin Production in the Isolated Perfused Dog Kidney."

Fisher, J.W. and Samuels, A.I.
1967 Proc. Soc. exp. Biol. Med. 125 : 482.
"Relationship Between Renal Blood Flow and Erythropoietin Production in Dogs."

Fisher, J.W., Sanzari, M.P., Birdwell, B.J. and Crook, J.J.
1962 "Erythropoiesis." Edited Jacobson, L.O. and Doyle, M. 1st Edition. Published - Grune & Stratton, New York. p.78. "The Role of the Kidney in Erythropoietin Production."

Fisher, J.W., Knight, D.B. and Couch, C.
1963 J. Pharmacol. exp. Ther. 141 : 113.
"The Influence of Several Diuretic Drugs on Erythropoietin Formation."

Fisher, J.W., Schofield, R. and Porteous, D.D.
1965a Brit. J. Haemat. 11 : 382.
"Effects of Renal Hypoxia on Erythropoietin Production."

Fisher, J.W., Taylor, G. and Porteous, D.D.
1965b Nature 205 : 611.
"Localisation of Erythropoietin in Glomeruli of Sheep Kidney by Fluorescent Antibody Technique."

Fisher, J.W., Roh, B.L. and Halvorsen, S.
1967a Proc. Soc. exp. Biol. Med. 126 : 97.
"Inhibition of Erythropoietic Effects of Hormones by Erythropoietin Antisera in Mildly Plethoric Mice."

Fisher, J.W., Samuels, A.I. and Langston, J.W.
1967b J. Pharmacol. exp. Ther. 157 : 618.
"Effects of Angiotensin and Renal Artery Constriction on Erythropoietin Production."

Fisher, R.A. and Yates, F.
1948 "Statistical Tables for Biological, Agricultural and Medical Research." 3rd Edition. Published - Oliver & Boyd, London and Edinburgh.

Fogh, J.
1966 Scand. J. clin. Lab. Invest. 18 : 33
"A Sensitive Erythropoietin Assay in Mice Exposed to CO-Hypoxia."

Forssell, J.
1954 Acta med. scand. 150 : 155.
"Polycythemia and Hypernephroma."

Forssell, J.
1958 Acta med. scand. 161 : 169.
"Nephrogenous Polycythaemia."

Freda, V.J.
1962 Amer. J. Obstet. Gynec. 84 : 1756.
"Placental Transfer of Antibodies in Man."

Freedman, B.J., and Penington, D.G.
1963 Brit. J. Haemat. 9 : 425.
"Erythrocytosis in Emphysema."

Frey, W.G.
1958 New Engl. J. Med. 258 : 842.
"Polycythemia and Hypernephroma."

Fried, W., Plzak, L., Jacobson, L.O. and Goldwasser, E.
1956 Proc. Soc. exp.Biol. Med. 92 : 203.
"Erythropoiesis. II. Assay of Erythropoietin in Hypophysectomised Rats."

Fried, W., Plzak, L.F., Jacobson, L.O. and Goldwasser, E.
1957 Proc. Soc. exp. Biol. Med. 94 : 237.
"Studies on Erythropoiesis. III. Factors Controlling Erythropoietin Production."

Fried, W., Ward, H.P. and Hopeman, A.R.
1968 Blood 31 : 813.
"Leiomyoma and Erythrocytosis: A Tumour Producing a Factor which Increases Erythropoietin Production.

Fried, W., Kilbridge, T., Krantz, S., McDonald, T.P. and Lange, R.D.
1969 J. Lab. clin. Med. 73 : 244.
"Studies on Extra-Renal Erythropoietin."

Fruhman, G.T. and Fischer, S.
1962 Experientia 18 : 462.
"The Short-Term Effects of a Single Dose of Erythropoietin upon Reticulocytes in Starved Rats."

Fujioka, S.
1966 J. Lab. clin. Med. 67 : 937.
"Effect of Erythropoietin upon Nucleic Acid Synthesis in Erythropoietic Tissue."

Fujita, A. and Iwatake, A.
1931 Biochem. Z. 242 : 43.
"Bestimmung des Echten Blutzuckers Ohne Hefe."

Gaddum, J.H.
1933 Special Report Series of Med. Res. Counc., London. No. 183.
"Reports on Biological Standards."

Gaddum, J.H.
1953 Pharmacol. Rev. 5 : 87.
"Bio-Assays and Mathematics."

Gallagher, N.I. and Donati, R.M.
1968 Ann. N.Y. Acad. Sci. 149 : 528.
"Inappropriate Erythropoietin Elaboration."

Gallagher, N.I., McCarthy, J.M., Hart, K.T. and Lange, R.D.
1959 Blood 14 : 662.
"Evaluation of Plasma Erythropoietic-Stimulating Factors in Anemic Uremic Patients."

Gallagher, N.I., McCarthy, J.M. and Lange, R.D.
1960 Ann. intern. Med. 52 : 1201.
"Observations on Erythropoietic Stimulating Factor (E.S.F.) in the Plasma of Uremic and Non-Uremic Anemic Patients."

Gallagher, N.I., Hagan, D.Q., McCarthy, J.M. and Lange, R.D.
1961a Proc. Soc. exp. Biol. Med. 106 : 127.
"Response of Starved Rats and Polycythemic Rats to Graded Doses of Erythropoietin."

Gallagher, N.I., McCarthy, J.M. and Lange, R.D.
1961b J. Lab. clin. Med. 57 : 281.
"Erythropoietin Production in Uremic Rabbits."

Gallien-Lartigue, O. and Goldwasser, E.
1965 Biochim. biophys. Acta 103 : 319.
"On the Mechanism of Erythropoietin-Induced Differentiation. I. The Effects of Specific Inhibitors on Hemoglobin Synthesis."

Garcia, J.F. and Schooley, J.C.
1963 Proc. Soc. exp. Biol. Med. 112 : 712.
"Immunological Neutralization of Various Erythropoietins."

Garcia, J.F. and Schooley, J.C.
1965 Proc. Soc. exp. Biol. Med. 120 : 614.
"Dose-Response Relationships for Erythropoietin Given in Serum or in Saline."

Gardner, E., Wright, C.S., Lewis, J.P. and Moores, R.R.
1966 J. clin. Invest. 45 : 1011.
"Erythropoietin Potentiation by Serum Proteins."

Gardner, E., Wright, C.S., Lewis, J.P. and Moores, R.R.
1967 Brit. J. Haemat. 13 : 317.
"Potentiation of Purified Erythropoietin with Serum Proteins."

Gelfand, M.M., Strean, G.J., Pavilanis, V. and Sternberg, J.
1960 Amer. J. Obstet. Gynec. 79 : 117.
"Studies in Placental Permeability."

Gibson, J.G., Weiss, S., Evans, R.D., Peacock, W.C., Irvine, J.W., Good, W.M. and Kipa, F.
1946 J. clin. Invest. 25 : 616.
"The Measurement of the Circulating Red Cell Volume by Means of Two Radioactive Isotopes of Iron."

Goldfarb, B. and Tobian, L.
1962 Proc. Soc. exp. Biol. Med. 111 : 150.
"The Inter-Relationship of Hypoxia, Erythropoietin and the Renal Juxtaglomerular Cell."

Goldfarb, B. and Tobian, L.
1963a Proc. Soc. exp. Biol. Med. 112 : 65.
"Relationship of Erythropoietin to Renal Juxtaglomerular Cells."

Goldfarb, B. and Tobian, L.
1963b Proc. Soc. exp. Biol. Med. 113 : 35
"Effect of High Oxygen Concentrations on Erythropoietin and the Renal Juxtaglomerular Cell."

Goldwasser, E.
1966 Current Topics in Developmental Biology 1 : 173.
"Biochemical Control of Erythroid Cell Development."

Goldwasser, E. and Kung, C.K.H.
1968 Ann. N.Y. Acad. Sci. 149 : 49.
"Progress in the Purification of Erythropoietin."

Goldwasser, E. and White, W.F.
1959 Fed. Proc. 18 : 236.
"Purification of Sheep Erythropoietin."

Goldwasser, E., Fried, W. and Jacobson, L.O.
1958 J. Lab. clin. Med. 52 : 375.
"Studies on Erythropoiesis. VIII. The Effect of Nephrectomy on Response to Hypoxic Anoxia."

Goldwasser, E., White, W.F. and Taylor, K.B.
1962 "Erythropoiesis." Edited Jacobson, L.O. and Doyle, M.
" 1st Edition. Published - Grune & Stratton, New York.
p.43. "On the Purification of Sheep Plasma Erythropoietin."

Goldwasser, E., Gallien-Lartigue, O. and Dukes, P.P.
1964 Fed. Proc. 23 : 168.
"The Effect of Actinomycin D on Erythropoietin Action In Vitro."

Gordon, A.S., Winkert, J., Dornfest, B.S. and Siegel, C.D.
1959 Ann. N.Y. Acad. Sci. 77 : 650.
"Studies on the Actions and Properties of the Circulating Erythropoietic Stimulating Factor."

Gordon, A.S., LoBue, J., Dornfest, B.S. and Cooper, G.W.
1962 "Erythropoiesis." Edited Jacobson, L.O. and Doyle, M. 1st Edition. Published - Grune & Stratton, New York. p.321. "Reticulocyte and Leucocyte Release from Isolated Perfused Rat Legs and Femurs."

Gordon, A.S., Cooper, G.W. and Zanjani, E.D.
1967 Seminars in Hematol. 4 : 337.
"The Kidney and Erythropoiesis."

Goudsmit, R., Krugers-Dagneaux, P.G.L.C. and Krijnen, H.W.
1967 Folia Med. Neerl. 10 : 39.
"Een Immunochemische Bepaling Van Erythropoietine."

Graham, L.A., Winzler, R.J. and Charles, H.E.
1963 Endocrinology 73 : 475.
"Separation and Partial Characterisation of Erythropoietin from Human Urine."

Grant, W.C., Linkenheimer, W.H., Patterson, E.L. and Berger, H.
1961 Proc. Soc. exp. Biol. Med. 107 : 221.
"Partial Purification of Erythropoietin by Alcoholic Fractionation."

Gross, M. and Goldwasser, E.
1969 Biochemistry 8 : 1795.
"On the Mechanism of Erythropoietin-Induced Differentiation. V. Characterisation of the Ribonucleic Acid Formed as a Result of Erythropoietin Action."

Gurney, C.W.
1965 Ann. Rev. Med. 16 : 169.
"Polycythemia Vera and Some Possible Pathogenetic Mechanisms."

Gurney, C.W. and Hofstra, D.
1963 Radiat. Res. 19 : 599.
"Assessment of Actinomycin and Radiation Damage of Stem Cells by the Erythropoietin Tolerance Test."

Gurney, C.W. and Pan, C.
1958 Proc. Soc. exp. Biol. Med. 98 : 789.
"Studies on Erythropoiesis. IX. Mechanism of Decreased Erythropoiesis in Experimental Polycythemia."

Gurney, C.W. and Pan, C.
1960 J. Lab. clin. Med. 55 : 67.
"Studies on Erythropoiesis. XIII. A Comparison of Methods of Bioassay of Erythropoietin in Human Plasma."

Gurney, C.W., Goldwasser, E. and Pan, C.
1957 J. Lab. clin. Med. 50 : 534.
"Studies on Erythropoiesis. VI. Erythropoietin in Human Plasma."

Gurney, C.W., Wackman, N. and Filmanowicz, E.
1961 Blood 17 : 531.
"Studies on Erythropoiesis. XVII. Some Quantitative Aspects of the Erythropoietic Response to Erythropoietin."

Hahn, P.F., Bale, W.F., Ross, J.F., Hettig, R.A. and Whipple, G.H.
1940 Science 92 : 131.
"Radio-Iron in Plasma Does not Exchange with Hemoglobin Iron in Red Cells."

Halvorsen, S.
1963 Acta paediat. 52 : 425.
"Plasma Erythropoietin Levels in Cord Blood and in Blood during the First Weeks of Life."

Halvorsen, S. and Finne, P.H.
1963 Brit. med. J. I : 1132.
"Transfer of Erythropoietin to Amniotic Fluid in Rh-Immunized Pregnant Women."

Hammond, G.D., Ishikawa, A. and Keighley, G.
1962 "Erythropoiesis." Edited Jacobson, L.O. and Doyle, M. 1st Edition. Published - Grune & Stratton, New York. p.351. "Relationship Between Erythropoietin and Severity of Anemia in Hypoplastic and Hemolytic States."

Hammond, D., Shore, N. and Movassaghi, N.
1968 Ann. N.Y. Acad. Sci. 149 : 516.
"Production, Utilisation and Excretion of Erythropoietin:
I. Chronic Anemias. II. Aplastic Crisis.
III. Erythropoietic Effects of Normal Plasma."

Hansen, P.
1963a Scand. J. clin. Lab. Invest. 15 : 357.
"Studies on Determination of Erythropoietin using ^{59}Fe Incorporation in Starved Rats."

Hansen, P.
1963b Scand. J. clin. Lab. Invest. 15 : 622.
"Studies on a Method of Concentrating Erythropoietin from Plasma and Kidney Extract."

Hansen, P.
1963c Scand. J. clin. Lab. Invest. 15 : 631.
"The Precipitation of Erythropoietin from Urine by Means of Tannic Acid."

Hansen, P.
1964a Acta path. microbiol. scand. 60 : 465.
"Polycythaemia Produced by Constriction of the Renal Artery in a Rabbit."

Hansen, P.
1964b Acta path. microbiol. scand. 61 : 514.
"Demonstration of Erythropoietin in Urine and in Kidney Extracts from Rabbits with Experimental Constriction of the Left Renal Artery."

Hartroft, P.M. and Hartroft, W.S.
1953 J. exp. Med. 97 : 415.
"Studies on Renal Juxtaglomerular Cells."

Hewlett, J.S., Hoffman, G.C., Senhauser, D.A. and Battle, J.D.
1960 New Engl. J. Med. 262 : 1058.
"Hypernephroma with Erythrocythemia."

Hirashima, K. and Takaku, F.
1962 Blood 20 : 1.
"Experimental Studies on Erythopoietin. II. The relationship between Juxtaglomerular Cells and Erythropoietin."

Hodgson, G.
1967 Proc. Soc. exp. Biol. Med. 124 : 1045.
"Synthesis of RNA and DNA at Various Intervals after Erythropoietin Injection in Transfused Mice."

Hodgson, G., Eskuche, I., Yudilevich, D., Hernandez, P. and Toha, J.
1957 Proc. Soc. exp. Biol. Med. 96 : 826.
"Effects of Plasma from Bled and Phenylhydrazine Treated Animals on Plasma Iron Turnover. A Test for 'hemopoietine'."

Hodgson, G., Perreta, M., Yudilevich, D. and Eskuche, I.
1958 Proc. Soc. exp. Biol. Med. 99 : 137.
"Assay of 'Hemopoietine' in Starved Animals: Properties of Urinary Hemopoietine."

Hodgson, G., Fischer, S., Perretta, M., Eskuche, I., Araya, G. and Dinamarca, M.
1960 Blood 16 : 1398.
"Separation and Properties of Human Urinary Hemopoietine."

Hoffman, G.C.
1968 Ann. N.Y. Acad Sci. 149 : 504.
"Human Erythropoiesis Following Kidney Transplantation."

Hudgson, P., Pearce, J.M.S., Yeates, W.K.
1967 Brit. med. J. I : 18.
"Renal Artery Stenosis with Hypertension and High Haematocrit."

Jacobson, L.O., Plzak, L., Fried, W. and Goldwasser, E.
1956 Nature 177 : 1240.
"Plasma Factor(s) Influencing Red Cell Production."

Jacobson, L.O., Goldwasser, E., Fried, W. and Plzak, L.
1957 Nature 179 : 633.
"Role of the Kidney in Erythropoiesis."

Jacobson, L.O., Marks, E.K., Gaston, E.O. and Goldwasser, E.
1959 Blood 14 : 635.
"Studies on Erythropoiesis. XI. Reticulocyte Response of Transfusion-Induced Polycythemic Mice to Anemic Plasma from Nephrectomised Mice and to Plasma from Nephrectomised Rats Exposed to Low Oxygen."

Jacobson, L.O., Goldwasser, E. and Gurney, C.W.
1960 Ciba Foundation Symposium. "Haemopoiesis." Edited Wolstenholme and O'Connor. Published - Churchill, London. p.423. "Transfusion-Induced Polycythaemia as a Model for Studying Factors Influencing Erythropoiesis."

Jaworski, Z.F. and Wolan, C.T.
1963 Amer. J. Med. 34 : 523.
"Hydronephrosis and Polycythemia."

Jepson, J.H. and Friesen, H.G.
1968 Brit. J. Haemat. 15 : 465.
"The Mechanism of Action of Human Placental Lactogen on Erythropoiesis."

Jepson, J.H. and Lowenstein, L.
1964 Proc. Soc. exp. Biol. Med. 116 : 921.
"Response of the Polycythemic Mouse and Polycythemic Guinea Pig to Human Erythropoietin."

Jepson, J. and Lowenstein, L.
1968a Brit. J. Haemat. 14 : 555.
"Hormonal Control of Erythropoiesis during Pregnancy in the Mouse."

Jepson, J.H. and Lowenstein, L.
1968b Canad. J. Physiol. Pharm. 46 : 573.
"Role of Erythropoietin and Placental Lactogen in the Control of Erythropoiesis during Pregnancy."

Jepson, J. and McGarry, E.E.
1968 Blood 32 : 370.
"Polycythaemia and Increased Erythropoietin Production in a Patient with Hypertrophy of the Juxtaglomerular Apparatus."

Jerne, N.K. and Perry, W.L.M.
1956 Bull. Wld. Hlth. Org. 14 : 167.
"The Stability of Biological Standards."

Jones, B. and Klingberg, W.G.
1960 J. pediat. 56 : 752.
"A Study of Erythropoietin in Two Types of Hemolytic Anemia - Erythroblastosis Fetalis and Sickle Cell Anemia."

Jones, N.F., Payne, R.W., Hyde, R.D. and Price, T.M.L.
1960 Lancet I : 299.
"Renal Polycythaemia."

Jones, R.T., Osgood, E.E., Brimhall, B. and Koler, R.D.
1967 J. clin. Invest. 46 : 1840.
"Hemoglobin Yakima. I. Clinical and Biochemical Studies."

Karlson, P.
1963 Perspectives in Biol. Med. 6 : 203.
"New Concepts on the Mode of Action of Hormones."

Keighley, G., Lowy, P.H., Borsook, H., Goldwasser, E., Gordon, A.S. Prentice, T.C., Rambach, W.A., Stohlman, F. and Van Dyke, D.C.
1960 Blood 16 : 1424.
"A Co-operative Assay of a Sample with Erythropoietic Stimulating Activity."

Korst, D.R. and Bethell, F.H.
1958 J. Lab. clin. Med. 52 : 356.
"Erythrocyte Radio-Iron Uptake in the Rat and the Effects of Cobalt."

Korst, D.R., Bishop, R.C. and Bethell, F.H.
1958 J. Lab. clin. Med. 52 : 364.
"Assay of Erythropoietic Factor(s) Using Radio-Iron Uptake in the Nitrogen Mustard Treated Rat."

Korst, D.R., Whalley, B.E. and Bethell, F.H.
1959 J. Lab. clin. Med. 54 : 916.
"Erythropoietic Activity of Plasma in Polycythemia."

Korst, D.R., Frenkel, E.P. and Wilhelm, J.E.
1962 "Erythropoiesis." Edited Jacobson, L.O. and Doyle, M. 1st Edition. Published - Grune & Stratton, New York. p.310. "Studies Utilising Short-Term Marrow Culture. I. Ferrokinetics and Erythropoietin."

Krantz, S.B.
1965 Life Sciences 4 : 2393.
"The Effect of Erythropoietin on Human Bone Marrow Cells In Vitro."

Krantz, S.B.
1967 Blood 30 : 848.
"In Vitro Studies of Polycythemia Vera."

Krantz, S.B.
1968 J. Lab. clin. Med. 71 : 999.
"Response of Polycythemia Vera Marrow to Erythropoietin In Vitro."

Krantz, S.B. and Goldwasser, E.
1965 Biochim. biophys. Acta 103 : 325.
"On the Mechanism of Erythropoietin-Induced Differentiation. II. The Effect on RNA Synthesis."

Krantz, S.B., Gallien-Lartigue, O. and Goldwasser, E.
1963 J. biol. Chem. 238 : 4085.
"The Effect of Erythropoietin upon Heme Synthesis by Marrow Cells In Vitro."

Krugers-Dagneaux, P.C.L.C.
1967 Thesis, University of Amsterdam.
"Erythropoietine. Een chemisch en immunologisch onderzoek."

Krugers-Dagneaux, P.C.L.C., Goudsmit, R. and Krijnen, H.W.
1968 Ann. N.Y. Acad. Sci. 149 : 294.
"Investigations on An Immunoassay of Erythropoietin."

Kulis, J.C.
1964 Illinois med. J. 125 : 537.
"Erythrocytosis Caused by a Renal Cyst with Remission of Polycythemia Following Its Removal."

Kuratowska, Z. and Lewartowski, B.
1962 "Erythropoiesis." Edited Jacobson, L.O. and Doyle, M. 1st Edition. Published - Grune & Stratton, New York. p.101. "Studies on the Active Principle Released by the Hypoxic Kidney into Tyrode's Solution."

Kuratowska, Z., Lewartowski, B. and Michalak, E.
1960 Bull. Acad. Pol. Sci. 8 : 77.
"Studies on the Production of Erythropoietin by the Isolated Hypoxic Kidney."

Kuratowska, Z., Lewartowski, B. and Michalak, E.
1961 Blood 18 : 527.
"Studies on the Production of Erythropoietin by Isolated Perfused Organs."

Kuratowska, Z., Kowalski, E., Lipinski, B. and Michalak, E.
1962 "Erythropoiesis." Edited Jacobson, L.O. and Doyle, M. 1st Edition. Published - Grune & Stratton, New York. p.58. "Human Blood as a Source for Preparation of Erythropoietin."

Kuratowska, Z., Lewartowski, B. and Lipinski, B.
1964 J. Lab. clin. Med. 64 : 236.
"Chemical and Biological Properties of an Erythropoietin-Generating Substance Obtained from Perfusates of Isolated Anoxic Kidneys."

Kurtides, E.S., Rambach, W.A. and Alt, H.L.
1965 Blood 25 : 292.
"Erythropoiesis in Rats with Acute Uremia and the Effect of Erythropoietin."

Lajtha, L.G. and Oliver, R.
1960 Ciba Foundation Symposium. "Haemopoiesis." Edited Wolstenholme and O'Connor. Published - Churchill, London. p.289. "Studies of the Kinetics of Erythropoiesis: A Model of the Erythron."

Landois, L.
1875 "Die Transfusion Des Blutes." Published - F.C.W. Vogel, Leipzig.

Landsteiner, K.
1900 Zbl. Bakt. I. Abt. Orig. 27 : 357.
"Zur Kenntnis Der Antifermentativen Lytischen und Agglutinierenden Wirkunzen Des Blutserums und der Lymphe."

Lange, R.D. and Gallagher, N.I.
1962 "Erythropoiesis." Edited Jacobson, L.O. and Doyle, M. 1st Edition. Published - Grune & Stratton, New York. p.361. "Clinical and Experimental Observations on the Relationship of the Kidney to Erythropoietin Production."

Lange, R.D., Gardner, E., Wright, C.S. and Gallagher, N.I.
1964 Brit. J. Haemat. 10 : 69.
"Neutralisation of the Biological Activity of Erythropoietin by Immune Sera."

Lange, R.D., Simmons, M.L. and Dibelius, R.
1966 Proc. Soc. exp. Biol. Med. 122 : 761.
"Polycythemic Mice Produced by Hypoxia in Silicone Rubber Membrane Enclosures: A New Technique."

Lange, R.D., O'Grady, L.F., Lewis, J.P. and Trobaugh, F.E.
1968 Ann. N.Y. Acad. Sci. 149 : 281.
"Application of Erythropoietin Antisera to Studies of Erythropoiesis."

Lange, R.D., McDonald, T.P. and Jordan, T.
1969 J. Lab. clin. Med. 73 : 78.
"Antisera to Erythropoietin: Partial Characterisation of Two Different Antibodies."

Lassen, N.A.
1964 Circulat. Res. 15 : Suppl. 2 : 1-183.
"The Metabolic Demand of the Kidney as Related to Variations of Renal Blood Flow."

Lawrence, J.H. and Donald, W.G.
1959 Ann. intern. Med. 50 : 959.
"Polycythemia and Hydronephrosis or Renal Tumors."

Lea, D.E.
1955 "Action of Radiations on Living Cells." 2nd Edition. Published Cambridge University Press.

Lewis, J.P., Gallagher, N.I., Carmody, S.E. and Lange, R.D.
1964 Proc. Soc. exp. Biol. Med. 116 : 742.
"Erythropoietic Activity in Human Urine Concentrate."

Lewis, J.P., Alford, D.A., Rathjen, J.H. and Lange, R.D.
1965a J. Lab. clin. Med. 66 : 987.
"Preparation of Human Urine Concentrate for Erythropoietin Studies."

Lewis, J.P., Gallagher, N.I., Carmody, S.E. and Lange, R.D.
1965b Biochim. biophys. Acta 104 : 218.
"Erythropoietin Activity and Protein-Protein Interactions in Human Urine."

Lewis, J.P., Alford, D.A., Wright, C.S., Gardner, E., Rathjen, J.H. and Moores, R.R.
1967 Acta Haemat. 38 : 372.
"Fractionation of Erythropoietin by Selective Membrane Permeability."

Lewis, J.P., Alford, D.A., Moores, R.R., Gardner, E., Wright, C.S., Smith, L.L., Scharnitzky, A. and Neal, W.A.
1969 J. Lab. clin. Med. 73 : 154.
"Dialysis as a Tool in the Study of Erythropoietin."

Linkenheimer, W.H., Grant, W.C., Berger, H. and Hall, R.H.
1959 Proc. Soc. exp. Biol. Med. 100 : 225.
"Effect of Erythropoiesis on ^{32}P-Uptake by Deoxyribonucleic Acid of Spleen in the Rat."

Linman, J.W., Korst, D.R. and Bethell, F.H.
1959 Ann. N.Y. Acad. Sci. 77 : 638.
"Some Observations on the Stimulation of Erythropoiesis by Humoral Factors."

Lockner, D., Sletten, K. and DeHevesy, G.
1963 Brit. J. Cancer 17 : 328.
"Studies on Cancer Anaemia."

Lorraine, J.A. and Bell, E.T.
1966 "Hormone Assays and Their Clinical Application."
2nd Edition. Published - Livingstone Ltd., Edinburgh.

Lowy, P.H. and Borsook, H.
1962 "Erythropoiesis." Edited Jacobson, L.O. and Doyle, M.
1st Edition. Published - Grune & Stratton, New York.
p.33. "Preparation and Properties of Erythropoietin Concentrates from Rabbit Plasma and Human Urine."

Lowy, P.H. and Keighley, G.
1961 Nature 192 : 75.
"Use of Phenol in the Isolation of Erythropoietic Glycoprotein."

Lowy, P.H. and Keighley, G.L.
1966 Clin. chim. Acta 13 : 491.
"Extraction of Erythropoietin from Human Urine and Rabbit Plasma."

Lowy, P.H. and Keighley, G.L.
1968 Fed. Proc. 27 : 394.
"Inactivation of Erythropoietin by Koshland's Tryptophan Reagent."

Lowy, P.H., Keighley, G.L., Borsook, H. and Graybiel, A.
1959 Blood 14 : 262.
"On the Erythropoietic Principle in the Blood of Rabbits made Severely Anemic with Phenylhydrazine."

Lowy, P.H., Keighley, G. and Borsook, H.
1960 Nature 185 : 102.
"Inactivation of Erythropoietin by Neuraminidase and by Mild Substitution Reactions."

Lucarelli, G., Howard, D. and Stohlman, F.
1964 J. clin. Invest. 43 : 2195.
"Regulation of Erythropoiesis. XV. Neonatal Erythropoiesis and the Effect of Nephrectomy."

Lucarelli, G., Rizzoli, V., Carnevali, C. and Ferrari, L.
1968 Abstr. XIIth Cong. Int. Soc. Haemat.
"Effect of Erythropoietin (EP) in Fasted Neonatal Rats."

Luke, R.G., Kennedy, A.C., Barr-Stirling, W. and McDonald, G.A.
1965 Brit. med. J. I : 164.
"Renal Artery Stenosis, Hypertension and Polycythaemia."

Lukowsky, W. and Painter, R.H.
1968 Canad. J. Biochem. 46 : 731.
"Molecular Weight of Erythropoietin from Anemic Sheep Plasma and Human Urine."

Manase, B. and Jepsen, J.
1969 Canad. Med. Ass. J. 100 : 687.
"Erythropoietin in Plasma and Urine during Human Pregnancy."

Mann, D.L., Gallagher, N.I. and Donati, R.M.
1967 Ann. intern. Med. 66 : 335.
"Erythropoytosis and Primary Aldosteronism."

Mantz, J.M., Hammann, B. and Warter, J.
1967 Path-Biol. 15 : 663.
"L'origine rénale de l'erythropoïétine."

Matoth, Y. and Ben-Porath, E.
1959 J. Lab. clin. Med. 54 : 722.
"Effect of Erythropoietin on the Mitotic Rate of Erythroblasts in Bone Marrow Cultures."

Matoth, Y. and Kaufmann, L.
1962 Blood 20 : 165.
"Mitotic Activity In Vitro of Erythroblasts Previously Exposed to Erythropoietin."

Matoth, Y. and Zaizov, R.
1969 J. clin. Invest. 48 : 57a.
"Regulation of Erythropoiesis in the Rat Fetus."

MacFadzean, A.J.S., Todd, D. and Tso, S.C.
1967 Blood 29 : 808.
"Erythrocytosis Associated with Hepatocellular Carcinoma."

McMain, P.B., Hoffman, G.C., Nakamoto, S. and Kolff, W.J.
1965 Canad. med. Ass. J. 93 : 241.
"Erythropoiesis and Renal Homotransplantation."

Melby, E.C., Murphy, G.P. and Mirand, E.A.
1965 Invest. Urol. 3 : 92.
"Erythropoietin Levels in Response to Ureteral Occlusion or Re-Insertion in the Rhesus Monkey."

Mirand, E.A.
1965 Invest. Urol. 2 : 579.
"Studies on Erythropoietin in Tamarins."

Mirand, E.A.
1968 South Afr. med. J. 42 : 462.
"Renal and Extra-Renal Relations to Erythropoietin Production in Animals and Man."

Mirand, E.A. and Prentice, T.C.
1957 Proc.Soc. exp.Biol. Med. 96 : 49.
"Presence of Plasma Erythropoietin in Hypoxic Rats with or without Kidney(s) and/or Spleen."

Mirand, E.A., Johnston, G.S., Huser, H.J. and Murphy, G.P.
1965a Invest. Urol. 2 : 574.
"Renal Transplantation in the Rhesus Monkey. V. Alterations in Erythropoietin Activity."

Mirand, E.A., Murphy, G.P., Weldon, G.S., Melby, E.V., Huser, H.J. and Johnston, G.S.
1965b Clin. Res. 13 : 279.
"Alterations in Erythropoietin Following Renal Transplantation in the Rhesus Monkey."

Mirand, E.A., Weintraub, A.H., Gordon, A.S., Prentice, T.C. and Grace, J.T.
1965c Proc. Soc. exp. Biol. Med. 118 : 823.
"Erythropoietic Activity of Untreated and Deproteinised Normal Human Plasma."

Mirand, E.A., Murphy, G.P., Johnston, G.S. and Gordon, A.S.
1967 J. surg. Res. 7 : 261.
"Erythropoietic Responses to Acute and Chronic Ureteral Occlusions in Dogs."

Mirand, E.A., Murphy, G.P., Steeves, R.A., Weber, H.W. and Retieg, F.P.
1968a Acta Haemat. 32 : 359.
"Extra-Renal Production of Erythropoietin in Man."

Mirand, E.A., Murphy, G.P., Bennett, T.B. and Grace, J.T.
1968b Life Sciences 7 : 689.
"Erythropoietin Response to Repeated Hemorrhage in Renal Allo-transplanted, Nephrectomized or Intact Dogs."

Mirand, E.A., Steeves, R., Van Zyl, J.J.W. and Murphy, G.P.
1968c Proc. Soc. exp. Biol. Med. 128 : 785.
"Erythropoietin Release in Baboons after Renal Allotransplantation."

Mirand, E.A., Murphy, G.P., Steeves, R.A., Groenewald, J.M. and DeKlerk, J.N.
1969 J. Lab. clin. Med. 73 : 121.
"Erythropoietin Activity in Anephric Allotransplanted, Unilaterally Nephrectomised and Intact Man."

Mitus, W.J. and Toyama, K.
1964 Arch. Path. 78 : 658.
"Experimental Renal Erythrocytosis. Role of the Juxtaglomerular Apparatus."

Mitus, W.J., Galbraith, P., Gollerkeri, M. and Toyama, K.
1964 Blood 24 : 343.
"Experimental Renal Erythrocytosis. I. Effects of Pressure and Vascular Interference."

Miura, Y., Mizoguchi, H., Takaku, F. and Nakao, K.
1968 Blood 31 : 433.
"In Vitro Effect of Erythropoietin on the Spleen of the Polycythemic Mouse. III. Limited Exposure to Erythropoietin and Actinomycin D."

Moores, R.R., Stohlman, F. and Brecher, G.
1963 Blood 22 : 286.
"Humoral Regulation of Erythropoiesis. XI. The Pattern of Response to Specific Therapy in Iron Deficiency Anemia."

Moores, R.R., Gardner, E., Wright, C.S. and Lewis, J.P.
1966 Proc. Soc. exp. Biol. Med. 123 : 618.
"Potentiation of Purified Erythropoietin with Serum Proteins. II. Serial Dose Response Relationships."

Morse, B.S. and Stohlman, F.
1966 J. clin. Invest. 45 : 1241.
"Regulation of Erythropoiesis. XVIII. The Effect of Vincristine and Erythropoietin on Bone Marrow."

Movassaghi, N., Shore, N.A. and Hammond, D.
1967 Proc. Soc. exp. Biol. Med. 126 : 615.
"Serum and Urinary Levels of Erythropoietin in Iron Deficiency Anemia."

Murphy, G.P., Melby, E.C., Mirand, E.A., Huser, H.J. and Weldon, C.S.
1965 Invest Urol. 3 : 244.
"Renal Transplantation in the Rhesus Monkey. VI. Observations in Homotransplanted Animals."

Murphy, G.P., Mirand, E.A., Wade, J.C. and Melby, E.C.
1967a Invest. Urol. 5 : 234.
"Erythropoietin Response to Renal Stress in the Chimpanzee."

Murphy, G.P., Mirand, E.A., Johnston, G.S., Gibbons, R.P., Jones, R.L. and Scott, W.W.
1967b Johns Hopk. med. J. 120 : 26.
"Erythropoietin Release Associated with Wilms' Tumor."

Murphy, G.P., Mirand, E.A., Steeves, R., Retief, F.P., Stockenström, J. Van Buuren, A.J., Keet, M.P., Retief, C.P., Van Zyl, J.J.W. and De Klerk, J.N.
1968a South Afr. med. J. 42 : 581.
"The Presence of Erythropoietin in an Anephric Human Before and After Renal Homo-Transplantation."

Murphy, G.P., Mirand, E.A., Johnston, G.S., Gibbons, R.P., Schirmer, H.K.A. and Scott, W.W.
1968b J. Urol. 99 : 802.
"Erythropoietin Alterations in Human Genitourinary Disease States: Correlation with Experimental Observations."

Naets, J.P.
1960a Proc. Soc. exp.Biol. Med. 103 : 129.
"Erythropoietic Factor in Kidney Tissue of Anemic Dogs."

Naets, J.P.
1960b Blood 16 : 1770.
"The Role of the Kidney in the Production of the Erythropoietic Factor."

Naets, J.P.
1963 Mem. Soc. Endocrin. 13 : 175.
"The Role of the Kidney in Erythropoiesis in the Dog."

Naets, J.P. and Heuse, A.F.
1962a Nature 195 : 190.
"Effect of Anaemic Anoxia on Erythropoiesis of Nephrectomized Dogs."

Naets, J.P. and Heuse, A.F.
1962b J. Lab. clin. Med. 60 : 365.
"Measurement of Erythropoietic Stimulating Factor in Anemic Patients with or without Renal Disease."

Naets, J.P. and Wittek, M.
1968 Lancet I : 941.
"Erythropoiesis in Anephric Man."

Nakao, K., Takaku, F. and Hirashima, K.
1960 Proc. Soc. exp. Biol. Med. 103 : 47.
"Clinical Studies on Erythropoietic Factor in Plasma."

Nakao, K., Shirakura, T., Azuma, M. and Maekawa, T.
1967 Blood 29 : 754.
"Studies on Erythropoietic Action of Angiotensin II."

Nies, B.A., Cohn, R. and Schrier, S.L.
1965 New Engl. J. Med. 273 : 785.
"Erythremia After Renal Transplantation."

Nixon, R.K., O'Rourke, W., Rupe, C.E. and Korst, D.R.
1960 Arch. intern. Med. 106 : 797.
"Nephrogenic Polycythemia."

Noyes, W.D., Domm, B.M. and Willis, L.C.
1962 Blood 20 : 9.
"Regulation of Erythropoiesis. I. Erythropoietin Assay as a Clinical Tool."

O'Grady, L.F., Lewis, J.P. and Trobaugh, F.F.
1968 J. Lab. clin. Med. 71 : 693.
"The Effect of Erythropoietin on Differentiated Erythroid Precursors."

Okcuoglu, A. and Jones, B.
1966a Acta pediat. 55 : 88.
"Erythropoietin in Children. I. Findings in Various Types of Anemia."

Okcuoglu, A. and Jones, B.
1966b Scand. J. Haemat. 3 : 99.
"Erythropoietin in Children. 2. Findings in Hypoxic Polycythemia."

Olesen, H. and Fogh, J.
1968 Scand. J. Haemat. 5 : 211.
"Apparent Molecular Weight of Erythropoietin Determined by Gel Filtration."

Osnes, S.
1958 Brit. med. J. 2 : 1387.
"An Erythropoietic Factor Produced in the Kidney."

O'Sullivan, M.B., Glekh, G.J. and Linman, J.W.
1969 J. clin. Invest. 48 : 61a.
"Studies on the Molecular Characteristics of Human Urinary Erythropoietin."

Ouchterlony, O.
1958 Prog. Allergy 5 : 1.
"Diffusion-in-gel Methods for Immunological Analysis."

Paul, J.
1969 Abst. 39th Gen. Meeting Brit. Soc. Haemat. + Nederlandse Vereniging Voor hematologie. No. 7 - "Erythropoietin."

Pavlovic-Kentera, V., Hall, D.P., Bragassa, C. and Lange, R.D.
1965 J. Lab. clin. Med. 65 : 577.
"Unilateral Renal Hypoxia and Production of Erythropoietin."

Payne, R.W.
1961 Brit. J. Haemat. 7 : 285.
"Plasma Erythropoietin Levels in Anaemia: Studies Using an Assay Method in Intact Rats."

Payne, P., Woods, H.F. and Wrigley, P.F.M.
1969 J. Obstet. Gynec. Brit. Comm. 76 : 845.
"Uterine Fibromyomata and Secondary Polycythaemia."

Peets, E.A. and Gordon, A.S.
1968 Life Sciences 7 : 561.
"Effect of Erythropoietin on Glucose Utilisation by Bone Marrow Cells."

Penington, D.G.
1961 Lancet I : 301.
"The Role of the Erythropoietic Hormone in Anaemia."

Penington, D.G.
1963 Mem. Soc. Endocrin. 13 : 201.
"Erythropoietin in Tissues: Features of the Renal Erythropoietin-Producing System."

Penington, D.G.
1965 Proc. roy. Soc. Med. 58 : 488.
"Polycythaemia in Neoplastic Diseases."

Perretta, M.A. and Thomson, R.Y.
1961 Nature 190 : 912.
"Effect of Erythropoietine on Incorporation of Formate Labelled with Carbon-14 into the Nucleic Acids of Normal Rabbit Tissues In Vitro."

Perretta, M.A. and Tirapegui, C.
1968 Experientia 24 : 680.
"Effect of Erythropoietine on Nucleic Acid Metabolism from Polycythemic Rat Bone Marrow."

Pieber-Perretta, Rudolph, W., Perretta, M. and Hodgson, G.
1965 Biochim. biophys. Acta 95 : 360.
"Effect of Erythropoietine on [^{14}C] Formate Incorporation into Rat Bone Marrow RNA."

Piha, R.S.
1962 Ann. Acad. Sci. fenn. Series A.V Medica 98 : 5.
"Demonstration of Erythropoietic Activity in Body Bluids, Organs and Tissues."

Piliero, S.J., Medici, P.T., Pansky, B., Luhby, A.L. and Gordon, A.S.
1956 Proc. Soc. exp. Biol. Med. 93 : 302.
"Erythropoietic Stimulating Effects of Plasma Extracts from Anemic Human Subjects."

Plzak, L.F., Fried, W., Jacobson, L.O. and Bethard, W.F.
1955 J. Lab. clin. Med. 46 : 671.
"Demonstration of Stimulation of Erythropoiesis by Plasma from Anemic Rats Using Fe^{59}"

Porteous, D.D. and Lajtha, L.G.
1966 Brit. J. Haemat. 12 : 177.
"On Stem-Cell Recovery After Irradiation."

Powsner, E.R. and Berman, L.
1967 Blood 30 : 189.
"Effect of Erythropoietin on DNA Synthesis by Erythroblasts In Vitro."

Preer, J.R.
1956 J. Immunol. 77 : 52.
"A Quantitative Study of a Technique of Double Diffusion in Agar."

Proom, H.
1943 J. Path. Biol. 55 : 419.
"The Preparation of Precipitating Sera for the Identification of Animal Species."

Quenouille, M.H.
1950 "Introductory Statistics." 1st Edition. Published - Pergamon Press, Ltd., London.

Rambach, W.A., Alt, H.L. and Cooper, J.A.D.
1957 Blood 12 : 1101.
"The Mode of Action and Nature of a Heat Stable Plasma Erythropoietic Factor."

Rambach, W.A., Cooper, J.A.D. and Alt, H.L.
1958a Proc. Soc. exp. Biol. Med. 98 : 602.
"Purification of Erythropoietin by Ion-Exchange Chromatography."

Rambach, W.A., Shaw, R.A., Cooper, J.A.D. and Alt. H.L.
1958b Proc. Soc. exp. Biol. Med. 99 : 482.
"Acid Hydrolysis Erythropoietin."

Rambach, W.A., Cooper, J.A.D. and Alt. H.L.
1959 Ann. N.Y. Acad. Sci. 77 : 623.
"The Nature of Erythropoietin."

Rambach, W.A., Alt, H.L. and Cooper, J.A.D.
1961 Proc. Soc. exp. Biol. Med. 108 : 793.
"Erythropoietic Activity of Tissue Homogenates."

Reeves, G., Lowenstein, L. and Sommers, S.C.
1963 Amer. J. med. Sci. 245 : 184.
"A Suggested Mechanism of Erythropoietic Control by Juxtaglomerular Cells."

Reich, E. and Goldberg, I.H.
1964 Prog. Nucl. Acid. Res. Mol. Biol. 3 : 183.
"Actinomycin and Nucleic Acid Function."

Reich, E., Franklin, R.M., Shatkin, A.J. and Tatum, E.L.
1961 Science 134 : 556.
"Effect of Actinomycin D on Cellular Nucleic Acid Synthesis and Virus Production."

Reichlin, M. and Harrington, W.J.
1960 Blood 16 : 1298.
"Studies on Erythropoietin. I. Demonstration of Erythropoietin in Normal Plasma."

Reissman, K.P.
1950 Blood 5 : 372.
"Studies on the Mechanism of Erythropoietin Stimulation in Parabiotic Rats during Hypoxia."

Reissman, K.R.
1963 Mem. Soc. Endocrin. 13 : 195.
"Erythropoietin Formation in the Kidney."

Reissman, K.R. and Nomura, T.
1962 "Erythropoiesis." Edited Jacobson, L.O. and Doyle, M. 1st Edition. Published - Grune & Stratton, New York. p.71. "Erythropoietin Formation in Isolated Kidneys and Liver."

Reissman, K.R., Nomura, T., Gunn, R.W. and Brosius, F.
1960 Blood 16 : 1411.
"Erythropoietic Response to Anemia or Erythropoietin Injection in Uremic Rats with or without Functioning Renal Tissue."

Reynafarje, C., Raymos, J., Faura, J. and Villavicencio, D.
1964 Proc. Soc. exp. Biol. Med. 116 : 649.
"Humoral Control of Erythropoietic Activity in Man During and After Altitude Exposure."

Riding, I.M.
1963 Ph.D. Thesis, University of Edinburgh.
"Preparation and Properties of the Components of the Fibrinolytic System of Human Plasma."

Rioux, E. and Erslev, A.J.
1968 J. Immunol. 101 : 6.
"Immunologic Studies of a Partially Purified Sheep Erythropoietin."

River, G.L.
1966 J. amer. med. Ass. 197 : 726.
"Erythroid Aplasia Following Thymomectomy."

Rosse, W.F. and Gurney, C.W.
1959 J. Lab. clin. Med. 53 : 446.
"Studies on Erythropoiesis. X. The Use of Bone Marrow Tissue Culture in Demonstrating Erythropoietin."

Rosse, W.F. and Waldmann, T.A.
1962 Blood 19 : 75.
"The Role of the Kidney in the Erythropoietic Response to Hypoxia in Parabiotic Rats."

Rosse, W.F. and Waldmann, T.A.
1964 Blood 24 : 739.
"A Comparison of Some Physical and Chemical Properties of Erythropoiesis - Stimulating Factors from Different Sources."

Rosse, W.F., Waldmann, T.A. and Houston, D.E.
1962 Proc. Soc. exp. Biol. Med. 109 : 836.
"Erythropoietin Assays Using Iron59 Incorporation into Blood and Spleen of the Polycythemic Mouse."

Rosse, W.F., Berry, R.J. and Waldmann, T.A.
1963a J. clin. Invest. 42 : 124.
"Some Molecular Characteristics of Erythropoietin from Different Sources Determined by Inactivation by Ionising Radiation."

Rosse, W.F., Waldmann, T.A. and Cohen, P.
1963b Amer. J. Med. 34 : 76.
"Renal Cysts, Erythropoietin and Polycythemia."

Rudolph, W. and Perretta, M.
1967 Proc. Soc. exp. Biol. Med. 124 : 1041.
"Effects of Erythropoietin on ^{14}C-Formate Uptake by Spleen and Bone Marrow Nucleic Acids of Erythrocyte-Transfused Mice.

Scaro, J.L. and Aggio, M.C.
1966 Rev. canad. Biol. 25 : 209.
"Erythropoietin in High Altitude Resident Animals."

Scheidegger, J.J.
1955 Int. Arch. Allergy 7 : 103.
"Une microméthode de l'immunoélectrophorèse."

Schleuter, R.J., Morgello, M. and White, W.F.
1960 Proc. Soc. exp. Biol. Med. 103 : 43.
"Erythropoietin. I. Analysis of Radio-iron Assay in Fasted Rats, Using Cobalt as a Reference Standard."

Schmid, J.R., Kiely, J.M., Harrison, E.J., Bayrd, E.D. and Pease, G.L.
1965 Cancer 18 : 216.
"Thymoma Associated with Pure Red Cell Agenesis."

Schooley, J.C.
1965 Blood 25 : 795.
"Responsiveness of Hematopoietic Tissue to Erythropoietin in Relation to the Time of Administration and Duration of Action of the Hormone.

Schooley, J.C. and Garcia, J.F.
1962 Proc. Soc. exp. Biol. Med. 109 : 325.
"Immuno-chemical Studies of Human Urinary Erythropoietin."

Schooley, J.C. and Garcia, J.F.
1965 Blood 25 : 204.
"Some Properties of Serum Obtained from Rabbits Immunised with Human Urinary Erythropoietin."

Schooley, J.C., Hayes, J.M., Cantor, L.N. and Havens, V.W.
1967 Radiat. Res. 32 : 875.
"Studies on the Behaviour of Erythropoietin-sensitive Cells in the Mouse During Recovery from 200 Roentgens of Whole-Body Irradiation."

Schooley, J.C., Garcia, J.F., Cantor, L.N. and Havens, V.W.
1967a United States A.E.C. University of California Radiation Lab. Publication No. UCRL-17481. p.110. "A Summary of Some Studies on Erythropoiesis Using Anti-Erythropoietin Immune Serum."

Sephton-Smith, R.
1963 Mem. Soc. Endocrin. 13 : 231.
"Some Properties of Red Blood Cells formed Following Erythropoietin Administration."

Shalet, M.F., Holder, T.M. and Walters, T.R.
1967 J. pediat. 70 : 615.
"Erythropoietin-Producing Wilms' Tumor."

Shaw, D.B. and Simpson, T.
1961 Quart. J. Med. 30 : 135.
"Polycythaemia in Emphysema."

Siri, W.E., Van Dyke, D.C., Winchell, H.S., Pollycove, M., Parker, H.G. and Cleveland, A.S.
1966 J. appl. Physiol. 21 : 73.
"Early Erythropoietin, Blood and Physiological Responses to Severe Hypoxia in Man."

Slater, J.D.H., Barbour, B.H., Henderson, M.H., Casper, A.G.T. and Bartter, F.C.
1963 J. clin. Invest. 42 : 1504.
"Influence of the Pituitary and Renin-Angiotensin System on the Secretion of Aldosterone, Cortisol and Corticosterone."

Slaunwhite, W.R., Mirand, E.A. and Prentice, T.C.
1957 Proc. Soc. exp. Biol. Med. 96 : 616.
"Probably Polypeptidic Nature of Erythropoietin."

Smith, H.W., Finkelstein, N., Aliminosa, L., Crawford, B. and Graber, M.
1945 J. clin. Invest. 24 : 388.
"The Renal Clearances of Substituted Hippuric Acid Derivatives and other Aromatic Acids in Dog and Man."

Snedecor, G.W.
1950 "Statistical Methods." 4th Edition. Published - Iowa State University Press, U.S.A.

Sokabe, H. and Grollman, A.
1962 Amer. J. Physiol. 203 : 991.
"Localisation of Blood Pressure Regulation and Erythropoietic Functions in Rat Kidney."

Stamatoyannopoulos, G., Yoshida, A., Adamson, J. and Heinenberg, S.
1968 Science 159 : 741.
"Hemoglobin Ranier (β^{145} Tyrosine-Histidine); Alkali-Resistant Hemoglobin with Increased Oxygen Affinity."

Stohlman, F.
1961a Proc. Soc. exp. Biol. Med. 107 : 884.
"Humoral Regulation of Erythropoiesis. VII. Shortened Survival of Erythrocytes Produced by Erythropoietine or Severe Anemia."

Stohlman, F.
1961b Proc. Soc. exp. Biol. Med. 107 : 751.
"Humoral Regulation of Erythropoiesis. VI. Mechanism of Action of Erythropoietin in the Irriadiated Animal."

Stohlman, F. and Brecher, G.
1956 Proc. Soc. exp. Biol. Med. 91 : 1.
"Stimulation of Erythropoiesis in Sub-Lethally Irradiated Rats by a Plasma Factor."

Stohlman, F. and Brecher, G.
1957 Proc. Soc. exp. Biol. Med. 95 : 797.
"Humoral Regulation of Erythropoiesis. IV. Relative Heat Stability of Erythropoietine."

Stohlman, F. and Brecher, G.
1959 Proc. Soc. exp. Biol. Med. 100 : 40.
"Humoral Regulation of Erythropoiesis. V. Relationship of Plasma Erythropoietine Level to Bone Marrow Activity."

Stohlman, F., Rath, C.E. and Rose, J.C.
1954 Blood 9 : 721.
"Evidence for a Humoral Regulation of Erythropoiesis."

Stohlman, F., Ebbe, S., Morse, B., Howard, D. and Donovan, J.
1968 Ann. N.Y. Acad. Sci. 149 : 156.
"Regulation of Erythropoiesis. XX. Kinetics of Red Cell Production."

Suki, W. and Grollman, A.
1960 Amer. J. Physiol. 199 : 629.
"Role of the Kidney in Erythropoiesis."

Takaku, S., Hirashima, A. and Nakao, K.
1962 J. Lab. clin. Med. 59 : 815.
"Studies on the Mechanism of Erythropoietin Production. 1. Effect of Unilateral Constriction of the Renal Artery."

Tarazi, R.C., Frohlich, E.D., Dustan, H.P., Gifford, R.W. and Page, I.H.
1966 Amer. J.Cardiol. 18 : 855.
"Hypertension and High Haematocrit."

Thomas, D.E., Lochte, H.L. and Stohlman, F.
1960 J. Lab. clin. Med. 55 : 311.
"Attempts to Develop an In Vitro System for the Assay of Erythropoietin."

Thurman, W.G., Grabstald, H. and Lieberman, P.H.
1966 Arch. intern. Med. 117 : 280.
"Elevation of Erythropoietin Levels in Association with Wilms' Tumor."

Till, J.E. and McCulloch, E.A.
1961 Radiat. Res. 14 : 213.
"A Direct Measurement of the Radiation Sensitivity of Normal Mouse Bone Marrow Cells."

Tobian, L., Thompson, J., Twedt, R. and Janecek, J.
1958 J. clin. Invest. 37 : 660.
"The Granulation of Juxtaglomerular Cells in Renal Hypertension, Adrenal Regeneration Hypertension and Adrenal Insufficiency."

Ullman, E.
1961 J. Physiol. 155 : 417.
"Acute Anoxia and the Excretion of Water and Electrolyte."

Van Dyke, D.C.
1960 Ciba Foundation Symposium "Haemopoiesis." Edited Wolstenholme and O'Connor. Published - J. & H. Churchill, London. p.397. "Sources and Properties of Human Urinary Erythropoietin."

Van Dyke, D.C., Layrisse, M., Lawrence, J.H., Garcia, J.F. and Pollycove, M.
1961 Blood 18 : 187.
"Relationship Between Severity of Anemia and Erythropoietin Titre in Human Beings."

Van Dyke, D.C., Mohr, M.L. and Lawrence, J.H.
1966 Blood 28 : 535.
"Erythropoietin in the Urine of Normal and Erythropoietically Abnormal Human Beings."

Vanier, T., Dulfano, M.J., Wu, C. and Desforges, J.F.
1963 New Engl. J. Med. 269 : 169.
"Emphysema, Hypoxia and the Polycythemic Response."

Vertel, R.M., Morse, B.S. and Prince, J.E.
1967 Arch. intern. Med. 120 : 54.
"Remission of Erythrocytosis after Drainage of a Solitary Renal Cyst."

Viault, M.F.
1890 C.R. Acad. Sci. 111 : 917.
"Sur L'Augmentation Considérable du Nombre des Globules Rouges dans le Sang chez les Habitants des Hauts Plateaux de L'Amérique du Sud."

Waldmann, T.A. and Bradley, J.E.
1961 Proc. Soc. exp. Biol. Med. 108 : 425.
"Polycythemia Secondary to a Pheochromocytoma with Production of an Erythropoiesis Stimulating Factor by the Tumour."

Waldmann, T.A. and Rosse, W.F.
1962 "Erythropoiesis." Edited Jacobson, L.O. and Doyle, M. 1st Edition. Published - Grune & Stratton, New York. p.87. "Sites of Formation of Erythropoietin."

Waldmann, T.A., Levin, E.H. and Baldwin, M.
1961 Amer. J. Med. 31 : 318.
"The Association of Polycythemia with a Cerebellar Haemangioblastoma."

Waldmann, T.A., Rosse, W.F. and Swarm, R.L.
1968 Ann. N.Y. Acad. Sci. 149 : 509.
"The Erythropoiesis-Stimulating Factors Produced by Tumors."

Ward, H.P.
1967 Proc. Soc. exp. Biol. Med. 125 : 370.
"An In Vitro Assay of Erythropoietin."

Ward, H.P., Gordon, B. and Pickett, J.C.
1969 J. Lab. clin. Med. 74 : 93.
"Serum Levels of Erythropoietin in Rheumatoid Arthritis."

Weil, J.V., Jamieson, G., Brown, D.W. and Grover, R.F.
1968 J. clin. Invest. 47 : 1627.
"The Red Cell Mass - Arterial Oxygen Relationship in Normal Man."

Weintraub, A.H., Gordon, A.S. and Camiscoli, J.F.
1963 J. Lab. clin. Med. 62 : 743.
"Use of the Hypoxia-Induced Polycythemic Mouse in the Assay and Standardisation of Erythropoietin."

Weisman, M., Martinson, D., Fried, W. and Gurney, C.W.
1967 J. Lab. clin. Med. 69 : 438.
"A Comparison of the Rates at which Colony Forming Units and Erythropoietin Responsive Cells Recover after 200r total Body X-Irradiation."

Westerman, M.P., Jenkins, J.L., Dekker, A., Kreutner, A. and Fisher, B.
1967 Lancet II : 755.
"Significance of Erythrocytosis and Increased Erythropoietin Secretion After Renal Transplantation."

White, W.F., Gurney, C.W., Goldwasser, E. and Jacobson, L.O.
1960 Recent Progr. Hormone Res. 16 : 219.
"Studies on Erythropoietin."

Winkert, J.W. and Gordon, A.S.
1960 Proc. Soc. exp. Biol. Med. 104 : 713.
"Biological Assay of Human Urinary Erythropoietic Stimulating Factor (ESF)."

Winkert, J.W., Gordon, A.S. and Winkert, E.
1962 "Erythropoiesis." Edited Jacobson, L.O. and Doyle, M. 1st Edition. Published - Grune & Stratton, New York. p.50. "Studies on the Purification and Structure of Human Urinary Erythropoietic Stimulating Factor (ESF)."

Wong, K.W., Sanjani, E.D., Cooper, G.W. and Gordon, A.S.
1968 Proc. Soc. exp. Biol. Med. 128 : 67.
"The Renal Erythropoietic Factor. V. Studies on its Purification."

Zangheri, E.O., Suarez, J.R.E., Fernandez, F.O., Campana, H., Silva, J.C. and Ponce, F.E.
1962 Nature 194 : 938.
"Erythropoietic Action of Tissue Extracts."

Zangheri, E.O., Campana, H., Ponce, F., Silva, J.C., Fernandez, F.O. and Suarez, J.R.E.
1963 Nature 199 : 572.
"Production of Erythropoietin by Anoxic Perfusion of the Isolated Kidney of a Dog."

Zanjani, E.D., Cooper, G.W., Gordon, A.S., Wong, K.K. and Scribner, V.A.
1967 Proc. Soc. exp. Biol. Med. 126 : 540.
"The Renal Erythropoietic Factor (REF). IV. Distribution in Mammalian Kidneys."

Zanjani, E.D., Schooley, J.C. and Gordon, A.S.
1968 Life Sciences 7 : 505.
"Renal Erythropoietic Factor (REF). Neutralisation of *In Vitro* Generated Erythropoietic Activity with Anti-Erythropoietin."