Exhibit 25

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

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Immunoreactive erythropoietin in serum

I. EVIDENCE FOR THE VALIDITY OF THE ASSAY METHOD AND THE PHYSIOLOGICAL RELEVANCE OF ESTIMATES

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SUMMARY. A radioimmunoassay for erythropoietin (Ep) in human serum is described. Evidence of assay validity includes parallelism of dilution curves of the 2nd International Reference Preparation (IRP) (used as standard) with test sera, a fall in serum immunoreactive Ep following blood transfusion and an increase in serum immunoreactive Ep with increasing severity of anaemia except in anaemias associated with severe renal failure. The geometric mean estimate of serum immunoreactive Ep in 93 normal men and women was 13·3 miu/ml above the low erythropoietin serum pool used as baseline for the assay. Extra-renal erythropoietin was detectable in the assay and gave a dilution curve parallel to the 2nd IRP.

Estimates of the biological activity of erythropoietin (Ep) in body fluids provide useful information for the elucidation of polycythaemic states and anaemias (Cotes & Wardle, 1979). However, bioassays for Ep are relatively insensitive and non-specific. Although *in vitro* systems can be more sensitive than *in vivo* assays, specificity remains a problem and both are unsuitable for serial studies or for the detection of small changes in serum levels of Ep nor are they practicable for handling large numbers of samples (Cotes & Wardle, 1979; Brandan *et al*, 1981). Thus the development of radioimmunoassays based on the recognition of Ep structure instead of on biological effects of the hormone might confer advantages in assay specificity and sensitivity. Two effective systems for radioimmunoassay of Ep in serum have already been described (Garcia *et al*, 1979; Sherwood & Goldwasser, 1979). Since it cannot be assumed that radioimmunoassays based on different antisera will have identical specificities, it is necessary to validate each new assay system. The present paper describes a third radioimmunoassay for Ep in human serum and provides evidence that estimates made with it reflect the biological activity of Ep in serum (Cotes *et al*, 1980). It is applicable to the estimation of extra-renal Ep from anephric subjects as well as the Ep in serum from subjects with functioning renal tissue which may be both renal and extra-renal in origin. The system

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is a classical radioimmunoassay with delayed addition of tracer and a double antibody separation.

METHODS AND MATERIALS

Erythropoietin. Preparations used were the First and Second International Reference Preparations of erythropoietin, human urinary for bioassay (Cotes & Bangham, 1966; Anable *et al*, 1972) respectively referred to as the 1st and 2nd IRP, a preparation of human urinary erythropoietin M-7-72-2 prepared by Miyake *et al* (1977) which was made available through the National Heart Lung and Blood Institute of the National Institutes of Health, U.S.A., and two preparations of human urinary erythropoietin (referred to as I and EH83) extracted and provided by Espada (1977). The biological potencies of these preparations were respectively 1, 5, 70,000, (I) 5290 and (EH83) 1000 iu per mg of peptide by bioassay *in vivo*.

Antiserum. In collaboration with Dr Espada, antiserum to erythropoietin was raised in New Zealand white rabbits by multiple intradermal injection (Vaitukaitis *et al*, 1971) of a total of some 530 iu per rabbit of human urinary erythropoietin, 5290 iu/mg. Antiserum R78/15, 231179 was used throughout the work reported here.

Donkey anti-rabbit IgG. A single donkey bleed HPD-9VF, referred to as DARS, was used. It was provided by Dr Brian Morris from Guildhay Reagents, Department of Biochemistry, University of Surrey.

Preparation and characterization of ^{125}I radioiodinated erythropoietin tracer. Human urinary erythropoietin, batch M-7-72-2, 0·7 μg was radioiodinated with ^{125}I sodium iodide 300 μCi (11·3 MBq) (IMS 30, from Radiochemical Centre, Amersham) and solid phase lactoperoxidase prepared and provided by Dr R. Edwards of Chemical Pathology Research, St Bartholomew's Hospital, London (Marchalonis, 1969; Thorell & Johansson, 1971). Unreacted iodide was separated by gel filtration on Sephadex G-50 and the protein peak (some 13–30% of the total ^{125}I) was further fractionated by gel filtration and sucrose density gradient ultracentrifugation. Gel filtration of radioiodinated erythropoietin was carried out on a column length 77 cm and volume 104 ml of Ultrogel AcA 44 acrylamide–agarose beads (LKB, Industries Biologique Française), in pH 7·5 buffer sodium chloride 0·15 M, sodium phosphate 0·04 M, ethylenediaminetetracetic acid disodium salt (EDTA) 0·01 M, sodium azide 1:10 000 w/v and human plasma protein fraction (PPF) (Lister, Elstree, Batch AD 628A) 2 ml/100 ml giving human serum albumin at a final concentration 1 mg/ml. This gave the typical elution pattern shown in Fig 1 in which fractions were characterized both by radioactivity and the ability of radioactive protein to bind to erythropoietin antiserum. Three main radioactive components were eluted, A, B and C, which showed respectively (i) with anti-erythropoietin serum high binding, poor binding and high binding and (ii) with control rabbit serum high, low and low non-specific binding. In occasional iodinations the proportion of peak B was increased and of C was less than that shown in Fig 1. Such tracers were relatively unsatisfactory for assay of erythropoietin in serum. The ^{125}I tracer erythropoietin used in assays was from peak C eluted off Ultrogel AcA 44 binding some 50–80% with anti-erythropoietin serum when tested at an antiserum dilution 1:400 (18–24 h incubation at 4°C in the buffer used for elution of the gel column and then precipitated in the presence of

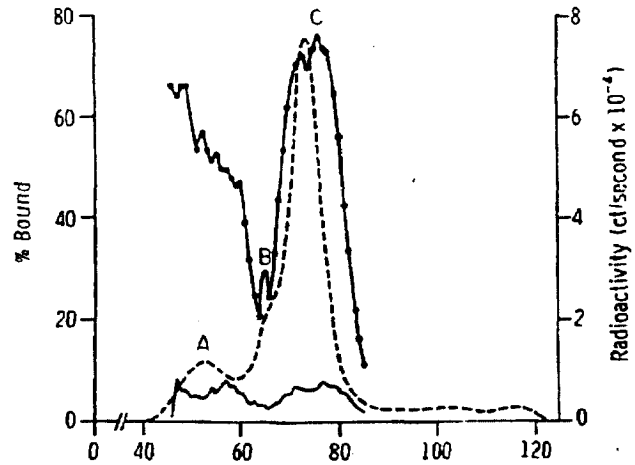

Fig 1. Elution of radioactivity of ^{125}I Ep off AcA 44 gel column (- - -), binding of fractions with anti-erythropoietin serum (●—●) and non-specific binding with control rabbit serum (——).

DARS 1:250 (v/v) and ammonium sulphate 0·8 M for 2 h at 20°C). Fractions eluted off AcA 44 gel were snap frozen in liquid nitrogen and stored at −40°C. They remained satisfactorily stable for use in assays for at least 5 weeks.

Fractions B and C were poorly resolved on Ultrogel AcA 44 but could be separated by sucrose density gradient ultracentrifugation under the conditions described by Cotes (1973). Fraction B sedimented in the same position as ^{125}I human serum albumin (IM 17P from the Radiochemical Centre, Amersham). Fraction C sedimented in the same position as both unlabelled human urinary erythropoietin EH83, characterized by bioassay of individual density gradient fractions in the polycythaemic mouse (Cotes & Bangham, 1961; Cotes, 1973), and ^{125}I human growth hormone (Medical Research Council 69/46).

Standard. The 1st and 2nd IRP were used as standards. Solutions of 10 iu/ml in water were snap frozen in liquid nitrogen and stored at −40°C until use.

Radioimmunoassay. Incubations were carried out at 4°C. Mixtures of 800 µl test sample or standard with anti-erythropoietin serum 0·08 µl and diluent buffer 3·2 ml (0·02 M veronal pH 8·6 containing calcium chloride 1 mM, sodium azide 1:10 000 w/v and PPF 2 ml per 100 ml) were incubated for 48 h. ^{125}I tracer erythropoietin 50 µl in diluent buffer, was added and, after a further 48 h, DARS, 4 µl in 1 ml pH 7·5 buffer containing veronal 0·02 M, EDTA 0·05 M, sodium azide 1:10 000 w/v and PPF 2 ml per 100 ml was added. After a total incubation of 116–120 h, mixtures were centrifuged at 2000 rev/min (1200 *g*) for 55 min at 4°C, supernatants were discarded and residues counted for ^{125}I radioactivity. Estimates of erythropoietin concentration in test samples were based on results from duplicate incubation tubes and derived from radioactivity count data using a computer program written by Dr R. Wootton (Clinical Research Centre) and based on the four parameter log-logistic dose–response curve described by Healy (1972). To ensure comparability of incubation mixtures for estimates on serum samples, all incubation mixtures contained 800 µl of serum, either test

sample or a low erythropoietin serum pool (LESP). The LESP was used in incubation mixtures with the standard and as a diluent for serum samples tested at volumes below 800 μl.

Low erythropoietin serum pool (LESP). LESP was prepared as a pool from selected sera of patients with polycythaemia rubra vera (primary polycythaemia). Sera were selected for inclusion in the LESP on the basis of having a low content of erythropoietin after being placed in ranking order of erythropoietin content. Ranking order was established by trial use of each serum as baseline for the assay (and diluent for the standard) and comparison of estimates of erythropoietin in a series of control sera assayed simultaneously against this series of different standard curves (Table I). Estimates of serum levels of erythropoietin were made in miu 2nd IRP per ml of serum above polycythaemic serum pools variously LESPI and LESPII. For the comparability of data all estimates are presented in miu 2nd IRP per ml of serum above LESPII. LESPI was made up from sera A, B, C, D and G shown in Table I. These individual sera appear to range in erythropoietin content from lowest A to highest G by about 5 miu/ml (see Table I). LESPII was a later pool containing 2 miu less Ep per ml than LESPI. A sample of serum from one patient with polycythaemia vera and iron deficiency (EH contributor of test serum 19 B, Table I) was found to have a lower level of Ep than other sera so far tested. To permit comparison of estimates made in assays using different batches of LESP successive batches of LESP have been calibrated in miu/ml above the test serum 19B. LESPI and LESPII contain respectively 10 and 8 miu Ep/ml more Ep than test serum 19B.

Serum samples for assay. Serum from blood samples allowed to clot at 20°C for 2–4 h or at 4°C for 16–20 h was snap frozen in liquid nitrogen and stored at −40°C until thawed for use.

Table 1. Estimates of Ep in miu/ml in test serum samples, using as baseline for the assay and diluent for the standard, samples of serum from patients with polycythaemia

Serum used as assay baseline	Test serum sample					
	19A	19B	19D	19E	19F	19H
A	18	<baseline	16	8	17	14
B	17	<baseline	15	9	16	13
C	16	<baseline	15	8	16	13
D	15	<baseline	13	7	14	11
E	15	<baseline	13	7	14	11
F	14	<baseline	12	7	13	11
G	13	<baseline	11	4	13	9
H	13	<baseline	11	4	12	9
I	10	<baseline	8	3	9	7
J	9	<baseline	7	<baseline	8	5
K	10	<baseline	7	<baseline	8	5
L	8	<baseline	6	<baseline	7	4
M	5	<baseline	4	<baseline	5	2
N	3	<baseline	1	<baseline	3	<baseline
P	1	<baseline	<baseline	<baseline	baseline	<baseline

RESULTS

Specificity of the assay system

Three preparations of human urinary Ep, purified to different extents (the 1st and 2nd IRP and M-7-72-2), gave parallel dose–response curves. Those for the 1st and 2nd IRP were essentially identical but that for the highly purified preparation M-7-72-2 was shifted to the left showing a 1·8-fold higher ratio of immunochemical *in vivo* biological activity for this preparation compared with the 1st and 2nd IRP. The scarcity of highly purified Ep precluded confirmation of this observation. Desialation of Ep (the 1st IRP) by treatment with neuraminidase under the conditions described by Sherwood & Goldwasser (1979) induced a decrease in radioimmunoassay potency and some change in the slope of the dose–response curve. Other substances tested and found to be without activity in the radioimmunoassay were Tamm and Horsfall urinary glycoprotein (2·5 μg) (kindly provided by Professor W. T. J. Morgan now at the Clinical Research Centre), serum α_1-acid glycoprotein 2·5 mg (Behring Institut, batch 8376), chorionic gonadotrophin 6·5 iu of the 1st IRP, human for immunoassay, chorionic gonadotrophin alpha subunit 700 miu of the 1st IRP, human for immunoassay and chorionic gonadotrophin beta subunit, 700 miu of the 1st IRP, human for immunoassay.

Suitability of the system for assay of human serum erythropoietin

Parallelism of standard and test. Dose–response curves given by the 1st IRP were parallel with those given by samples of serum tested in the assay at a series of dilutions in LESPI (Fig 2). The response curves given by dilutions of serum samples from a patient with chronic pulmonary disease and secondary polycythaemia (Fig 2a), from an anephric patient tested as a source of extra-renal erythropoietin (Fig 2b) and from a patient with rheumatoid arthritis (Fig 2b) each showed parallelism with the 1st IRP tested at the same time. Serum from a normal subject also showed parallelism and, tested at doses 800 and 400 μl, gave estimates of 21 and 20 miu/ml respectively above LESPI.

Reproducibility of estimates. The mean within and between assay coefficients of variation of estimates carried out using a single batch of LESP in a series of assays (on samples in the range 11–62 miu/ml) were respectively 7·8% (six samples tested twice and three samples tested four times) and 15·7% (two samples examined in eight assays and one sample examined in seven assays). Systematic between assay differences in estimates can arise with change from one batch of LESP to another. This may be avoided by calibration of successive batches of LESP and application of a baseline correction for each batch.

The stability of erythropoietin in samples for assay. Estimates of Ep in serum samples from blood stored 20–24 h at 4°C before separation were 94·5% (mean of six samples ±SEM 2·5%) of levels found in sera from each of the same blood samples kept for 2–4 h at 20°C before separation. Estimates on plasma samples separated within 30 min of collection of heparinized blood samples cooled immediately to 4°C gave a mean recovery of 95·1% of

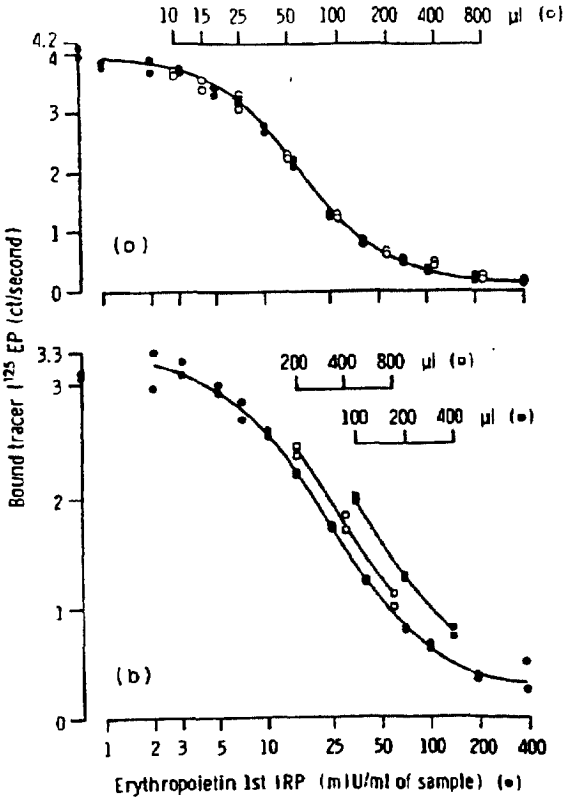

Fig 2. Radioimmunoassay dose–response curves given by the 1st IRP human urinary erythropoietin (●) and (a) serum from a patient with secondary polycythaemia associated with chronic pulmonary disease (○) and (b) serum from an anephric patient (□) and from a patient with anaemia in association with rheumatoid arthritis (■).

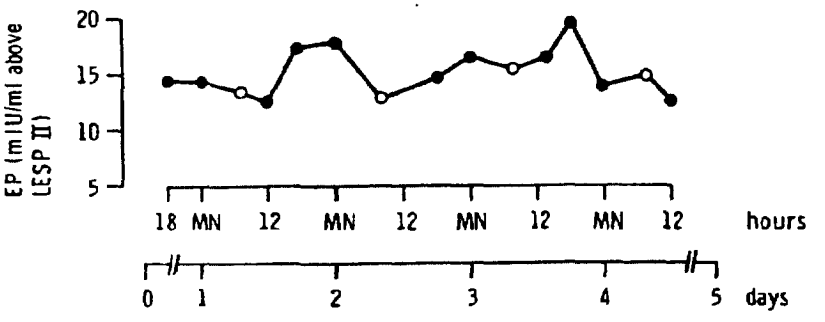

Fig 3. Estimates of immunoreactive erythropoietin in serum samples collected during a 4 d period from a normal subject (P.M., ♀, age 54 years) when fasting (○) or non-fasting (●).

estimates on serum separated from blood kept 2–4 h at 20°C (from observations on 86 paired samples).

The stability of serum levels of Ep in vivo. The variability of serum levels of erythropoietin in a normal female subject during a 4 d period is shown in Fig 3. Serum was separated from blood samples stored at 4°C for 4–20 h. Estimates showed no obvious diurnal variation nor any consistent difference between levels of Ep in the fed and fasted states.

The effect of blood transfusion on serum levels of immunoreactive Ep. Partial correction of anaemia by blood transfusion induced a fall in serum levels of immunoreactive Ep when pre-transfusion levels were compared with levels found between 24 and 48 h after completion of a transfusion (Fig 4).

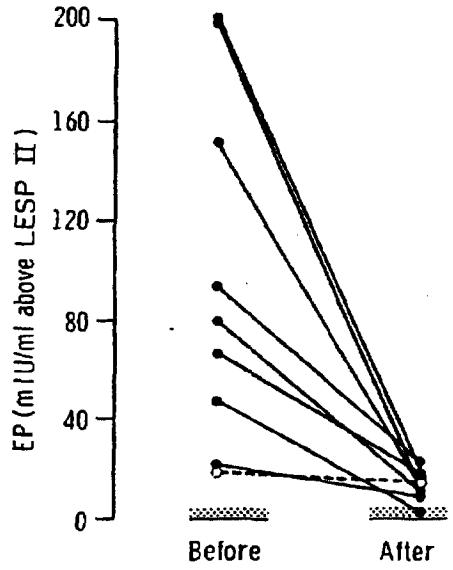

Fig 4. Serum levels of immunoreactive erythropoietin before and 24–48 h after blood transfusion. In one patient (O) anaemia was associated with renal failure. Potency estimates were not assigned below 5 miu/ml above LESPII (the hatched area in the figure).

Serum levels of immunoreactive Ep in normal subjects

Estimates of immunoreactive Ep were made on serum samples from 93 normal volunteer subjects (52 men and 41 women, age ranges 21–69 and 20–58 years with medians 38·5 and 36 years respectively). Any who acknowledged that they were either regular donors of blood or plasma or occasional donors estimated to have given more than 450 g of blood during the 6 months prior to sampling, and pregnant women were excluded. Estimates ranged from <4 to 35 miu/ml above LESPII (median 13·2) and were essentially log normally distributed (Kolmogorov-Smirnov test) with geometric mean potency 13·3 miu/ml above LESPII with standard deviation calculated from the logarithm of the potency estimates, 1·6. The distribution of estimates is shown in Fig 5(a). There was no difference between estimates in

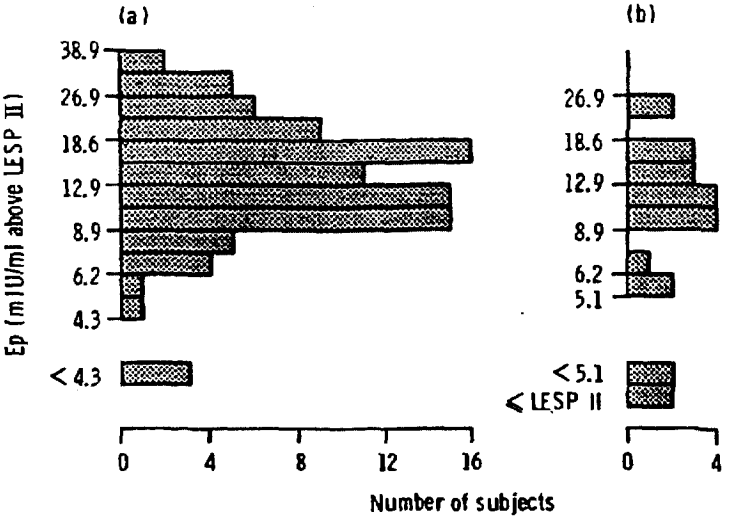

Fig 5. Histograms derived from log-potency estimates to show the distribution of estimates of serum immunoreactive erythropoietin in (a) 93 normal men and women, excluding regular blood or plasmapheresis donors and subjects who had given more than 450 g of blood by venesection in the past 6 months, and (b) 23 patients with polycythaemia rubra vera.

men and women nor any significant relation between serum Ep and age. In these normal non-anaemic men and women, serum Ep was not related to haemoglobin level (Fig 6).

The effect of anaemia on serum levels of immunoreactive Ep

In serum samples from 58 patients who were investigated because of anaemia, excepting anaemias associated with renal disease or pregnancy, estimates of serum immunoreactive Ep were inversely related to haemoglobin levels (Fig 6). In 22 patients with renal failure who were maintained by regular haemodialysis and who were anaemic (Hb $<8{\cdot}5$ g/dl, mean $6{\cdot}9 \pm$ SD $1{\cdot}0$ g/dl) the mean estimate of serum immunoreactive Ep was 14·6 miu/ml above LESPII (SD 10·5). This may be compared with estimates of immunoreactive Ep in 32 patients with 'non-renal' anaemias and haemoglobin $<8{\cdot}5$ g/dl (mean $7{\cdot}1 \pm$ SD $1{\cdot}1$ g/dl) and which in 30 out of 32 (91%) cases were >46 miu/ml above LESPII.

In eight anephric patients maintained by haemodialysis, serum immunoreactive Ep was detectable (range of estimates in 14 samples tested <5–99 miu/ml above LESPII) but at lower levels than in patients with non-renal anaemias with similar haemoglobin levels. In these patients with extra-renal Ep only, levels of immunoreactive Ep were essentially unrelated to the severity of the associated anaemia although in one patient a fall in haemoglobin from 5 to 3·5 g/dl was associated with a change in Ep from <5 to 99 miu/ml above LESPII.

Serum immunoreactive Ep in polycythaemia rubra vera

Estimates of serum immunoreactive Ep in samples from 23 patients with polycythaemia

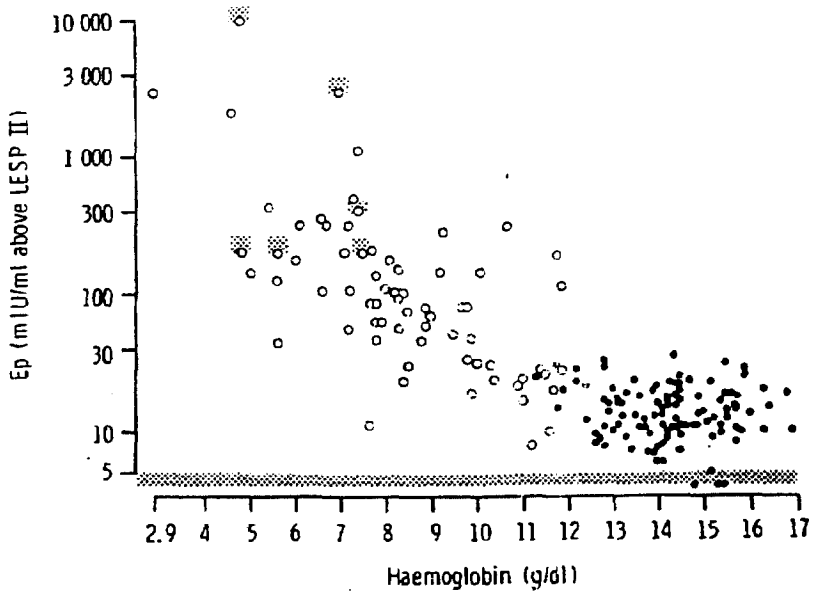

Fig 6. Estimates of serum immunoreactive erythropoietin and haemoglobin in normal men and women (●) and in patients with anaemia of various aetiologies (excluding renal disease and pregnancy associated anaemias) (○). Points with a small hatched area above show the lower limit of Ep concentration for samples which were insufficiently diluted to provide a precise estimate. Potency estimates were not assigned below 5 miu/ml above LESPII (the hatched area in the figure).

rubra vera according to the criteria developed by the Polycythemia Vera Study Group (Berlin, 1975) are shown in Fig 7(*b*). Samples were collected randomly at different stages of the disease and at least 8 months after treatment with ^{32}P or 4 weeks since venesection. Estimates of Ep were not inversely related to haemoglobin levels and were closely comparable with levels found in normal individuals but with a higher proportion of estimates at relatively low levels. Sera selected for their particularly low content of Ep were used as the baseline for the assay.

DISCUSSION

This study provides evidence for the validity of the assay method and its physiological relevance. The minimum requirement for assay validity is the demonstration of parallelism of assay dose–response curves given by the standard and by test sera. This has been shown with normal sera and with sera which contain increased amounts of renal and perhaps of extra-renal Ep (Fig 2a) and with serum containing extra-renal Ep only (Fig 2b, serum from an anephric patient). Two glycoproteins Tamm and Horsfall urinary glycoprotein and serum α_1-acid glycoprotein which might be associated with Ep extracted from urine and serum respectively did not cross react in the assay. Physiological manoeuvres known to induce changes in Ep biological activity are associated with corresponding changes in serum

immunoreactive Ep. Thus blood transfusion induced a fall in serum immunoreactive Ep (Fig 4).

Pathological conditions known to be associated with changes in serum or plasma Ep biological activity showed similar changes in serup Ep immuno-activity. Thus most anaemias excluding those associated with renal disease are associated with increased levels of serum Ep biological activity and samples from such patients tested in the immunosystem show increased levels of serum immunoreactive Ep (Fig 6). Anaemias associated with renal disease usually show impaired secretion of biologically active Ep and we found that sera from patients with renal failure and an associated anaemia and who are maintained by haemodialysis show low levels of immunoreactive Ep. Indeed in these patients levels of serum immunoreactive Ep seem to be unrelated to severity of anaemia (Cotes, Mansell & Stellan, unpublished).

The system has the disadvantage that, for strict comparability of incubation conditions for standard and test sera, the standard is diluted in serum. The diluent serum, LESP, is selected to contain a low concentration of Ep and is the baseline for the assay. Successive batches of LESP are therefore calibrated in terms of previous batches in order to have continuity of estimates. This disadvantage will be overcome and a zero baseline established when serum from which Ep has been removed is used in place of LESP.

No consistent pattern of diurnal change was found in estimates of serum immunoreactive Ep in samples collected over a 4 d period from a normal subject (Fig 3) so samples for assay were collected at randomly selected times. In serum samples from 93 normal subjects, the geometric mean estimate of immunoreactive Ep was 13·3 miu/ml above LESPII and similar to the estimates already reported for 218 blood and plasmapheresis donors (Cotes *et al*, 1980). These estimates without baseline correction are close to Koeffler & Goldwasser's (1981) mean estimates of 15 miu/ml (± SD 4) for serum erythropoietin in 26 normal volunteers. If the test serum 19B is taken as baseline the mean estimate becomes approximately 21 miu/ml which may be compared with estimates by radioimmunoassay of erythropoietin in pools of serum from normal subjects reported by Garcia *et al* (1979) and Sherwood & Goldwasser (1979). These were respectively 23 miu/ml (male), 21 miu/ml (female) and 20 miu/ml. The closeness of these various estimates suggests that the specificity of each of the three radioimmunoassays is similar.

In a preliminary study of random samples from 16 patients with anaemia associated with rheumatoid arthritis (Cotes *et al*, 1980) concentrations of serum immunoreactive Ep seemed to be of the same order as concentrations found in patients with anaemias of other aetiology (excluding renal disease and pregnancy) and results from these 16 patients with rheumatoid arthritis are included in Fig 6. This is in contrast to the suggestion that production of Ep is lower in patients with anaemias of chronic disorders than is to be expected from the severity of their anaemia (Wallner *et al*, 1977). The inverse relation between estimates of serum immunoreactive Ep and haemoglobin concentration (Fig 6) is similar to the inverse relation between haematocrit and estimates of plasma erythropoietin biological activity obtained by bioassay *in vivo* in polycythaemic mice (Erslev, 1977; Erslev *et al*, 1980). This similarity suggests that estimates of Ep by this radioimmunoassay may reflect a part of Ep structure associated with biological activity.

In patients with renal failure maintained by haemodialysis, estimates of serum

immunoreactive Ep were lower than in patients with anaemias of other aetiology. These results are in concurrence with the bioassay evidence that the anaemia of renal failure is associated with impaired secretion of Ep.

There is inconsistency between the present findings of some <5–99 miu/ml immunoreactive Ep in serum from eight anephric patients and the finding (Caro *et al.* 1979) that in 11 anephric patients plasma Ep biological activity was less than 6 miu/ml by *in vivo* biological assay. This may be explained by the uncertain loss of extra-renal Ep during acid heat treatment and concentration of plasma or it might reflect a higher ratio of immunopotency to biological activity in extra-renal compared with renal Ep as postulated by Sherwood & Goldwasser (1979).

At polycythaemic stages of polycythaemia rubra vera (primary polycythaemia) endogenous secretion of immunoreactive Ep was seldom completely suppressed and this might occur if unsuppressed Ep were extra-renal in origin. Our finding of low and normal levels of serum immunoreactive Ep in polycythaemia rubra vera (irrespective of haemoglobin level) is similar to that of Koeffler & Goldwasser (1981) who used a different radioimmunoassay system. In contrast, Adamson (1968), using an *in vivo* bioassay, found that urinary excretion of Ep in patients with polycythaemia rubra vera was less than excretion in normal subjects and not detectable in the assay system. Similarly, Erslev *et al* (1980) found less biologically active Ep in concentrates of plasma from patients with polycythaemia rubra vera than from normal subjects. Thus usually biologically active but only sometimes immunoreactive Ep is below normal levels in polycythaemia rubra vera.

In conclusion, the radioimmunoassay described here for estimation of Ep in human serum has specificity which is relatively similar to the *in vivo* polycythaemic mouse bioassay and sensitivity adequate for estimation of Ep in unconcentrated serum. In contrast to *in vivo* bioassay, the radioimmunoassay is easily applied to test many samples; moreover it provides the possibility of routine tests of function of the Ep system, screening in polycythaemic states to detect ectopic or other abnormal secretion of Ep, monitoring the response to treatment of Ep secreting tumours and new studies of the control of erythropoietin secretion in man. Bioassays and immunoassays respectively reflect functional and structural characteristics of erythropoietin and estimates by these two different types of system are unlikely to be identical. For example, precursors and degradation products of erythropoietin as well as the native serum hormone (which is itself almost certainly heterogeneous) may react in a radioimmunoassay. Thus radioimmunoassays are likely to contribute new information about the physiology of the erythropoietin system which will be complementary to information obtainable by bioassay.

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REFERENCES

ANNABLE, L.M., COTES, P.M. & MUSSETT, M.V. (1972) The second international reference preparation of erythropoietin, human, urinary, for bioassay. *Bulletin of the World Health Organisation*, 47, 99–112.

BERLIN, N.I. (1975) Diagnosis and classification of the polycythemias. *Seminars in Hematology*, 12, 339–351.

BRANDAN, N.C., ESPADA, J. & COTES, P.M. (1981) *In vitro* assay of erythropoietin in fetal mouse liver cultures. II. Effects of human transferrin bound iron and of serum on the stimulation of incorporation of ^{3}H-thymidine into DNA. *British Journal of Haematology*, 47, 469–478.

CARO, J., BROWN, S., MILLER, O., MURRAY, T. & ERSLEV, A.J. (1979) Erythropoietin levels in uremic, nephric and anephric patients. *Journal of Laboratory and Clinical Medicine*, 93, 449–458.

COTES, P.M. (1973) Erythropoietin 3. Measurement. 3.1 Bioassay, 3.2 Radioimmunoassay. *Methods in Investigative and Diagnostic Endocrinology*. Vol. 2B (ed. by S. A. Berson and R. S. Yalow), pp. 1110–1123. North Holland Publishing Company, Amsterdam.

COTES, P.M. & BANGHAM, D.R. (1961) Bioassay of erythropoietin in mice made polycythaemic by exposure to air at a reduced pressure. *Nature*, 191, 1065–1067.

COTES, P.M. & BANGHAM, D.R. (1966) The international reference preparation of erythropoietin. *Bulletin of the World Health Organisation*, 35, 751–760.

COTES, P.M., BROZOVIC, B., MANSELL, M. & SAMSON, D.M. (1980) Radioimmunoassay of erythropoietin in human serum: validation and application of an assay system. *Experimental Haematology*, 8, Suppl. 8, 292.

COTES, P.M. & WARDLE, D.E.G. (1979) Erythropoietin. *Hormones in Blood*, 1 (ed. by C. H. Gray and V. H. T. James), 3rd edn, pp. 473–497. Academic Press, London.

ERSLEV, A.J. (1977) The clinical usefulness of erythropoietin measurements. *Kidney Hormones. II. Erythropoietin* (ed. by J. W. Fisher), pp. 571–584. Academic Press, London.

ERSLEV, A.J., CARO, J., MILLER, O. & SILVER, R. (1980) Plasma erythropoietin in health and disease. *Annals of Clinical and Laboratory Science*, 10, 250–257.

ESPADA, J. (1977) Chemistry and purification of erythropoietin. *Kidney Hormones. II. Erythropoietin* (ed. by J. W. Fisher), pp. 31–71. Academic Press, London.

GARCIA, J.F., SHERWOOD, J. & GOLDWASSER, E. (1979) Radioimmunoassay of erythropoietin. *Blood Cells*, 5, 405–419.

HEALY, M.J.R. (1972) Statistical analysis of radioimmunoassay data. *Biochemical Journal*, 130, 207–210.

KOEFFLER, H.P. & GOLDWASSER, E. (1981) Erythropoietin radioimmunoassay in evaluating patients with polycythemia. *Annals of Internal Medicine*, 94, 44–47.

MARCHALONIS, J.J. (1969) An enzymic method for the trace iodination of immunoglobulins and other proteins. *Biochemical Journal*, 113, 299–305.

MIYAKE, T., KUNG, C.K-H. & GOLDWASSER, E. (1977) Purification of human erythropoietin. *Journal of Biological Chemistry*, 252, 5558–5564.

SHERWOOD, J.B. & GOLDWASSER, E. (1979) A radioimmunoassay for erythropoietin. *Blood*, 54, 885–893.

THORELL, J.I. & JOHANSSON, B.G. (1971) Enzymatic iodination of polypeptides with ^{125}I to high specific activity. *Biochimica et Biophysica Acta*, 251, 363–369.

VAITUKAITIS, J., ROBBINS, J.B., NIESCHLAG, E. & ROSS, G.T. (1971) A method for producing specific antisera with small doses of immunogen. *Journal of Clinical Endocrinology and Metabolism*, 33, 988–991.

WALLNER, S.F., KURNICK, J.E., VAUTRIN, R.M., WHITE, M.J., CHAPMAN, R.G. & WARD, H.P. (1977) Levels of erythropoietin in patients with the anemias of chronic diseases and liver failure. *American Journal of Hematology*, 3, 37–55.

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