EFFECTS OF CYCLIC ADENOSINE 3',5'-MONOPHOSPHATE ON ERYTHROPOIESIS IN VITRO

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PREFACE

In the wake of Sutherland's *et al.* (1965) "second messenger" hypothesis, it has been established that many hormones exert their effects on target tissues by changing the cellular concentration of cyclic adenosine 3',5'-monophosphoric acid (cAMP or cyclic AMP). Sutherland's subsequent Nobel Prize and the interest he stimulated about this molecule have prompted numerous research reports, recent reviews (Robison *et al.*, 1968; Breckenridge, 1970; Rasmussen, 1970; Hardman *et al.*, 1971; Pastan and Perlman, 1971) and a monograph on the subject by Sutherland himself (Robison *et al.*, 1971).

Sutherland's hypothesis can be summarized as follows. There exist on the cell membrane receptor sites to which hormones attach. Hormone attachment to a receptor site causes an increase in the activity of a membrane-bound adenyl cyclase. Increase in adenyl cyclase activity causes an increase in conversion of adenosine triphosphate (ATP) to cAMP. Cyclic AMP initiates metabolic responses to a given hormone.

That cAMP is the second messenger of many polypeptide hormones has encouraged investigation of erythropoietin (EPO) for mediation by cAMP. Further impetus has been given by two preliminary papers read at national meetings that reported that the dibutyryl derivative of cAMP stimulated the incorporation of radioiron into red blood cells (Gorshein and Gardner, 1970; Winkert and Birchette, 1970).

To date, all attempts by researchers in this field to show an effect of cAMP on erythropoiesis, with iron incorporation into heme as

the index of erythropoiesis, have failed. The research reported in this dissertation will demonstrate that cAMP does have an effect on heme synthesis of rat bone marrow cells *in vitro* under a modified Goldwasser system (Goldwasser and Gross, 1969).

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INTRODUCTION

SYNOPSIS

Erythropoiesis is the process by which mature erythrocytes, or red blood cells, are produced. Erythropoiesis is controlled by the hormone erythropoietin (EPO), presumed to originate in the kidney in response to reduced oxygen tension (White *et al.*, 1960). EPO regulates the rate of development of committed cells into mature erythrocytes, may direct stem cell differentiation and hastens the release of reticulocytes into circulation. EPO, however, is such a large glycoprotein that one would expect it to exceed the limits of permeability of its target cells in bone marrow. Since many relatively small polypeptide hormones are mediated by the second messenger, cyclic 3',5'-adenosine monophosphate (cyclic AMP or cAMP) (Robison *et al.*, 1971), it is reasonable to wonder if EPO is also. Clinically and experimentally, steroid metabolites, androgens and estrogens modify erythropoiesis (Gordon *et al.*, 1970; Byron, 1971), but their relationships, if any, to EPO or cAMP remain unclear; interpretations of research in this field must be viewed, therefore, with caution. Cyclic AMP affects differentiation, mitosis, post-confluence inhibition of cell division and cell agglutinibility in non-hemopoietic culture systems (Bürk, 1968; Otten *et al.*, 1971; Prasad and Sheppard, 1972; Sheppard, 1972). These facts and its controversial effects on erythroid cells warrant investigation of the relationships of

cAMP and erythropoiesis.

ERYTHROPOIETIN

Control of erythropoiesis by a humoral mechanism was first suggested by Carnot and Deflandre in 1906. They observed that plasma from anemic rabbits raised peripheral erythrocyte counts of normal rabbits and correctly inferred the existence of a blood-borne factor that stimulates erythropoiesis. They provisionally named the agent *hémopoïétine*. The physiologic significance of the factor was revealed when Reissmann (1950) found that the induction of anoxia in one parabiotic rat stimulated erythrocyte production in both. After other authors (Erslev, 1953; Borsook *et al.*, 1954; Gordon *et al.*, 1954) confirmed the observation of Carnot and Deflandre, the function of the humoral factor was found to be involved exclusively in erythrocyte production and the name erythropoietin (EPO) was adopted (White *et al.*, 1960).

Researchers' attempts to identify the site of EPO production by excision of various organs ultimately focused on the kidney when Jacobson *et al.* (1957) demonstrated a marked reduction in circulating EPO titers in bilaterally nephrectomized rats and rabbits. The idea that the kidney controls erythropoiesis by producing EPO and acting as an oxygen sensor has been so widely accepted that Erslev (1971) refers to it as the "central dogma in erythropoietin research." A growing body of evidence, however, now constitutes a serious challenge to this dogma.

Parenthetic but of interest is a review of the controversy surrounding the proposed sites of EPO production. Such indirect evidence as Jacobson's *et al.* (1957; 1959) forms the strongest argument for inferring a major role of the kidney in regulating erythropoiesis; empirically,

appreciable amounts of EPO have yet to be extracted from kidney homogenates. Although Gordon *et al.* (1967) could not find EPO in kidney homogenate, they did extract what they called renal erythropoietic factor (REF) which stimulated erythropoiesis--but only after incubation with normal plasma. They speculated that REF is an enzyme that under normal and hypoxic conditions is released from the kidney and interacts with a serum precursor of EPO to produce active circulating EPO. An extremely efficient inactivator of EPO, a lipid substance extracted from the kidney, led its discoverers (Erslev and Kazal, 1968; Erslev *et al.*, 1971) to infer that inactive EPO may, indeed, be stored in the kidney; but physiologic EPO-releasing factors elude discovery. This evidence does not preclude the kidney as a site of EPO production. When, however, Erslev (1960) and Jacobson (1962) noted that normal erythropoiesis continued in nephrectomized animals, the existence of at least one extrarenal source of EPO or an EPO analogue became clear. In addition, increased rates of erythropoiesis in anephric laboratory animals in response to hypoxia (Fried *et al.*, 1969; Peschle *et al.*, 1972) proved the necessity of extrarenal oxygen sensors. Among various reports of sites of extrarenal erythropoietic control, two recent ones present particularly intriguing concepts.

In one, Perris and Whitfield (1971), using polycythemic and nephrectomized rats, detected increases in several indices of erythropoiesis in response to elevated plasma concentrations of calcium via injection of calcium chloride or parathyroid hormone. Conversely, thyroparathyroidectomized rats manifested parallel decreases in plasma calcium and the same indices of erythropoiesis, both effects reversible by injection of calcium chloride or parathyroid hormone. The authors acknowledged

the importance of EPO in dictating erythroid differentiation in consort with the calcium homeostatic system's determination of subsequent erythropoietic amplification.

In the other report, Tramezzani *et al.* (1971) found that the carotid body, too, is an erythropoietic organ. One reason to suspect its implication in erythropoiesis is its long-known function as an oxygen-carbon dioxide chemoreceptor. In another line of evidence, bilateral resection of the carotid body of cats resulted in anemia; induced anemia in intact cats caused weight increase in the carotid body and concomitant morphological changes that indicate increased protein synthesis. Furthermore, extracts and efferent blood from the carotid body stimulated radio-iron incorporation into the erythrocytes of polycythemic rats. Tramezzani *et al.* inferred from their data, including a dose-response curve, that one secretion of the carotid body may be none other than EPO.

Regardless of which of these or any new hypotheses is finally proved correct, it is certain from studies on anephric individuals (Nathan *et al.*, 1964; Fried and Gurney, 1968; Peschle *et al.*, 1972) that the kidney plays an important role in the circumscription of erythropoiesis.

A review of the effects of EPO on hemopoietic tissue *in vivo* and *in vitro* will illustrate its central function in the control of erythropoiesis. Evidence indicates that EPO *in vivo* causes stem cells to differentiate (Alpen and Cranmore, 1959; Hrinda and Goldwasser, 1969). It probably also controls the rate of development into mature erythrocytes by hastening red cell maturation, augmenting hemoglobin production and speeding up the release of reticulocytes into circulation (Alpen and Cranmore, 1959; Schooley, 1965; Hillman and Finch, 1967). An early

increase occurs in bone marrow RNA and DNA synthesis *in vivo* (Borsook, 1964; Hodgson, 1967) and *in vitro* where it is characterized by the production of a large molecular-weight RNA (150s) within fifteen minutes after stimulation by EPO (Krantz and Goldwasser, 1965a; Goldwasser and Gross, 1969). EPO has been implicated not only in such control of nucleic acids during cell division and maturation, but also in the stimulation of hemoglobin synthesis, as indicated by incorporation of radiolabeled valine into globin (Goldwasser and Gross, 1969) and by radiolabeled iron incorporation into heme (Krantz *et al.*, 1963). The activity of the presumed rate-limiting enzyme in heme biosynthesis, δ-aminolevulinic acid synthetase (ALAase), is enhanced *in vitro* by EPO (Bottomley and Smithee, 1969). Other functions of EPO include stimulation of stroma production as indicated by incorporation of glucosamine (Dukes *et al.*, 1964) and regulation of cellular iron accumulation into a non-hematinic compound. Seventy-seven percent of this cellular uptake in five hours is non-hematinic, but both total radioiron incorporation and that into heme alone are linear and proportional to the log of EPO concentration in the cultures in the range of 0.004 to 0.2 units[1] per milliliter (Krantz *et al.*, 1963; Hrinda and Goldwasser, 1969).

Recently much emphasis has been placed on isolating and purifying EPO (Goldwasser and Kung, 1968; 1971a; 1972) because of its possible benefit in the treatment of production anemias. Lack of clinically useful

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[1]According to Camiscoli and Gordon (1970), one "International Unit is defined as the activity contained in 1.48 mg of the IRPE [International Reference Preparation of Erythropoietin] which is equivalent to . . . one Cobalt Unit (CoU); the CoU activity being equal to that obtained with 5 μmoles of $CoCl_2 \cdot 6H_2O$ in the fasted rat assay system (Goldwasser et al., 1958)."

quantities of EPO has necessitated development of a substantial literature on the use of androgens to provoke erythropoiesis (Gordon *et al.*, 1966; Sanchez-Medal *et al.*, 1969). In most cases it appears that androgens directly affect the kidney or other organ to increase the production of native EPO. A study by Byron (1971) is one of the few that allow one to assess the direct effects of these compounds on hemopoietic cells. He demonstrated that an early action of testosterone and some of the chemically related steroids on bone marrow cells *in vitro* is to increase proliferation of spleen colony-forming units. 5β-H steroid metabolites may directly stimulate bone marrow cells (Gordon *et al.*, 1970; Gorshein and Gardner, 1970); but to conclude that the androgenic steroids that have been successful in the treatment of acquired aplastic anemias are a direct stimulus, EPO-mediated or a combination of the two would be premature.

Steroid factors, therefore, may constitute an insidious element in any investigation of erythropoiesis and should be taken into account, first in experimental design and then in interpretation of results.

No single organism can yield the extremely large quantities of EPO necessary for purification. Two sources of EPO are commonly used (Krantz and Jacobson, 1970), urine from patients with acute anemia and plasma from animals made severely anoxic by hemolytic agents. From the second source, EPO has been purified 1,180,000-fold with a two-percent yield by a combination of chromatography, ammonium sulfate precipitation and dialyzation against calcium phosphate gels. Extremely small quantities of this yield, still insufficient for direct analysis, are electrophoretically pure (Goldwasser and Kung, 1971a).

EPO has been identified as a sialic acid-containing glycoprotein.