Studies on Hormonal Induction of Embryonic Erythropoiesis

by

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It has been established that the hormone, erythropoietin, is concerned in the control of normal adult erythropoiesis in mammals; it is known to act by stimulating maturation of progenitor cells. The part played by erythropoietin during foetal life is not clear; its mode of action on sensitive cells is also uncertain. The hormone may be assayed *in vitro* by measuring the incorporation of ^{59}Fe into protein-bound haem by erythroid cell cultures.

The erythroid activity and response to erythropoietin of cells from rat yolk sac, foetal liver and foetal spleen were investigated; similar studies were carried out on rabbit foetal liver cells. The pattern of *in vivo* activity and sensitivity to erythropoietin observed during gestation suggested that, in both species, foetal erythropoiesis was controlled, in part, by erythropoietin; it was concluded that erythropoietin, probably of foetal origin, controlled red cell production except during yolk sac erythropoiesis.

In many mammals the foetus produces haemoglobin distinct

from the adult haemoglobin. Using starch gel electrophoresis the nature of the haemoglobins in circulating cells during gestation of mouse and rabbit foetuses was determined. Two haemoglobins were found in the blood of young rabbit foetuses; three were found in young mouse foetuses. In both species these disappeared during gestation and were replaced by a single adult haemoglobin. The site of production of the different haemoglobins was determined by starch gel electrophoresis of ^{59}Fe-labelled haemoglobin synthesised by erythroid cells in vitro. Only foetal haemoglobins were made by mouse yolk sac cells while only adult haemoglobin was made by foetal liver cells. Erythropoietin, to which only foetal liver cells were sensitive, stimulated only adult haemoglobin synthesis; it did not appear to affect haemoglobin synthesis directly, in circulating cells.

Two haemoglobins were found in the blood of young rat foetuses; three others appeared during the early stages of foetal liver erythropoiesis. After birth, the amount of one of the components first present decreased to very low levels in adult animals. The relative rates of synthesis of the haemoglobins were characteristic of the stage of gestation and remained the same during much, if not all, of cell maturation. It was found that erythropoietin treatment of foetal or adult erythroid cells had only a quantitative effect on haemoglobin synthesis;

it did not alter the relative amounts of each haemoglobin present. As in mouse, it appeared to have no direct effect on haemoglobin synthesis by circulating cells.

The effects of inhibitors, colchicine, FUdR, actinomycin D and puromycin, on the response to erythropoietin of mouse foetal liver cells indicated that syntheses of DNA, RNA and protein, but not cell division, were essential for erythropoietin-induced haemoglobin synthesis. Further studies indicated that cells usually divided after erythropoietin treatment but that this was not mandatory for haemoglobin synthesis.

The technique was altered to measure synthesis of DNA, RNA and total protein, as well as haem synthesis after erythropoietin treatment. These studies, including the effect of inhibitors upon the response, suggested that the first detectable result of erythropoietin treatment was mRNA synthesis; this permitted protein synthesis which was essential for DNA synthesis. These events occurred very quickly and DNA synthesis doubled within 1 hr. of erythropoietin treatment. Afterwards, cell maturation, including haemoglobin synthesis, could proceed. The time-course of the response suggested that erythropoietin might stimulate maturing cells, capable of DNA synthesis, as well as progenitor cells; the responding cells, whatever their nature, appeared to be sensitive to erythropoietin during the G_1 period of their cycle. These results were discussed in relation to <u>in vitro</u> studies on other differentiating cells.

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Hormonal Induction of Embryonic Erythropoiesis.

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in the Faculty of Science

by

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CONTENTS

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ABBREVIATIONS

ALA	δ -aminolaevulinic acid
BSS	balanced salt solution
CFC	colony-forming cells
ESC	erythropoietin-sensitive cells
FUdR	5-fluorodeoxyuridine
PBG	porphobilinogen
PCA	perchloric acid
P.P.O.	2,5 diphenyloxazole
P.O.P.O.P.	1,4-di{2-(5 phenyloxazolyl)} benzene
mRNA	messenger RNA
rRNA	ribosomal RNA
tRNA	transfer RNA
SLS	sodium lauryl sulphate
u.	units

Other abbreviations were used as specified in Biochem.J., 102, 1 (1967).

I. INTRODUCTION

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Introduction

1. Mammalian embryonic erythropoiesis

In mammalian embryos erythroid differentiation takes place successively in the yolk sac, general mesenchyme, liver, spleen and bone marrow (Le Douarin, 1966). It first occurs in the extra-embryonic blood islands surrounding the umbilical vesicle. The blood-forming cells arise from the splanchnopleuric connective tissue interspersed between the endodermal and mesodermal layers (Sorenson, 1961; Block, 1946); the centrally placed cells become the primitive blood cells, while the peripheral cells flatten out to form a sinusoidal lining. By the 5-6 somite stage haemoglobin synthesis has begun, a network of anastomosing sinusoids is established extra- and intra-embryonically, and the heart is beating (Le Douarin, 1966). At the 30-40 somite stage small blood-forming foci appear in the mesenchyme and erythropoiesis in the yolk sac begins to decrease (Sorenson, 1961). The mesenchymal phase is brief except in the liver which becomes the main erythropoietic organ.

The liver arises from interdigitation of the foregut-floor endoderm with mesenchyme from the septum transversum and recruitment of cells from the coelomic mesothelium. Erythroid cells are derived from the septum mesenchyme and the coelomic mesothelium which is continuous with the splanchnic mesoderm of the yolk sac (Wilson *et al.*, 1963). Erythroid maturation takes place extravascularly in close

contact with the hepatocytes (Sorenson, 1960; Jones, 1959; Grasso, et al., 1962); the maturing cells are grouped together with the youngest placed centrally (Fish, 1960). Before maturation is complete the cells may enter the sinusoids, either through discontinuities present in the sinusoidal wall or by rupturing it (Zamboni, 1964; Grasso, et al., 1962), where maturation may continue. The cell population in the sinusoidal lumens becomes more mature as liver erythropoiesis proceeds, while the extravascular population remains relatively immature (Fish, 1960). The liver is the most important erythropoietic organ of embryonic life because of its size and of the length of time it remains erythropoietic, until birth in many species (Le Douarin, 1966).

The spleen becomes erythropoietic during the later stages of liver erythropoiesis; it later becomes lymphopoietic and in only a few species, e.g. rodents, does it retain a capacity for erythropoiesis (Le Douarin, 1966; Russell and Bernstein, 1966). Erythropoiesis in the liver generally outlasts that in the spleen. The spleen arises as a condensation of mesenchymal cells on the left dorso-lateral portion of the mesentery at its junction with the stomach wall (Thiel and Downey, 1921); erythroid maturation proceeds extravascularly in the same way as in the liver (Le Douarin, 1966; Thiel and Downey, 1921).

The bone marrow medulla becomes erythropoietic even later in gestation, the extent to which it does so depending upon the length of gestation (Le Douarin, 1966). It increases in activity progressively, as the activity of other sites decreases, until it is the major and

usually the only, source of erythroid cells in the adult animal.

2. Maturation of erythroid cells

The morphological changes during erythroid maturation have been intensively studied; the changes observed are very similar in adult and foetal erythroid tissues from all mammalian species studied (Pease, 1956; Fish, 1960; Jones, 1959; Zamboni, 1964; Le Douarin, 1966; Sorenson, 1960, 1963; Ackerman et al., 1961; Grasso et al., 1962). Only the maturation of yolk sac cells is significantly different (Sorenson, 1961; Marks and Kovach, 1966). Many terminologies have been applied to the different maturation stages; that used here is, in order of increasing maturation, pronormoblast, basophilic normoblast, polychromatic normoblast, acidophilic normoblast, reticulocyte, erythrocyte. These terms represent stages in a continuous process and no clear-cut line can be drawn between most of them; intermediate stages are frequently seen.

In general, immature cells are large and have a high nucleus-to-cytoplasm ratio; they have several nucleoli and very many cytoplasmic organelles. During maturation the size of the cell and the nucleus-to-cytoplasm ratio decrease, the nucleoli quickly disappear and the cytoplasmic organelles decrease progressively in number; the nucleus itself is extruded, transforming the acidophilic normoblast into the reticulocyte. The variety of proteins synthesised decreases until haemoglobin synthesis constitutes over 90% of total protein synthesis.

DNA synthesis continues only until the maturing cell approaches the polychromatic normoblast stage (Grasso et al. 1963; Cronkite, 1964); cell division does not occur beyond the polychromatic normoblast stage (Lajtha, 1964; Borsook, 1964; Grasso et al., 1963). Cell division is estimated to occur at least three times as the pronormoblast matures (Lajtha, 1964; Borsook, 1966) and the length of the division cycle is probably 12-20 hr. (Lajtha, 1964; Cronkite, 1964). After DNA synthesis and cell division cease the nucleus continues to disintegrate and is eventually extruded (Jones, 1959; Grasso et al., 1962). The appearance of haemoglobin in the nucleus at these later stages may contribute to the disintegration of the nucleus (Marks and Kovach, 1966); the haemoglobin may be synthesised within the nucleus or enter from the cytoplasm through the nuclear annuli (Grasso et al., 1962).

The pronormoblast contains very many ribosomes which are mainly associated as polysomes (Rifkind et al., 1964); most ribosomal synthesis occurs before or during this stage (De Bellis et al., 1964). This is in accord with the rapid breakdown of nucleoli after this time (Grasso et al., 1962). As maturation proceeds the number of ribosomes decreases and, in the later stages, the proportion of polysomes also decreases (Rifkind et al, 1964; Grasso et al., 1962); there is evidence that some reticulocyte polysomes may be inactive (Ulowacki and Millette, 1965; Marks et al., 1965).

The number of mitochondria also decreases during maturation although the enzymatic activity associated with each remains constant (Pease, 1956; Ackerman, 1962); the mitochondrial content of the cells

falls faster than the ribosomal content (Grasso et al., 1962; Ackerman, 1962).

RNA synthesis occurs primarily during the pronormoblast and basophilic normoblast stages and cannot be detected after the polychromatic normoblast stage (Grasso et al., 1963; De Bellis et al., 1964). The concentration of RNA decreases until the cell reaches the polychromatic stage, remains constant thereafter until the nucleus is extruded and then decreases once more (Grasso et al., 1963); during early maturation the cellular RNA appears to be turning over quite rapidly. The decrease in RNA concentration late in maturation may be effected by enzymatic degradation to nucleosides and pentose (Marks and Kovach, 1966; Adachi et al., 1964).

The rate of protein synthesis changes little until the polychromatic normoblast stage (Grasso et al., 1963; Borsook, 1964); since the protein concentration also remains relatively constant, the cellular proteins appear to be turning over fairly rapidly (Grasso et al., 1963; Borsook, 1966). Haemoglobin synthesis can first be detected in late basophilic or early polychromatic normoblasts (Grasso et al., 1963) although some globin may be present earlier than this (Ackerman, 1962; Wilt, 1962). As haemoglobin synthesis increases the overall rate of protein synthesis is at first unchanged and then decreases as synthesis of non-haemoglobin proteins declines (Borsook, 1966). Since synthesis of haemoglobin, and of some other proteins, continues after RNA synthesis has stopped, the mRNAs involved must be stable; the haem

synthesising enzymes or their mRNAs must also be stable.

The cells are usually released as reticulocytes; those released by foetal erythroid tissue are often less mature than in the adult (Fisher et al., 1965a; Le Douarin, 1966). There is evidence that a small proportion of the cells is always released prematurely although containing near-normal amounts of haemoglobin, perhaps by by-passing or accelerating a stage of maturation prior to nuclear loss (Borsook et al., 1968; Stohlman et al.,1964; Borsook et al., 1962). The time taken to reach the reticulocyte stage after cell division has stopped is between 30-50 hr. (Lajtha, 1964); reticulocyte maturation may take another 48-72 hr. (Stohlman et al., 1964).

Yolk sac erythroid maturation. The maturation of the primitive erythrocytes in the yolk sac follows a similar but not identical pattern. The cells are larger throughout but have smaller, more granular nuclei; the nucleus is not extruded and may undergo mitosis even in the circulation (Sorenson, 1961; Craig and Russell, 1964). Ribosomes and mitochondria are lost progressively and eventually completely (Sorenson, 1961; Kovach et al., 1967); the loss of ribosomes has been correlated with a decrease in haemoglobin synthesis (Kovach et al., 1967). As foetal liver erythropoiesis proceeds, the yolk sac cells in circulation are replaced by non-nucleated erythrocytes and rarely persist until birth (Le Douarin, 1966; Wintrobe and Shumacker, 1936).

3. Stem cells

Erythroid cells are destroyed and replenished in considerable quantities throughout the life of an animal, but identifiable erythroid cells are not self-propagating (Alpen and Cranmore, 1959; Lajtha, 1964). Furthermore, suppression of erythropoiesis until no recognisable erythroid precursors are found does not destroy the erythropoietic capacity of the animal (Jacobson et al., 1957). Such observations imply that there exists a pool of undifferentiated cells, which are both self-maintaining and capable of differentiation when required, called stem cells (Patt and Quastler, 1963). The pool must be able to maintain the erythroid mass at its correct size not only under normal conditions but also in conditions of stress, such as rapid foetal growth, changes in oxygen tension or large loss of blood (Boggs, 1965).

Models of stem cell function. Osgood (1957, 1959) suggested that stem cells were capable of two kinds of division: symmetric, to maintain themselves, or asymmetric, to produce differentiated cells as well as more stem cells. Such a model would explain many aspects of erythropoiesis, but the very rapid replenishment of the system after massive blood loss is less well explained. Asymmetric cell division is a rare occurrence and is unequivocally present only during the early stages of development of certain fertilised eggs (Brachet, 1967); in this case the asymmetry appears to be determined by the

cytoplasm rather than the nucleus. No means of switching to one or other kind of division has been demonstrated and there is no direct evidence for this model.

It has been postulated that stem cells divide continuously at a set rate and that unwanted cells die (Cronkite, 1964); this model might lead to complete depletion of the pool during a prolonged demand for a high rate of differentiation, an event which has not been reported. The model most accepted at present is an extension and refinement of these, proposed by Lajtha and co-workers (Lajtha and Oliver, 1961; Lajtha, 1964).

According to this model, self-maintenance and differentiation are independently controlled and affect each other only indirectly; the former is regulated by the size of the stem cell population, while the latter is influenced by exogenous factors. A certain fraction of the cells is always actively cycling at a given time; these cells cannot be stimulated to mature, so that complete depletion of the pool does not occur. After partial depletion the remaining cells do not pass through a resting phase and cannot be stimulated to mature; thus, depletion of the stem cell pool leads to a more rapid production of stem cells until there is once more a sufficient number to allow maturation of further cells. The rate of replenishment of the pool may be steadily maintained at several different levels.

The number of erythroid cells is steadily maintained at different oxygen tensions (Boggs, 1965). There is evidence of more rapid pro-

liferation of precursor cells following massive bleeding in rats (Hanna, 1967); an increase in the proportion of cells actively cycling has also been reported to reduce their ability to colonise irradiated recipients (Becker et al., 1965). These observations lend support to this model. In addition, the model was used to predict the kinetics of the recovery from irradiation (Lajtha and Oliver, 1961) and the predictions were later confirmed (Gurney et al., 1962).

Haematopoietic colony formation in the spleen of irradiated hosts. The stem cell content of haematopoietic tissue may be estimated by its capacity to form colonies in the spleen of irradiated recipients (Till and McCulloch, 1961); the colonies are clonal in nature (Becker et al., 1963). Both granulocytic and erythropoietic elements are found in some colonies, and the cells of homogeneous colonies of either cell line can produce all the kinds of colony produced by bone marrow cells (Lewis and Trobaugh, 1964). While none of the spleen colonies is lymphoid, they contain cells capable of repopulating lymphoid tissue (Curry et al., 1967); lymph node tissue does not support recovery from irradiation (Cudkowitz et al., 1964a). Colony forming cells (CFC) thus exhibit both self-maintenance and the capacity to differentiate into one or more cell lines. Whether they are identical with stem cells is, however, not certain.

Identity and source of stem cells. Stem cells have been found in foetal and adult haematopoietic tissue (Feldman, 1967) and their presence in peripheral blood has also been reported (Goodman and Hodgson, 1962). It has been suggested that a cell morphologically identical with a small lymphocyte may be a stem cell (Fliedner et al., 1964, Cudkowitz et al., 1964b) but direct evidence for this is lacking. On morphological grounds, it has been suggested that the reticular cells in haematopoietic tissue are stem cells (Pease, 1956) but again there is no direct evidence and the identity of the stem cell remains unknown.

During foetal development the new blood-forming foci may result from differentiation of cells already present or from colonisation of the site by cells from previous erythroid sites. Stem cells are probably capable of colonisation, but differentiation of cells in situ has not been ruled out. The cells produced in the yolk sac are so different from the cells produced elsewhere as to suggest that they do not colonise later sites.

Different stem cell varieties. Although there is now good evidence for the existence of pluripotent stem cells, it is not known whether these differentiate directly into the various blood cell lines or whether a separate stem cell stage, limited to only one cell line, intervenes. In vivo each cell line is controlled independently (Boggs, 1965) and suppression of erythropoiesis does not affect the output of

the other cell lines (Jacobson et al., 1961; Morse et al., 1964). Serial transplantation of spleen colonies or bone marrow cells through irradiated hosts leads to a progressive decline and eventual loss of colony-forming ability, although the latest donor animals in the series can still effect normal haematopoietic control (Lajtha, 1966; Siminovitch et al., 1964). These observations suggest at least a separation of stem cell function; further studies on spleen colony formation influenced by erythropoietin, described in section 6, suggest that the different functions are associated with separate cell types.

4. Structure and synthesis of haemoglobin

During maturation, as has been seen, the variety of proteins synthesised by erythroid cells is reduced until over 90% of the total protein synthesised is haemoglobin; the mature cells contain relatively few other proteins. Haemoglobin is a tetramer of polypeptide chains, usually arranged in unlike pairs of identical chains, although varieties containing four identical chains have been found (Huehns et al., 1964a; Braunitzer et al., 1964; Schroeder, 1963). Each chain bears a haem group, located near the surface of the tetramer (Perutz, 1965); the haem is always composed of protoporphyrin IX combined with Fe^{++} (Lascelles, 1964). The molecular weight of the whole is 68,000 (Ingram, 1963; Schroeder, 1963).

The polypeptide chains of the major component of human adult haemoglobin are termed α-chains, containing 141 amino acids, and

β-chains, containing 146 amino acids; this terminology has been extended to animal haemoglobins as it became clear that their structure was very similar to that of human haemoglobin (Russell and Bernstein, 1966; Peruts, 1965; Huisman et al., 1965). β -like chains found in normal variants of human haemoglobin have been termed γ-, δ- and ε-chains but this practice has not been followed for animal haemoglobins.

The complete sequence of amino acids in several human haemoglobins is known (Braunitzer et al., 1964) as is that of some mouse haemoglobins (Russell and Bernstein, 1966); the sequence of several other animal haemoglobins is also completely or partially known (Braunitzer et al., 1964; Schroeder, 1963; Huisman et al., 1965).

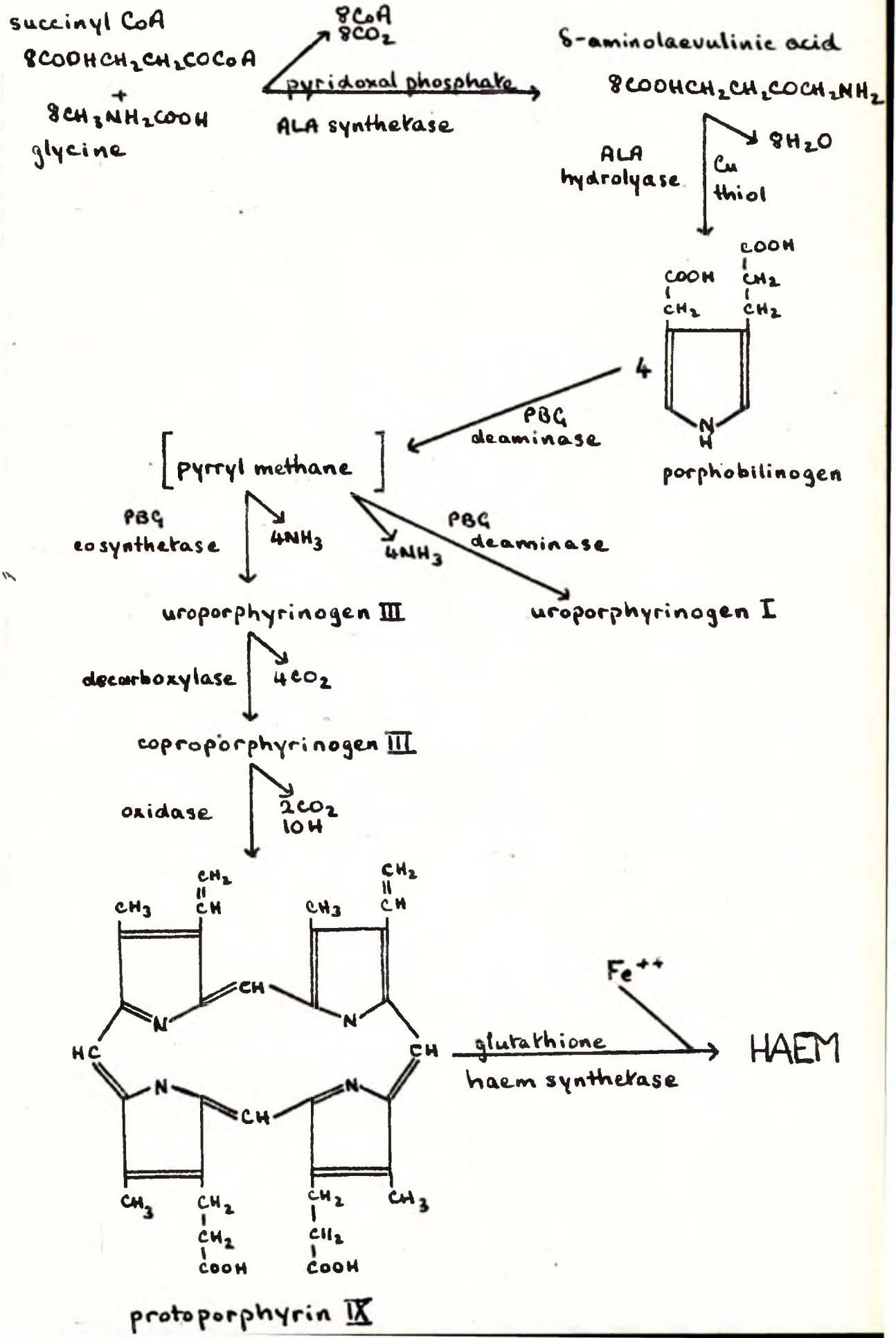
The synthesis of so complex a molecule requires the cooperation of three main pathways: the supply of iron, the synthesis of porphyrins, and the synthesis of globin.

Supply of iron. In order to enter erythroid cells iron is first bound to transferrin, a serum glycoprotein with a molecular weight of 85,000 (Bothwell and Finch, 1962), which can bind two atoms of iron to each molecule (London et al., 1964). Transferrin is bound preferentially to maturing erythroid cells all of which can absorb iron; in different species maximal iron absorption occurs at different maturation stages (Alpen, 1962; Bothwell and Finch, 1962; Russell and Bernstein, 1966).

Transport of iron across the cell membrane is rapid and energy-dependent (London et al., 1964; Allen and Jandl, 1960); it is independent

Figure 1.

The pathway of haem biosynthesis



of haemoglobin synthesis (Finch, 1957) and of the total amount of plasma iron except at very low levels (Bothwell and Finch, 1962). Transferrin does not itself enter the cells (Gitlin and Janeway, 1960).

Inside the cell the iron may be stored in the cytoplasm or taken up by the mitochondria, another energy-dependent step (Harris, 1964), prior to its insertion into haem (London et al., 1964). Although a mitochondrial enzyme catalyses the insertion of iron into haem (Lascelles, 1964) the bound iron must first be released from the mitochondria by a process requiring pyridoxal phosphate (Cooper et al., 1963). It seems likely that iron transport into the cells, its combination with and release from mitochondria are enzymatic processes, but there is no direct evidence for this.

Synthesis of haem. Many organisms synthesise tetrapyrroles from simple precursors and those so far studied utilise the same synthetic pathway, an outline of which is shown in Figure 1. In animal cells formation of δ-aminolaevulinic acid (ALA) and conversion of coproporphyrinogen III to haem take place in the mitochondria while the intermediate steps take place in the cytoplasm (Mahler and Cordes, 1966; Lascelles, 1964).

Glycine is probably derived mainly from exogenous sources; succinylcoenzyme A (succinyl-CoA) is derived either from exogenous succinate or by oxidative decarboxylation of α-oxoglutarate. Operation

of all or part of the tricarboxylic acid cycle is required for the latter at least and this is in accord with the mitochondrial location of ALA synthetase. It has been reported that glycine is activated by pyridoxal phosphate before being combined with the succinyl residue (Shemin, 1957).

Formation of uroporphyrinogen III from porphobilinogen (PBG) requires the simultaneous presence of PBG deaminase and PBG cosynthetase; the deaminase alone leads to the formation of uroporphyrinogen I upon which the cosynthetase cannot act (Bogorad, 1963). A pyrryl methane intermediate which may be a substrate for both enzymes has been postulated (Mahler and Cordes, 1966; London et al., 1964; Lascelles, 1964).

The decarboxylation of uroporphyrinogen III is known to proceed in stages and it is possible that more than one enzyme is involved (Mauzerall and Granick, 1958). The oxidation of coproporphyrinogen III is also multi-stage (Porra and Falk, 1963); the hydrogen acceptor of this reaction is oxygen which cannot be replaced by other acceptors (Lascelles, 1964).

The activity of the enzymes is normally well controlled since intermediates do not accumulate; in abnormal conditions the intermediates may be present in amounts greater than those of the functional products (Drabkin, 1951; Mauzerall and Granick, 1956). In tissues actively synthesising haem, e.g. marrow, the enzymes are present in greater amounts (Gibson et al., 1955; Sano and Granick, 1961) but variations in enzyme synthesis alone would probably not provide sufficiently fine control.

Since part of the pathway is cytoplasmic one means of regulation may be the rate of passage of ALA from, and of coproporphyrinogen III into, mitochondria, perhaps mediated by specific permeases (Granick and Mauzerall, 1961). The intra-mitochondrial concentration of ALA might also affect its rate of release; this, in turn, is determined by the availability of its precursors, especially succinyl-CoA. This compound is the substrate for several enzymes which compete with ALA synthetase; the tricarboxylic acid cycle is important for the supply of succinyl-CoA and alterations in its activity have been found to affect haem synthesis (Shemin and Kumin, 1952; Granick and Urata, 1963).

End products of the pathway such as haem or haemin, but not protophorphyrin IX, have been shown to inhibit haem synthesis *in vitro* (Levere and Granick, 1965; London *et al.*, 1964) and *in vivo* (Gallo, 1965) probably through their effect on ALA synthetase which is the overall rate-limiting enzyme (Lascelles, 1964). Haem or haemin have also been shown to inhibit ALA hydro-lyase, as does haemoglobin to a lesser degree (Calissano *et al.*, 1966); this enzyme may also be inhibited by copper deficiency (Cartwright *et al.*, 1957).

Synthesis of globin. The mechanism of protein synthesis is currently arousing a good deal of attention; much of the work has been carried out on reticulocytes, but the basic mechanism appears to be universally similar. Only a brief outline of the current model will be discussed since the means by which it may be controlled in maturing erythroid

cells is of more relevance.

The amino acid sequence of a protein is determined by the sequence of bases in the DNA corresponding to the protein by means of a code of non-overlapping base triplets (Crick et al, 1961; Bennett and Dreyer, 1964). The sequence of bases of the DNA is transcribed into a corresponding sequence in RNA; this type of RNA is known as messenger RNA (mRNA) (Singer and Leder, 1966). The mRNA attaches to a ribosome, in the cytoplasm in the case of globin synthesis, and the base sequence is translated into the amino acid sequence. The primary structure of the protein determines its three-dimensional structure (Anfinsen, 1963).

Amino acids are linked through their carboxyl groups to the adenylic moiety of the terminal cytidylyl-cytidylyl-adenylate sequence of transfer RNA (tRNA) after enzymatic activation, forming aminoacyl tRNA complexes; for each amino acid there is a specific activating enzyme and one, or more, specific tRNA (Moldave, 1965; Berg, 1961; Schweet and Heintz, 1966). The aminoacyl tRNA may then be bound to the mRNA/ribosome complex; the tRNA for each amino acid is thought to possess a 'recognition triplet' corresponding to the triplet for the same amino acid possessed by mRNA. This correspondence would ensure that the amino acids are assembled in the correct sequence (Schweet and Heintz, 1966; Singer and Leder, 1966).

The peptide chain is formed from the N-terminal end (Dintzis, 1961; Williamson and Schweet, 1965), the C-terminal end remaining attached to the tRNA on the ribosome until the chain is completed.

The α-amino group of the incoming aminoacyl tRNA reacts with the carboxyl carbon of the attached chain to form a peptide bond; the chain remains attached through the tRNA of the newly incorporated residue and the tRNA previously present is released. Repetition of this reaction results in synthesis of the completed peptide chain; as the chain grows, further ribosomes may attach to the mRNA to form polysomes and so increase the efficiency of translation of each mRNA molecule (Warner et al., 1963; Hardesty et al., 1963; Moldave, 1965).

It is obviously essential that complete peptide chains should be synthesised; in bacterial systems there is evidence that N-formyl-methionine acts as an initiation 'signal' for chain synthesis and is later removed from the completed chain (Leder and Bursztyn, 1966; Marcker et al., 1966). This does not appear to happen in erythroid cells (Rich et al., 1966) and it may be that the N-terminal residue of the globin chain itself determines chain initiation.

It has been suggested that completed β-chains are necessary to release completed α-chains (Colombo and Baglioni, 1966) or that completed α-chains are necessary to release β-chains (Cline and Bock, 1966) but free α-chains or free β-chains have been found when synthesis of the other is retarded (Weatherall et al., 1965; Ingram, 1963). In any case, such hypotheses do not account for the completion and release of the chains necessary to release subsequent chains. Nor is chain release dependent upon the formation of stable tetramers since free α-chains have been found in rabbit and human cells (Shaeffer et al., 1967; Winslow and Ingram, 1966) and free α-chains do not form

tetramers (Fessas and Loukopoulous, 1964). It has been concluded that chain completion is both necessary and sufficient for chain release (Williamson and Schweet, 1965), although it is possible that tetramerisation might enhance subsequent chain synthesis.

It has been found that the rate of amino acid incorporation decreases as the peptide chain lengthens in rabbit (Dintzis, 1961) and human cells (Winslow and Ingram, 1966; Weatherall et al., 1965); the rates of incorporation are such that this cannot be attributed to a scarcity of a particular type of tRNA (Dintzis, 1961; Englander and Page, 1965). The attachment of only one of thirty leucine residues in rabbit globin to a particular tRNA has been reported but is not rate-limiting (Weisblum et al., 1965). The decrease in rate has been attributed to steric hindrance as the lengthening chain folds up (Cline and Bock, 1966) or to insertion of haem at the point of slowing (Winslow and Ingram, 1966); there is no direct evidence that haem is inserted before the globin chains are released, but both explanations are possible.

Alteration of the DNA to produce less mRNA or less easily translatable mRNA would decrease the rate of globin synthesis; it is possible that this is the case in human thalassaemia (section 5). A less stable mRNA would also reduce the amount of globin synthesised; this is thought to occur in some human cells also (section 5).

Haem and globin interaction. In normal conditions haem and globin are synthesised at equivalent rates both in marrow cells (Morell et al., 1958), where many other proteins are being made, and in reticulocytes (Kruh and Borsook, 1956), where most of the protein made is haemoglobin. There is some immunological evidence for the presence of globin in chick embryo cells before haem synthesis can be detected (Wilt, 1962) and some histochemical evidence in rabbit foetal liver cells (Ackerman, 1962) has been interpreted to show globin synthesis preceding that of haem. Continued globin synthesis after inhibition of haem synthesis has also been found (Morell et al., 1958). It has been suggested that haem synthesis is thus unnecessary for globin synthesis, but the evidence is not conclusive.

Iron, haem and haem precursors have been shown to stimulate globin synthesis in vivo and in vitro (Grayzel et al., 1966; Waxman and Rabinowitz, 1965; Levere and Granick, 1965; Bruns and London, 1965); it appears that the effect of iron and of haem precursors depends on their incorporation into haem. Although haem does not appear to be essential for the release of globin chains from polysomes, it has been suggested that haem may facilitate chain release perhaps by stabilising the released chains (Grayzel et al., 1966; Gribble and Schwartz, 1965).

In chick embryos, where globin mRNA is transcribed some hours before haemoglobin synthesis can be detected (Wilt, 1965), the addition of ALA can not only increase haemoglobin synthesis but also initiate it earlier than normal (Wainwright and Wainwright, 1967). The delay

in haem synthesis in the absence of exogenous ALA may be due to low levels of some of the necessary enzymes or to diversion of the earliest precursors into other metabolic pathways at this stage. Similar studies have not been done on the earliest stages of mammalian erythropoiesis.

Increased levels of globin have been shown to stimulate haem synthesis (Schwartz et al., 1961); inhibition of globin synthesis has been shown to reduce haem synthesis (Grayzel et al., 1967). These effects are thought to be mediated by the control exerted by haem upon its own synthesis (Karibian and London, 1965).

5. Haemoglobin varieties

Human haemoglobins. In normal adult individuals 95 - 98% of the total haemoglobin is haemoglobin A, composed of α- and β-chains; the remainder is haemoglobin A_2, composed of α- and δ-chains (Ingram, 1963; Baglioni, 1963). A third component, haemoglobin A_3, found in ageing red cells appears to be a complex of haemoglobin A with glutathione (Braunitzer et al., 1964). Many abnormal variants of these, especially of haemoglobin A, have been described (Braunitzer et al., 1964; Schroeder, 1963; Baglioni, 1963), which may differ only in one amino acid of either chain and which are genetically controlled (Baglioni, 1963); they are usually synthesised more slowly than their normal counterparts (Jukes, 1966; Baglioni, 1963).

In very young foetuses two haemoglobins have been found, one of

which is a tetramer of ϵ-chains, while the other is composed of α-chains and ϵ-chains (Huehns et al., 1964a); their disappearance has been correlated with the disappearance of primitive erythrocytes from the foetal circulation (Butler et al., 1960). In later foetuses a further haemoglobin variety is found, haemoglobin F, composed of α-chains and γ-chains; small amounts of γ-chain tetramers are also found during the initial period of haemoglobin F synthesis (Huehns et al., 1964b). The rate of synthesis of α-chains in early foetuses does not appear to keep pace with that of ϵ-chains and γ-chains.

It has been suggested that the site of erythropoiesis may determine the nature of the haemoglobin produced (Kunzer, 1957) and it may be that ϵ-chains are produced only during yolk sac erythropoiesis. α-chains, however, are present at all stages studied and β-chain synthesis begins shortly after the onset of γ-chain synthesis (Thomas et al., 1960). In addition, both haemoglobin A and haemoglobin F have been found within the same cells (Kleihauer et al., 1957).

The structure of each chain is under separate genetic control (Baglioni, 1963; Ingram, 1963), the α-chain gene being distant from the others and possibly on a different chromosome (Neel, 1961; Motulsky, 1962). The β-chain and δ-chain genes are closely linked and may be adjacent (Baglioni, 1963); the γ-chain gene appears to be linked to these but less closely (Baglioni, 1963; Burka and Marks, 1964).

The rates of synthesis of human globin chains have often been studied and some genetically determined control points which may be

of general relevance have been detected. δ-chains are assembled much more slowly than α- or β-chains; this is not due to a limiting requirement for a particular tRNA (Reider and Weatherall, 1965; Winslow and Ingram, 1966). Such a requirement has not been ruled out in the case of abnormal variants upon which detailed studies have not been performed. The cause may be defective mRNA production or increased hindrance of assembly as the altered chain lengthens. Evidence for defects in mRNA production is found in thalassaemic cells in which the rate of chain synthesis is much reduced (Weatherall et al., 1965) although the ribosomes are functionally normal (Bank and Marks, 1966) and the chains are structurally unaltered (Guidotti, 1964).

The synthesis of haemoglobin A_2 relative to that of haemoglobin A is greater in immature than in mature cells (Reider and Weatherall, 1965); similarly, at birth the synthesis of haemoglobin F relative to that of haemoglobin A is greater in immature than in mature cells (Wecheles et al., 1965; Burka and Marks, 1964). These observations suggest that the mRNA of the different chains may have different life spans.

There is also some evidence that the activity of some genes may depress the activity of other genes. Homozygotes for persistence of high haemoglobin F synthesise no haemoglobin A or A_2 and are not anaemic (Baglioni, 1963; Marks and Kovach, 1966). Homozygotes for combined β/δ-chain thalassaemia, who produce small amounts of haemoglobins A and A_2, are anaemic despite compensation by increased

haemoglobin F synthesis (Brancati and Baglioni, 1966). This suggests that the activity of the β-chain and δ-chain genes may suppress that of the γ-chain gene.

It has been suggested that as erythroid stem cells replicate they lose the capacity to synthesise γ-chains; during periods of rapid erythropoiesis, as in the foetus and in anaemic conditions, the stem cells differentiate while still able to synthesise γ-chains (Baglioni, 1963). Thus, the disappearance of haemoglobin F during late foetal and early neonatal life is attributed to the decrease in the rate of erythroid differentiation; the cells in the stem cell pool replicate for long enough to lose the capacity for γ-chain synthesis. The clonal distribution of haemoglobin F in compensated anaemias (Thompson et al., 1961) provides some evidence for this hypothesis; the apparent linkage of the activity of γ-chain genes to that of β- and δ-chain genes also lends some support.

Any or all of the control mechanisms detected in human haemoglobin synthesis may operate in other animal cells also; indeed, where other mammals have been as closely studied some of the controls have already been detected.

Mouse haemoglobins. Some mouse strains (Hb^s/Hb^s) possess only one adult haemoglobin while others (Hb^d/Hb^d) possess two or more in unequal quantities (diffuse pattern); some of the variations observed in diffuse patterns may be due to differences in technique or to aggregation of

some of the components (Riggs, 1965; Russell and Bernstein, 1966). Some Hb^s/Hb^s strains possess electrophoretically identical haemoglobins; none of those so far compared is common to Hb^d/Hb^d strains also. Some Hb^d/Hb^d strains having two components have α-chains in common with an Hb^s/Hb^s strain; none of the β-like chains has been found to be identical (Hutton et al., 1962; Schwartz and Gerald, 1967; Russell and Bernstein, 1966). The structure and interrelation of the different varieties is not yet clear.

Three foetal haemoglobins were found in Hb^s/Hb^s mice; their disappearance from the circulation was correlated with the decrease in the proportion of nucleated erythrocytes (Craig and Russell, 1964). Yolk sac cells were later shown to synthesise only these haemoglobins while foetal liver cells synthesised only adult haemoglobin (Kovach et al., 1967). Foetal haemoglobins were also found in Hb^d/Hb^d mice; they were replaced by adult haemoglobin before birth, but more slowly than those of Hb^s/Hb^s mice (Craig and Russell, 1963).

The structure of mouse haemoglobins is also genetically controlled and some of the genes have been mapped (Russell and Bernstein, 1966). As in man, the β-like chain genes appear to be closely linked, while the α-chain gene is separate (Hutton et al., 1964; Russell and Bernstein, 1966).

No haemoglobinopathies have been found in mice, but several genetically controlled anaemias have been studied; they appear to be due to defects in cell proliferation as, e.g. W-series anaemias

(McCulloch et al., 1964; Keighley et al., 1966) or sideroocytic anaemias associated with flex-tailed (f) characteristics (Thompson et al., 1966; Russell and Bernstein, 1966). Sl-series anaemias are due to inhibition of cell proliferation by their environment (McCulloch et al., 1965).

Rat haemoglobins. Early studies on rat haemoglobin indicated the presence of several components (Gratzer and Allison, 1960). More recently, 7 components were found in random-bred Wistar rats (Brada and Tobiska, 1963), using CM-cellulose chromatography; it was reported that the relative rates of ^{59}Fe incorporation into the different components were unchanged as erythrocytes matured.

Four components were found in two inbred rat strains, one an inbred Wistar strain and one an inbred Wistar/black hybrid (Marinkovic et al., 1967); three components were common to both. Random-bred Wistar rats were found to have five components, electrophoretically identical to the components found in the two inbred strains. It was suggested that the five-component pattern was a combination of the two four-component patterns.

No information is available on foetal rat haemoglobins nor on the control of rat haemoglobin synthesis.

Rabbit haemoglobins. Only one component has been found in adult rabbit haemoglobin (Gratzer and Allison, 1960). A difference was found in the acid solubility of the haemoglobins in the nucleated and in non-nucleated erythrocytes of foetal blood (Ackerman, 1962), but no further studies on foetal haemoglobins have been reported. Several of the control mechanisms of globin synthesis have been observed in rabbit cells (Section 4); no information on the genetic control of rabbit haemoglobins is available.

6. Control of erythroid maturation.

The maturation of erythroid cells may be controlled at many points. The supply of stem cells, the pressure upon them to differentiate and their replacement thereafter is one point; environmental, hormonal and proliferative control factors may affect this stage. The process of maturation must also be controlled; little is known of specific control mechanisms at this stage, save those affecting haemoglobin synthesis itself. The limitations on haemoglobin synthesis, other than nutritional deficiency, so far determined appear to be genetically controlled; almost every aspect of synthesis may be affected.

This work is concerned with the hormone, erythropoietin, which is one of the factors controlling erythropoiesis.

Nature and production of erythropoietin. Erythropoiesis is markedly stimulated by tissue anoxia caused by excessive bleeding, anaemias or exposure to low oxygen tension; the stimulation has been found to be mediated by a humoral factor, erythropoietin (Gordon, 1959; Jacobson and Doyle, 1962). The hormone has not yet been isolated but studies on partially purified preparations indicate that it is a glycoprotein with a molecular weight of 60 – 70,000 (Goldwasser, 1966). There is little or no species specificity in its action on mammalian erythroid tissues, but it can act as an antigen (Goldwasser, 1966; Garcia and Schooley, 1963).

It is produced mainly by the kidney (Waldmann and Rosse, 1962; Erslev, 1962), but is not stored there in an active form (Kuratowska et al., 1964); small amounts may be produced or stored in other sites in some species (Reissmann and Momura, 1962; Erslev, 1964a). Lowering the oxygen tension in the renal blood supply causes increased production of erythropoietin (Fisher et al., 1965b). Serum factors have been found to enhance, or even be essential for, erythropoietin activity (Contrera et al., 1965; Kuratowska et al., 1964); it has been reported that the factor produced by the kidney reacts enzymatically with a serum factor to produce active erythropoietin (Zanjani et al., 1967). Inhibitory factors in kidney and serum have also been reported (Reynafarje et al., 1964; Kuratowska et al., 1964). In this work erythropoietin is used to denote the active humoral principle.

Role of erythropoietin in normal adult erythropoiesis. The assay methods currently available are not sufficiently sensitive to measure accurately the concentration of erythropoietin in normal serum; qualitative studies on the effect of normal plasma on polycythaemic rats and mice, however, have indicated that some erythropoietin is present (Jacobson et al., 1957; Reichlin and Harrington, 1960). Injection of antiserum to erythropoietin into normal animals has been shown to provoke an anaemia (Schooley and Garcia, 1962). In addition, the cells produced in response to moderate doses of exogenous erythropoietin are morphologically and functionally normal (Nakao et al., 1966; Orlic et al., 1965; Gurney et al., 1961; Ito and Reissmann, 1966). It seems most likely that erythropoietin is intimately concerned in erythropoietic regulation in normal animals.

In vivo studies on erythropoietin. No recognisable erythroid precursors can be seen in animals made polycythaemic by hypertransfusion (Gallagher et al., 1963; Jacobson et al., 1957). Injection of exogenous erythropoietin produces a wave of erythroid maturation, the magnitude of which is proportional to the dose of erythropoietin within fairly wide limits (Nakao et al., 1966; Pilmanowicz and Gurney, 1961; Orlic et al., 1965). These results suggest that erythropoietin acts by stimulating maturation of erythroid stem cells. This is substantiated by autoradiographic data indicating that higher numbers of cells enter the erythroid maturation pathway after erythropoietin stimulation; although the mitotic index

of erythroid cells increases, the generation time remains unchanged (Alpen and Cranmore, 1959; Alpen et al., 1962; Matoth and Kaufmann, 1962; Gurney et al., 1961).

As the dose of erythropoietin given increases, a level is reached at which no further increase in the response can be elicited by a variety of doses; subsequent doses given to the same animal, however, can provoke a response greater than that found if the total amount of erythropoietin were given as a single dose, (Keighley et al., 1964; Schooley, 1965; Gurney and Fried, 1965). There is probably a limited number of sensitive cells available at any given time (Schooley, 1965) and removal of these by differentiation stimulates the replenishment of the pool; there appears to be an initial over-replenishment to make possible the augmented response elicited by the later doses of hormone. The recovery of the pool of sensitive cells is probably not affected directly by erythropoietin (Erslev, 1964a); it has been suggested that a further humoral factor stimulates replenishment of the pool (Gurney and Fried, 1965).

Continued injection of higher than normal doses of erythropoietin does not lead to exhaustion of the erythroid tissue or to the production of abnormal cells (Keighley et al., 1964) suggesting that the activity of the stem cell pool can be adjusted to cope with conditions of stress. Injection of very high doses or massive bleeding, however, leads to the production of short-lived macrocytes (Stohlman, 1961); such doses may also accelerate reticulocyte release from erythroid tissue (Fruhman

and Fischer, 1962; Fisher et al., 1964). The production of macrocytes may be due to a reduction in the number of divisions at the polychromatic normoblast stage (Suit et al., 1957) or to by-passing the acidophilic normoblast stage (Borsook et al., 1962). It has been shown that high doses of erythropoietin may affect cells peripheral to the stem cell compartment (Borsook et al., 1968) but lower doses have not been shown to do so (Erslev, 1964b). It is possible that the production of such abnormal cells is a 'panic' response only and that, in normal circumstances, erythropoietin acts mainly, if not wholly, on stem cells.

The response to erythropoietin of polycythaemic mice can be inhibited by actinomycin D, probably because of stem cell damage (Gurney and Hofstra, 1963; Keighley and Lowy, 1966); the recovery time depends on the amount of the inhibitor given and the timing of events is consistent with erythropoietin stimulation of stem cells leading to early and complete mRNA synthesis, followed by maturation at normal rates (Keighley and Lowy, 1966). This would not exclude the possibility of an erythropoietin effect on later maturation stages in different conditions.

Erythropoiesis is also suppressed in rats by starvation, probably owing to suppression of erythropoietin synthesis (Reissmann, 1964); after erythropoietin treatment an increase in the activity of ALA hydro-lyase and of enzymes involved in nucleic acid synthesis was observed (Fischer, 1962). Few samples were taken soon after erythropoietin treatment so that the increase observed may reflect only the

increased erythropoietic activity rather than a direct effect of erythropoietin. An increase in incorporation into RNA was observed before any increase in DNA incorporation in normal rat marrow after injection of erythropoietin; the incorporation was measured 4 hr. after erythropoietin treatment (Pieber-Perretta et al., 1965).

Effect of erythropoetin on spleen colonies. The formation of erythroid colonies in the spleens of irradiated polycythaemic hosts is suppressed, but later administration of erythropoietin not only permits growth of erythroid colonies but also the formation of erythroid foci in granuloid colonies already present (Curry et al., 1964, 1967; Bruce and McCulloch, 1964; Schooley, 1966). During the initial phase of colony growth the cells are refractory to erythropoietin (Liron and Feldman, 1965; Gurney et al., 1962); the number of cells present in the colonies also suggests that cells do not mature at this time, but are replicating (Schooley, 1966). The number of colony-forming cells (CFC) and of erythropoietin-sensitive cells (ESC) found in developing colonies suggests that ESC are progeny of CFC (Bruce and McCulloch, 1964).

It was suggested that the apparent differences between CFC and ESC are artefacts due to transplantation (Porteous and Lajtha, 1966). However, the colonies formed in the spleens of mice irradiated with one femur shielded are derived from endogenous marrow cells; this eliminates artefacts due to transplantation. The kinetics of colony formation and the pattern of erythropoietin-sensitivity are the same

in these animals as in animals seeded with exogenous marrow cells (De Gowin, 1967). In addition, freezing and thawing of marrow cells destroys their ability to respond to erythropoietin while they can still afford radiation protection (Erslev, 1962). Microscopic colonies of undifferentiated cells are found in the spleens of polycythaemic hosts; after erythropoietin treatment leading to formation of erythroid colonies, the smaller colonies are not found (Curry et al., 1967; Till et al., 1967). This suggests a separation of replication and differentiation of CFC. It seems most likely that the functional differences observed reflect the presence of two cell types, one the CFC, capable of self-maintenance and differentiation into several haematopoietic cell lines, and one the ESC capable of limited self-maintenance and able to form only erythroid cells.

The importance of the environment to differentiation has also been stressed; some cells may be partially or wholly shielded from the action of erythropoietin (Playfair and Cole, 1965; Bruce and McCulloch, 1964; Bleiberg et al., 1967; Curry et al., 1967). Furthermore, Sl anaemic mice have been shown not to support adequate colony growth (McCulloch et al., 1965) even in the presence of erythropoietin. In some conditions erythropoietin may be able to supercede other influences on progenitor cells since erythroid foci may be produced by it even in colonies previously composed of other cell types (Schooley, 1966); erythroid maturation appears to take precedence over other types of maturation when the number of cells injected is limiting (Urso and

Congdon, 1957). It has been suggested that the production by CFC of ESC or of the progenitors of other cell lines is a random process; only after it has occurred do exogenous factors influence the numbers of cells produced (Till et al., 1964). There remain, however, many points to be confirmed and/or elucidated.

In vitro studies on erythropoietin. While studies on the effect of erythropoietin in vivo are essential for a complete picture of erythropoiesis, the effects seen may be confused by the intervention of factors so far unrecognised; in vitro studies on erythroid tissue are more suitable to determine the direct effects of erythropoietin upon maturation. Explants of erythroid tissue contain morphologically normal cells (Matoth, 1962) and respond to erythropoietin in a manner similar to that observed in vivo (Goldwasser, 1966) during 48-60 hr., although complete maturation may not occur (Erslev, 1962).

Iron uptake and haem synthesis in vitro are good indices of haemoglobin formation during erythroid maturation (Gallien-Lartigue and Goldwasser, 1964). The response elicited by identical amounts of erythropoietin was found to be greater in marrow from polycythaemic animals than in normal marrow (Erslev, 1964b) indicating that the effect of erythropoietin upon stem cells can be observed in vitro. The magnitude of the response is proportional to the dose of erythropoietin (Krantz et al., 1963). No utilisation of erythropoietin has been detected in marrow cultures (Krantz and Goldwasser, 1965a)

or by isolated femurs (Fisher et al., 1965a); the amount absorbed by the cells may well be below the level of sensitivity of the assay systems available. In vivo studies suggest that increased marrow activity affects the rate of clearance of erythropoietin from plasma (Stohlman and Howard, 1962), but the point remains to be elucidated. The increased clearance rate in vivo need not mean the marrow itself removes erythropoietin from the circulation.

Treatment of marrow cultures with erythropoietin stimulates iron absorption very quickly (Hrinda and Goldwasser, 1966); haem synthesis (Gallien-Lartigue and Goldwasser, 1964) and stroma synthesis are stimulated more slowly (Dukes and Goldwasser, 1965). All of these are inhibited by actinomycin D, the increase in haem synthesis becoming less sensitive to the inhibitor with time (Gallien-Lartigue and Goldwasser, 1965), as was found in vivo (Keighley and Lowy, 1966). mRNA for stroma synthesis appears to have a shorter lifetime than mRNA for globin synthesis (Goldwasser, 1966). An early stimulation of RNA synthesis has been observed (Krantz and Goldwasser, 1965b; Pieber-Perretta et al., 1965), the newly formed RNA having sedimentation properties similar to those proposed for mRNA (Singer and Leder, 1966; Krantz and Goldwasser, 1965b).

Erythropoietin was found to increase the number of normoblasts synthesising DNA; the activity of each cell was unchanged (Powsner and Berman, 1965). Increased DNA synthesis was not found in the early stages of the response (Pieber-Perretta et al., 1965; Goldwasser, 1966)

suggesting that the increase seen is not due to the primary effect of erythropoietin. Colchicine does not completely inhibit the increase in haem synthesis suggesting that cell division augments the response but is not essential to it (Gallien-Lartigue and Goldwasser, 1965).

Role of erythropoietin in foetal erythropoiesis. Although erythropoietin is clearly implicated in the regulation of adult erythropoiesis, its role during foetal erythropoiesis is less clear. Mice made polycythaemic throughout pregnancy produced erythropoietically normal offspring; the mothers showed no erythropoiesis at any time suggesting that any erythropoietin produced by the foetus does not cross the placenta (Jacobson et al., 1959). If erythropoietin is involved in foetal erythropoiesis, then it must be produced by the foetus itself.

Although mouse yolk sac cells were found to be resistant to erythropoietin, liver cells were sensitive until day 15 of gestation; later than this no response could be seen and the control rates of haem synthesis were very much higher than before. The pattern of haem synthesis was consistent with the hypothesis that a pool of ESC accumulates in the liver until foetal erythropoietin is produced around day 14; the cells are then stimulated to differentiate and the pool is depleted and replenished in a cyclic manner (Cole and Paul, 1966). The appearance of resistance to erythropoietin coincides with the period of maximum release of non-nucleated cells into the foetal circulation (Craig and Russell, 1964). The evidence thus suggests

that foetal erythropoiesis, in mice, may be regulated by erythropoietin produced by the foetus.

Nephrectomy of neonatal rats was found to have little or no effect on erythropoiesis during the first three weeks after birth; the possibility that erythropoietin was transferred from the mothers during suckling (Grant, 1955) was excluded by making the mothers polycythaemic (Stohlman et al., 1964). It was suggested that in the rat, which is quite immature at birth, erythropoiesis is controlled by factors peculiar to the foetus until about three weeks after birth, when adult-type erythropoiesis begins. Although the kidney was eliminated as the source of foetal and neonatal erythropoietin, other sites of production are possible. In addition, the animals survived nephrectomy for only 2-3 days; if the rate of clearance of erythropoietin from the blood were slower at this stage than later, little effect upon erythropoiesis would be seen.

Rats made anaemic during pregnancy produced erythropoietically normal foetuses (Block, 1946) suggesting that, in the rat also, maternal erythropoietin does not cross the placenta. The role of erythropoietin in foetal rat erythropoiesis remains unclear.

7. Erythroid maturation and cytodifferentiation

The development of the fertilised egg is characterised by a progressive loss of totipotency and the production of increasingly specialised cells; as the embryo develops the cells become more

restricted in their capacity for further, or alternative, modes of differentiation (Waddington, 1956; Saxen and Toivonen, 1963; Brachet, 1967). There is a considerable similarity in the molecular biology of widely varying organisms; it seems likely that the basic mechanisms of cytodifferentiation in higher organisms are also broadly similar, although the specific details differ (Bell, 1965; De Reuck and Knight, 1967; Davidson, 1965). The maturation of erythroid stem cells may thus be considered as a particular example of a generally occurring phenomenon (Borsook, 1966). The analogy cannot be overextended, however, since the nucleus is lost during erythroid maturation; complete nuclear breakdown is rare in other systems. Only the initial stages of maturation could be truly analogous to most other differentiating cells.

Examples of cytodifferentiation accompanied by nuclear breakdown are found in some invertebrates; in these organisms only the germ cells retain the entire genetic complement (White, 1950; Wilson, 1925). Most of the somatic cells in a given vertebrate species, however, contain identical chromosome complements and similar amounts of DNA (Brachet, 1957). Since the determination of specific cell characteristics may require only a small loss of DNA, the techniques available may not be sufficiently sensitive to detect such variations. Differentiation by the loss of genes cannot therefore be excluded by these measurements.

There is evidence that differentiation need not be accompanied by the loss of DNA. The DNA of various mouse organs was compared with

whole mouse embryo DNA using molecular hybridisation; the embryonic DNA presumably contained the DNA of all the organs. No difference could be detected between any of the DNA preparations (McCarthy and Hoyer, 1964). This suggests that, although small amounts of DNA may be lost, most of the DNA is retained as the cells differentiate.

When nuclei from differentiated cells in Xenopus tadpoles are transplanted into eggs whose nuclei have been inactivated, the eggs can develop into normal tadpoles (Gurdon, 1963) and in some cases into fertile adult toads (Gurdon and Uehlinger, 1966). As the age of the donor increases, there is a progressive decrease in the ability of transplanted nuclei to support normal development of the eggs; this may reflect the progressive decrease in cellular totipotency during embryonic development (Waddington, 1956). Nonetheless, these experiments demonstrate that normal cytodifferentiation can occur without loss of genes.

The presence of redundant genes in differentiated cells indicates that the cells can suppress gene activity selectively and often permanently. In bacterial cells a model of gene repression has been proposed to explain the induction and loss of enzymes (Jacob and Monod, 1961; Stent, 1964). In this model repressors are produced by regulator genes; the repressors can act through the cytoplasm to affect the activity of other genes which are grouped together into operons. The repressors interact with operator genes so as to inhibit the activity of the associated operon; inducers have a greater affinity

for the repressors than do the operator genes. The interaction of inducer and repressor thus permits the operon to become active and enzyme synthesis takes place. There is genetic evidence for this model (Pardee et al., 1959). Furthermore, the repressor of β-galactosidase (EC.3.2.1.23) synthesis in E. coli has been isolated by means of its affinity for the inducer (Gilbert and Mueller-Hill, 1966); it appears to be protein in nature.

The model thus seems well-established for bacterial cells; there is, however, no direct evidence that it exists in higher cells. In addition the cells of higher organisms differ from bacterial cells in a number of ways vital to the operation of this or similar models.

Enzyme induction in bacteria is generally reversible and dependent upon the presence of the inducer; its effect is often merely to increase the concentration of an enzyme already present in minute amounts. Differentiation of higher cells, however, is irreversible and may continue after only a brief period of stimulation; many proteins are totally absent from differentiated cells. In addition, most, if not all, bacterial DNA appears to be available for transcription in vivo (McCarthy and Bolton, 1964), whereas little of the DNA in mammalian cells may be transcribed (Paul and Gilmour, 1966a). Thus, although this form of regulation may be involved in reversible changes in the character of higher cells, such as pigment production in retinal cells (Coon and Cahn, 1966), it is less likely to be the sole determinant of stable differentiation.

Selective gene action in higher cells. The DNA in higher cells is in complex association with RNA and protein material, forming chromatin, and it is possible that the nature of the association may be responsible for the activity or otherwise of the genes. Chromatin occurs in a condensed (heterochromatic) and an uncondensed (euchromatic) form; although up to 80% of the chromatin may be heterochromatic most RNA synthesis occurs in euchromatic regions (Allfrey et al., 1963; Frenster et al., 1963; Berlowitz, 1965). Also, only 5-10% of the DNA of chromatin appears to be available for transcription; different portions of the DNA are so available in different organs (Paul and Gilmour, 1966b). This suggests that some portions of the DNA in differentiated cells are permanently repressed in a specific way, while others are active to a degree which may be influenced reversibly by the environment.

Polytene chromosomes present in some Dipteran species provide further evidence for selective utilisation of genetic information. These structures, the result of continued replication without mitotic segregation, carry localised swellings, or puffs; the distribution of puffs is characteristic of the stage of development and of the tissue (Beermann, 1959; Clever, 1962). During pupation there is sequential alteration in the pattern of puffs, which can be mimicked in vitro by treatment with the pupating hormone, ecdysone (Clever, 1966) or by altering the concentration of divalent ions in the medium (Kroeger and Lezzi, 1966). The puffs have been shown to be associated with

RNA synthesis while other regions are not (Pelling, 1959; 1964); the character of the RNA formed suggests that it may be mRNA (Edstrom and Beermann, 1962).

The fact that fertilised eggs do not immediately begin to synthesise all the proteins whose genes it contains (Brachet, 1967; Bell, 1965) may also argue for selective repression of gene activity. Similarly, stem cells do not synthesise haemoglobin; it therefore seems possible that the stem cell maturation, prior to nuclear breakdown, may reflect the changes occurring during other types of cytodifferentiation.

8. Aim of the present work

The growth *in vitro* of erythroid cells to study the action of erythropoietin has several advantages over *in vivo* systems; the environment of the cells can be readily controlled and there is no loss of maturing cells. In addition, the number of cells initially present can be measured precisely; the dose of erythropoietin actually reaching the cells is also known precisely.

The main advantage of using foetal cells rather than marrow cells is that a greater response of foetal cells to erythropoietin has been reported (Cole and Paul, 1966; Goldwasser, 1966). The differences between control and hormone-treated cultures are thus larger and more easily detected.

There has been, and still is, some dubiety as to the role of erythropoietin in foetal erythropoiesis. The first parts of the work

were designed to study the course of foetal erythropoiesis, especially foetal haemoglobin synthesis, more closely and to investigate the part which erythropoietin may play in its control. Different species were studied to reduce the possibility that the results obtained were of limited validity.

Erythroid cell cultures also constitute a system in which differentiation may be induced at will by erythropoietin treatment. The second part of the work is a preliminary investigation of the mode of action of the hormone; its effect was studied in some detail and an attempt was made to identify its primary effect.

II. MATERIALS AND METHODS

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Unless otherwise stated, all chemicals used were British Drug Houses Ltd., "AnalaR" grade; radioactively labelled chemicals were obtained from the Radiochemical Centre, Amersham.

1. Production of foetuses

Random-bred albino mice (Porton strain) were maintained on Oxoid Diet 41B and unlimited water. Males were kept either in individual cages or in groups; two 10-12 week-old females were introduced to each male and left overnight. On the following morning the females were removed and examined for mating plugs. This method permits accurate timing of foetal age, the day upon which plugs were found being designated day 0. Mating may occur at any time from 9 p.m. to 9 a.m. if the females are introduced in the later afternoon (Marshall, 1956), so that variations in foetal age of up to 12 hours may be found. The frequency of pregnancy was 10-15%.

Greater numbers of foetuses may be obtained by treating the females with hormones before mating. Ovulation was induced by intraperitoneal injection of 10 u. serum follicle stimulating hormone ('Gestyl', Organon Ltd.), followed 36 hours later by intraperitoneal injection of 10 u. chorionic luteinising hormone ('Pregnyl', Organon Ltd.). The females were then placed with the males. The incidence of pregnancy rose to 25-30% and the litter size increased from an average of 7.5 to an average of 14. A slight increase in the number

of retarded foetuses was also found.

Random-bred albino rats (Wistar strain) were maintained on the same diet and water allowance. Since excessive fat reduced the incidence of pregnancy the females were starved overnight twice a week. Males were kept in individual cages; a single 3 - 6-month old female was introduced to each and left overnight. It was found that, if more than one female was introduced, the male mated usually only with one female during this time. Again this permits timing of foetal age to within half a day and the morning upon which the females were removed from the males was designated day 0. Although mating plugs were found, the occurrence of pregnancies did not always correspond to the distribution of plugs. This may be due to absorption of the plug which can occur within eight hours of mating (Marshall, 1956) so that animals mated around midnight could have lost their plugs by morning. Plugs were also observed in the cage litter. Routine examination for plugs was not, therefore, carried out. The incidence of pregnancy was 20-25% and the average litter size was 7.

Hormone treatment with 15 u. of each hormone was attempted as described for mice. Again the incidence of pregnancy and litter size increased, but a high proportion of the foetuses was retarded. Reduction of the dose to 10 u. of each hormone produced a similar increase in pregnancies and litter size, but again a high proportion of retarded foetuses. Accordingly, hormone treatment was not used on rats.

Random-bred rabbits (New Zealand White) were maintained on Oxoid

Diet 18 with unlimited water and supplemented with carrot and cabbage. All were kept in individual cages. 6 - 9-month old does were introduced to the males and left until they had mated at least three times. Ovulation is stimulated by mating (Marshall, 1956), and was further stimulated by intravenous injection of 10 u. of chorionic luteinising hormone ('Pregnyl', Organon Ltd.) immediately after mating. The time of mating was designated time 0.

Rats and mice were killed by cervical dislocation, rabbits by intravenous injection of Nembutal (Abbot Laboratories Ltd.). The abdomen was soaked in 70% alcohol and the skin was reflected from the abdominal wall. The uteri containing the foetuses were cut out and washed several times in sterile Hanks BSS (Paul, 1965). The uterine wall was cut open and the foetuses and their membranes were removed. The membranes were discarded and the foetuses washed again in BSS. Any abnormal or retarded foetuses were rejected.

2. Culture procedure

Foetal organs were removed aseptically in Hanks BSS and treated overnight at 4°C with 0.5% trypsin (EC. 3.4.4.4. 1:250 Difco Corpn.). The trypsin was dissolved in 0.6% NaCl/0.29% Na citrate solution containing 0.3% CM-cellulose (Edifas B. ICI Ltd.) at pH 7.8. The following day the supernatant trypsin was removed and the tissue was incubated in the remainder at 37°C for 5 minutes. Disaggregation was completed by gentle pipetting in medium.

The medium used was Waymouth's medium MB 752/1 (Waymouth, 1959) based on Hanks BSS and supplemented with 10% serum. It was equilibrated to 5% CO_2 in air using 6.6% $NaHCO_3$ to adjust the pH to 7.4. 50 u./ml. benzoylpenicillin (Glaxo Ltd.) was added routinely.

The cell suspension was checked for clumping and for viability by exclusion of 0.1% naphthalene black (Paul, 1965) and counted by haemocytometer; only nucleated cells were counted. Single-cell suspensions 90 - 95% viable were obtained; omission of the CM-cellulose from the trypsin solution reduced the viability by some 20 - 30%. Organs removed at an early stage of their development are smaller and looser so that the trypsin can penetrate in about 8 hr.; more highly developed organs require a longer exposure and can be left for up to 24 hr. without reducing the viability of the cells. The cultures were set up in 15 ml. tubes at 0.5 - 3 x 10^6 cells in 1 ml. of medium and incubated at 37°C in 5% CO_2 in air.

Most of the serum used was foetal bovine serum (Flow Laboratories Ltd.) but homologous female serum was used as a source of transferrin which is species specific (Bothwell and Finch, 1962). Blood obtained by bleeding from the jugular vein was allowed to clot for ½ - 1 hr. at room temperature and was then placed at 4°C until the clot had retracted, some 4 - 6 hr. later. The serum was removed and spun at 1500 g for 30 min.. The supernatant was diluted 1:1 with Hanks BSS and filtered through a 0.22 μ Millipore membrane.

Before use the sera were examined for toxicity to foetal liver

cells. Cultures were set up as described in medium containing the serum to be tested; exclusion of 0.1% naphthalene black was checked after 24 hr. in culture. Sera supporting less than 80% viability were discarded. Maximum survival was found with 10 - 30% serum whether homologous, foetal bovine or a mixture of both. 10% serum was routinely added to the medium.

3. Measurement of haem synthesis

Haem synthesis was studied by labelling with ^{59}Fe. Since iron is absorbed by the cells from homologous transferrin (Bothwell and Finch, 1962) the ^{59}Fe was incubated at 37°C in 50% homologous female serum in BSS for at least 4 hr.. It has been shown that, in these conditions, $^{59}FeCl_3$ has reached equilibrium with the transferrin in less than 1 hr., although ferric citrate takes up to 8 hr. to do so (Bates et al., 1967). 0.05 - 0.1 ml. of the equilibrated serum containing 1 - 2 μc ^{59}Fe was added to each culture at the requisite time and the cells were allowed to incorporate the isotope for a period appropriate to each experiment. The length of the pulse is indicated for each experiment as it is discussed.

After labelling, the cells were washed twice in BSS; the supernatant then contained no detectable activity. The pellet was taken up in 1 ml. of a solution of 2 parts water:1 part Drabkin's solution which contained 1 g. $NaHCO_3$, 50 mg. KCN and 200 mg. $K_3Fe(CN)_6$/l. of water before dilution. The haemoglobin in the cells was thus converted to

cyanmethaemoglobin (Wintrobe, 1961). The cells were lysed by freezing and thawing three times; the lysate was acidified with 0.1 ml. IN-HCl and the haem was extracted into 1.2 ml. of ice-cold butanone (Teale, 1959). The mixture was spun at 500 g for 5min. The use of these volumes left a supernatant layer of 1 ml. of butanone containing the labelled haem, from which an aliquot was taken for counting.

^{59}Fe is an active isotope and may be counted with an end-window gas-flow counter, for which purpose aliquots were dried on to stainless steel planchettes. Standard samples of ^{59}Fe showed a counting efficiency of 30 - 50%. In experiments involving a large number of samples the efficiency was checked by counting standards several times in the series; a variation of $\pm$ 3% was sometimes observed during 6 - 8 hours of constant use. Larger variations were found when discrete series of samples were counted, but allowance was made for this by the inclusion of a standard sample at least once in any experimental series. The results were calculated on the basis of the activity detected in the standard samples. A reagent blank was always included.

A Nuclear Chicago Mark I scintillation counter was used for much of the work. In this case, aliquots were dried on to Whatman GF/C 2.5 cm. circles at 80°C for at least one hour. Up to 0.5 ml. of liquid could be added to the circles. Toluene-based scintillation fluid was used; this contained 4.23 g. P.P.O. (2,5diphenyloxazole) and 200 mg. P.O.P.O.P. (1,4-di(2-(5-phenyloxazolyl))-benzene)/l. of toluene. The activity of ^{59}Fe is such that very little scintillation fluid was required and a maximum efficiency of 95% was attained using

TABLE 1

The effect of different volumes of toluene-based scintillation fluid and the presence of butanone upon the efficiency of counting ^{59}Fe.

Volume of scintillation fluid ml.	$^{59}FeCl_3$ in 50% serum		$^{59}FeCl_3$ in 50% serum + 0.5 ml. butanone	
	counts/min.	efficiency %	counts/min.	efficiency %
0.5	3255.2	83.5		
1.0	3702.8	95.0	3751.4	96.2
1.5	3679.2	94.4		
2.0	3727.2	95.6	3629.8	93.1
2.5	3694.2	94.8		
3.0	3751.4	96.2	3800.9	97.5
3.5	3661.3	93.9		
4.0	3687.1	94.6	3762.3	96.5

Calculated activity present - 3897.6 disintegrations/min.

only 1.0 ml. Little or no quenching occurred in the presence of butanone (Table 1). Reagent blank and standard samples were included in each experimental series. The level of activity detected by both types of counter in extracts of cultured cells was two or more times the level of background activity in all experiments.

Cyclohexanone may also be used to extract haem and the efficiency of extraction should be less temperature-dependent than that of butanone (Krantz, 1967). Tests were conducted to compare the efficiency of the two solvents. 0.1 ml. of $^{59}FeCl_3$ equilibrated with 50% serum was added to 1 ml. of diluted Drabkin's solution; the mixture was acidified and extracted in 1.2 ml. of either solvent in the cold. In both cases, less than 1% of the ^{59}Fe present could be detected in the solvent; this indicates that iron, free or transferrin-bound, was not absorbed by either of the solvents.

Cultures of foetal cells were prepared and ^{59}Fe equilibrated with 50% homologous serum was added. Some of the cultures were immediately washed in BSS and their haem was extracted into one or other solvent. The remaining cultures were incubated for 4 hr. before being washed and were then divided into several groups. Haem was extracted from two of the groups into butanone in the cold or at room temperature; two further groups were extracted with cyclohexanone in the cold or at room temperature. One group was extracted with cold butanone in the absence of HCl. The cell lysates from the last group were spun at 1500 g. for 5 min. (Cole and Paul, 1966) and the supernatants were extracted

TABLE 2

The effect of incubation and of the method of extraction on the amount of ^{59}Fe recovered from cultures of 13-day mouse foetal liver cells.

Length of incubation hr.	Solvent	Temperature	Vol. N-HCl ml.	Spun before extraction	counts/min. in extract
-	butanone	ice	0.1	-	15.5
-	cyclohexanone	ice	0.1	-	20.9
4	butanone	ice	0.1	-	930.7
4	butanone	room temp.	0.1	-	464.5
4	cyclohexanone	ice	0.1	-	309.6
4	cyclohexanone	room temp.	0.1	-	315.7
4	butanone	ice	0.1	5 min. at 1500 g	324.3
4	butanone	ice	-	-	27.9

Reagent blank - 16.1 counts/min.

Each result is the average of 3 cultures

with cold butanone . The extracts were counted, with the results shown in Table 2.

Very little activity was found in any extracts of unincubated cells; this confirmed that neither solvent absorbed significant amounts of unincorporated ^{59}Fe. Although cyclohexanone was indeed found to extract similar amounts of haem in the cold and at room temperature, its efficiency was much less than that of butanone in the cold. Cyclohexanone was not used further. The effect of temperature upon the efficiency of butanone extraction of haem was clearly demonstrated. Non-acidified extracts contained almost as little activity as was found in extracts of unincubated cells, confirming that most of the haem was bound to protein. When the lysate had been spun before extraction, the activity was greatly reduced; since unincorporated ^{59}Fe was not absorbed by the solvent, the loss must be of haem. The lysate was not spun at this stage in any of the experiments. These results are in accord with those obtained with adult bone marrow cells (Krantz et al., 1963) in which it was found that 95% of the labelled haem was derived from haemoglobin.

The medium described by Cole and Paul (1966) contained 5% foetal bovine serum and 5% homologous female serum. It has been reported, however, (Gallien-Lartigue and Goldwasser, 1964) that inclusion of homologous serum reduced the amount of ^{59}Fe incorporated owing to dilution of the labelled transferrin added by unlabelled transferrin in the medium. This would lead to low estimates of haem synthesis

TABLE 3

The effect upon haem synthesis in cell cultures of the presence of homologous female serum in the culture medium

		15-day rat foetal liver cells		15-day mouse foetal liver cells	
Foetal bovine serum %	Homologous female serum %	Time hr.	μμ moles haem/ 10^6 cells/hr.	Time hr.	μμ moles haem/ 10^6 cells/hr.
10		0	39.7	0	54.3
		24	13.3	23	40.2
	10	0	32.1	0	41.4
		24	8.9	23	33.6
5	5	0	35.3	0	48.9
		24	11.3	23	37.6

Each result is the average of 3 cultures

TABLE 4

The effect upon haem synthesis in cell cultures of the addition of $FeCl_3$ to the culture medium.

15-day rat foetal liver cells			15-day mouse foetal liver cells		
m M-$FeCl_3$ added	Time hr.	μμ moles haem/ 10^6 cells/hr.	m M-$FeCl_3$ added	Time hr.	μμ moles haem/ 10^6 cells/hr.
0	0	35.7	0	0	52.7
0	25	11.3	0	23	39.6
0.001	0	36.1	0.005	0	53.4
0.001	25	10.9	0.005	23	38.2
0.005	0	33.8	0.01	0	53.1
0.005	25	9.4	0.01	23	37.5
0.01	0	30.5	0.02	0	52.2
0.01	25	9.5	0.02	23	37.5
0.02	0	27.8			
0.02	25	8.6			

Each result is the average of 3 cultures.

by the cells. Haem synthesis by both rat and mouse foetal liver cells was measured in the presence of 10% foetal bovine serum, 10% homologous female serum or 5% of each.

It was found that more ^{59}Fe was incorporated in the presence of 10% foetal bovine serum than in that of 10% homologous female serum; cultures containing 5% of each incorporated amounts intermediate between the two (Table 3). The system may thus be made more sensitive to changes in haem synthesis by using foetal bovine serum alone in the culture medium and adding homologous serum only when labelling; this was done throughout.

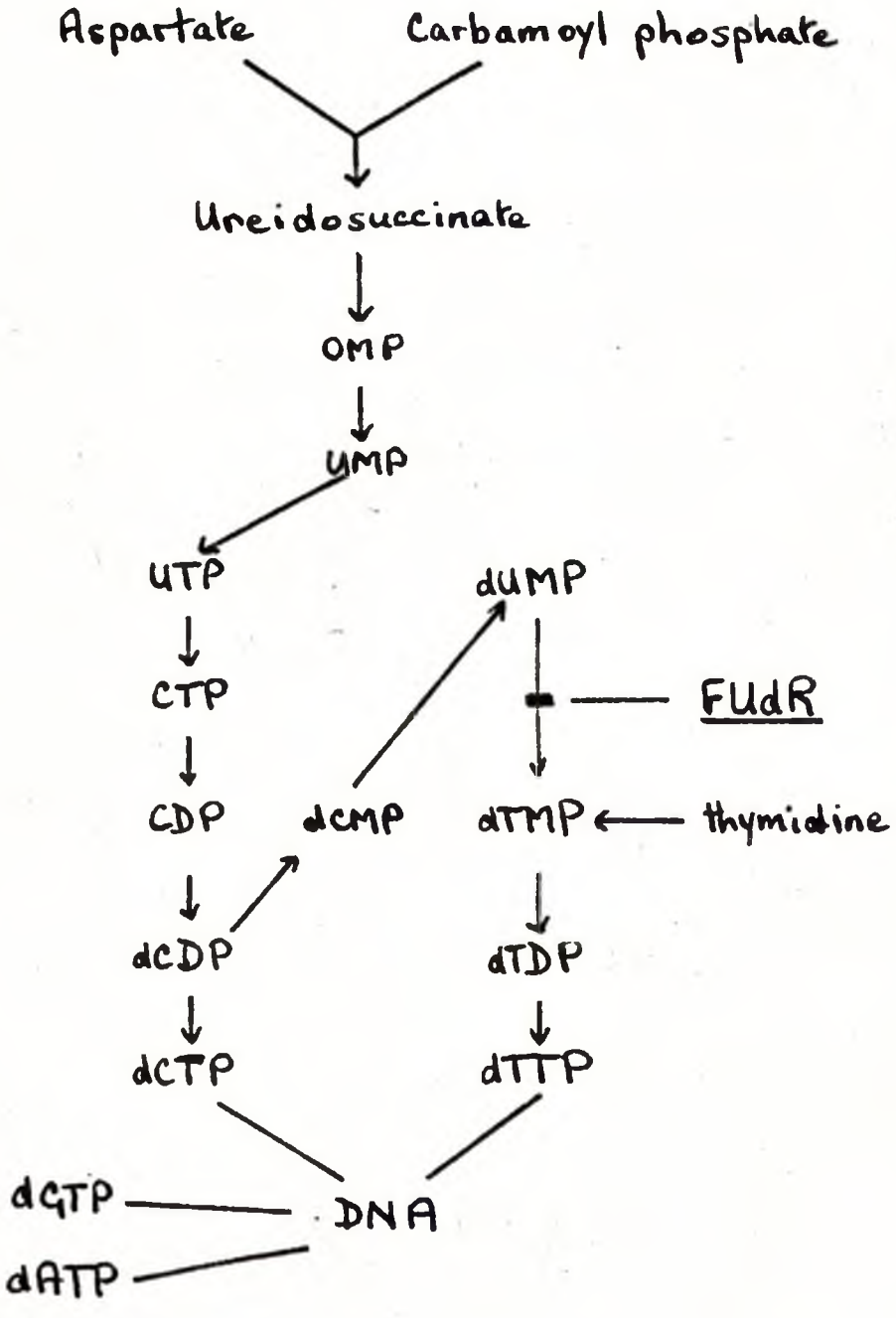
Cole and Paul (1966) also recommended adding 0.01 m M-$FeCl_3$ to the culture medium to avoid iron deficiency. When varying amounts of $FeCl_3$ were added to cultures of rat foetal liver cells it was found that not only was sufficient iron already present in the medium but that the addition of more than 0.005 m M-$FeCl_3$ actually inhibited haem synthesis. This is shown in Table 4 which also shows the result of a similar test performed on cultures of mouse foetal liver cells. It can be seen that addition of up to 0.02 m M-$FeCl_3$ had no effect on haem synthesis. No extra iron was added to the medium in any experiments except where the work was a direct continuation of that of Cole and Paul; this will be fully described later.

4. Measurement of DNA synthesis

DNA synthesis in cell cultures was studied by incorporation

Figure 2

Site of action of FUdR on DNA synthesis



of [methyl-^{3}H] thymidine in the presence of 10^{-4}M-thymidine and 10^{-4}M-5-fluorodeoxyuridine (FUdR) (Schwartz Bio Research Inc.). As shown in Figure 2, the inhibitor prevented de novo synthesis of thymine, while the addition of unlabelled thymidine enabled DNA synthesis to continue. Thus, the incorporation of labelled thymidine was proportional to DNA synthesis. At suitable times during culture the cells were pulsed for 1 hr. with 5 µc [methyl-^{3}H] thymidine (5000 c/mole) after which they were washed twice in BSS.

Nucleic acids were extracted from the cells by a modification of the Schneider principle (Schneider, 1945). The cell pellet was washed twice in 0.5 ml. ice-cold 2N-perchloric acid (PCA) to remove unincorporated thymidine; the washings were discarded. The residue was extracted twice with 0.5 ml. 2N-PCA at 70°C for 20 min. to hydrolyse the DNA and release the incorporated isotope. The pooled extracts were neutralised with 2N-KOH and the precipitate of $KClO_4$ was spun down. 0.5 ml. of the supernatant was added to 10 ml. of dioxane-based scintillation fluid for counting. The scintillation fluid contained 7 g. P.P.O., 300 mg. P.O.P.O.P. and 100 g. naphthalene/l. of dioxane. The volume of the supernatant remaining was measured and the activity detected in 0.5 ml. was corrected to represent that present in the total. The activity present in the samples was always four or more times the background activity.

A Nuclear Chicago Mark I scintillation counter was used to count all tritium-labelled samples; the efficiency was always 15 - 20%. Standard samples were included in each experiment; little variation

TABLE 5

The removal of activity from [methyl-^{3}H]thymidine-labelled DNA during treatment with 2N-PCA

	Washes in cold 2N-PCA					Extractions in hot 2N-PCA		Residue	Original sample
	1	2	3	4	5	1	2		
a. counts/min.	1956.2	732.1	183.9	184.0	178.0	6452.7	2113.9	1555.5	13324.3
% original activity	14.7	5.5	1.4	1.4	1.3	48.4	15.9	11.6	100
b. counts/min.	385.4	145.6				1523.5	395.5	316.0	2785.5
% original activity	13.8	5.2				54.7	14.2	11.3	100

in efficiency occurred during any one series, but there was some variation between separate series. It was found that 0.5 ml. of the PCA/KOH supernatant caused some 5% quenching of standard samples; the standards used to calculate the efficiency of counting of experimental samples always contained 0.5 ml. of the supernatant. The experimental background was the activity observed in 0.5 ml. of PCA/KOH supernatant.

Although washing the cell pellet in cold PCA was necessary to remove unincorporated thymidine, it was also possible that loss of incorporated thymidine occurred either by hydrolysis or by exchange between the tritium and the hydrogen in the supernatant (Okita and Spratt, 1961). To assess the magnitude of the loss, tests were conducted on a sample of DNA prepared by chloroform/octanol precipitation (Marmur, 1961) and purified with ribonuclease (EC. 2.7.7.16) and pronase; this sample was prepared from Landschutz ascites tumour cells labelled for 20 hr. with [methyl-^{3}H] thymidine in the absence of FUdR.

A known amount was washed several times in 0.5 ml. of ice-cold 2N-PCA and the washings retained. The residue was extracted twice in 0.5 ml. 2N-PCA at 70°C for 20 min. and the extracts kept separate. The washings and the extracts were neutralised with 2N-KOH and counted. The residue was taken up in 10% sodium lauryl sulphate (SLS) (see section 6), added to 0.5 ml. PCA/KOH supernatant, and counted. The results are shown in Table 5a. 20.1% of the activity was found in the first two washes; a further 1.3 - 1.4% was found in each of the following

washes. A total of 64.1% of the activity was found in the extracts, while 11.6% remained in the residue.

The test was repeated, washing only twice with cold 2N-PCA before extraction. A similar pattern was found, (Table 5b). 19% of the activity was found in the washes, 68.9% in the extracts and 11.3% in the residue. Since DNA is reported to be stable in cold 2N-PCA (Munro and Fleck, 1966) most of the loss of activity during washing was probably due to tritium exchange. It has been reported that tritium attached to carbon atoms, as in this case, can exchange at extremes of pH (Okita and Spratt, 1961). The source of the activity remaining in the residue is not known; activity refractory to release by deoxyribonuclease II (EC. 3.1.4.6) was also found to be characteristic of this sample (Becker, unpublished observations). It may be that the longer pulse and the absence of FUdR permitted tritium incorporation into other materials, an event which is unlikely to occur in a pulse lasting only 1 hr. with FUdR present.

It can be seen that the rate of loss of activity slowed considerably after two washes in cold PCA and that the amount extracted by hot PCA was quite consistent. In experimental work, the activity present in replicate samples was sufficiently consistent to have confidence in the results; although RNA was also extracted from the cells by this procedure, the labelling conditions used ensured that little or none of the activity was incorporated into RNA.

TABLE 6

The loss of activity from (5-^{3}H) uridine-labelled RNA during treatment with PCA

		Washes in cold 0.5N-PCA					Extractions in hot 2N-PCA		Residue	Original sample
		1	2	3	4	5	1	2		
a.	counts/min.	12180.2	7661.6	1236.4	1427.3	1330.6	101,111.5	15391.4	5490.4	204,913.6
	% original activity	5.9	3.7	0.6	0.7	0.6	78.6	7.5	2.7	100
b.	counts/min.	11312.9	4877.4				101,753.0	10873.4	4395.6	133,737.2
	% original activity	8.4	3.6				76.1	8.1	3.3	100

5. Measurement of RNA synthesis

RNA synthesis in cell cultures was measured by pulsing for 1 hr. with 2 µc [5-^{3}H] uridine (30,000 c/mole); after labelling the cells were washed twice in BSS. Again, the Schneider extraction principle was used (Schneider, 1945). The cell pellet was washed twice in 0.5 ml. ice-cold 0.5 N-PCA to remove unincorporated uridine; the residue was extracted twice in 0.5 ml. 2N-PCA at 70°C for 20 min.. The pooled extracts were neutralised with 2N-KOH and the precipitate spun down. The counting procedure was the same as that described in the preceding section. Standard background samples containing 0.5 ml. PCA/KOH supernatant were included in each experimental series. The counting efficiency was 15-20% and the activity present in experimental samples was always at least five times the background activity.

To assess the proportion of incorporated tritium lost during washing, tests were carried out on a sample of [5-^{3}H] uridine-labelled RNA prepared by phenol extraction (Hiatt, 1962). A known amount was washed several times in 0.5 ml. ice-cold 0.5N-PCA and the washings retained; the residue was extracted twice in 0.5 ml. 2N-PCA at 70°C for 20 min. and the extracts kept separate. The washings and extracts were neutralised with 2N-KOH and counted. The residue after extraction was taken up in 10% SLS, added to 0.5 ml. PCA/KOH supernatant and counted. The results are shown in Table 6a. 9.6% of the activity was found in the first two washes and less than 1% in each of the following washes; 85.7% was extracted by hot PCA and less than 3%

remained in the residue.

The test was repeated, washing only twice with cold PCA and a similar pattern was found, Table 6b. 12.1% was removed by washing in the cold, 84.2% by hot extraction and 3.2% remained in the residue. It can be seen that the rate of loss was much reduced after two washes and that a quite consistent proportion of the activity was extracted into hot PCA; the activity found in replicate samples in experimental work was also quite consistent.

During a pulse of 1 hr. some of the [^{3}H] uridine may be incorporated into DNA as well as into RNA (Davidson, 1965); the extent to which this occurred in the foetal liver cells is not known. Since the extraction method used does not separate the two nucleic acids, the results may not represent RNA synthesis only. Alkaline extractions to separate the nucleic acids is inadvisable for tritium-labelled material since up to 90% of the activity may be lost by exchange and the rate of loss does not level off in the manner found here (Paul, unpublished observations). The patterns of incorporation of [^{3}H]-thymidine and [^{3}H] uridine after erythropoietin treatment are such as to suggest that little or no diversion of [^{3}H] uridine to DNA synthesis occurs in foetal liver cells during 1 hr. (see section III,9).

6. Measurement of total protein synthesis

Protein synthesised in cell cultures was labelled with [^{14}C]-labelled algal hydrolysate for 1 hr.; the cells were then washed twice in BSS. The pellet was extracted twice in 2N-PCA at 70°C for

TABLE 7

The amount of [^{14}C] radioactivity detected in the residue of PCA-extracted cells after suspension in different liquids

Liquid 0.5 ml.	Cell residue	counts/min.
Water	-	84.3
10% SLS	-	85.7
Water	+	566.0
10% SLS	+	1330.5

Each result is the average of 3 cultures

TABLE 8

The effect of the volume of toluene-based scintillation fluid and of the presence of 10% SLS on the efficiency of counting ^{3}H

Volume of scintillation fluid ml.	[4,5(n)-^{3}H] lysine in 0.5 ml. H_2O		[4,5(n)-^{3}H] lysine in 0.5 ml. 10% SLS	
	counts/min.	efficiency %	counts/min.	efficiency %
1	68741.5	5.6	69514.7	5.6
2.5	115623.1	9.4	124953.8	10.2
5	201357.0	16.4	199425.6	16.2
7.5	197526.4	16.1	192573.3	15.6
10	189432.7	15.4	202167.9	16.5

Calculated activity present-1,227,634.4 disintegrations/min.

20 min. to remove nucleic acids which may also be labelled in these conditions. The residue was taken up in 0.5 ml. water or 0.5 ml. 10% SLS and the whole was dried on to Whatman GF/C 2.5 cm. circles for 2 hr. at 80°C. 5 ml. toluene-based scintillation fluid was added for counting; 0.5 ml. water and 0.5 ml. 10% SLS were counted to determine background activity. The results are shown in Table 7.

It can be seen that taking up the residue in 10% SLS instead of in water doubled the activity recovered.

Since the specific activity of the algal hydrolysate was dubious, the rest of the work was done using [4,5(n)-^{3}H] lysine; 5 µc was added to the cultures for 1 hr. and the same extraction and counting procedures were used. Experimental samples always contained activity four or more times the background activity. Standard samples were always included and the background activity was that present in 0.5 ml. 10% SLS.

A number of standard samples was dried on to glass fibre circles and varying amounts of toluene-based scintillation fluid were added. It was found (Table 8) that maximum activity was detectable using 5 ml. scintillation fluid. As can be seen, little or no quenching was observed in the presence of 0.5 ml. 10% SLS. The efficiency of counting was 15-20% in these conditions. The PCA residue taken up in 10% SLS could also be added directly to 10 ml. dioxane-based scintillation fluid without reducing the efficiency of counting. Since this method required more scintillation fluid it was not used

TABLE 9

The loss of activity from a 70% ethanol precipitate of [4,5(n)-^{3}H] lysine-labelled cells during treatment with 2N-PCA

Sample		Extractions in hot 2N-PCA					Residue	Total
		1	2	3	4	5		
1	counts/min.	7,246.3	4,218.8				14,371.2	25,836.3
	% total	28	16.3				55.6	100
2	counts/min.	6,319.3	3,661.4	1,357.6			9.079.8	20,358.1
	% total	31	17.7	6.7			44.6	100
3	counts/min.	6,027.4	3,261.3	1,024.6	253.5		12,057.3	22,634.1
	% total	26.6	14.4	4.5	1.1		53.3	100
4	counts/min	6,310.6	3,913.6	873.5	352.7	384.8	12,273.9	23,809.1
	% total	26.5	16.4	3.6	1.5	1.6	51.5	100

Activity in unextracted cell pellet - 23,456.1 counts/min.

routinely. It was, however, used in the tests described in sections 4, 5 and 6 so that the results should be comparable throughout.

Extraction in hot PCA, apart from removing non-protein material, may lead to loss of incorporated isotope by tritium-exchange or by protein hydrolysis. Cells were labelled with 5 μc [^{3}H] lysine for 1 hr. and washed twice in BSS. The cells were lysed and proteins and nucleic acids were precipitated by the addition of ice-cold 70% v/v ethanol. Samples were extracted a number of times in 0.5 ml. 2N-PCA at 70°C for 20 min.; the extracts were neutralised with 2N-KOH and counted. The residue was taken up in 10% SLS, added to 0.5 ml. PCA/KOH supernatant and counted. The results are shown in Table 9.

It can be seen that 45% of the activity present was removed in the first two extractions, 3-6% in the third and just over 1% in the following extractions. The activity remaining in the residue was just over 50% of the original activity present in the ethanol precipitate.

The amount of lysine incorporated into nucleic acids in a pulse lasting 1 hr. was probably very small; most of the loss of activity during PCA extraction was probably due to extraction of unincorporated lysine and to tritium-exchange. The proportion of the activity remaining in the residue was relatively constant and consistent levels of activity were found in replicate samples in experimental work.

7. Use of inhibitors

Cell division was inhibited by the addition of 10^{-4}M-colchicine (Hopkin and Williams Ltd.) which prevents spindle formation and so halts division in metaphase; it has been reported to permit chromosomal duplication (Dustin, 1962). Although its effect upon the cells is not known in detail, the concentration used was much greater than that generally used in cell cultures (Paul, 1965).

DNA synthesis was inhibited by adding 10^{-4}M-FUdR whose site of action is shown in Figure 2. Its effect could be reversed by the addition of 10^{-4}M-thymidine but not by 10^{-4}M-uridine (see section III, 9).

10 µg./ml. actinomycin D (Mann Research Laboratories Ltd.) was used to inhibit DNA-primed RNA synthesis. Although its effect was almost immediately evident, it did not completely inhibit uridine incorporation within 1½ hr. (Figure 75, section III, 9); it may be that a sufficient intra-cellular concentration takes some time to build up. Since it was present in the culture medium for long periods other cell processes, such as DNA synthesis or cell division, may later be affected by this concentration (Reich and Goldberg, 1964). Only cellular activity affected in the early stages of culture may be regarded as being due to the inhibition of DNA-primed RNA synthesis.

Protein synthesis was inhibited by adding 10^{-4}M-puromycin (Nutritional biochemical Corp.) which prevents the assembly of amino-acyl tRNA on ribosomes (Williamson and Schweet, 1965).

Since these substances are not supplied sterile, 150 µg./ml. kanamycin was added to the medium when they were used. This did not affect the response (Figure 6) and no contamination occurred in any of the experiments. The inhibitors were added either at the same time as erythropoietin or 1 hr. prior to adding erythropoietin, the latter procedure being adopted when the effect of erythropoietin was observed before that of the inhibitors. The timing is specified in each experiment.

8. Dose-Response of foetal liver cells to erythropoietin

All the erythropoietin preparations used were derived from plasma of phenylhydrazine-treated sheep (White et al., 1960). Since most of them were purified to varying degrees, it was necessary to discover the concentration required to elicit a maximal response. It has been shown that the effect of erythropoietin can be modified by the number of cells present (Krantz and Goldwasser, 1965); although these authors used adult bone marrow cells at a much higher concentration than were the cells in this study, it was felt that the concentration of erythropoietin should be based on cell numbers. Thus, the erythropoietin concentration is expressed throughout as u./10^6 cells. The hormone was dissolved in Hanks BSS before use and stored in small volumes at -70°C.

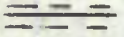
Early experiments were performed using highly purified erythropoietin (N.I.H. Haematology Study Section, Step 4, Lot K103,217A) previously studied by Cole and Paul (1966). This was found by them to be very active

FIGURE 3.

Dose-response curves of erythropoietin (N.I.H. Haematology Study Section, step 2, Lot R 041, 245) on

a) 14-day rat foetal liver cells

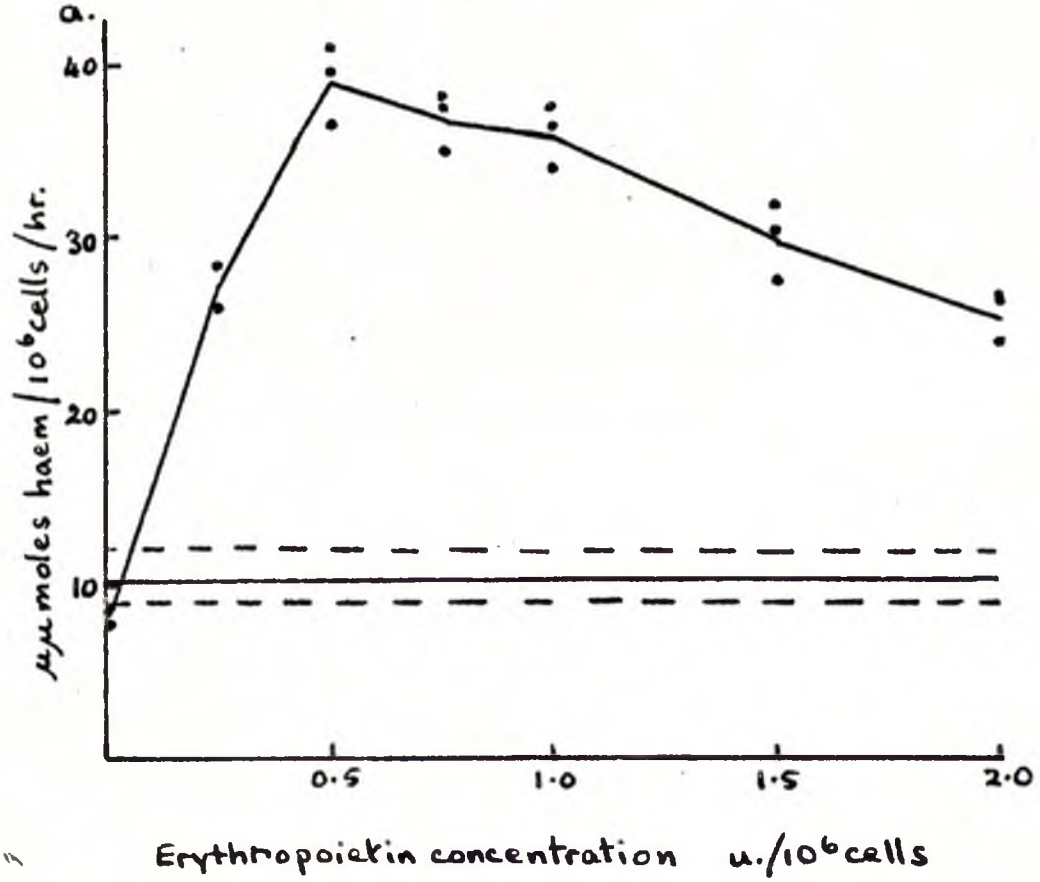
b) 13-day mouse foetal liver cells



initial rate of haem synthesis

rate of haem synthesis after 24 hr. treatment with erythropoietin at the concentrations shown.

Figure 3


a.
40
30
20
10
μμmoles haem / 10⁶cells / hr.
0·5
1·0
1·5
2·0
Erythropoietin concentration u./10⁶cells

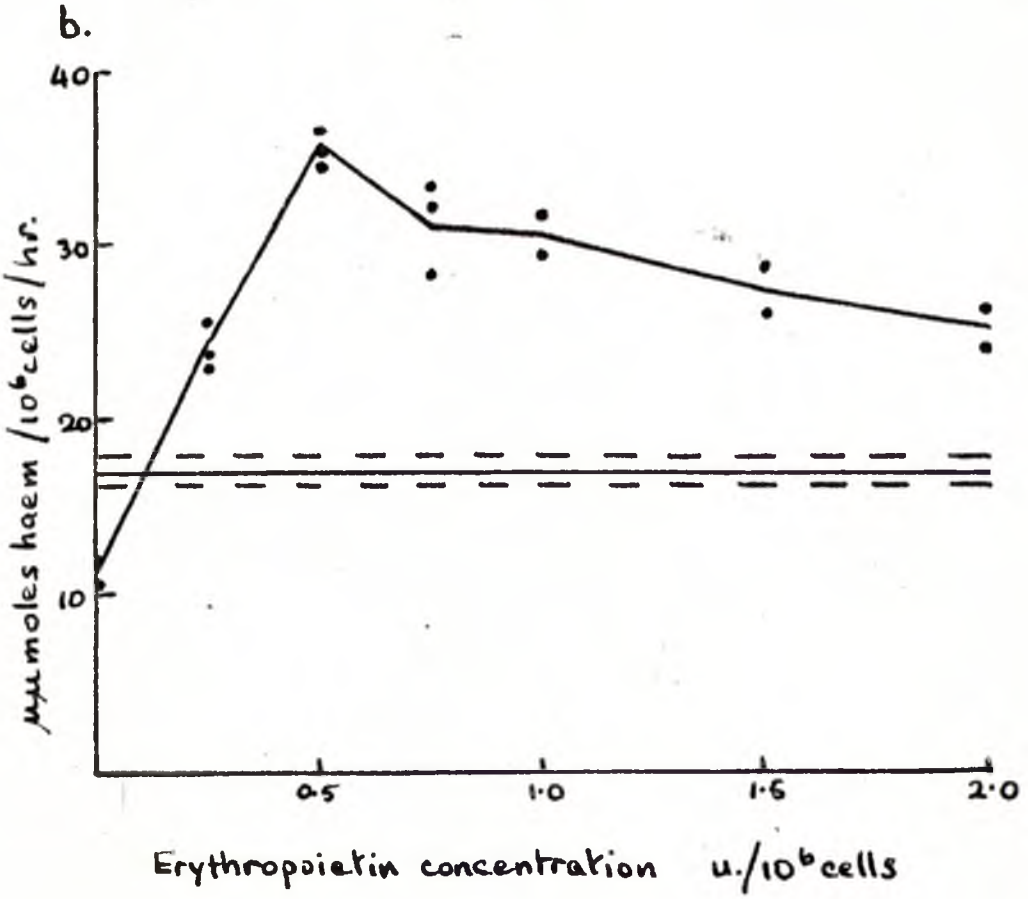
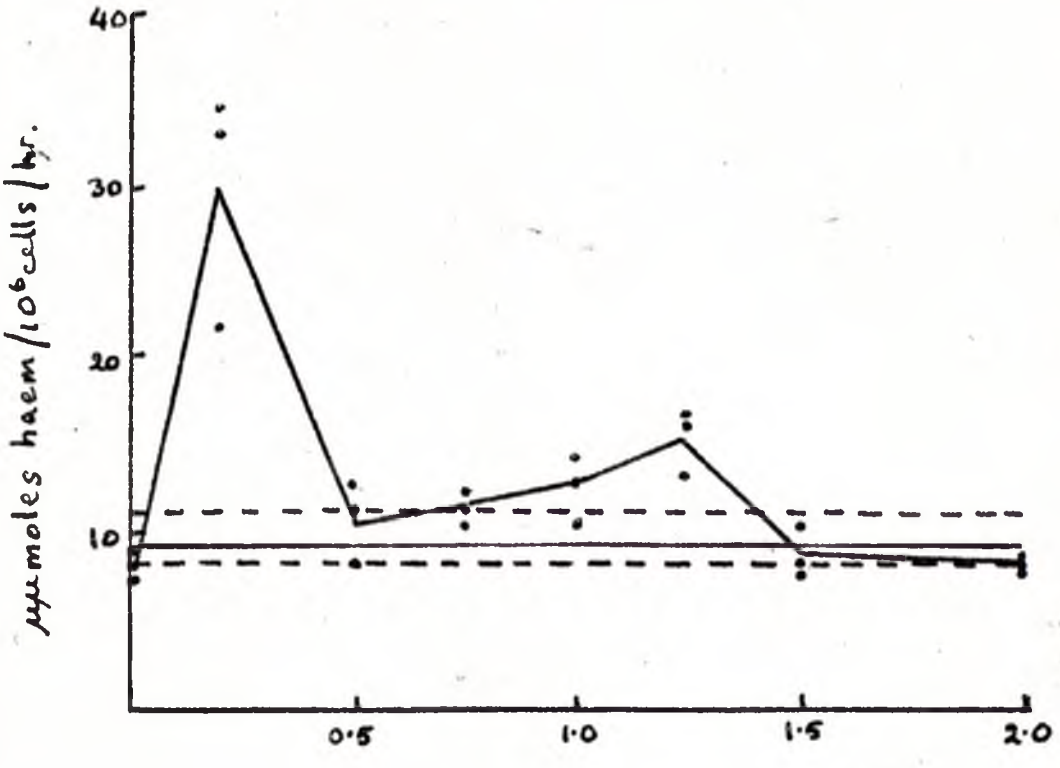

b.
40
30
20
10
μμmoles haem /10⁶cells / hr.
0·5
1·0
1·6
2·0
Erythropoietin concentration u./10⁶cells

FIGURE 4.

Dose-response curve of step 1 erythropoietin, Lot 1/4 (Connaught Laboratories Ltd.) on 14-day rat foetal liver cells.

Legend as Figure 3.

Figure 4


40
30
20
10
μμmoles haem/10⁶cells/hr.
0·5
1·0
1·5
2·0
Erythropoietin concentration u./10⁶cells

at 0.1 u./10^6 cells and was used at this concentration to discover at what stage of gestation rat foetal liver cells best responded. As will be discussed later in more detail, the greatest response was found in 14-day liver cells and these were used to determine the optimal dosage of later batches of erythropoietin for experiments on rat cells. 13-day mouse foetal liver cells, which also responded very well to erythropoietin, were used to find the optimal dose for mouse cells.

The effect of the hormone was tested by setting up cell cultures and treating them with a range of erythropoietin concentrations. The initial level of haem synthesis was measured and the effect of erythropoietin treatment on it was measured after overnight incubation.

A less highly purified erythropoietin preparation (N.I.H. Haematology Study Section, Step 2, Lot R041,245) was tested on both rat and mouse cells. As shown in Figure 3, the pattern of the response was the same in both cases, with a maximum at 0.5 u./10^6 cells. 0.75 or 1.0 u./10^6 cells provoked a slightly reduced response and at higher concentrations the response was even smaller. This was probably due to the presence in the erythropoietin preparation of inhibitory or toxic substances whose active concentration corresponded to 0.8 u. erythropoietin/10^6 cells or more. Mouse cells were slightly more sensitive than rat cells to this effect. This batch of hormone was used at 0.5 u./10^6 cells.

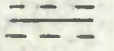
Connaught Laboratories Ltd. have produced a very impure erythropoietin preparation, corresponding to Step 1 of the N.I.H. preparations. One batch of this (Lot 1/4) was tested only on 14-day rat cells (Figure 4). The greatest response was elicited by only 0.2 u./10^6 cells and

FIGURE 5.

Dose-response curves of step 1 erythropoietin, Lot 117/1 (Connaught Laboratories Ltd.) on:

a) 14-day rat foetal liver cells

b) 13-day mouse foetal liver cells

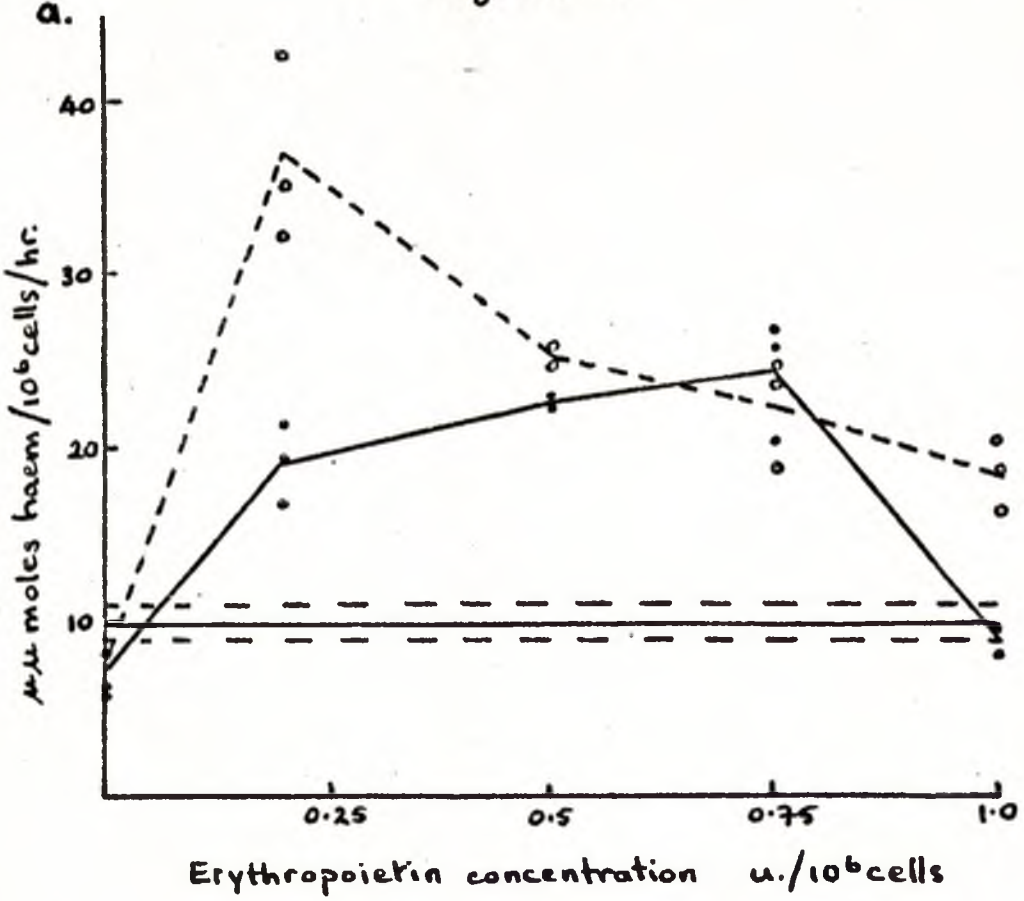


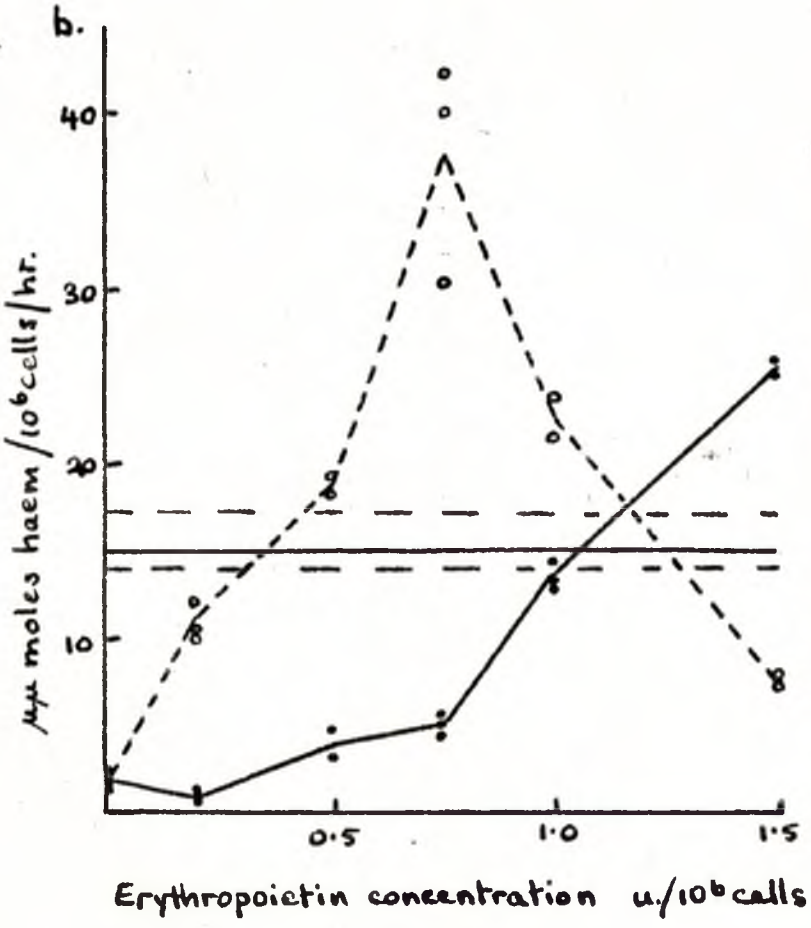
initial rate of haem synthesis.

rate of haem synthesis after 24 hr. treatment with erythropoietin at the concentrations shown.

rate of haem synthesis after 24 hr. treatment with erythropoietin, after precipitation of impurities at pH 4.3, at the concentrations shown.

Figure 5


a.
40
30
20
10
μμ moles haem/10⁶cells/hr.
0·25
0·5
0·75
1·0
Erythropoietin concentration u./10⁶cells


b.
40
30
20
10
μμ moles haem/10⁶cells/hr.
0·5
1·0
1·5
Erythropoietin concentration u./10⁶cells

the response decreased sharply at higher concentrations. This batch was used at 0.2 u./10^6 cells. Since it was not used for any experiments on mouse cells its effect upon them was not tested.

Some of the impurities present in this type of preparation can be precipitated at pH 4.3 (Goldwasser, 1967) and a later batch (Lot 117/1) was so treated. The crude material was added to a mixture of 1 part BSS:1 part 0.15 M-acetate buffer at pH 4.3 and the precipitate formed was removed by spinning at 1500 g for 15 min. The concentration of erythropoietin in the supernatant was taken as that present before precipitation but it is not known how much erythropoietin was precipitated together with the impurities. The effect of the precipitated hormone on both rat and mouse cells was compared with that of unprecipitated material on the same cell suspension with the results shown in Figure 5.

0.75 u./10^6 cells of unprecipitated material were required to elicit a maximal response from rat cells and the response decreased sharply at higher concentrations. It is evident that different Lots of this material vary considerably in activity. In this case a higher concentration of hormone was necessary to provoke a lower maximum response from the cells than had the previous batch. It is of interest that the pattern of the response seen after precipitation of impurities was more similar to that produced by Lot 1/4. The maximum response occurred at 0.25 u./10^6 cells.

There was a considerable difference in sensitivity between rat and mouse cells. So much unprecipitated hormone was evidently required

FIGURE 6.

Dose-response curve of step 1 erythropoietin, Lot 1/4 (Connaught Laboratories Ltd.) on 14-day rat foetal liver cells grown in medium with or without 150 µg./ml. kanamycin.

= = = initial rate of haem synthesis

•——• rate of haem synthesis after 24 hr. treatment with erythropoietin at the concentrations shown, in medium containing no kanamycin.

o— — —o rate of haem synthesis after 24 hr. treatment with erythropoietin at the concentrations shown, in medium containing 150 µg./ml. kanamycin.

FIGURE 7.

Dose-response curve of erythropoietin (N.I.H. Haematology Study Section, step 3, Lot K 147,048) on 13-day mouse foetal liver cells.

Legend as Figure 3.

Figure 6

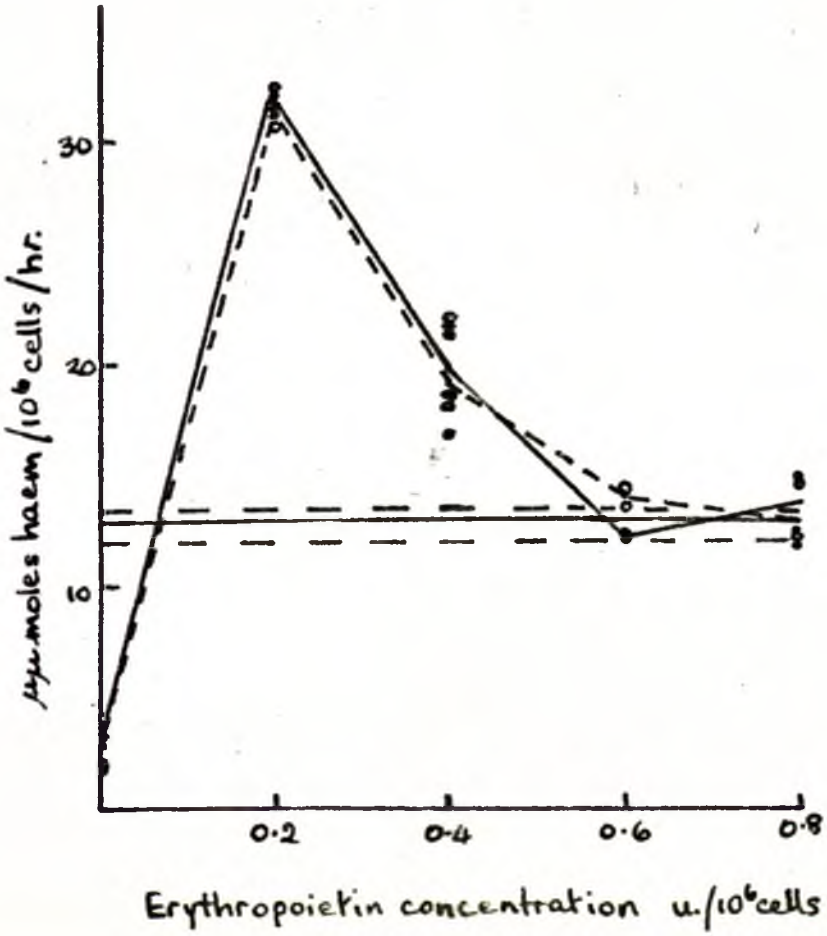


Figure 7

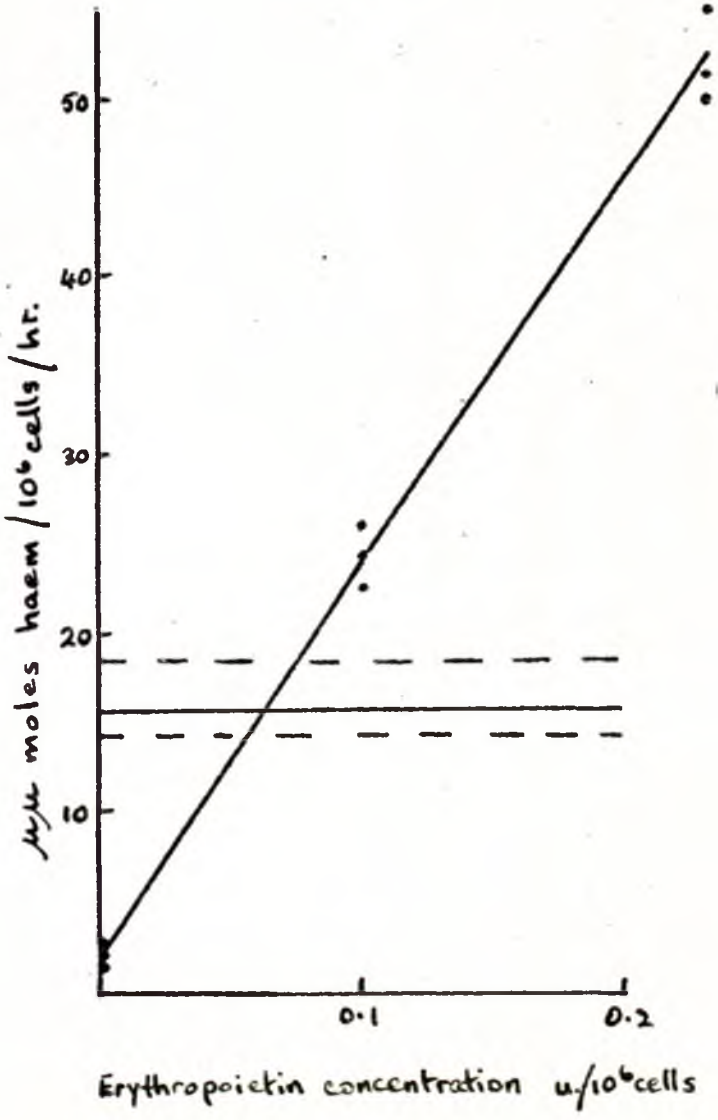


TABLE 10

A comparison between the dose and purification of different erythropoietin preparations and the maximum response produced by rat and mouse foetal liver cells

Degree of purification (White et al., 1960)	14-day rat foetal liver cells				13-day mouse foetal liver cells			
	μμ moles haem/10^6 cells/hr			Erythropoietin u./10^6 cells	μμ moles haem/10^6 cells/hr			Erythropoietin u./10^6 cells
	Initial rate	Maximum rate	Ratio		Initial rate	Maximum rate	Ratio	
Step 1. 1/4	9	30	3.3	0.2				
Step 1. 117/1	10	25	2.5	0.75	14	25	1.8	1.5
Step 1. 117/1 pH 4.3 supernatant	10	37	3.7	0.25	14	38	2.7	0.75
Step 2	10	39	3.9	0.5	12	35	3.0	0.5
Step 3					15	51	3.4	0.2
Step 4	10	86	8.6	0.1	15	61	4	0.1

Each result is the average of 3 cultures

to elicit a maximal response in mouse cells that it may not have fallen within the range of concentrations tested. Only at 1.5 u./10^6 cells did the treated cells exceed the initial rate of haem synthesis. A greater response was provoked by precipitated material with a maximum at 0.75 u./10^6 cells but the response was found over a very narrow range of erythropoietin concentrations. This batch was used, after precipitation of impurities at pH 4.3, at 0.25 u./10^6 foetal rat cells and at 0.75 u./10^6 foetal mouse cells.

The Step 1 preparation was not sterile and cultures containing it were frequently contaminated. After filtration through a 0.22 μ Millipore membrane, much of its activity was lost. The addition of 150 μg./ml. of kanamycin ('Kanamyn', Bayer Ltd.) effectively prevented contamination; as shown in Figure 6 the response was unaffected. In all experiments involving Step 1 material, kanamycin was added to cultures with and without erythropoietin.

A batch of more purified erythropoietin (N.I.H. Haematology Study Section, Step 3, Lot K147,048) was also tested. Since supplies of effective hormone were limited a complete dose-response was not determined. It was tested on 13-day mouse cells at 0.1 and 0.2 u./10^6 cells (Figure 7). The effect of 0.2 u./10^6 cells was judged sufficient to study the response and this concentration was used routinely.

A comparison of the concentration of erythropoietin which produced a maximal response with the degree of purification showed that each purifying step, later than step 1, reduced the dose required (Table 10). It also showed that more highly purified preparations provoked a greater

maximum response. Since later work suggested that the magnitude and duration of the response may be related to the initial level of haem synthesis, the maxima are expressed in terms of this level. Purification to step 2 did not greatly increase the activity of the hormone but the preparation was more stable. As can be seen the step 1 preparations were very variable and the activity of Lot 117/1 was also found to be affected by storage.

It is probable that the effects of the impurities present in the hormone preparations, especially in the crude preparations, varied considerably. The responses displayed by cells from different sources are not directly comparable unless the same hormone preparation was used; in the case of the crude preparations, the results obtained after storage of the hormone may not be comparable to those previously obtained.

9. Starch gel electrophoresis

A suspension in buffer of hydrolysed starch (Connaught Laboratories Ltd.) was heated to boiling, with constant swirling to avoid burning; the amount of starch required per 100 ml. of buffer to give optimum separation is indicated by the makers for each batch. The suspension was allowed to boil for 1 min. after which dissolved air may be removed by a brief application of negative pressure. The hot starch was poured into a perspex tray measuring 0.7 x 8 x 14 cm. An alternative method used was to pour the starch without first removing

the air; as the starch cooled, the air formed bubbles which rose to the surface and were trapped in the skin which formed there. The layer of skin holding the bubbles could be swept off without disturbing the lower part of the starch. The gels produced by both methods appeared identical; the pattern of electrophoresis in gels made by each method was also identical. After a little cooling the tray was covered with a perspex sheet, held in place by a weight, to give the gel a flat surface. Gels were used 3 - 6 hr. after pouring.

Haemoglobin samples were soaked on to pieces of Whatman 3MM paper trimmed to the depth of the gel and about 0.7 cm. long; these were inserted into slits cut in the gel transversely to the flow of the current. Care was taken to make close contact between the paper and the gel. The slits could be widened to hold several such papers behind one another to apply larger amounts of very dilute samples; this did not affect separation of the components, although it occasionally caused a slight overall retardation.

The gels were run horizontally at 4°C (Smithies, 1955) between two baths containing buffer; the gel and the baths were connected by wicks consisting of nine thicknesses of Whatman "Separa" paper. The first of these, in direct contact with the gel, was applied separately so as to ensure completely even contact; the others were folded together to give eight thicknesses and applied carefully to the first. A sheet of polythene was placed over the gel and the wicks nearly to the surface of the buffer to prevent any drying out during electrophoresis. Carbon

or platinum electrodes were placed in the buffer baths and 8-10 volts/cm. of gel were applied from a D.C. mains transformer until a good separation of the components was achieved. The time necessary for this depended on the buffer system used.

Staining procedures. After electrophoresis the position of the bands was noted before staining, but components present in small quantities were not always visible at this time. Two staining methods were used, one which stained haemoglobin specifically and one which stained proteins in general. The specific stain used was o-dianisidine (O'Brien, 1961). This method depends on the catalase activity of haemoglobin; a deep orange colour is produced by the subsequent oxidation of o-dianisidine.

The staining solution was made up as follows:

100 mg. o-dianisidine in 70 ml. absolute, redistilled ethanol	4 parts
distilled water	1.5 parts
0.1 M-acetate buffer, pH 4.6	1 part
30% v/v solution of H_2O_2 (100 vol.)	0.2 parts

This was applied to the gel for 15 minutes and then washed off with water. The o-dianisidine and diluted H_2O_2 solutions were made up immediately before use.

When successful this method made visible components present in very small concentrations, but the results obtained were variable.

Attempts to make it more consistent by varying the proportions of the components or the pH were unsuccessful. Changing the buffer in the gel had no effect. In consequence, the method was used only until each sample had been stained at least once; each component described later was, therefore, verified by o-dianisidine staining but was not always so stained.

The non-specific protein stain used was the naphthalene black method of Smithies (1955). A saturated solution of naphthalene black (Gurr Ltd.) was made up in a solvent containing water, methanol and glacial acetic acid in the proportions 5:5:1. This was applied to the gel for 30 sec. and surplus stain was washed out over some hours in several changes of the same solvent. This stain was also very sensitive but not specific for haemoglobin. To compare its effect with that of o-dianisidine, gels were sliced horizontally to produce two identical parts; one of these was stained with naphthalene black and the other with o-dianisidine. The staining pattern produced by each was identical provided that the blood cells had been adequately washed. It was judged that routine use of naphthalene black, after initial checking with o-dianisidine, was unlikely to prove misleading.

Buffer systems. Three buffer systems were used; they were assessed according to their ability to separate the multiple components of rat haemoglobin. The buffers used were:

A. 0.05 M-tris (Sigma Ltd.), 0.005 M-ethylenediaminetetra-acetic acid, disodium salt (EDTA), 0.03 M-boric acid, pH 8.6. This was used to make the gel and was also placed in the baths for electrophoresis. It gave good separation and clearly defined bands; 4 components were found.

B. 0.01 M-phosphate buffer, pH 6.2. This was also used in both the gel and the baths. The separation was less good and the bands less well-defined; 3 components were found.

C. 0.076 M-tris, 0.005 M-citric acid, pH 8.6, was used to make the gel. The baths contained 0.3 M-boric acid, 0.05 M-NaOH, pH 8.6 (Poulik, 1957). This gave good separation and clearly defined bands, sometimes bow-shaped instead of straight; 5 components were found in some samples, especially from late foetuses.

Attempts were made to modify buffer A, with which misshapen bands never occurred, to separate the fifth component found with buffer C. Keeping the pH at 8.6, the concentrations of the three components were varied in a number of ways and the effect on the separation of rat haemoglobin components was tested. None separated more than four components and one or two separated fewer. Despite the occasional distortion of bands found with buffer C, it was used for much of the work on rat haemoglobin.

Extra bands may be found owing to formation of disulphide bonds between haemoglobin molecules (Riggs, 1965) or between haemoglobin and glutathione (Baglioni, 1963). To exclude this possibility 1.0% v/v

mercaptoethanol was added to the buffer. This had no effect on the electrophoretic pattern of rat or rabbit haemoglobins, but it did alter that of mouse, especially foetal mouse, haemoglobin. 1.0% mercaptoethanol was used only in studies on mouse material.

10. Preparation of haemoglobin samples

To prevent clotting, blood was collected in BSS containing a small amount of EDTA, adjusted to pH 7 with 0.1N-NaOH. Adult animals were bled from the jugular vein; foetuses were washed free of maternal blood with BSS, decapitated, and allowed to bleed from the cord and body blood vessels. The blood cells were washed three times in BSS and lysed with 10 vol. distilled water. Lysis was complete within minutes in all samples. Non-haem proteins and other cell components were precipitated with an equal or greater volume of chloroform (Weiss, 1960) and removed by spinning for 10 min. at 1500 g. The precipitate formed a compact disc between the chloroform and the clear, aqueous supernatant containing the haemoglobin.

The haemoglobin samples were stored in the oxyhaemoglobin form at 4°C. Adult mouse, rabbit and all foetal samples could be kept in this way for up to three weeks without alteration of their electrophoretic pattern, but fresh samples were made as often as possible and used whenever available. Adult rat haemoglobin, especially at high concentrations, was less stable and a precipitate often formed within four days. The precipitate did not appear to be of constant

composition, since the electrophoretic pattern changed in a variety of ways after its formation in different samples. The precipitate may be redissolved by bubbling CO_2 through the solution (Brada and Tobiska, 1963) when the original electrophoretic pattern was apparently restored. In general, however, fresh samples were made as required.

To ensure that chloroform did not precipitate any haemoglobin components, blood samples from rat, mouse and rabbit were lysed in distilled water and the lysates split into two parts. One part of each was treated with chloroform and both parts were electrophoresed. The gel was cut into two parts so as to compare the pattern shown by o-dianisidine and by naphthalene black staining. In each case many bands stainable with naphthalene black were found in the unprecipitated sample which were not found in the chloroform-treated sample. The pattern of o-dianisidine staining was identical in precipitated and unprecipitated samples from each animal.

Other haem proteins may also remain in the aqueous layer after chloroform extraction. Both cytochrome c (EC.1.11.1.5) and catalase (EC.1.11.1.6) are more stable in the presence of chloroform than is haemoglobin itself (Boyer et al., 1963). At pH 8.6, however, cytochrome c migrates to the cathode while haemoglobin migrates to the anode. Catalase, on the other hand, also migrates to the anode and stains very strongly with o-dianisidine. Although its concentration in erythrocytes is less than one thousandth of that of haemoglobin (Lemberg and Legge, 1949), its much greater enzymatic activity

might make it detectable even at that concentration. In buffer B at pH 6.2 haemoglobin migrates to the cathode and catalase to the anode. Samples from several different animals were electrophoresed at several concentrations in this buffer system and the gels were stained with o-dianisidine. No staining was visible between the origin and the anode, suggesting that catalase, if present, was at too low a concentration to interfere with the results.

11. Electrophoresis of haemoglobin made in cell cultures

Although the amount of haemoglobin synthesised by cultured cells is too small to be visible after electrophoresis, its nature can be determined by labelling with ^{59}Fe and studying the distribution of the radioactivity in starch gels after electrophoresis.

Cell cultures were set up containing a maximum of 3×10^6 cells/ml. and incubated at 37°C. To obtain detectable radioactivity in all types of haemoglobin made, the length of the ^{59}Fe pulse was extended to at least six hours and several replicate cultures were used to prepare each sample. After labelling, the cells from the replicate cultures were pooled and washed three times in BSS. The pellet was lysed in 0.5 ml. distilled water by freezing and thawing three times. Mild homogenisation with a motor-driven teflon-on-glass homogeniser was used in some early experiments but it was found that up to 30% of the activity was lost by this method. The lysate was extracted with 1 ml. of chloroform and spun at 1500 g for 5 minutes. The colourless

TABLE 11

The effect of the counting procedure on the efficiency of counting ^{59}Fe in starch gels

Crushed	Hydrolysis in hot HCl	Evaporation at 80°C	Glass fibre circles	counts/min.	% total activity
-	-	-	-	3209.2	15.6
+	-	-	-	3719.8	18.1
-	+	+	-	6599.1	32.0
-	+	+	+	18540.3	90.0

Calculated activity in each slice - 20,601.2 disintegrations/min.

Each result is the average of 2 samples.

supernatant was removed and to it was added 0.2 ml. of a chloroform-extracted adult or foetal haemoglobin preparation so that the position of the bands on the gel could be seen.

0.05 ml. of the labelled sample was electrophoresed; several different haemoglobin samples were run on the same gel for comparison. After electrophoresis the strip of gel containing the labelled sample was cut out and the position of the unstained bands in this and in the other samples on the gel was noted. The strip was transversely sliced mechanically into 1.5 mm. slices. Each slice was hydrolysed in 0.4 ml. 'AnalaR' HCl at 95°C and the whole hydrolysate was dried on to Whatman GF/C 2.5 cm. circles at 80°C for 1-2 hr.. 1 ml. of toluene-based scintillation fluid was added and the samples were counted.

To determine the most efficient method of counting ^{59}Fe in starch gels, a gel was prepared in buffer containing a known amount of ^{59}Fe. Slices of standard size were cut from it; some were placed directly into scintillation fluid while others were crushed before doing so. The rest were hydrolysed in hot HCl and the hydrolysates were evaporated at 80°C in scintillation vials with or without glass fibre circles. As shown in Table 11, 90% of the activity present could be detected in hydrolysed samples dried on to glass fibre circles; the amount detected by other methods was very much less.

A known amount of ^{59}Fe was made up to 0.5 ml. in water or in 50% serum in BSS. The samples in water and some of those in serum were treated with 1 ml. of chloroform; the rest of those in serum

TABLE 12

The effect of equilibration of $^{59}FeCl_3$ with 50% serum in BSS upon the activity remaining in the supernatant after chloroform precipitation

^{59}Fe diluent	Incubated at 37°C hr.	counts/min.	% total
water	-	334,877.7	24.1
50% serum	-	326,697.0	23.5
50% serum	5	333,333.3	24

Total activity added 1,388,887.5 counts/min.

Each result is the average of 3 samples

TABLE 13

Comparison of the activity present in extracts of foetal liver cell cultures after precipitation with chloroform or extraction in acid-butanone.

Length of incubation hr.	Extraction procedure		Cells washed in BSS	counts/min.	% of total activity added
	Chloroform	Butanone			
0	+	-	-	68,937.7	21.2
0	+	-	+	423.8	1.3
0	-	+	+	28.5	
0	+	+	+	26.9	
6	+	-	+	5,427.8	
6	-	+	+	647.3	
6	+	+	+	627.4	

Total activity added - 324,674.8 counts/min.

Background activity - 24.2 counts/min.

Each result is the average of 3 cultures.

were incubated at 37°C for 5 hr. before chloroform treatment. It was found (Table 12) that 21-23% of the activity remained in the aqueous layer in all cases. Since transferrin is precipitated by chloroform (Gitlin and Janeway, 1960) these results suggest that it was ^{59}Fe which remained in solution.

A suspension of foetal liver cells was prepared and equilibrated ^{59}Fe was added to it; a number of cultures was set up. Some were extracted with chloroform immediately; others were washed twice in BSS before extracting. The aqueous layers from the latter were split into two equal parts. One of the parts was counted while the other was extracted in acid-butanone; the whole butanone extract was also counted. Some cultures were extracted with acid-butanone only. The rest of the cultures were incubated for 6 hr. at 37°C, after which a similar series of extracts was prepared. The results are shown in Table 13.

At zero time 21-22% of the ^{59}Fe added remained after extraction of unwashed cells with chloroform; only 1-2% remained after washing the cells. The activity present in butanone extracts was the same whether or not the cells had previously been treated with chloroform. These results suggest that the extra activity present in the chloroform extracts was due to unbound iron. Any iron applied to the gel would migrate to the cathode, away from the haemoglobin. The results of butanone extraction indicate that all of the labelled haemoglobin remained in the supernatant after chloroform extraction.

Cultures of 16-day rat foetal liver cells, which had been found very active in haemoglobin synthesis, were set up and labelled with ^{59}Fe for 7 hr.. The cells were lysed and extracted with chloroform; a chloroform-treated preparation of haemoglobin from the same litter was added as marker. The sample was electrophoresed in buffer C and the position of the bands was noted. The strip of gel containing the sample was cut out and the full length from anode to cathode was cut into slices. Each band seen comprised one slice and the area between and beyond them was cut into 0.5 cm. slices. Where this was not possible, as between the bands, the width of the slice was noted. The slices were hydrolysed and counted. No activity was found between the origin and the cathode, nor between the fastest haemoglobin band and the anode. Apart from some trailing, the activity found between the origin and the fastest band corresponded to the positions of visible bands.

The sample was also electrophoresed in buffer B and the distribution of radioactivity checked in the same way. No activity was found between the origin and the anode, indicating that detectable amounts of catalase were not present. Again the activity found corresponded to the positions of the visible bands.

III. EXPERIMENTAL

III. EXPERIMENTAL

FIGURE 8.

The response of 10-day rat yolk sac cells to step 2 erythropoietin, at 0.5u./10^6 cells.

•——• rate of haem synthesis in untreated cultures

o——o rate of haem synthesis in erythropoietin-treated cultures.

The thickened portions of the abscissa indicate the periods of incubation during which the cultures were pulsed with ^{59}Fe; the results are plotted at the mid-points of the pulses with ^{59}Fe.

FIGURE 9.

The response of 12-day rat foetal liver cells to step 2 erythropoietin, at 0.5 u./10^6 cells.

Legend as Figure 8.

FIGURE 10.

The response of 13-day rat foetal liver cells to step 2 erythropoietin, at 0.5 u./10^6 cells.

Legend as Figure 8.

Figure 8

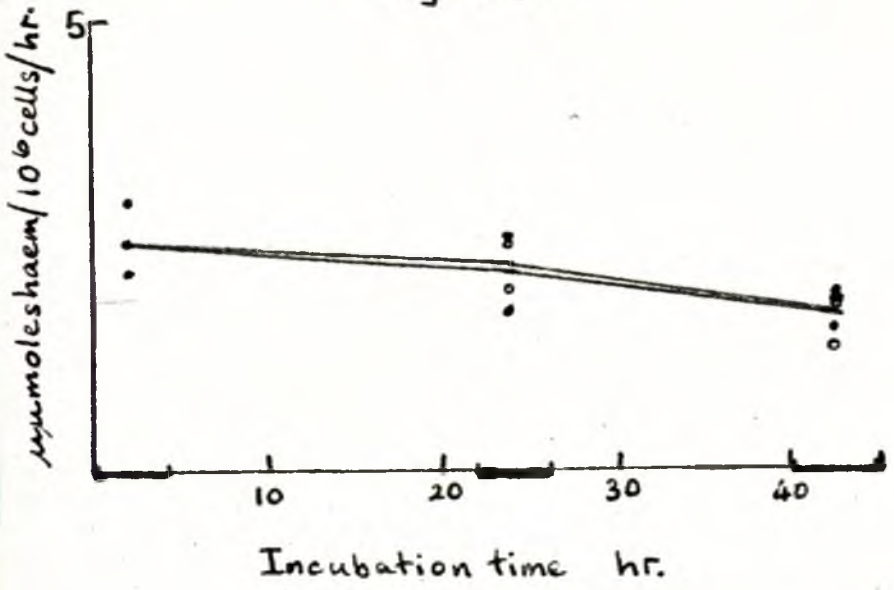


Figure 9

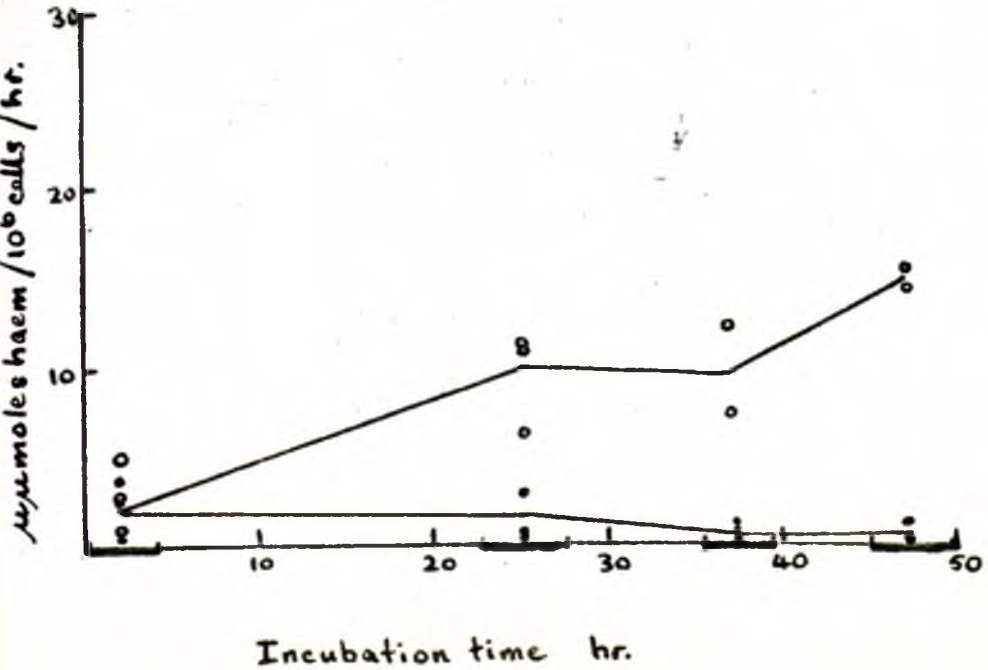
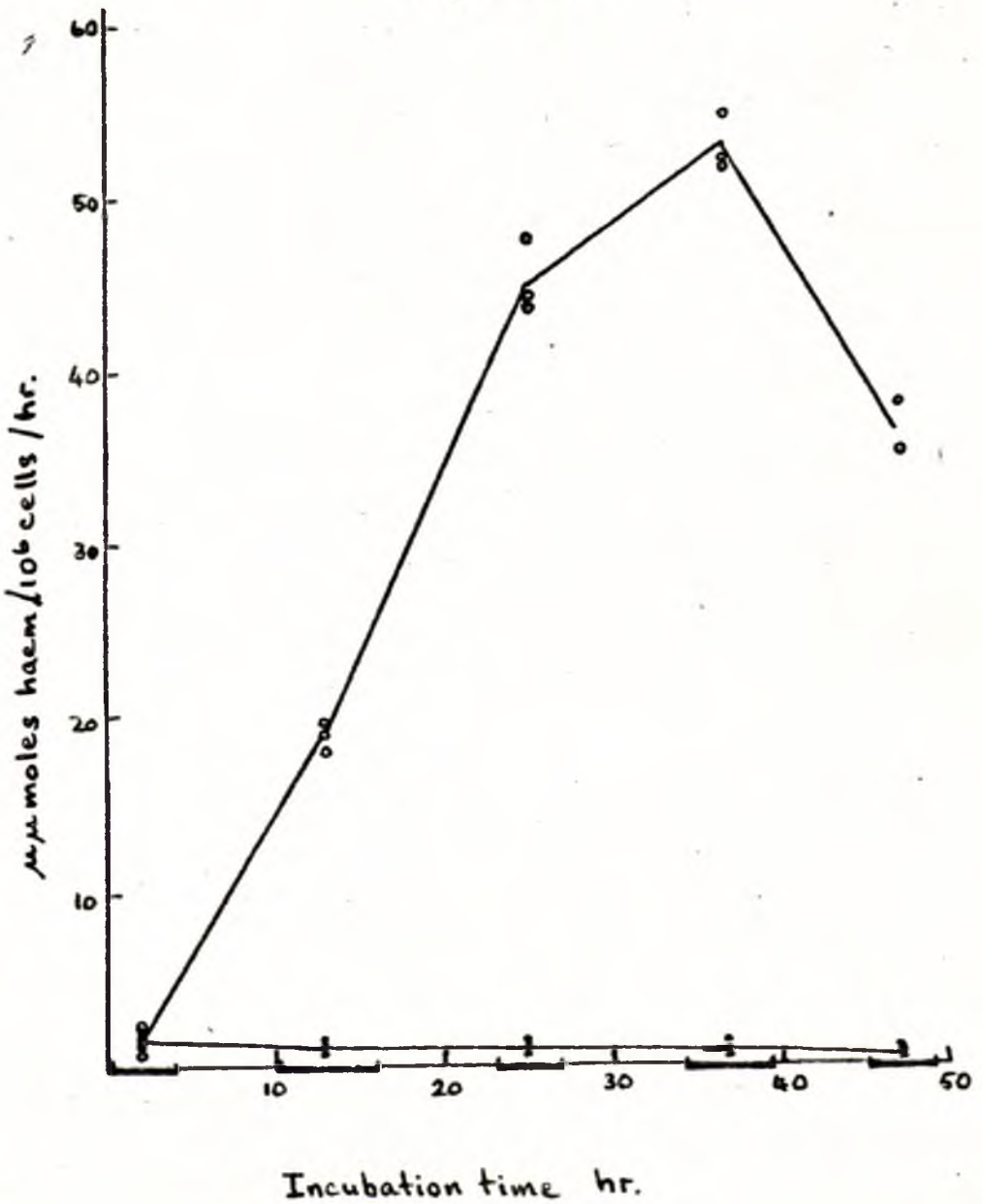


Figure 10



III EXPERIMENTAL

1. The effect of erythropoietin on foetal rat erythroid cells

It has been suggested that foetal rat erythropoesis is not controlled by erythropoietin (Stohlman et al., 1964). This series of experiments was designed to determine whether foetal erythroid cells were sensitive to erythropoietin; the location and persistance of sensitive cells during foetal development was also investigated. The age of the foetuses was counted in days from the morning on which the mothers were separated from the males (section II, 1).

Yolk sac cells. After removing the ectoplacental cone, 24 10-day rat embryos were trypsinised; the embryos, pooled from 3 litters, contained 5 - 20 somites. The cells were incubated in medium with or without erythropoietin and haem synthesis was measured at the times shown in Figure 8. The rate of haem synthesis was low throughout incubation and was unaffected by erythropoietin treatment. Since ESC were found to survive trypsinisation (Figure 19) this suggested that there were no ESC in yolk sac in vivo. This may mean either that yolk sac erythroid cells were inherently insensitive to erythropoietin at all times, or that they were fully stimulated by endogenous erythropoietin before explantation.

Liver cells

The liver was first visible during day 11; the four-lobed structure began to develop during day 12 when the organ was still completely white. Later on day 12 a faint stippling of pink appeared

Table 14

The number of nucleated cells found after trypsinisation of livers from rat foetuses of different ages.

Foetal age days	Number of nucleated cells/liver x 10^6
12	0.5
13	1.5
14	4.25
15	10.6
16	28.0
17	50.1
18	95.4
19	160.0
20	225.0
neonatal	270.0

at the points of the developing lobes. At 13 days the lobes were clearly defined and only a few white patches remained. From 14 days the lobes increased in size and the red colour deepened until day 18. The number of nucleated cells obtained from the livers after trypsinisation reflected these morphological changes (Table 14).

Liver cells from three 12-day litters were pooled and their response to erythropoietin was investigated; haem synthesis was measured at the times shown in Figure 9. The initial rate of haem synthesis was low and decreased in control cultures; in the presence of erythropoietin the rate increased slowly for 25 hr., remained constant at that level until 37 hr., and then increased once more until, after 47 hr. in culture, it stood at 2½ times the initial rate.

At 13 days, the livers from two litters provided enough cells to measure the response to erythropoietin. Again, the initial rate of haem synthesis was low and decreased in the control cultures (Figure 10). The erythropoietin-treated cultures showed a considerable increase in rate after 8 hr. in culture; the increase continued until, after 36 hr., the rate of haem synthesis was 25 times the initial rate. Thereafter it decreased quite quickly, but was still, after 47 hr. incubation, 18 times the initial rate.

The livers of 14-day, or older, foetuses contained a larger number of cells; the subsequent experiments were performed on the pooled

FIGURE 11.

The response of 14-day rat foetal liver cells to step 4 erythropoietin, at 0.1 u./10^6 cells.

Legend as Figure 8.

Figure 11

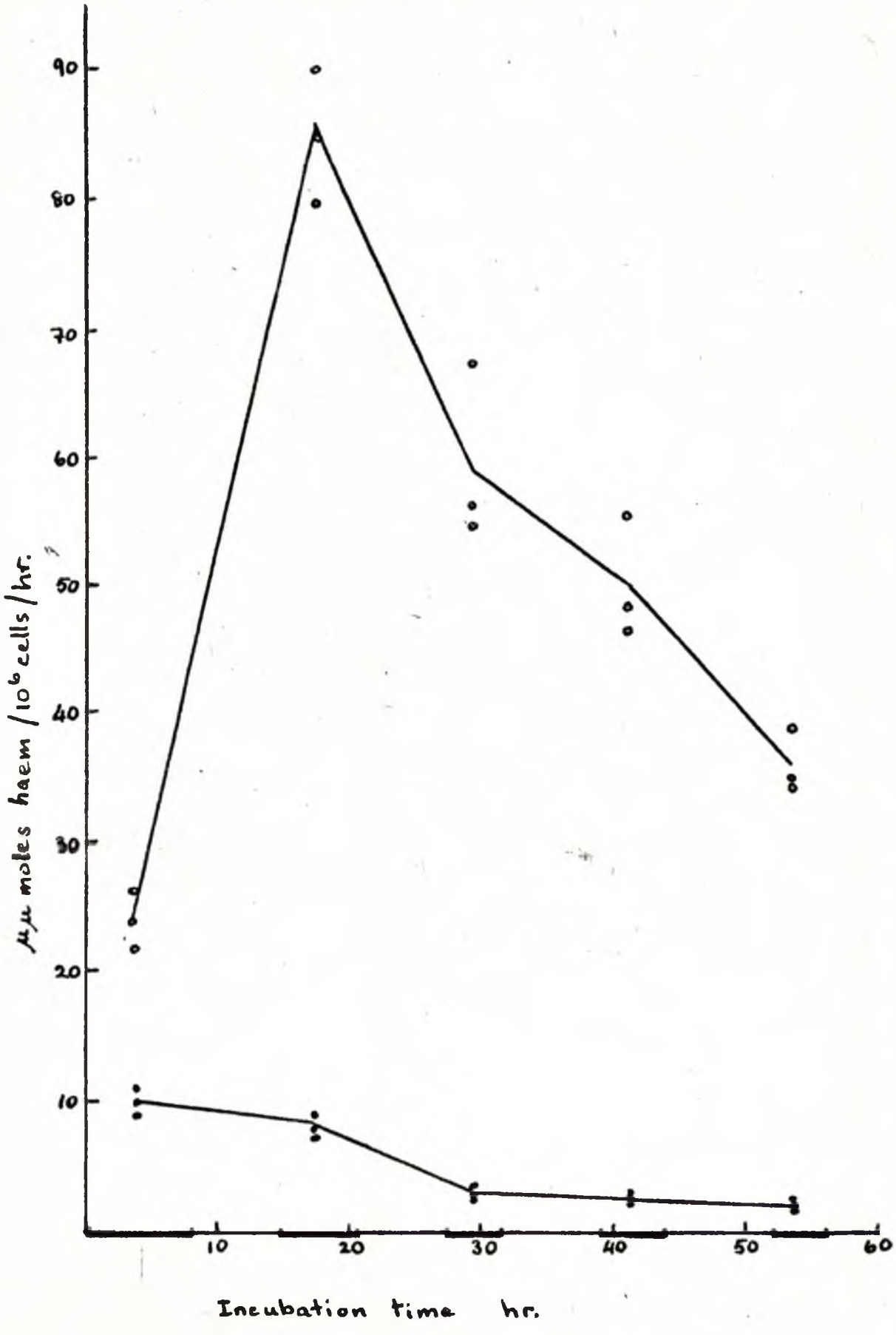
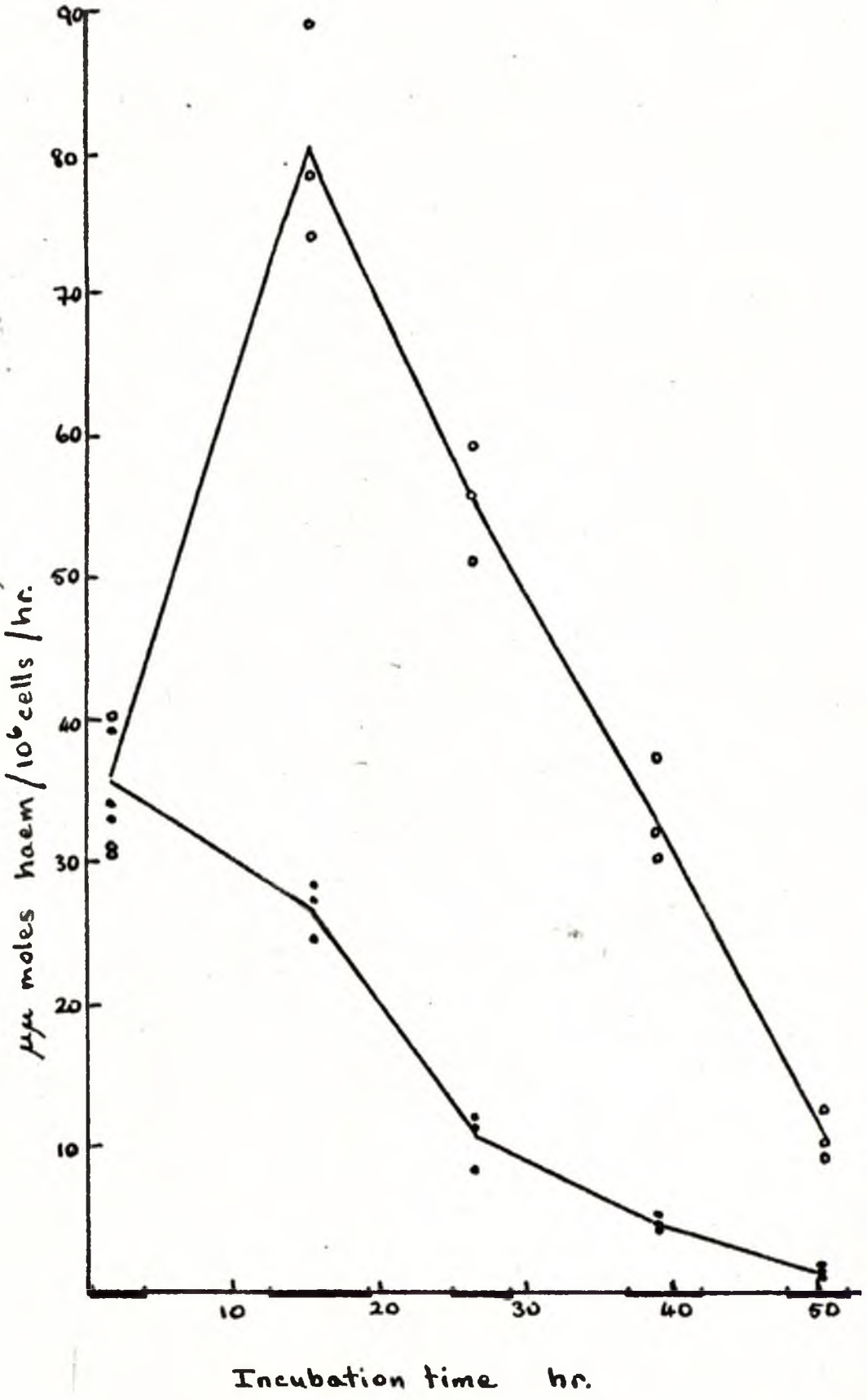

90
80
70
60
50
40
30
20
10
μμ moles haem /10⁶ cells /hr.
10
20
30
40
50
60
Incubation time hr.

FIGURE 12.

The response of 15-day rat foetal liver cells to step 2 erythropoietin, at 0.5 u./10^6 cells.

Legend as Figure 8.

Figure 12


90
80
70
60
50
40
30
20
10
μμ moles haem / 10⁶ cells / hr.
10
20
30
40
50
Incubation time hr.

liver cells from single litters. The response of 14-day foetal liver cells to erythropoietin was next studied; haem synthesis was measured at the times shown in Figure 11. The initial rate of haem synthesis was higher in these cultures than in cultures of younger liver cells; in the absence of erythropoietin the rate decreased during incubation. The rate of haem synthesis in erythropoietin-treated cultures during the first pulse with ^{59}Fe was more than twice the rate in control cultures; this was probably due to the considerable length of this pulse (8 hr.) and to the large and rapid increase in the rate of haem synthesis produced by erythropoietin treatment. After 18 hr. treatment with erythropoietin the rate of haem synthesis reached a maximum of 8 times the initial rate; after this time it decreased but was still, after 54 hr. incubation, 3.6 times the initial rate.

In 15-day foetal liver cell cultures the initial rate of haem synthesis was again higher; it decreased continually in the absence of erythropoietin (Figure 12). In erythropoietin-treated cultures the rate of haem synthesis rose to a level near that achieved by 14-day cells after erythropoietin treatment, although this was only 2.2 times the initial rate of synthesis in the present cultures; the maximum response was observed after 15 hr. in culture and the rate of synthesis decreased thereafter. No response was visible during the first pulse, probably because of its relatively short length.

The initial rate of haem synthesis in 16-day foetal liver cell cultures was once more slightly higher; only a slight response to

FIGURE 13.

The response of 16-day rat foetal liver cells to step 4 erythropoietin, at 0.1 u./10^6 cells.

Legend as Figure 8.

FIGURE 14.

The response of 17-day rat foetal liver cells to step 4 erythropoietin, at 0.1 u./10^6 cells.

Legend as Figure 8.

Figure 13

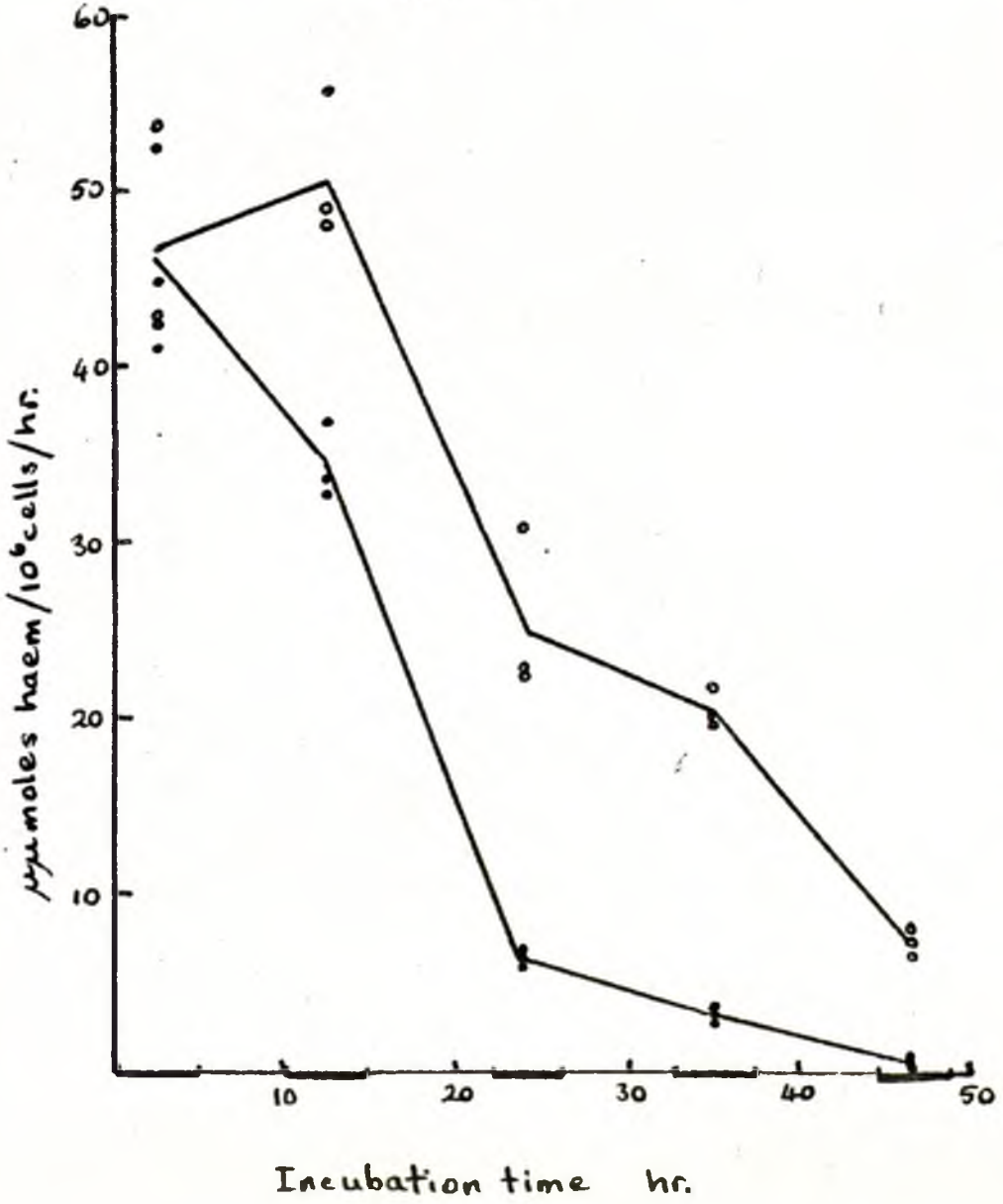


Figure 14

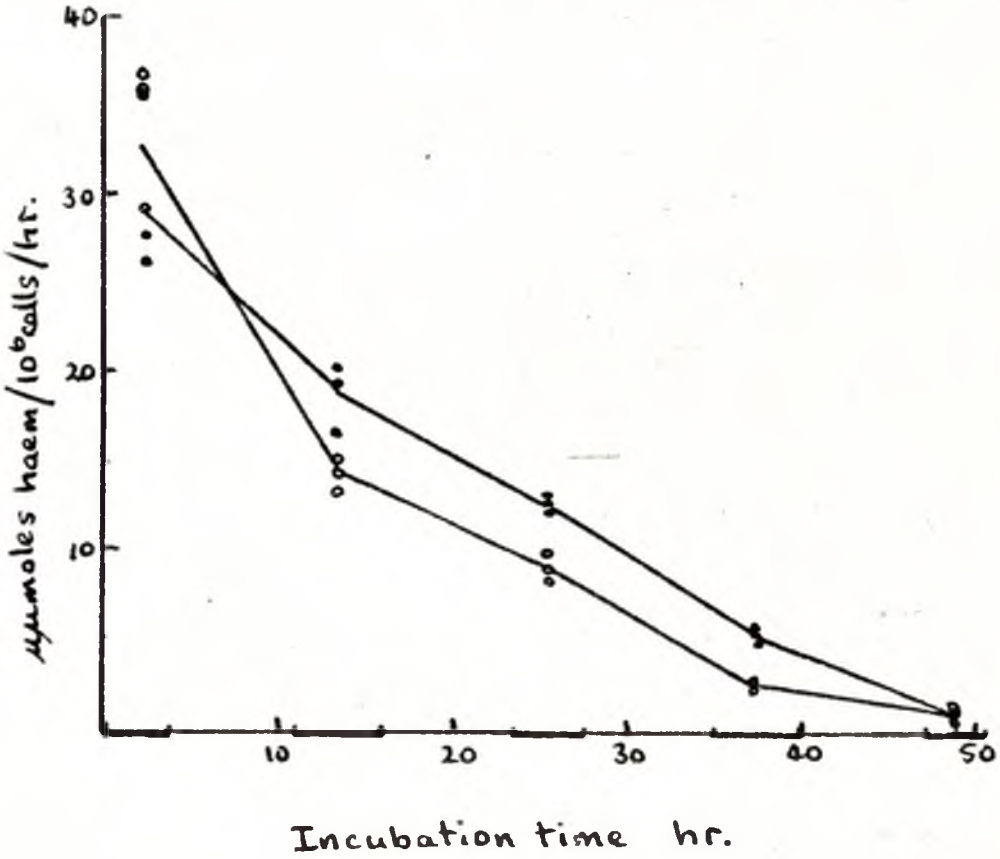


FIGURE 15.

The response of 18-day rat foetal liver cells to step 4 erythropoietin, at 0.1 u./10^6 cells.

Legend as Figure 8.

FIGURE 16.

The response of 19-day rat foetal liver cells to step 2 erythropoietin, at 0.5 u./10^6 cells.

Legend as Figure 8.

FIGURE 17.

The response of 20-day rat foetal liver cells to step 2 erythropoietin, at 0.5 u./10^6 cells.

Legend as Figure 8.

FIGURE 18.

The response of neonatal rat liver cells to step 1 erythropoietin (Lot 1/4), at 0.2 u./10^6 cells.

Figure 15

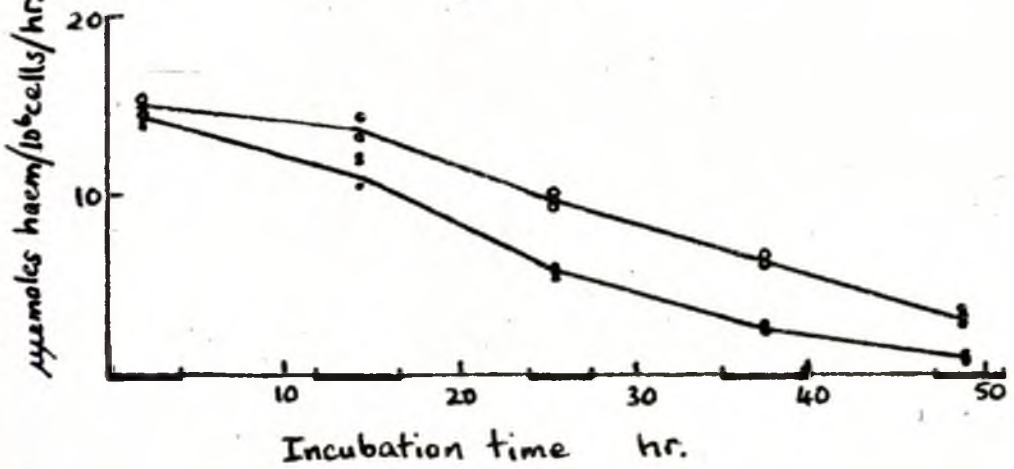


Figure 16

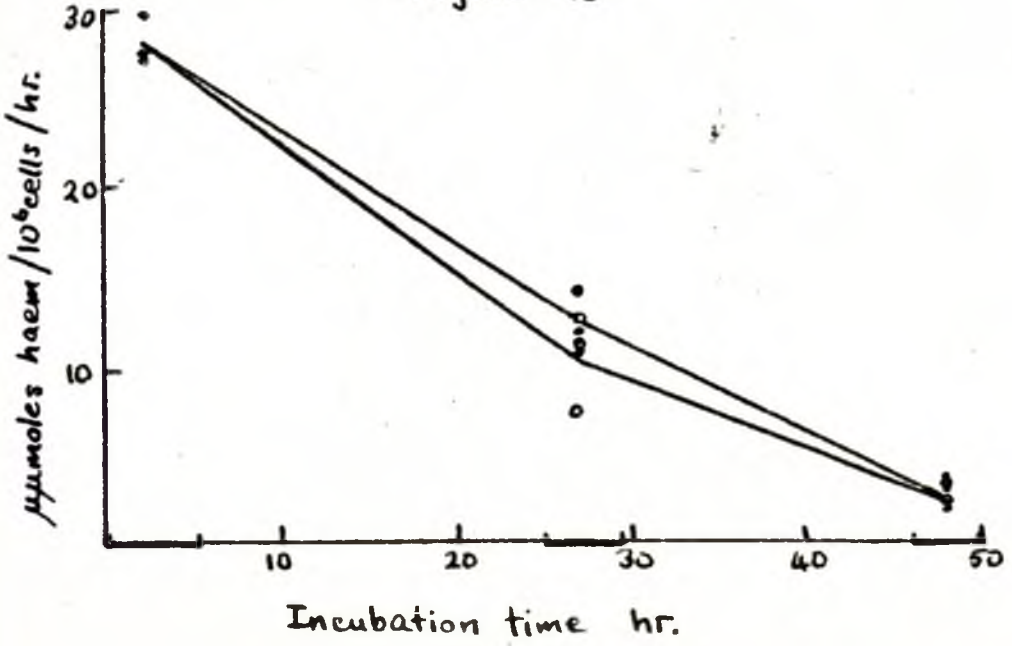


Figure 17

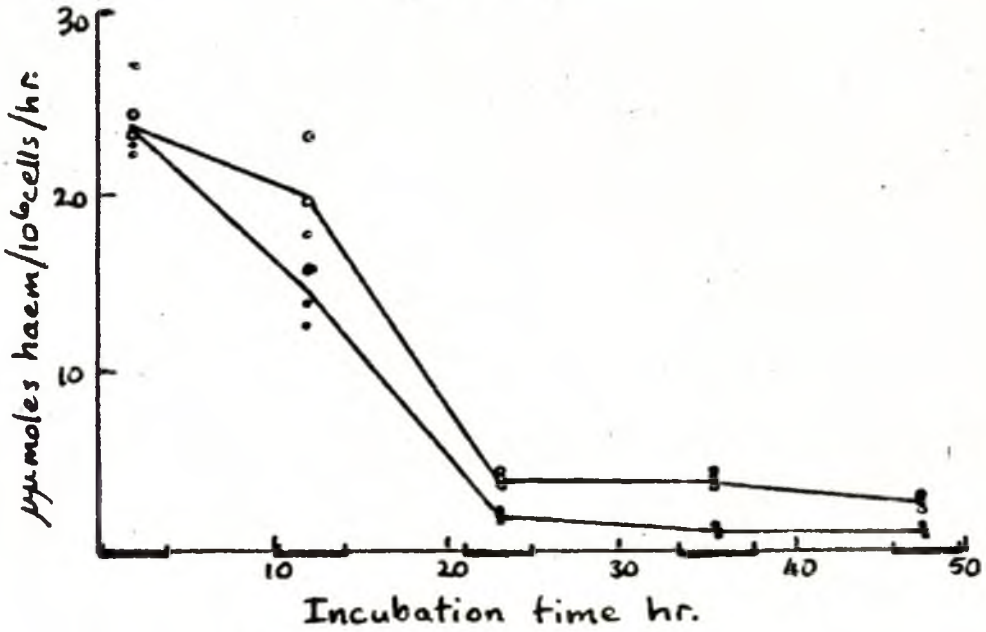
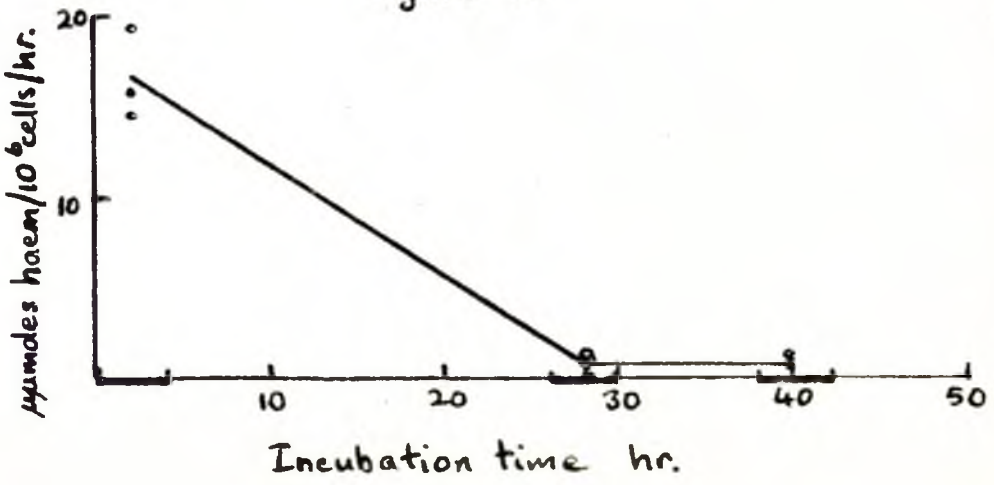


Figure 18



erythropoietin was observed (Figure 13). The maximum response, 1.1 times the initial rate of synthesis, was seen after 12 hr. in culture, after which the rate of haem synthesis decreased. At no time was the rate of haem synthesis as high as that observed following treatment of 14-day or 15-day foetal liver cells with erythropoietin.

In cultures of 17-day foetal liver cells the initial rate of haem synthesis was lower than in 16-day cell cultures (Figure 14) and was even lower in 18-day foetal liver cell cultures (Figure 15); there was no response to erythropoietin in either of these experiments. The initial rate of haem synthesis in 19-day foetal liver cell cultures was almost the same as that in 17-day foetal liver cell cultures (Figure 16); erythropoietin treatment had no effect and the rate decreased during incubation in both treated and untreated cultures. The initial rate of haem synthesis in cultures of 20-day foetal liver cells (Figure 17) was lower than that observed in 19-day foetal liver cell cultures; it was lower still in cultures of neonatal liver cells (Figure 18). Neonatal cells were obtained from livers explanted during the first day after birth. Erythropoietin had no effect on either 20-day foetal liver cells or neonatal liver cells; in each case the rate of haem synthesis in both untreated and erythropoietin-treated cultures decreased during incubation.

It was possible that treatment with cold trypsin for up to 20 hr. might affect the response of liver cells to erythropoietin. Pooled 13-day foetal livers were finely chopped and divided into two aliquots; one aliquot was treated with cold trypsin overnight and

FIGURE 19.

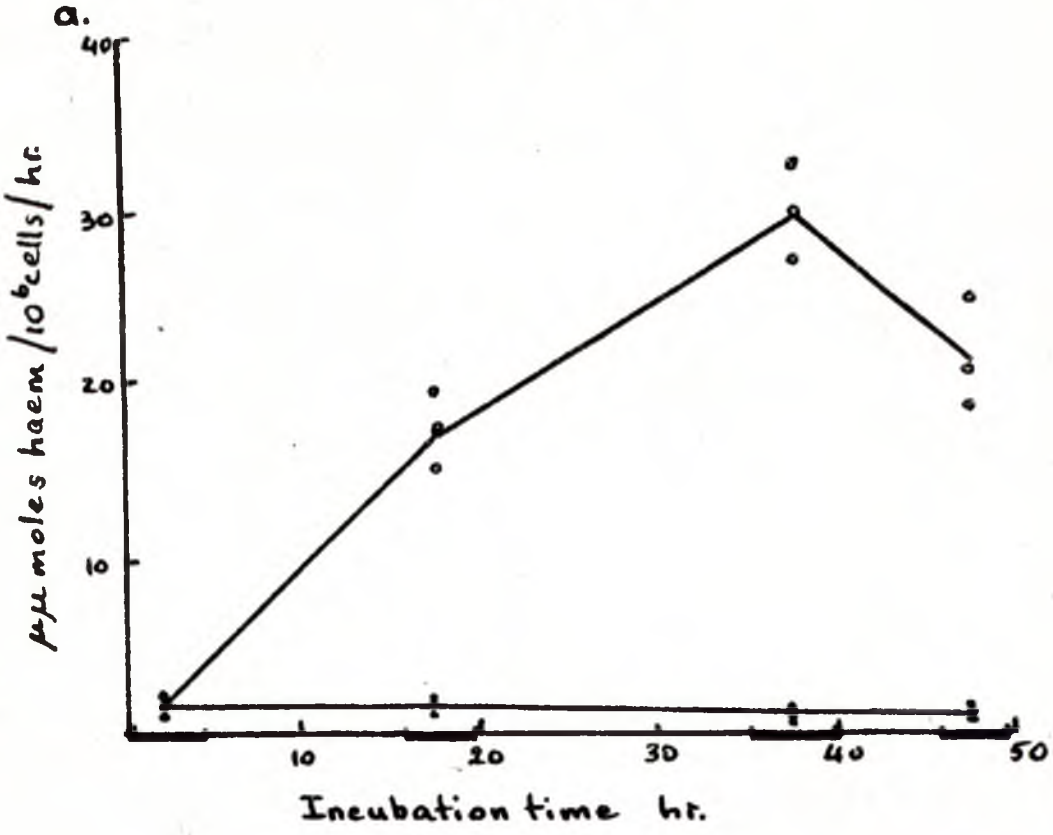
The response of 13-day rat foetal liver cells to step 1 erythropoietin (Lot 117/1), at 0.25 u./10^6 cells, after precipitation of impurities at pH 4.3.

a. The liver cells were treated with cold trypsin overnight and were exposed to erythropoietin on the day following explantation.

b. The liver cells were disaggregated by gentle pipetting in medium and were exposed to erythropoietin on the day of explantation.

Legend as Figure 8.

Figure 19


a.
40
30
20
10
μμ moles haem /10^b cells/ hr.
10
20
30
40
50
Incubation time hr.

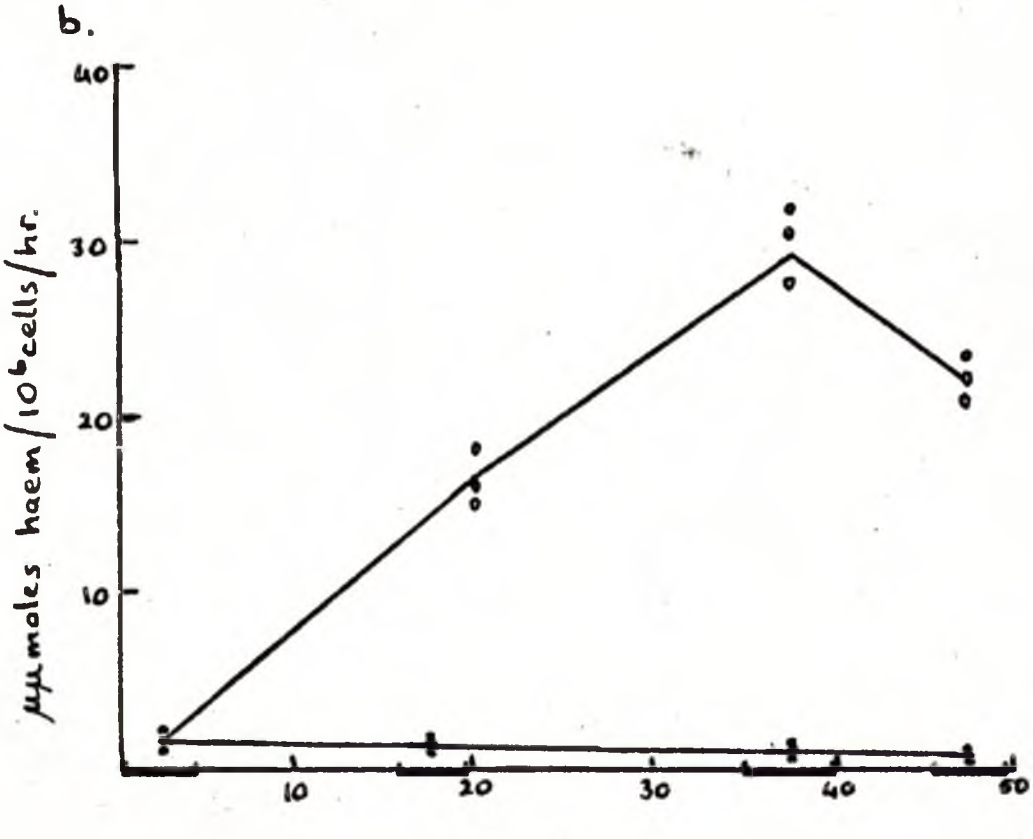

b.
40
30
20
10
μμ moles haem /10^b cells/ hr.
10
20
30
40
50
Incubation time hr.

FIGURE 20.

Summary of the erythroid activity and response to erythropoietin of rat foetal liver cells of different ages.

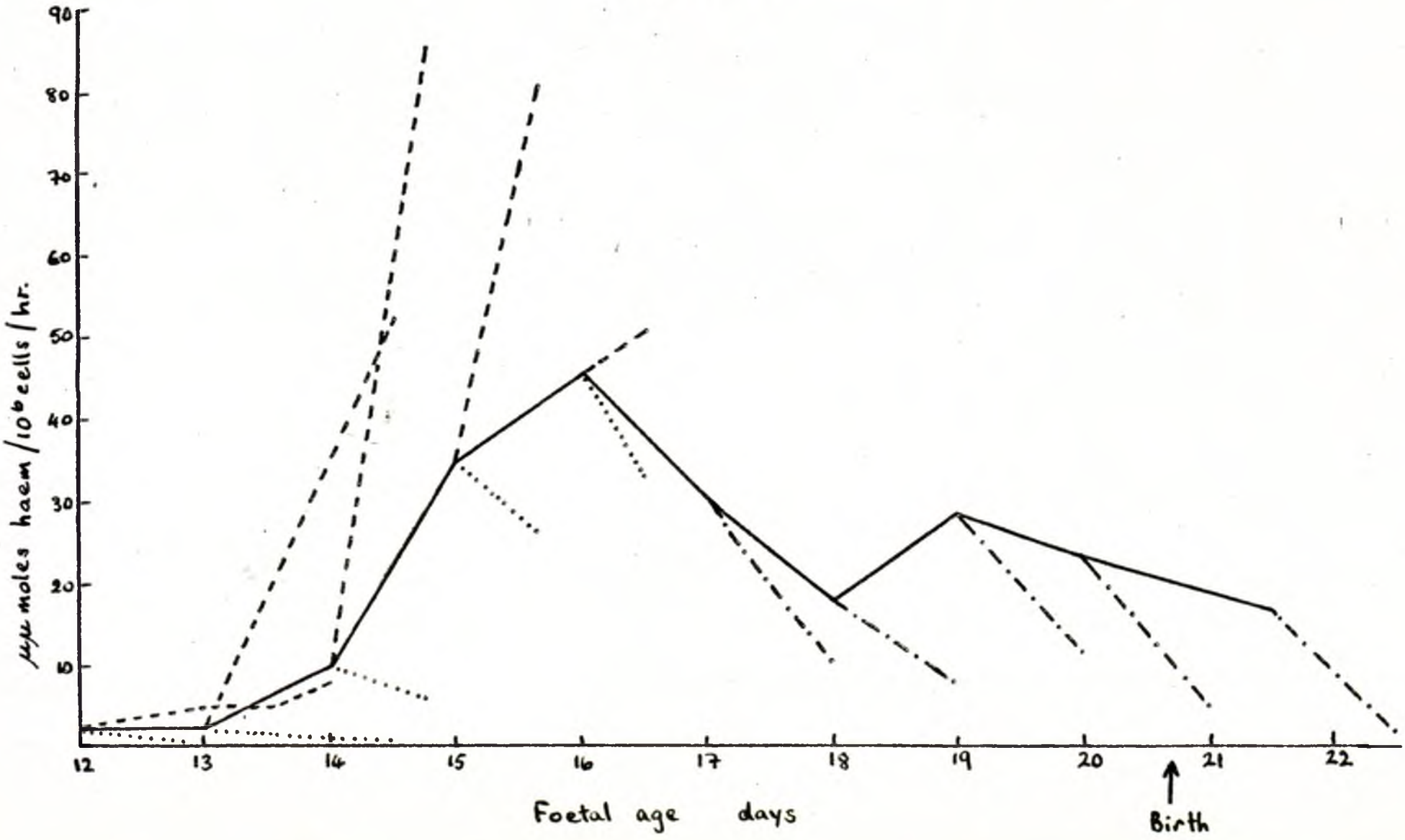
———— initial rate of haem synthesis in cultures of foetal liver cells explanted at the foetal ages shown. The period during which the cells were treated with cold trypsin is neglected and the results represented as if the cells had been placed in culture immediately following explantation.

– – – – rate of haem synthesis after treatment with erythropoietin for the times shown.

..... rate of haem synthesis in untreated cultures after incubation for the periods shown.

–.–.–. rate of haem synthesis in untreated cultures and in cultures which did not respond to erythropoietin after incubation for the periods shown.

Figure 20


90
80
70
60
50
40
30
20
10
μμ moles haem / 10⁶ cells / hr.
12
13
14
15
16
17
18
19
20
21
22
Foetal age days
Birth

incubation in medium with or without erythropoietin was begun on the following day. The cells of the second aliquot were disaggregated by gentle pipetting in medium and incubation in medium with or without erythropoietin was begun immediately. 13-day foetal livers are loose in structure and the cell suspension made in this way contained few clumps, so that the cells could be counted quite accurately. Haem synthesis in the two groups of cultures was measured at the times shown in Figure 19.

The initial rate of haem synthesis was low irrespective of trypsinisation, and remained low in control cultures. In both groups of erythropoietin-treated cultures the rate increased steadily until, after 37 hr. culture, it was 15 times the initial rate; the rate of haem synthesis decreased after this time. It can be seen that the response elicited by the Step 1, Lot 117/1, erythropoietin preparation, even after precipitation at pH 4.3, was less than that provoked by Step 2 erythropoietin from 13-day foetal liver cells (Figure 10). It was also evident that trypsinisation had no effect on the response to erythropoietin or on the cells' inherent rate of haem synthesis; it appeared that foetal liver erythroid cells survived trypsinisation undamaged.

The response to erythropoietin of foetal liver cells of different ages is summarised in Figure 20. The initial rate of haem synthesis increased until day 16 and then decreased through day 18; it increased on day 19 only to decrease once more thereafter. During incubation

in the absence of erythropoietin the rate of haem synthesis never exceeded the initial rate; instead it decreased to very low levels. The magnitude of the response to erythropoietin increased until day 14; it remained high on day 15, but was much smaller on day 16; cells explanted from foetuses more than 16 days old did not respond to erythropoietin. The maximum response was observed after progressively shorter incubation periods as the liver developed between day 12 and day 16.

The initial rate of haem synthesis reflected the in vivo activity of the erythroid cells during foetal liver development; since the results were expressed in terms of the total number of nucleated cells present after trypsinisation, the values would be influenced by the number of non-erythroid cells and of non-nucleated erythroid cells in the livers. Most of the maturing cells are released from the liver before or soon after extrusion of the nucleus (Le Douarin, 1966); in addition, only a small and relatively constant proportion of non-nucleated cells was observed after trypsinisation. The proportion increased slightly in older livers. The proportion of non-erythroid cells is reported to remain relatively constant until late in gestation, when it begins to increase; as the liver develops the erythroid cell population becomes more mature (Fish, 1959; Jones, 1960; Conkie, unpublished observations).

It is likely that the alterations in the initial rate of haem synthesis were due mainly to alterations in the activity of the erythroid cells rather than to a change in the proportion of non-

erythroid cells. Towards the end of gestation, however, an increase in the proportion of non-erythroid cells may have led to artificially low estimates of the erythroid activity. The increase in the initial rate of haem synthesis between day 12 and day 16 probably indicated an increase in maturing cells; that the rate of haem synthesis in control cultures continually decreased suggested that many of the maturing cells initially present had achieved their maximum rate of haem synthesis before incubation began. It may also be that the medium lacked a factor necessary to maintain a high rate of haem synthesis in maturing cells. This factor is unlikely to be nutritional, as the high rates of haem synthesis following erythropoietin treatment demonstrate; it may be erythropoietin itself.

The magnitude of the response to erythropoietin reflected the number of sensitive cells in the foetal liver at explantation; it appeared that most, if not all, of these survived trypsinisation. The increase in the magnitude of the response between day 12 and day 14 suggested that ESC were accumulating in the liver during this time. Since the cells were able to respond *in vitro* to exogenous erythropoietin it seemed that little endogenous erythropoietin was available to them *in vivo*. Also, the increase in the initial rate of haem synthesis concurrent with a decreased response to erythropoietin on days 15 and 16 suggested that endogenous erythropoietin may become available in significant amounts after 13-14 days of gestation. After day 16, it appeared that there was sufficient endogenous erythropoietin to stimulate ESC as they were formed in the liver.

The erythroid activity was measured in terms of haem synthesis in these experiments; in explants of chick embryos it has been shown that the supply of haem precursors is limiting in the early stages of haemoglobin synthesis in erythroid foci (Wainwright and Wainwright, 1967). It was possible that the increase in *in vivo* erythroid activity between day 12 and day 16 was due to an increased supply of haem precursors rather than to the production of endogenous erythropoietin. Since the medium used in the experiments was able to support a high rate of haem synthesis for considerable periods, the supply of haemoglobin precursors was evidently not limiting *in vitro*; if such limitations were present *in vivo* their effect should not persist in culture. It thus seemed unlikely that the increase in the initial rate of haem synthesis as the liver developed was due to a more plentiful supply of haem precursors *in vivo*; an increased amount of endogenous erythropoietin appeared a more likely explanation.

If all of the ESC detected in 15-day foetal liver cell cultures were stimulated by erythropoietin *in vivo* it might be anticipated that the rate of haem synthesis in 16-day liver cell cultures would equal that observed after erythropoietin treatment of 15-day cells. This was not observed, nor did erythropoietin, although highly purified, provoke a comparably high rate of synthesis in 16-day liver cell cultures. It is possible that livers of an intermediate age might have shown a higher rate of synthesis. The decrease in the erythroid potential of the livers between day 15 and day 16 was, however, quite large; this suggested that many erythroid cells had been lost from the

Table 15

The number of nucleated cells found after trypsinisation of spleens from rat foetuses of different ages.

Foetal age days	Number of nucleated cells/spleen x 10^6
16	0.75
17	1.8
18	3.8
19	5.9
20	9.4
neonatal	15.3

FIGURE 21

The response of 16-day rat foetal spleen cells to step 1 erythropoietin (Lot 117/1), at 0.25 u./10^6 cells, after precipitation of impurities at pH 4.3.

•——• rate of haem synthesis in untreated cultures

o——o rate of haem synthesis in erythropoietin-treated cultures

The thickened portions of the abscissa indicate the periods of incubation during which the cultures were pulsed with ^{59}Fe; the results are plotted at the mid-points of the pulses with ^{59}Fe.

FIGURE 22

The response of 17-day rat foetal spleen cells to step 1 erythropoietin (Lot 117/1), at 0.25 u./10^6 cells, after precipitation of impurities at pH 4.3.

Legend as Figure 21.

Figure 21

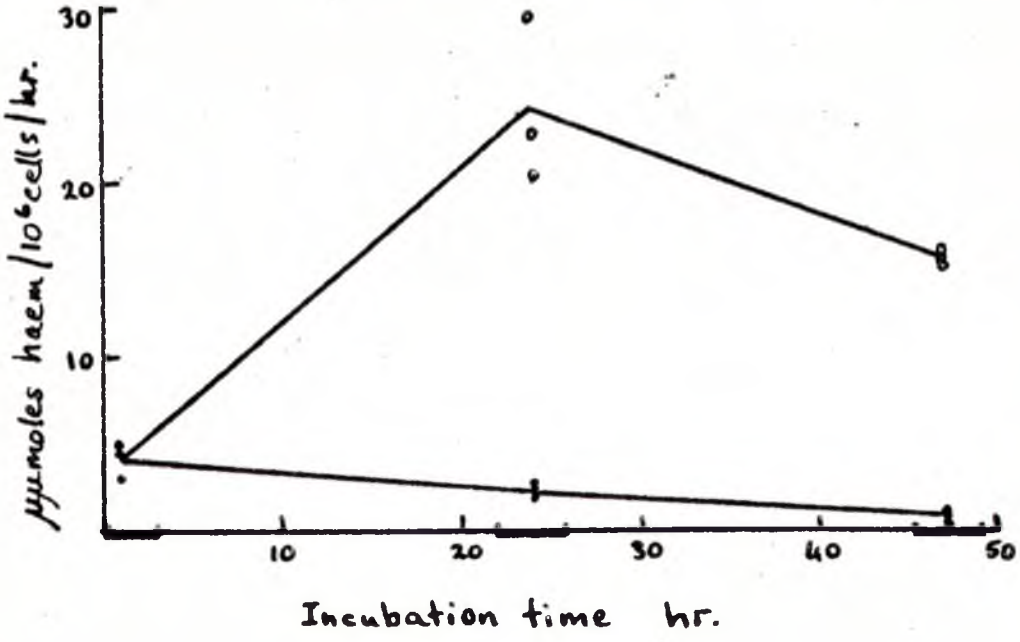
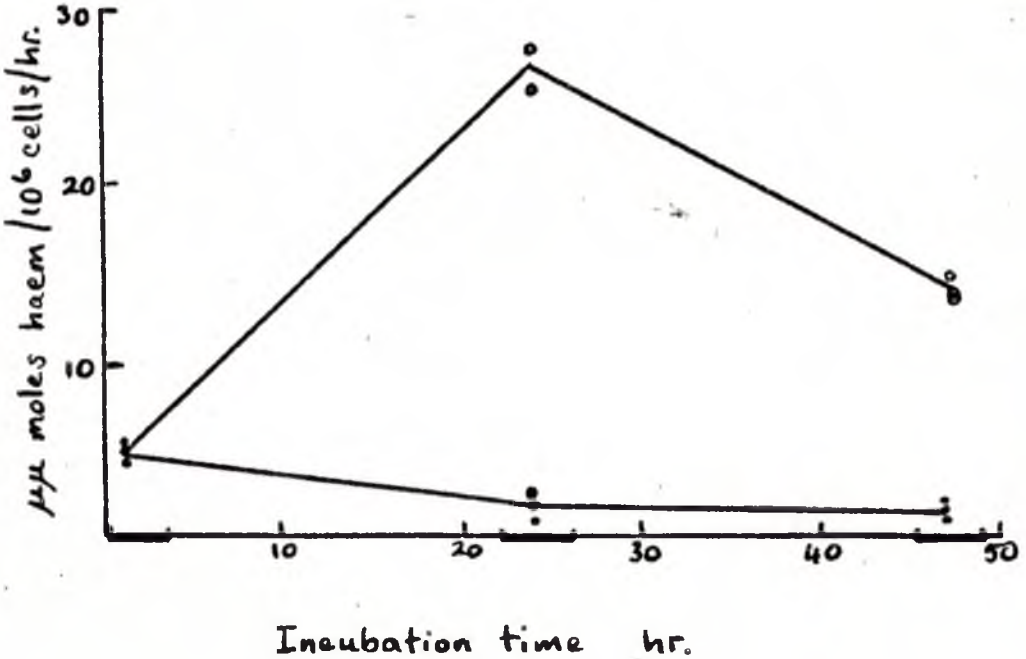


Figure 22



liver. It may be that some ESC were released to 'seed' the erythroid sites developing in the spleen. It is also possible that maturing cells were released from 15-16 day foetal livers at an earlier stage of maturation or in greater numbers than they were released from younger livers.

Spleen cells. The spleen was first visible on day 15; it had doubled in size by day 16, but was still almost completely white. Patches of red were seen on day 17; these coalesced during day 18 and the colour deepened until day 20. The size of the spleen increased continually until after birth (Table 15).

The effect of erythropoietin on the cells pooled from the spleens of three 16-day litters is shown in Figure 21. The initial rate of haem synthesis was low and in control cultures it decreased further. The rate of haem synthesis in erythropoietin-treated cultures increased to 6 times the initial rate after 24 hr. incubation; it decreased thereafter but was still, after 47 hr., 4 times the initial rate.

A similar pattern was observed in 17-day cells, pooled from the spleens of 2 litters (Figure 22); the initial rate of haem synthesis was higher than in 16-day spleen cell cultures and again it decreased during incubation. After 24 hr. treatment with erythropoietin the rate was 5.4 times the initial rate; it decreased later until, after 47 hr., it was 2.8 times the initial rate.

FIGURE 23.

The response of 18-day rat foetal spleen cells to step 1 erythropoietin (Lot 1/4), at 0.2 u./10^6 cells.

Legend as Figure 21.

FIGURE 24.

The response of 19-day rat foetal spleen cells to step 1 erythropoietin (Lot 1/4), at 0.2 u./10^6 cells.

Legend as Figure 21.

Figure 23

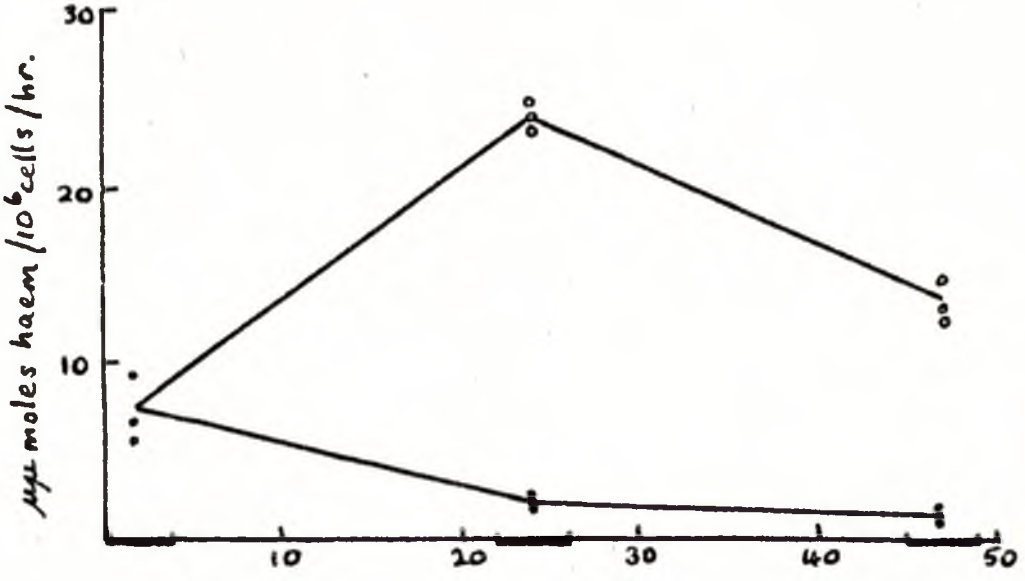


Figure 24

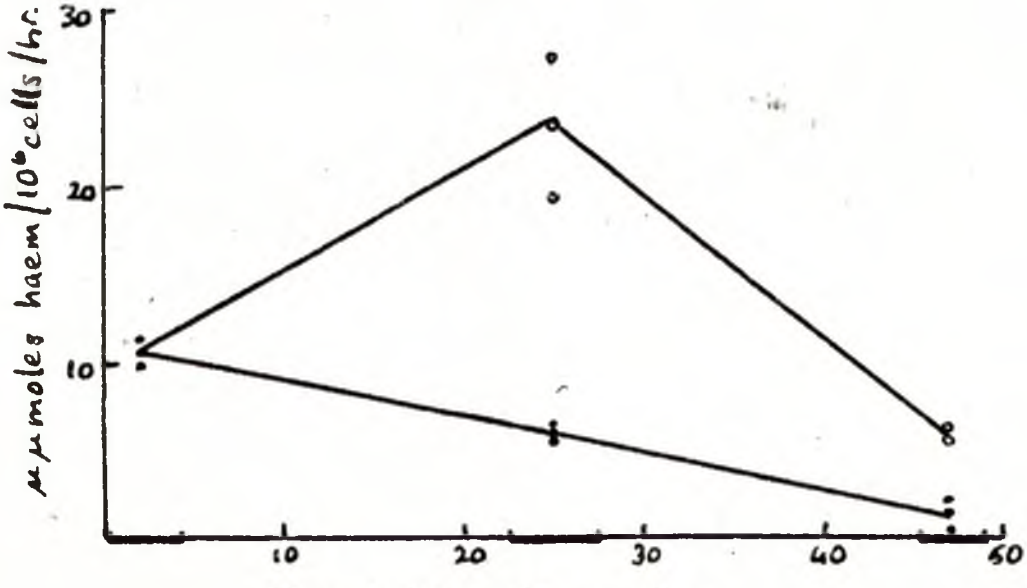


FIGURE 25.

The response of 20-day rat foetal spleen cells to step 2 erythropoietin, at 0.5 u./10^6 cells.

Legend as Figure 21.

FIGURE 26.

The response of neonatal rat spleen cells to step 1 erythropoietin, (Lot 117/1), at 0.25 u./10^6 cells, after precipitation of impurities at pH 4.3.

Legend as Figure 21.

Figure 25

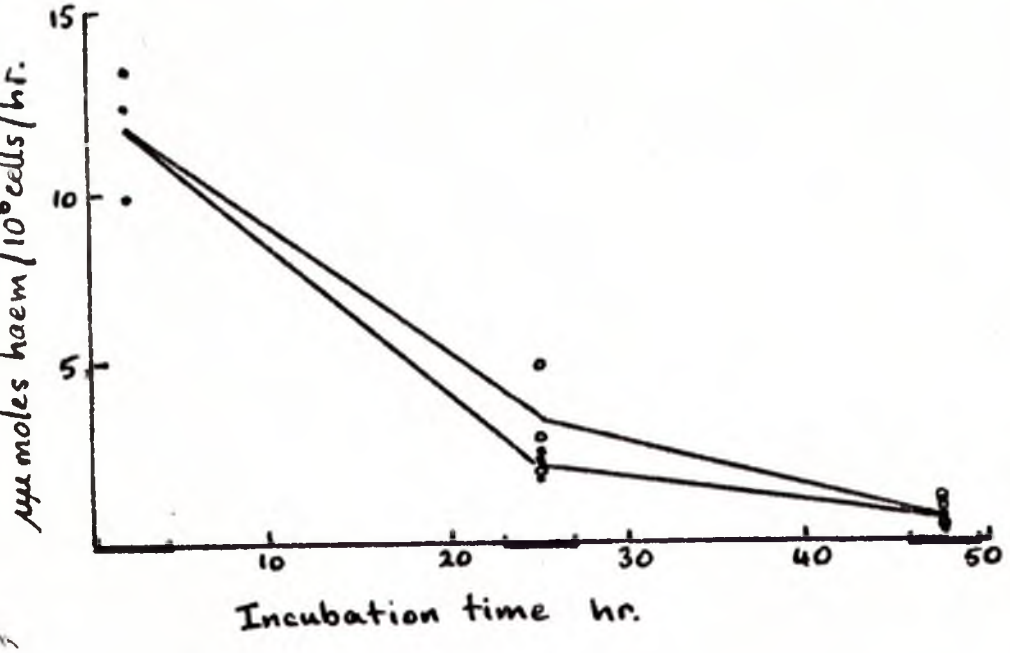


Figure 26

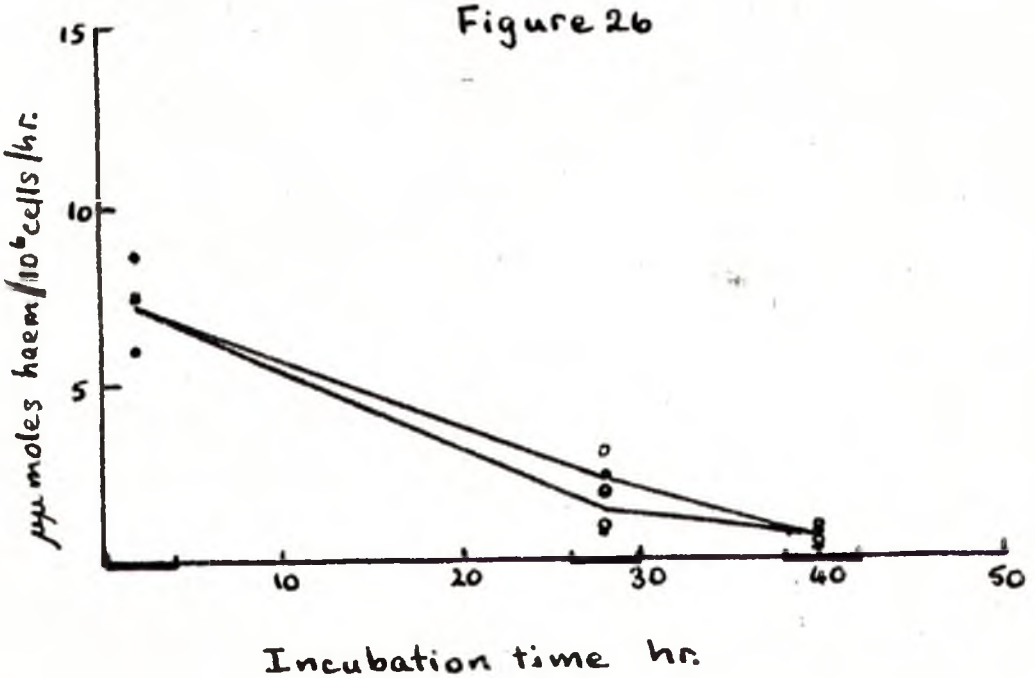


FIGURE 27.

Summary of the erythroid activity and response to erythropoietin of rat foetal spleen cells of different ages.

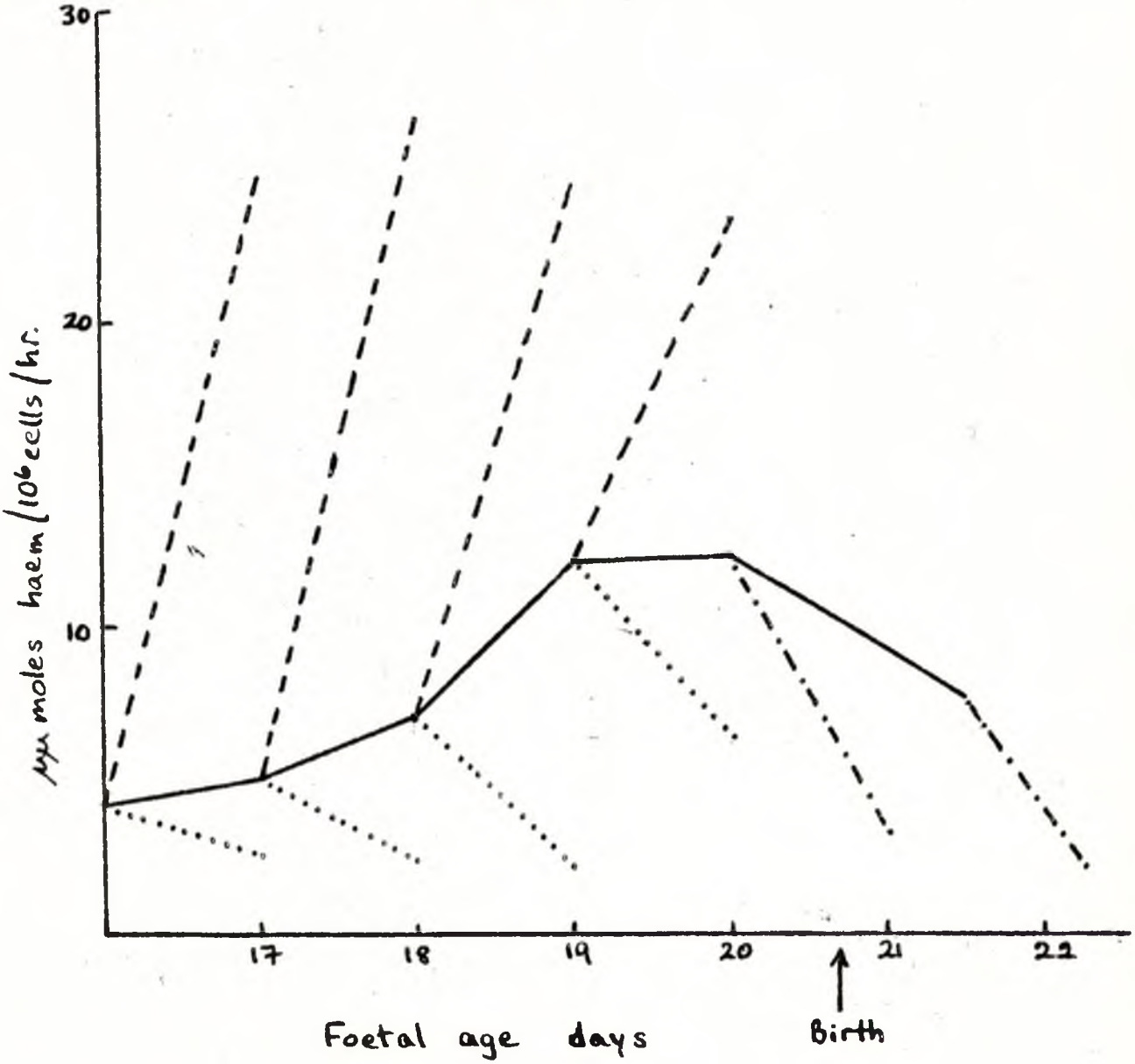
———— initial rate of haem synthesis in cultures of foetal spleen cells explanted at the foetal ages shown. The period during which the cells were treated with cold trypsin is neglected and the results represented as if the cells had been placed in culture immediately following explantation.

– – – – rate of haem synthesis after treatment with erythropoietin for the times shown.

..... rate of haem synthesis in untreated cultures after incubation for the periods shown.

–.–.–. rate of haem synthesis in untreated cultures and in cultures which did not respond to erythropoietin, after incubation for the periods shown.

Figure 27


30
20
10
μμ moles haem / 10⁶ cells / hr.
17
18
19
20
21
22
Foetal age days
Birth

From day 18 the spleens of single litters provided enough cells to study the effect of erythropoietin. The initial rate of haem synthesis in 18-day foetal spleen cell cultures was higher (Figure 23) and still higher in 19-day spleen cell cultures (Figure 24); in both experiments the rate decreased in control cultures during incubation. After 24 hr. treatment with erythropoietin the rate of haem synthesis in 18-day spleen cell cultures was 3.5 times the initial rate; in 19-day spleen cell cultures it was 2.3 times the initial rate. In each case the rate decreased between 24 and 47 hr..

In cultures of 20-day foetal spleen cells the initial rate of haem synthesis was lower (Figure 25); it was lower still in cultures of neonatal spleen cells (Figure 26). Erythropoietin had no effect in either case; the rate of haem synthesis decreased in both control and erythropoietin-treated cultures at the same rate.

These results are summarised in Figure 27. As the spleen developed, the initial rate of haem synthesis increased, until day 19; it decreased thereafter. The maximum rate of haem synthesis after erythropoietin treatment was similar in all cultures in which a response was detected, suggesting that the total erythroid potential of the spleen remained relatively constant between day 16 and day 19. The ratio of ESC to maturing cells appeared to decrease during this period and no ESC were detectable in older spleens. Fewer samples were taken during incubation in this series of experiments; it may be that samples at different times would have demonstrated a variation in the maximum rate

of haem synthesis after erythropoietin treatment as the spleen developed. It may also be that the true maximum was not reached after 24 hr. in all cultures.

As in liver, it appeared that not all ESC detected on day 19 had been stimulated in vivo by day 20; again, the decrease in the erythroid activity may indicate that some ESC were released from the spleen, perhaps to 'seed' marrow erythropoiesis. It is also possible that the increasing production of other haematopoietic cells by the spleen at this stage (Le Douarin, 1966) may lead to an artificially low estimate of the erythroid activity.

2. The effect of erythropoietin on rabbit foetal liver cells

The effect of erythropoietin on rat foetal liver cells during development was broadly similar to the pattern observed in mouse foetal livers (Cole and Paul, 1966). However, the rat and the mouse are closely related and it cannot be inferred that a similar pattern would occur in other species. The studies were therefore extended to the rabbit which belongs to a separate order and whose gestation period is some 10-12 dy. longer than that of the rat or the mouse. The age of the foetuses was counted in days from the time of mating.

The foetal rabbit liver was first visible on day 11; during the next two days pink patches appeared, which coalesced by day 14 and the colour deepened until day 18. The lobed structure began

Table 16

The number of nucleated cells found after trypsinisation of livers from rabbit foetuses of different ages.

Foetal age days	Number of nucleated cells/liver $\times 10^6$
13	1.2
14	3.5
15	8.7
16	18.8
17	38.5
18	73.4
21	332.0

FIGURE 23.

The response of 13-day rabbit foetal liver cells to step 2 erythropoietin at 0.5 u./10^6 cells.

•——• rate of haem synthesis in untreated cultures

•——o rate of haem synthesis in erythropoietin-treated cultures.

The thickened portions of the abscissa indicate the periods of incubation during which the cultures were pulsed with ^{59}Fe; the results are plotted at the mid-points of the pulses with ^{59}Fe.

Figure 28

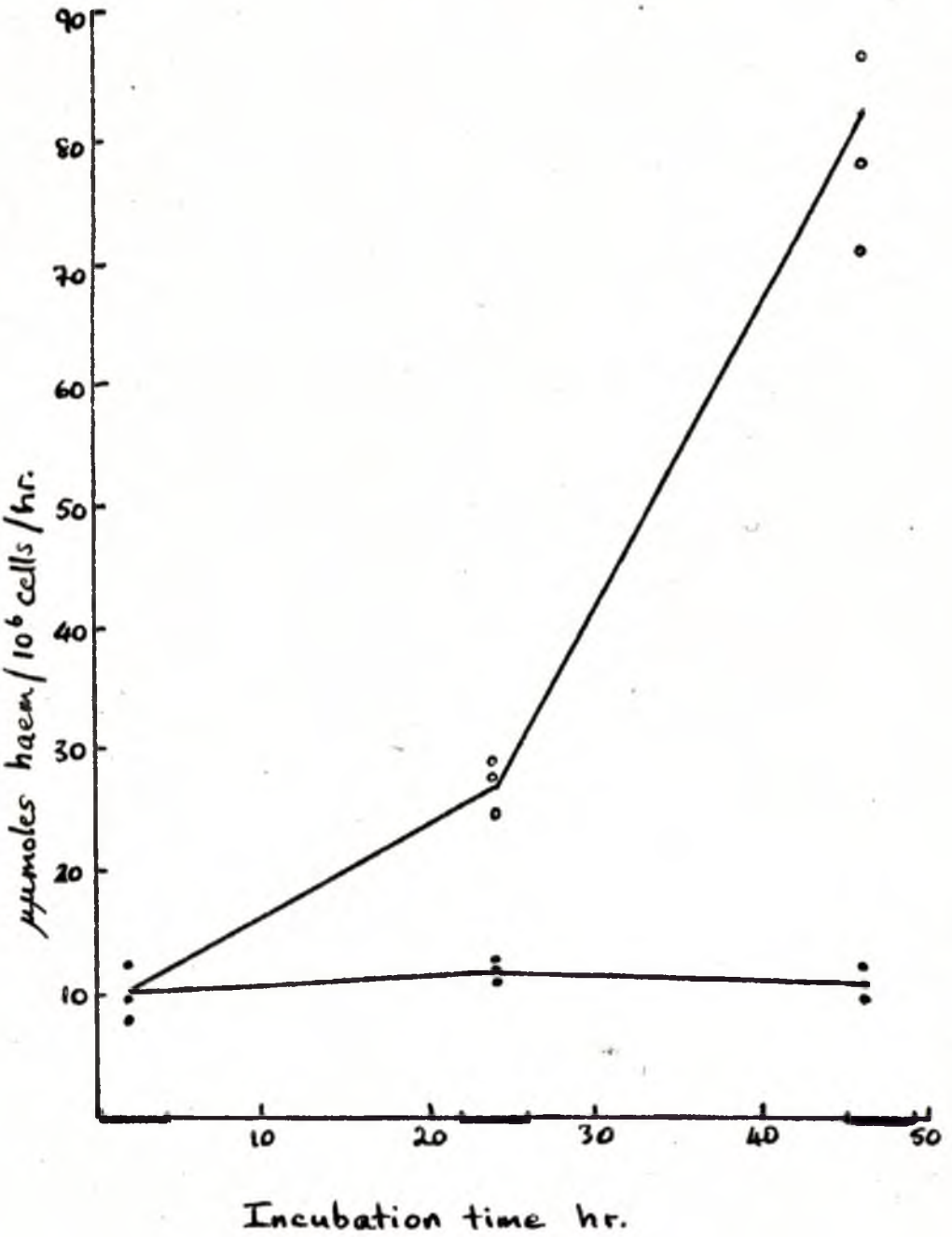
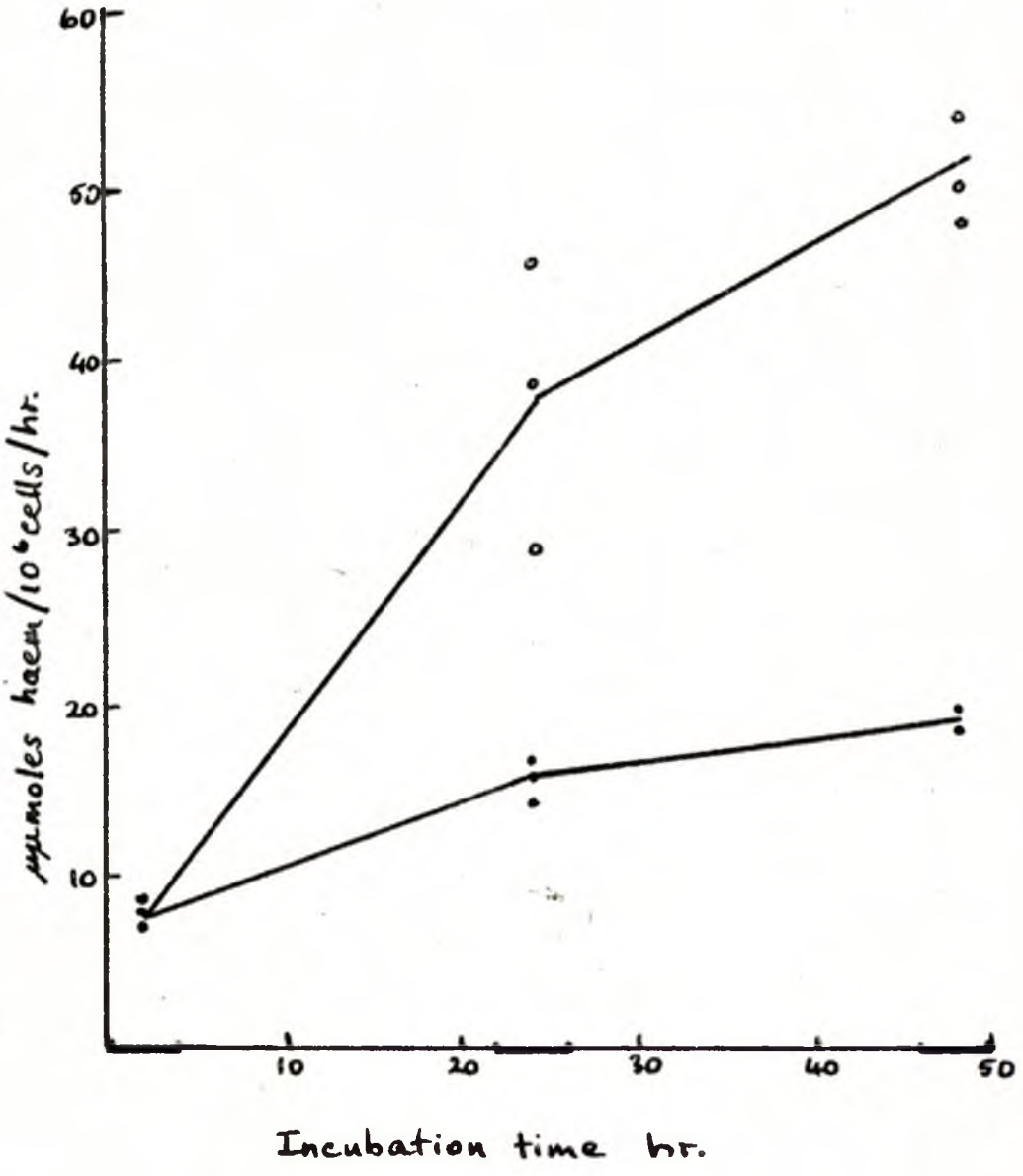

90
80
70
60
50
40
30
20
10
μμmoles haem / 10⁶ cells / hr.
10
20
30
40
50
Incubation time hr.

FIGURE 29.

The response of 14-day rabbit foetal liver cells to step 1 erythropoietin (Lot 117/1), at 0.5 u./10^6 cells, after precipitation of impurities at pH 4.3.

Legend as Figure 28.

Figure 29


60
50
40
30
20
10
μμmoles haem/10⁶cells/hr.
10
20
30
40
50
Incubation time hr.

to form on day 12 and was clearly defined by day 14. The liver increased in size throughout the period studied; the number of nucleated cells recovered after trypsinisation reflected the growth of the liver (Table 16). The time-course of the morphological development of the liver was similar to that observed in rat foetuses; during this period, day 12 - day 17, the livers of both species yielded similar numbers of cells. After day 17 the rabbit liver increased in size more rapidly than the rat liver.

All of this series of experiments were performed on liver cells from a single litter. The effect of erythropoietin on 13-day foetal liver cells is shown in Figure 28. The initial rate of haem synthesis was low; it increased very slightly in control cultures during 24 hr. incubation and then decreased. Treatment with erythropoietin provoked a much greater increase in rate, which accelerated between 24 and 46 hr. incubation; after 46 hr. the rate of haem synthesis was 7 times the initial rate.

In 14-day foetal liver cell cultures the initial rate of haem synthesis was still low; in control cultures it increased throughout incubation until, after 48 hr., it was 2.4 times the initial rate (Figure 29). After treatment with erythropoietin, the rate of haem synthesis increased much more; in these cultures, the rate of increase slowed between 24 and 48 hr. incubation. At its maximum, the rate of haem synthesis in erythropoietin-treated cultures was 6.8 times the initial rate, but only 2.7 times the control rate at the same

FIGURE 30.

The response of 15-day rabbit foetal liver cells to step 2 erythropoietin, at 0.5 u./10^6 cells.

Legend as Figure 28.

Figure 30

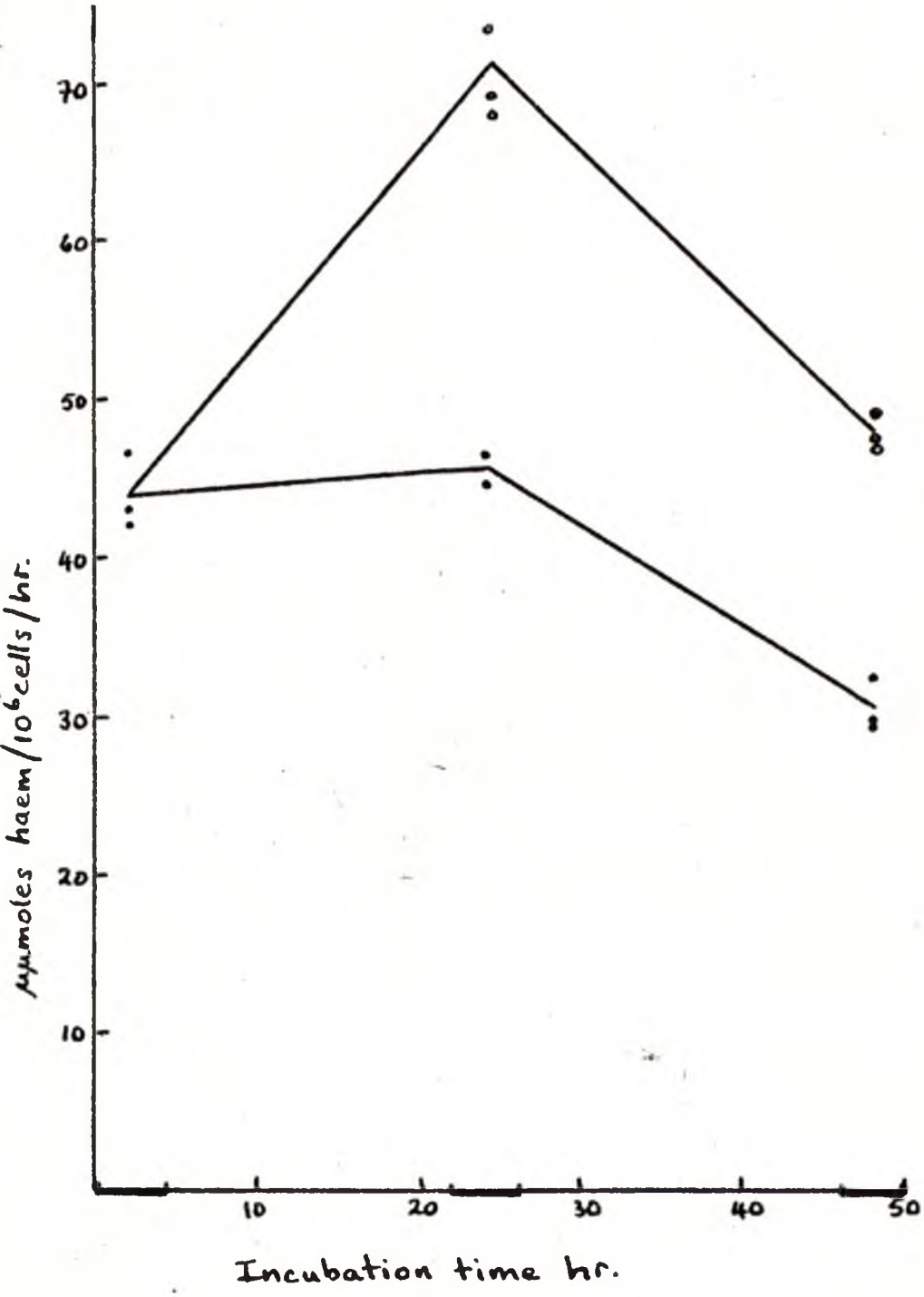

70
60
50
40
30
20
10
μmoles haem/10⁶cells/hr.
10
20
30
40
50
Incubation time hr.

FIGURE 31.

The response of 16-day rabbit foetal liver cells to step 4 erythropoietin at 0.1 u./10^6 cells.

Legend as Figure 28.

FIGURE 32.

The response of 17-day rabbit foetal liver cells to step 2 erythropoietin at 0.5 u./10^6 cells.

Legend as Figure 28.

Figure 31

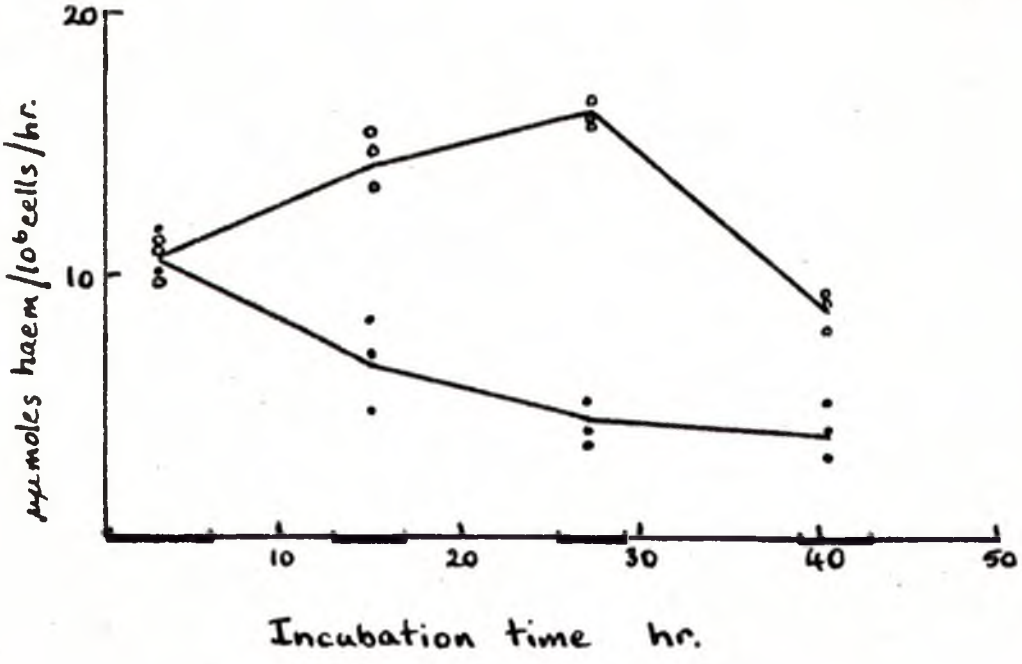


Figure 32

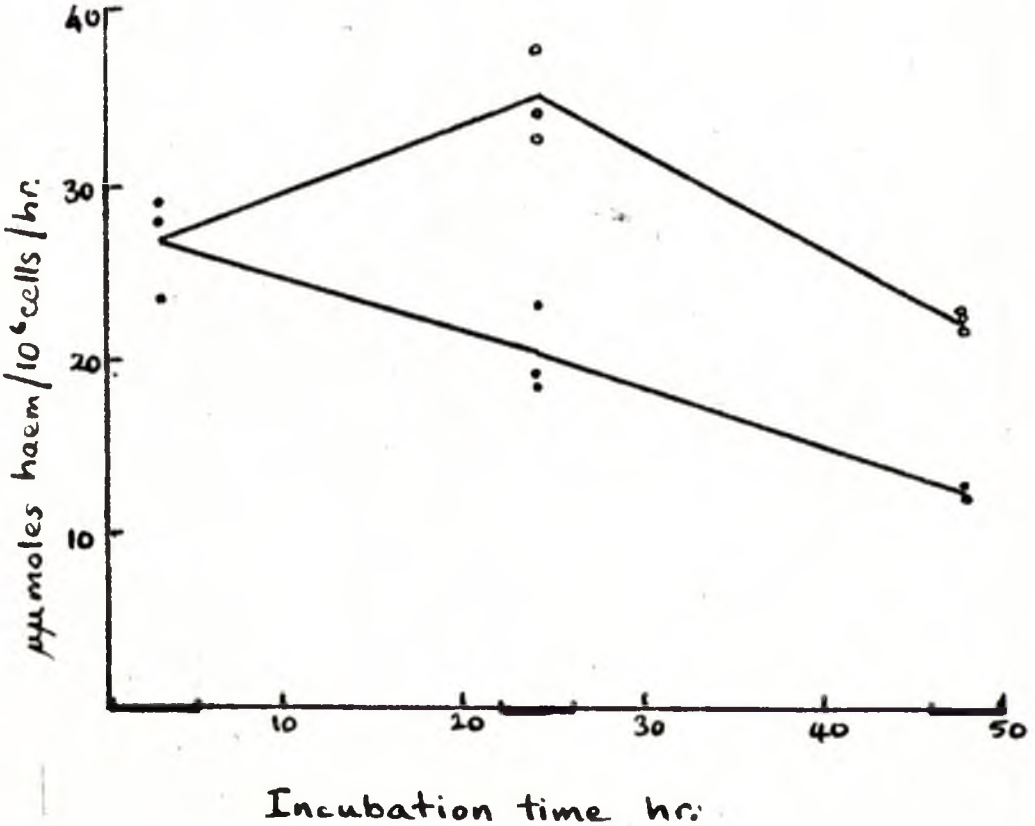


FIGURE 33.

The response of 18-day rabbit foetal liver cells to step 1 erythropoietin (Lot 117/1) at 0.5 u./10^6 cells, after precipitation of impurities at pH 4.3.

Legend as Figure 28.

FIGURE 34.

The response of 21-day rabbit foetal liver cells to step 1 erythropoietin (Lot 117/1), at 0.5 u./10^6 cells, after precipitation of impurities at pH 4.3.

Legend as Figure 28.

Figure 33

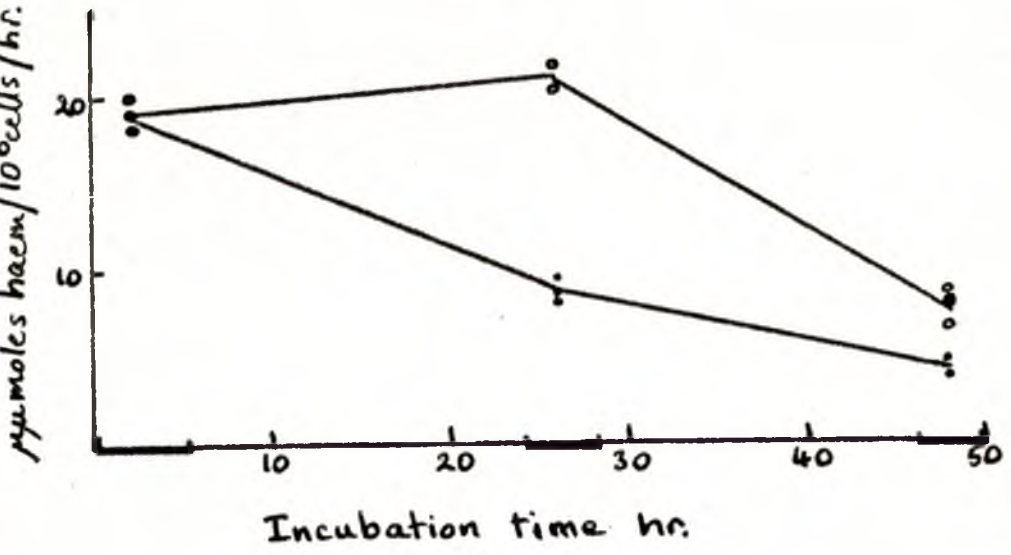
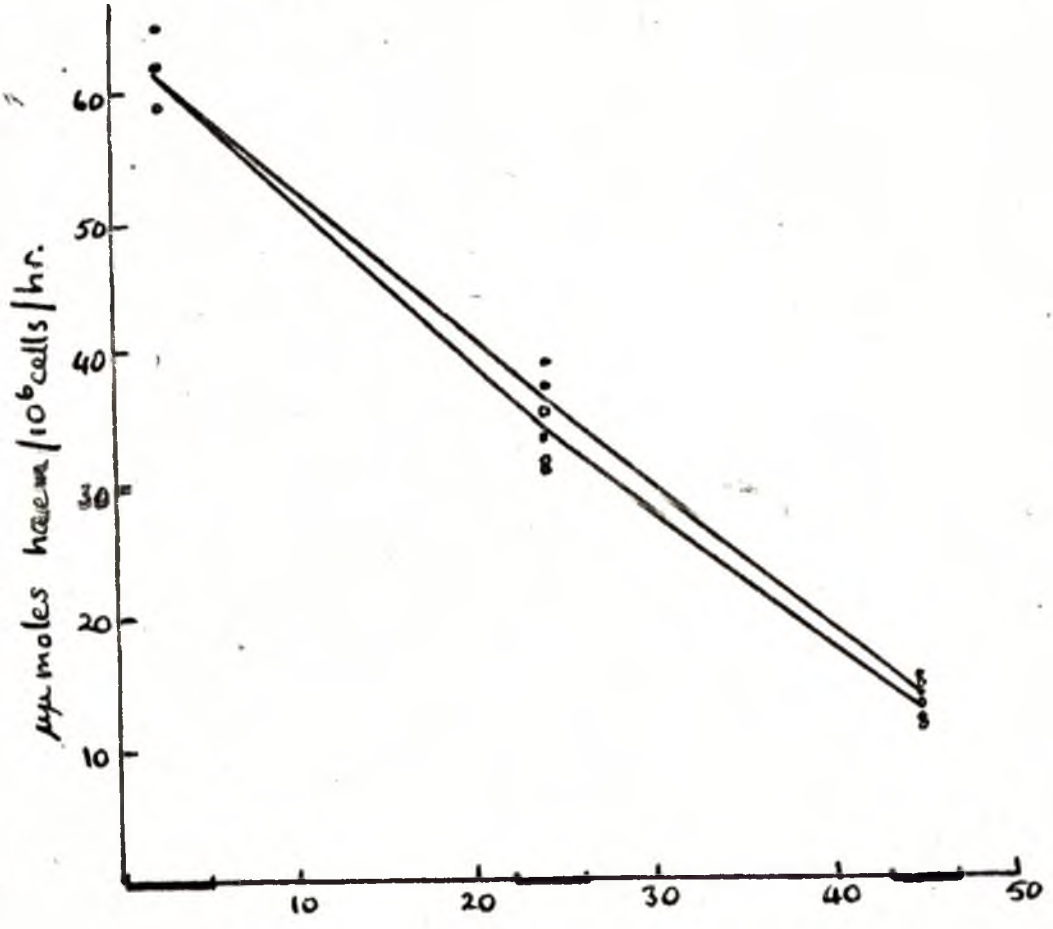


Figure 34



time.

The initial rate of haem synthesis in 15-day cell cultures was much higher; it increased slightly in control cultures and then decreased between 24 and 48 hr. incubation (Figure 30). A greater increase occurred after erythropoietin treatment, reaching a maximum at 24 hr.; between 24 and 48 hr. the rate of haem synthesis decreased once more. After 24 hr. the rate was 1.6 times the initial rate of haem synthesis.

In 16-day foetal liver cell cultures the initial rate of haem synthesis was lower (Figure 31) and decreased throughout incubation in control cultures. There was a small response to erythropoietin treatment; after 27 hr. incubation, the maximum rate observed was 1.5 times the initial rate. The initial rate of haem synthesis was higher in 17-day cell cultures (Figure 32); after 24 hr. treatment with erythropoietin the rate was 1.4 times the initial rate. In 18-day cultures the initial rate of haem synthesis was again lower (Figure 33); after 24 hr. treatment with erythropoietin the rate was 1.2 times the initial rate. In both these experiments also, the rate of haem synthesis in control cultures decreased throughout incubation. The initial rate of haem synthesis in 21-day cell cultures was almost as high as that observed in cultures of younger cells after erythropoietin treatment (Figure 34); there was no response to erythropoietin and the rate decreased in both treated and untreated cultures at the same rate during incubation.

These results are summarised in Figure 35. The initial rate

FIGURE 35.

Summary of the erythroid activity and response to erythropoietin of rabbit foetal liver cells of different ages.

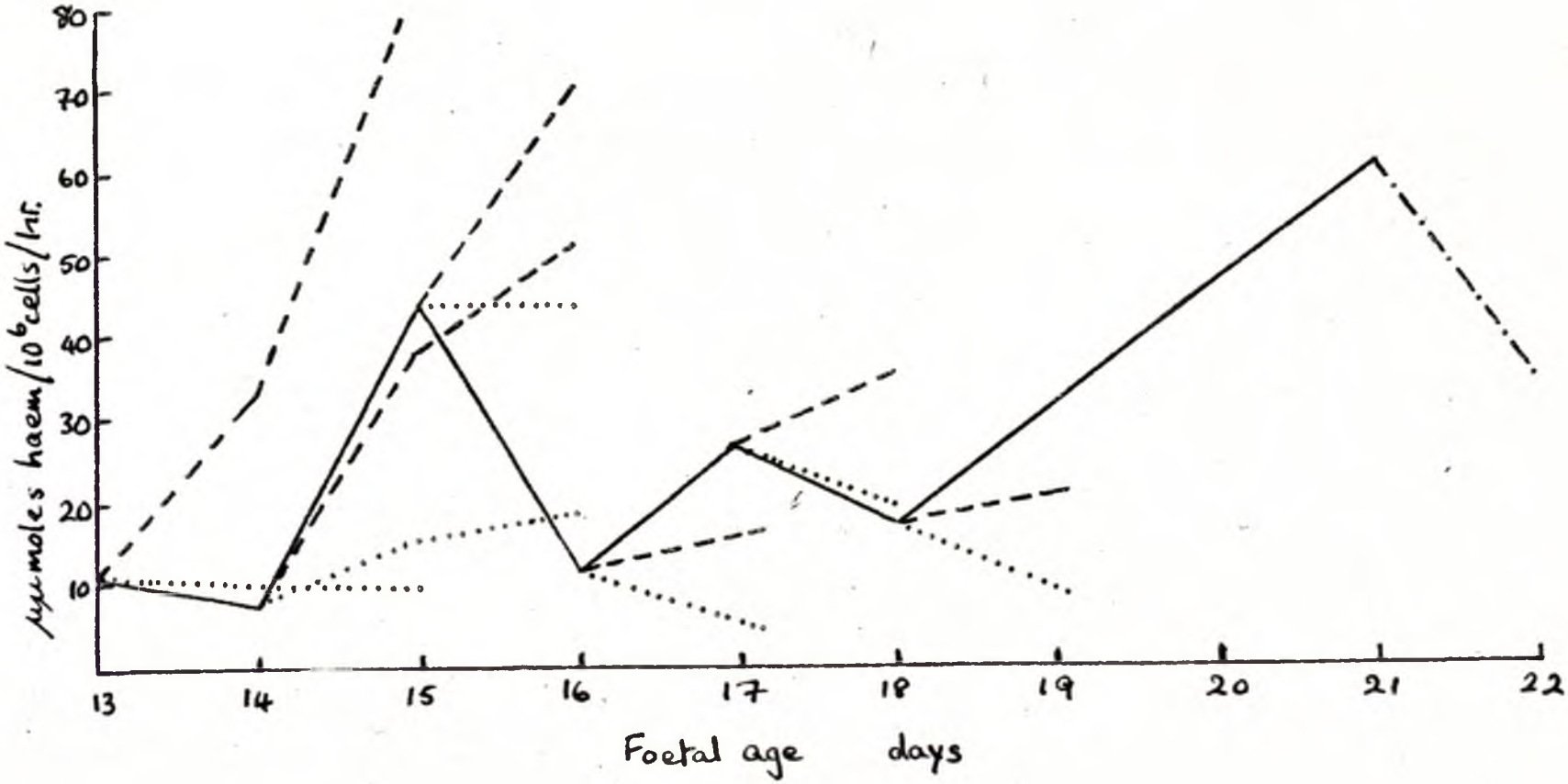
_____ initial rate of haem synthesis in cultures of foetal liver cells explanted at the ages shown. The period during which the cells were treated with cold trypsin is neglected and the results represented as if the cells had been placed in culture immediately following explantation.

\- - - - rate of haem synthesis after treatment with erythropoietin for the times shown.

..... rate of haem synthesis in untreated cultures after incubation for the periods shown.

_._._. rate of haem synthesis in untreated cultures and in cultures which did not respond to erythropoietin after incubation for the periods shown.

Figure 35


μμmoles haem/10⁶cells/hr.
80
70
60
50
40
30
20
10
13
14
15
16
17
18
19
20
21
22
Foetal age days

of haem synthesis increased until day 15 and then decreased; it increased again on day 17 only to decrease once more on day 18. On day 21 it had increased considerably; it is not known whether this high level of activity is maintained until birth. Sensitivity to erythropoietin persisted until day 18 and possibly later, although the magnitude of the response was considerably reduced after day 15. The rate of haem synthesis after erythropoietin treatment was lower in 14-day cell cultures than in either 13-day or 15-day cell cultures. This reduced response may have been characteristic of the cells themselves; it seemed more likely, however, to have been caused by the use of a less active erythropoietin preparation (Step 1, Lot 117/1).

During incubation of cultures of cells from 13-15-day foetal livers the rate of haem synthesis increased, even without erythropoietin treatment; this was most marked in 14-day cell cultures and was not observed later than day 15. This is in contrast with the pattern found in rat foetal liver cells. It is possible that younger rabbit liver cells were reversibly damaged by trypsinisation, so that the rates of haem synthesis observed initially were lower than the rates <u>in vivo</u>. It is also possible that the maturing cells in these young livers had not achieved their maximum rate of haem synthesis when they were explanted. The increased rate achieved in the absence of erythropoietin may or may not be the maximum rate which the cells would have reached <u>in vivo</u>. The increased rate of haem synthesis in these cultures was too small to be confused with the increase in

rate after erythropoietin treatment, or with the increase in the initial rate between day 14 and day 15.

The results suggest that ESC accumulated in the liver until day 14; the increased initial rate on day 15 concomitant with a reduced response to erythropoietin suggested that endogenous erythropoietin may appear or increase in amount at this stage of gestation. ESC persisted in small numbers until at least day 18, suggesting that the amount of endogenous erythropoietin available was limiting; it is also possible that these cells were protected in some manner from any endogenous hormone available.

The potential erythroid activity of 16-day livers was much less than that of 15-day livers; as in the rat, this may suggest that ESC, and maturing cells also, had been released from the liver in considerable amounts. The erythroid potential remained quite low until at least day 18 but had increased again by day 21. It may be that cells were being released throughout this period; it is also possible that the number of cells released from the liver between days 15 and 16 was so large that their replacement required several days. Spleen and marrow erythropoiesis were not studied; it is thus not known whether ESC were present in these sites following the decrease of detectable ESC in the liver.

PLATE 1

Starch gel electrophoresis of haemoglobins, stained with naphthalene black.

a. Mouse haemoglobins, separated in buffer A containing 1.0% v/v mercaptoethanol.

   i. adult (12 weeks old).

   ii. 16-day foetal.

b. Rat haemoglobins, separated in buffer C.

   i. adult (2½ months old).

   ii. 19-day foetal.

c. Rabbit haemoglobins, separated in buffer C.

   i. 18-day foetal.

   ii. 17-day foetal.

   iii. adult (9 months old).

Plate 1

a.

origin

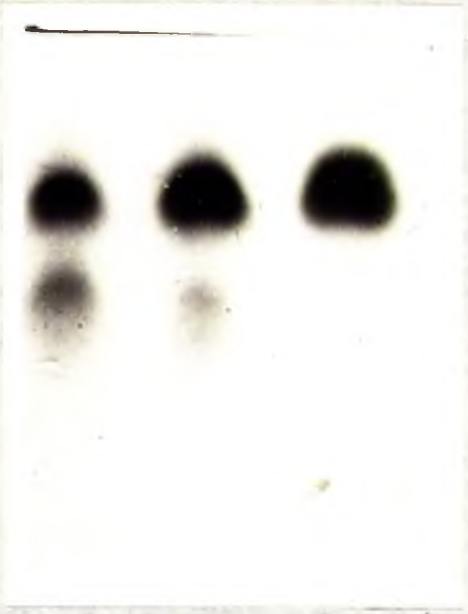
i. ii.

b.

origin

i. ii.

c.



origin

i. ii. iii.

3. Haemoglobins present in foetal blood during gestation

In this section the haemoglobins are separated only electrophoretically; it is not proven that each component separated was homogeneous. In one instance an apparently homogeneous band was separated into two components after electrophoresis in a different buffer system. Although the components will be discussed as being homogeneous, it may be that further investigation would reveal that they are not. Specimens of the separation achieved of mouse, rat, and rabbit haemoglobins are shown in Plate 1.

It is known that the foetuses of many species contain haemoglobins distinct from those of the adult and that the pattern of haemoglobin synthesis alters in a characteristic manner during gestation (Gratzer and Allison, 1960; Craig and Russell, 1963, 1964; Huehns et al., 1964a,b; Ingram, 1963). The factors controlling the changes in the type of haemoglobin synthesised are unknown. In this section the pattern of foetal haemoglobins during gestation was examined in rat, mouse and rabbit; the effect of erythropoietin on the pattern in mouse and rat foetuses was determined.

4. Mouse haemoglobins and the effect of erythropoietin on their synthesis.

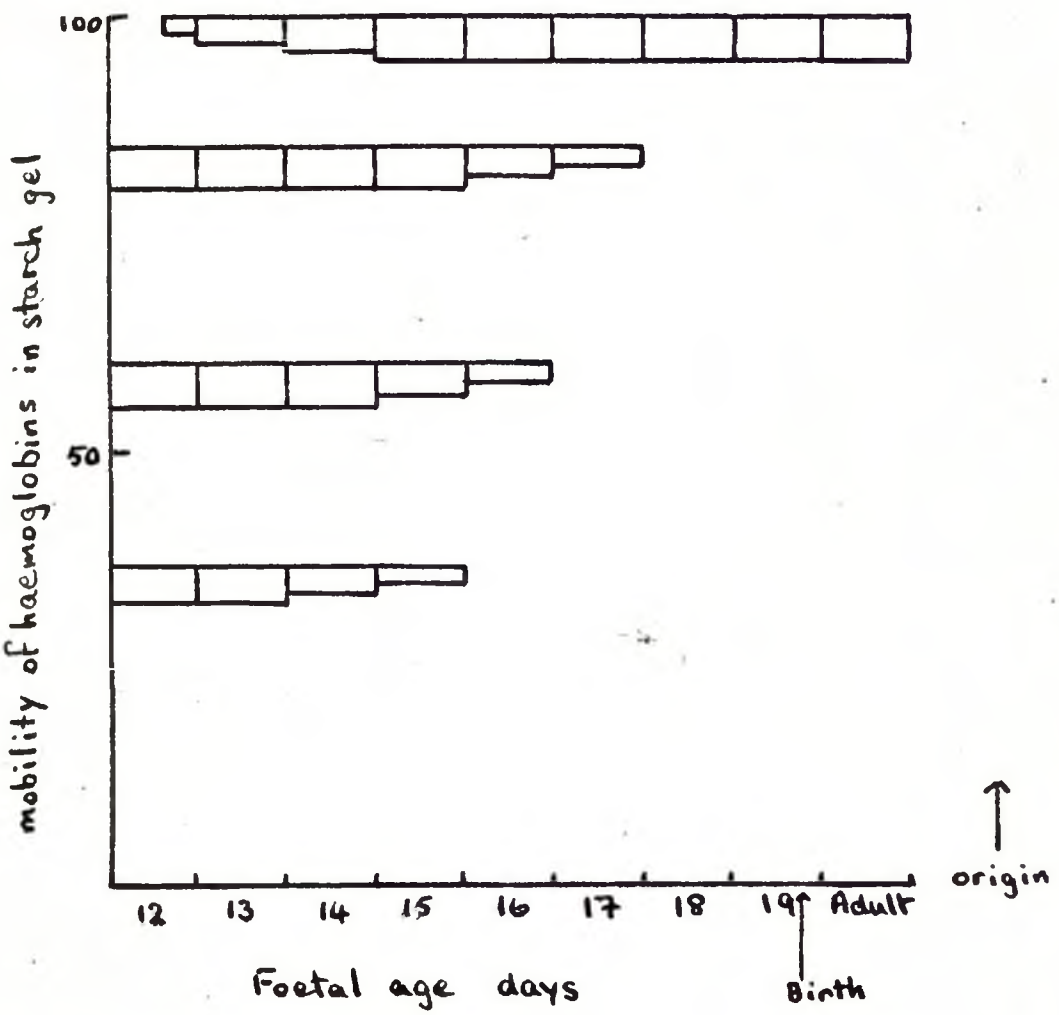
Adult mice of the Porton strain studied possessed a single haemoglobin component which was electrophoretically identical to that of adult C57/BL mice. This component had a mobility greater than that

FIGURE 36.

Haemoglobins in foetal mouse blood during gestation, separated by starch gel electrophoresis in buffer A containing 1% v/v mercapotoethanol.

position on the gel of the visible bands in haemoglobin samples from foetuses of the ages shown; the mobility of each haemoglobin is expressed as a percentage of the mobility of the leading haemoglobin, adult haemoglobin.

Figure 36


100
50
mobility of haemoglobins in starch gel
origin
12
13
14
15
16
17
18
19
Adult
Foetal age days
Birth

of any of the foetal varieties found and was used as a standard. Electrophoresis of mouse haemoglobins was always carried out in buffer A containing 1.0% mercaptoethanol.

12-day foetal blood samples contained three haemoglobin components, having mobilities 85%, 60% and 36% respectively of that of the adult haemoglobin. In some 12½-day samples small amounts of adult haemoglobin were found after staining. At 13 days a low concentration of adult haemoglobin was consistently found; the amounts of the three slower components had not altered. As the foetus developed these three components disappeared from the blood at varying times. The slowest of the three was markedly reduced in amount on day 14, was barely detectable on day 15 and had completely disappeared by day 16. The amount of the second slowest component was slightly reduced on day 15; it was very faint on day 16 and was never found on day 17. The fastest of the three remained constant in quantity until day 16 when a slight decrease was seen; on day 17 its concentration was even lower and by day 18 it had completely disappeared. Throughout this time the amount of adult haemoglobin present was rapidly increasing. These results are represented in Figure 36.

This pattern was very similar to that found in other mouse strains (Craig and Russell, 1963, 1964) and it seemed likely that the three foetal components were produced by yolk sac cells, while adult haemoglobin was produced by foetal liver cells (Kovach et al., 1967).

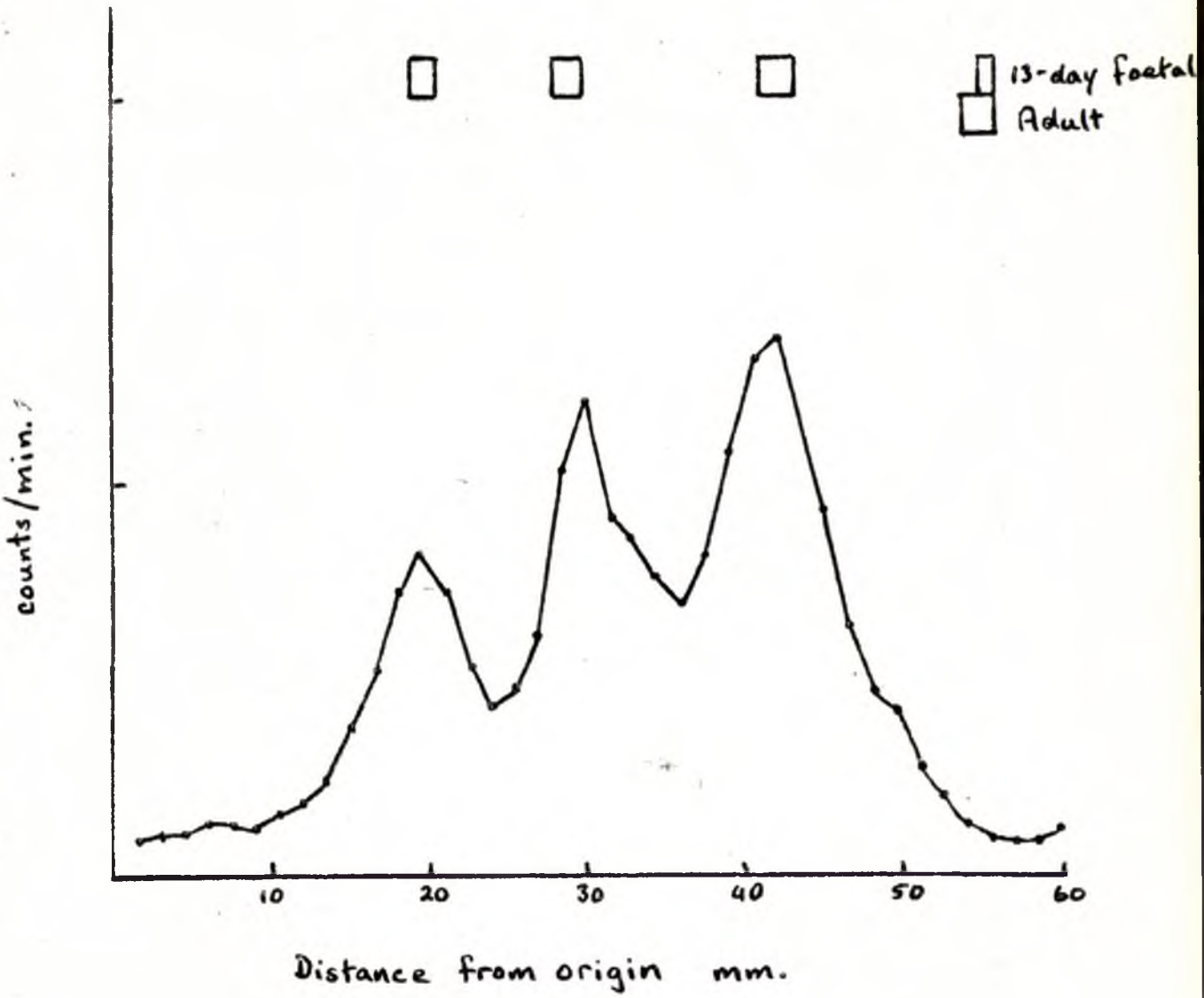
FIGURE 37.

The pattern of haemoglobin synthesis in 9-day mouse yolk sac cells, after treatment with step 2 erythropoietin at 0.5 u./10^6 cells. The cells were pulsed with ^{59}Fe between 0 and 21 hours incubation. The haemoglobin components were separated by starch gel electrophoresis in buffer A containing 1.0% v/v mercaptoethanol; 13-day foetal mouse haemoglobin was added as marker.

counts/min. in 1.5 mm. slices of gel after electrophoresis of haemoglobin from cultures incubated with or without erythropoietin.

position on the gel of the visible bands separated in the haemoglobin samples shown.

Figure 37


13-day foetal
Adult
counts/min.
10
20
30
40
50
60
Distance from origin mm.

Yolk sac cells. It is not known whether liver erythroid foci are 'seeded' by cells from the yolk sac, or whether they are formed by differentiation of cells *in situ*. One of the ways in which yolk sac and liver erythroid cells differ is in the kind of haemoglobin they contain; if yolk sac cells could be stimulated to synthesise adult haemoglobin, this would suggest that they might be able to 'seed' erythroid foci in the liver. Erythropoietin markedly stimulated haem synthesis in foetal liver cells (Cole and Paul, 1966); although it had no quantitative effect on yolk sac cells, it was possible that erythropoietin might induce these cells to synthesise adult haemoglobin at the expense of the other varieties.

After removing the ectoplacental cone, 22 9-day embryos were trypsinised; the embryos, pooled from 3 litters, contained 2-18 somites. The cells were incubated in medium with and without erythropoietin; 0.01 mM-$FeCl_3$ was added to the medium (Cole and Paul, 1966). The cultures were incubated with ^{59}Fe for 21 hr.. The distribution of the activity after electrophoresis of the cell extracts is shown in Figure 37; 13-day foetal haemoglobin was added as marker and adult haemoglobin was run on the same gel. It was found that all of the activity was associated with the foetal haemoglobins; treatment with erythropoietin did not alter the pattern in any way. In order of decreasing mobility, the porportions of each component synthesised, irrespective of erythropoietin treatment, were 45%, 33% and 22% of the total. Thus, erythropoietin was unable to change the pattern

FIGURE 38.

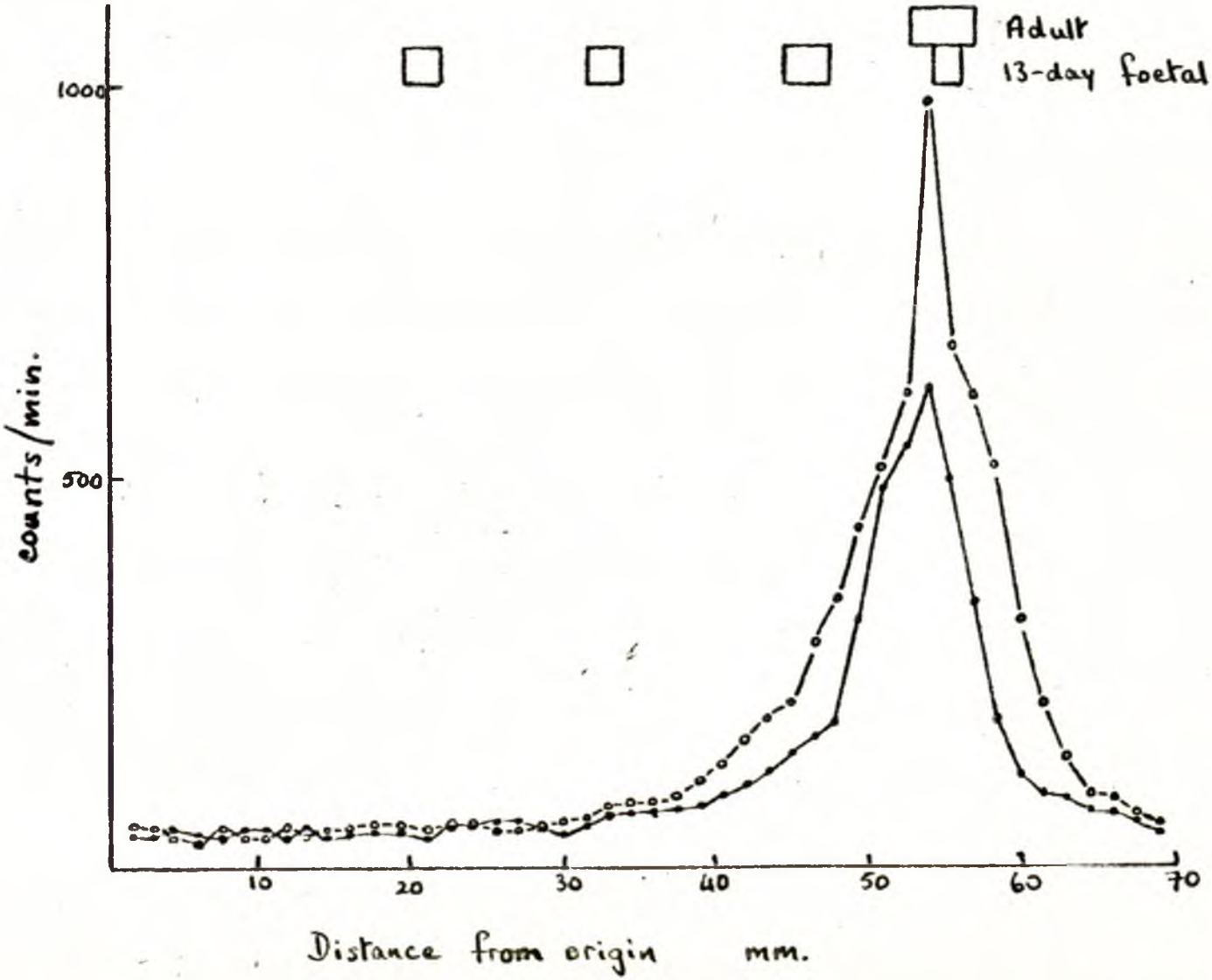
The pattern of haemoglobin synthesis in 13-day mouse foetal liver cells, after treatment with step 2 erythropoietin at 0.5 u./10^6 cells. The cells were pulsed with ^{59}Fe between 0 and 20 hours incubation. The haemoglobin components were separated by starch gel electrophoresis in buffer A containing 1.0% v/v mercaptoethanol; 13-day foetal mouse haemoglobin was added as marker.

•——• counts/min. in 1.5 mm. slices of gel after electrophoresis of haemoglobin from untreated cultures.

∘——∘ counts/min. in 1.5 mm. slices of gel after electrophoresis of haemoglobin from erythropoietin-treated cultures.

□ position on the gel of the visible bands separated in the haemoglobin samples shown.

Figure 38


Adult
13-day foetal
1000
500
counts/min.
10
20
30
40
50
60
70
Distance from origin mm.

of haemoglobin synthesis by mouse yolk sac cells. If later erythroid cells are derived from yolk sac cells, it would appear that the change is not brought about by the action of erythropoietin alone. This result confirms the finding of Kovach et al. (1967) that foetal haemoglobins are made by yolk sac cells, while adult haemoglobin is not.

Foetal liver cells. Erythropoietin markedly increased the synthesis of haemoglobin by foetal liver cells (Cole and Paul, 1966); it has been reported that only adult haemoglobin is produced by these cells (Kovach et al., 1967). To confirm that foetal liver cells from the Porton strain mice used in this laboratory also synthesised adult haemoglobin, the electrophoretic pattern of the haemoglobin synthesised in foetal liver cell cultures was determined. The effect of erythropoietin on the haemoglobin made was also determined.

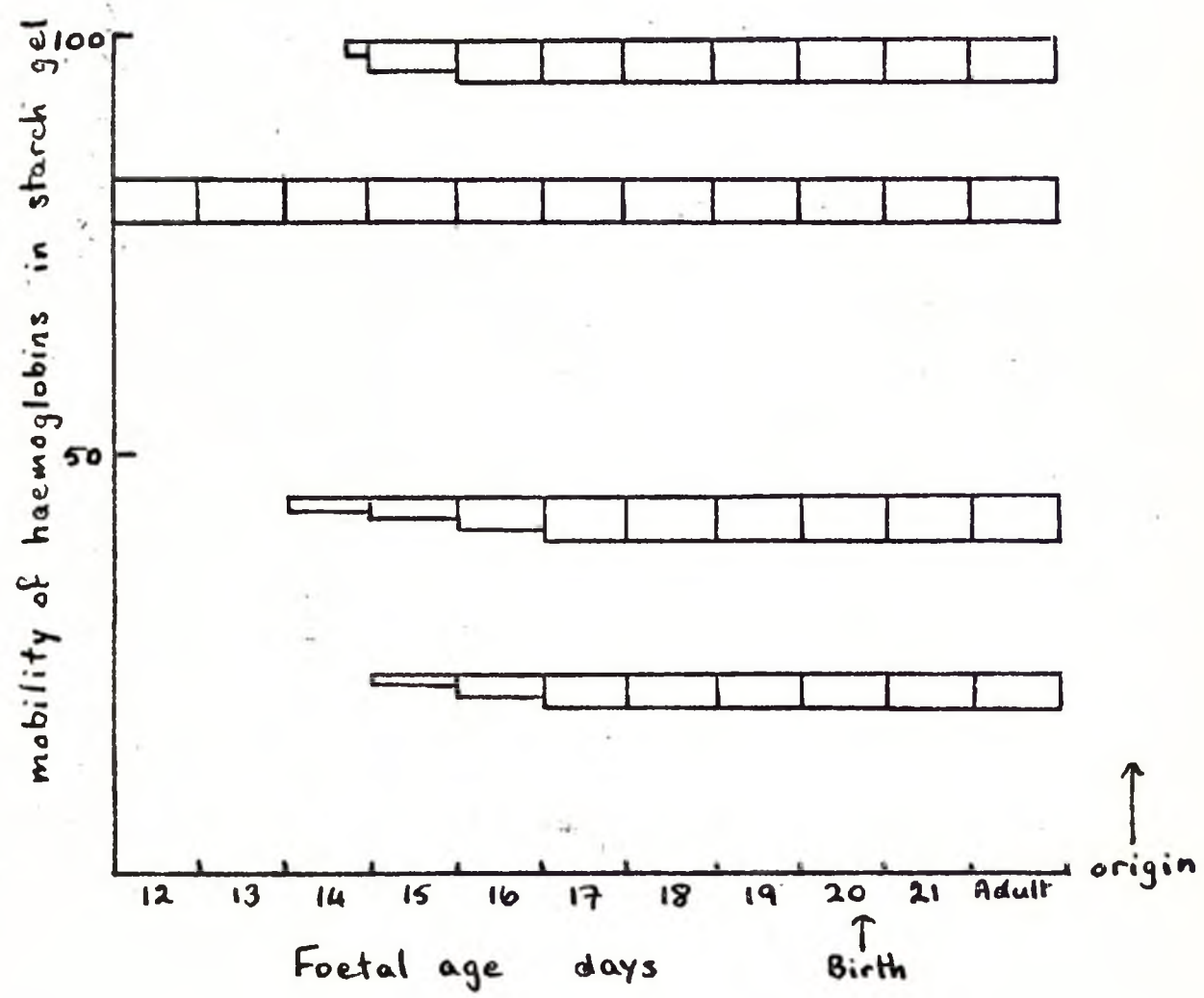
Cultures of 13-day cells were labelled with ^{59}Fe for 20 hr. with and without erythropoietin treatment; 0.01 mM-$FeCl_3$ was added to the medium. The distribution of activity after electrophoresis of the cell extracts is shown in Figure 38. No activity was found in any foetal component, irrespective of erythropoietin treatment. All of the activity was associated with adult haemoglobin; the amount synthesised increased by 65% after erythropoietin treatment. This result is in agreement with those of Kovach et al. (1967) that only adult haemoglobin is made by foetal liver cells. It appeared that erythropoietin might be concerned in the quantitative control

Figure 39

Haemoglobins in foetal rat blood during gestation, separated by starch gel electrophoresis in buffer A.

position on the gel of the visible bands in haemoglobin samples from foetuses of the ages shown; the mobility of each component is expressed as a percentage of the mobility of the leading component of adult rat haemoglobin, haemoglobin '1'.

Figure 39


mobility of haemoglobins in starch gel
100
50
origin
12
13
14
15
16
17
18
19
20
21
Adult
Foetal age days
Birth

of mouse foetal erythropoiesis; the hormone did not, however, affect the nature of the haemoglobin synthesised.

5. Rat haemoglobins and the effect of erythropoietin on their synthesis

After electrophoresis in buffer A, four components were found in adult rat haemoglobin samples. The fastest, also the most dense, was taken as a standard; it was termed haemoglobin '1'. The mobilities of the other components, termed haemoglobins '2', '3' and '4', were respectively 83%, 45% and 24% of that of haemoglobin '1'. Haemoglobin '2' was slightly less dense than haemoglobin '1', while haemoglobin '3' was only ½ as dense. Haemoglobin '4' was very faint and was sometimes not visible before staining.

12-day and 13-day foetal blood contained only haemoglobin '2'; haemoglobin '3' was first found on day 14 and increased slowly in quantity until day 18. Later on day 14 haemoglobin '1' was detected; it increased rapidly in quantity until day 18. Haemoglobin '4' was first seen on day 15 and increased little in quantity thereafter. Haemoglobin '2' remained dense throughout, although its relative concentration decreased as the other components appeared. From day 18 the proportion of each component appeared constant and similar to that found in adult blood. This pattern is represented in Figure 39.

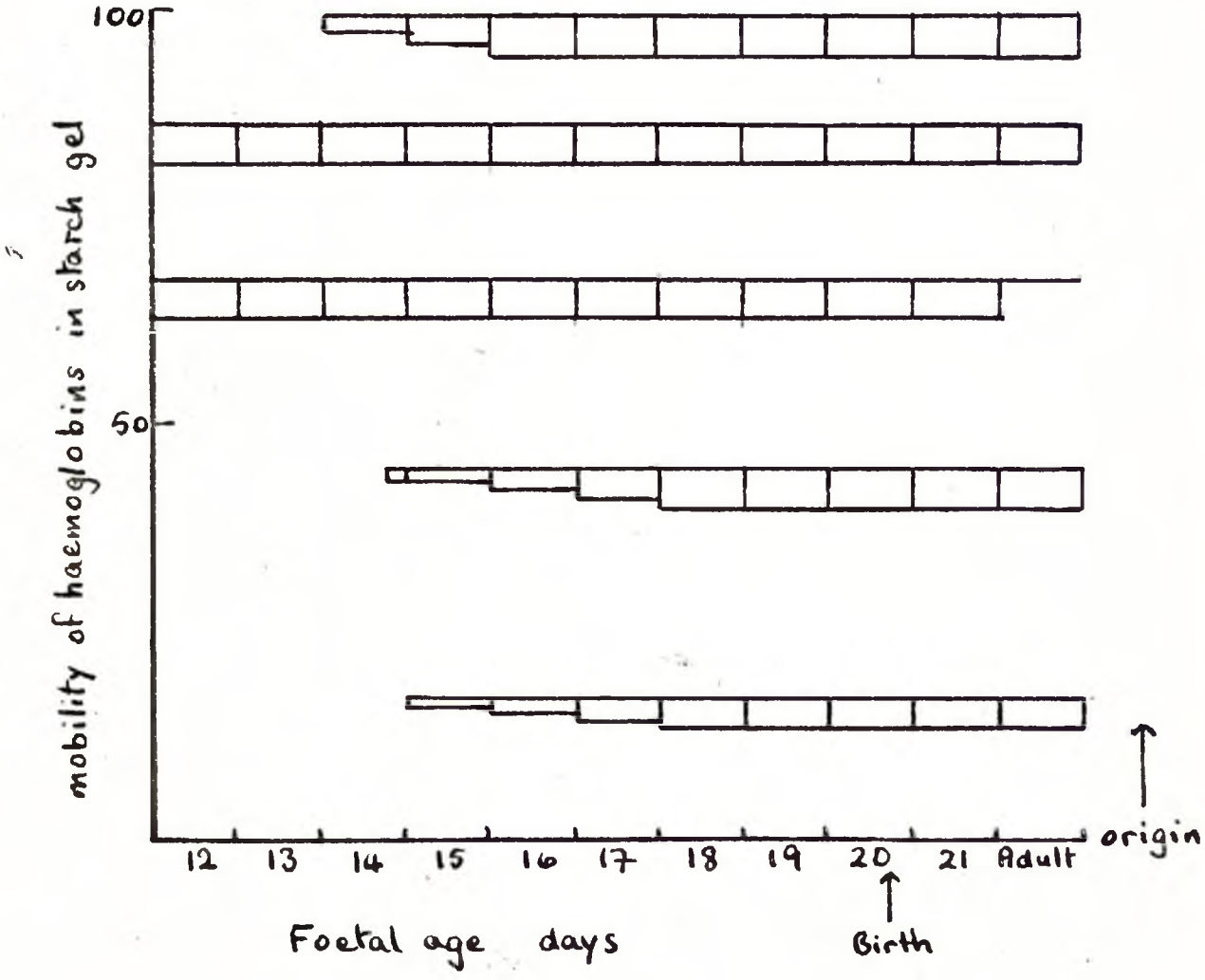
Five components were found after electrophoresis in buffer C; the leading component, also the most dense, was again taken as a standard

Figure 40

Haemoglobins in foetal rat blood during gestation, separated by starch gel electrophoresis in buffer C.

position on the gel of the visible bands in haemoglobin samples from foetuses of the ages shown; the mobility of each component is expressed as a percentage of the mobility of the leading component of adult rat haemoglobin, haemoglobin 'a'.

Figure 40


100
50
mobility of haemoglobins in starch gel
origin
12
13
14
15
16
17
18
19
20
21
Adult
Foetal age days
Birth

and was termed haemoglobin 'a'. The mobilities of the other four components, termed haemoglobins 'b', 'c', 'd' and 'e', were respectively 87%, 68%, 45% and 17% of that of haemoglobin 'a'. In adult samples haemoglobin 'b' was slightly less dense than haemoglobin 'a' while haemoglobin 'd' was ½ as dense. Haemoglobin 'e' was faint and not always visible before staining. Haemoglobin 'c' was detected in less than half of the samples and was rarely visible before staining. 12-day and 13-day foetal blood contained haemoglobins 'b' and 'c', the former being the fainter of the two. Haemoglobin 'a' was first detected early on day 14; it increased slowly in amount until day 16 and more rapidly thereafter. Haemoglobin 'd' appeared late on day 14 and increased only slowly in quantity. Haemoglobin 'e' was found late on day 15 and increased little in quantity thereafter. The amount of haemoglobin 'b' increased continually, slowly until day 16 and more rapidly later. Haemoglobin 'c' remained dense until day 18, after which it decreased in amount. From day 19 the relative proportions of haemoglobins 'a', 'd' and 'e' appeared constant and similar to the adult pattern. The proportion of haemoglobin 'b' was less at 19 days than in adult samples; it increased slowly through birth and weaning. The foetal and adult patterns are represented in Figure 40.

Haemoglobin 'c' was consistently found in neonatal and suckling animals; it decreased steadily in quantity as the animals developed. By one month post-weaning it was very faint indeed and was detected less frequently in older animals. It was detectable in a proportion of animals aged up to 2½ years; it is not known whether it was completely

absent from other animals or whether it was present in undetectable amounts.

The patterns observed with the two different buffers were broadly similar; the extra component resolved in buffer C and a change in the order of the appearance of the components in the foetal blood during gestation were the major differences. Since the rats studied with buffer C, although also Wistar strain, were derived from a separate random-bred colony, it was possible that the disparities were due to differences in the rats themselves. When the haemoglobins from these rats were separated in buffer A, 4 components were found; their mobilities corresponded to those of the components previously resolved in buffer A (Figure 39). The order in which the components appeared in the foetal blood, however, corresponded to the pattern shown in Figure 40, i.e. haemoglobin '1' was found earlier than haemoglobin '3' and only haemoglobin '2' was found in 12-day and 13-day blood. Thus, the stages of foetal development at which the different components appeared differed in the two rat colonies. It is probable, however, that haemoglobins 'a', 'd' and 'e' may be identified with haemoglobins '1', '3' and '4', haemoglobins 'b' and 'c' combining in buffer A to produce haemoglobin '2'. This was substantiated by studies on the haemoglobin components synthesised by foetal liver cell cultures.

Yolk sac cells. The presence of haemoglobins 'b' and 'c' in the circulation on day 12 when the erythropoietic activity of the liver

Figure 41

The pattern of haemoglobin synthesis in 10-day rat yolk sac cells, after treatment with step 1 erythropoietin (Lot 117/1), at 0.25 u/10^6 cells, after precipitation of impurities at pH 4.3.

The cells were pulsed with ^{59}Fe between 0 and 15 hr. incubation.

The haemoglobin components were separated by starch gel electrophoresis in buffer C; 17-day foetal rat haemoglobin was added as marker.

•——• counts/min. in 1.5 mm. slices of gel after electrophoresis of haemoglobin from cultures incubated with or without erythropoietin.

□ position on the gel of the visible bands separated in the haemoglobin samples shown.

Figure 42

The pattern of haemoglobin synthesis in 12-day rat foetal blood cells.

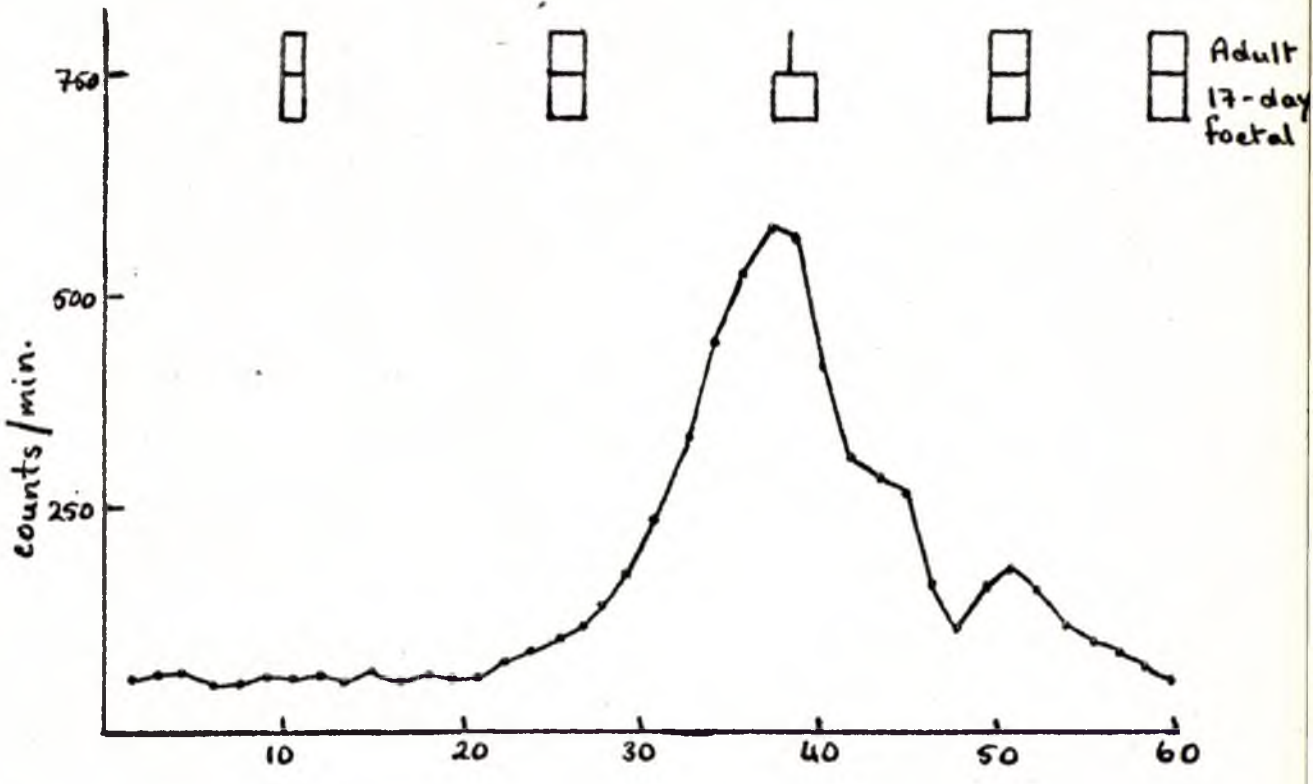
The cells were pulsed with ^{59}Fe between 0 and 8 hr. incubation.

The haemoglobin components were separated by starch gel electrophoresis in buffer C; no marker haemoglobin was added.

•——• counts/min. in 1.5 mm. slices of gel after electrophoresis.

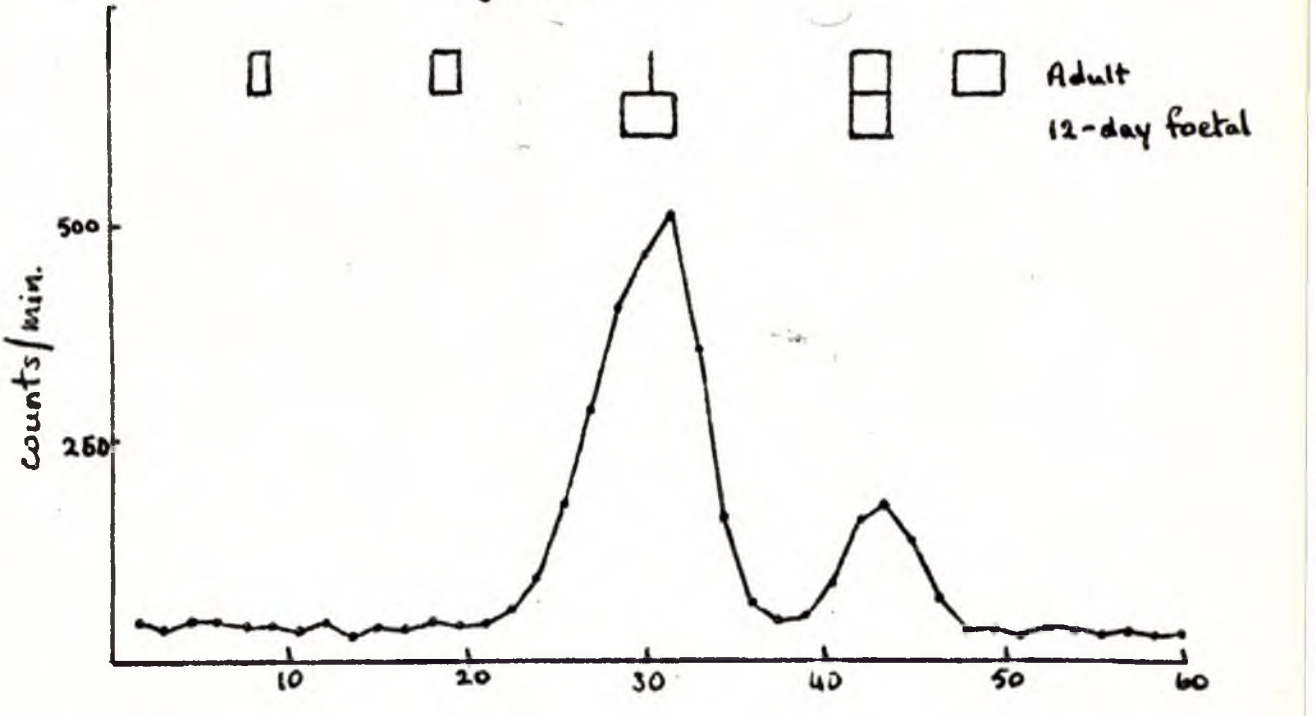
□ position on the gel of the visible bands separated in the haemoglobin samples shown.

Figure 41



Distance from origin mm.

Figure 42



Distance from origin mm.

was very low suggested that these haemoglobins were made by yolk sac cells; the increase in the total amount of each after yolk sac erythropoiesis has stopped suggested that they were also made in liver cells. If this were the case, it would appear that the yolk sac and liver erythroid cells of rat foetuses are more similar than those of mouse foetuses. Although there was no quantitative effect of erythropoiesis upon rat yolk sac haemoglobin synthesis, (Figure 8), it is possible that some qualitative change might be induced.

Cultures of 10-day yolk sac cells were labelled for 15 hr. with ^{59}Fe, with and without erythropoietin treatment. 17-day haemoglobin was added to the cell extracts as marker; the pattern of radioactivity after electrophoresis in buffer C was determined and is shown in Figure 41. All of the activity detected was associated with haemoglobins 'b' and 'c'; erythropoietin had no visible effect. Thus, yolk sac cells synthesised haemoglobins electrophoretically identical to two of those produced by liver cells, but did not respond to erythropoietin.

Haemoglobin synthesis by circulating yolk sac cells. It was possible that small amounts of the other haemoglobin components might be produced as yolk sac erythropoiesis continued. Since the liver begins to develop during day 11, cell cultures of whole 11-day embryos may contain liver erythroid cells as well as yolk sac cells. On day 12 the circulating cells were all nucleated suggesting that no liver erythroid cells had

yet been released.

The nature of the haemoglobin produced by 12-day blood cells during 8 hr. incubation with ^{59}Fe was determined; addition of marker haemoglobin was unnecessary since the haemoglobins were visible after electrophoresis. Haemoglobin samples prepared from the blood of older foetuses were run in the same gel for comparison. The results are shown in Figure 42. Again, all of the activity found was associated with haemoglobins 'b' and 'c'; the proportion of each component was similar to that found in 10-day yolk sac cells. This result suggests that only haemoglobins 'b' and 'c' are synthesised during yolk sac erythropoiesis; there appeared to be no alteration in their relative rates of synthesis as the cells matured.

It was possible that the ^{59}Fe-labelled haemoglobin synthesised by 10-day yolk sac cells might have become associated with the marker haemoglobin after it was added. If this were the case, it might be anticipated that some activity would become associated with all the components of the marker haemoglobin; instead only haemoglobins 'b' and 'c' were active. In addition, circulating yolk sac cells on day 12 showed a similar incorporation pattern in the absence of added marker haemoglobin. The distribution of radioactivity in the gels was thus a true reflection of the nature and relative amounts of the components synthesised by the cells.

Figure 43

The pattern of haemoglobin synthesis in 13-day rat foetal liver cells, after treatment with Step 2 erythropoietin, at 0.5 u./10^6 cells.

The cells were pulsed with ^{59}Fe between 0 and 21 hr. incubation.

The haemoglobin components were separated by starch gel electrophoresis in buffer A; adult haemoglobin was added as marker.

•——• - counts/min. in 1.5 mm. slices of gel after electrophoresis of haemoglobin from untreated cultures.

o——o - counts/min. in 1.5 mm. slices of gel after electrophoresis of haemoglobin from erythropoietin-treated cultures.

 - position on gel of the visible bands separated in the haemoglobin samples shown.

Figure 43

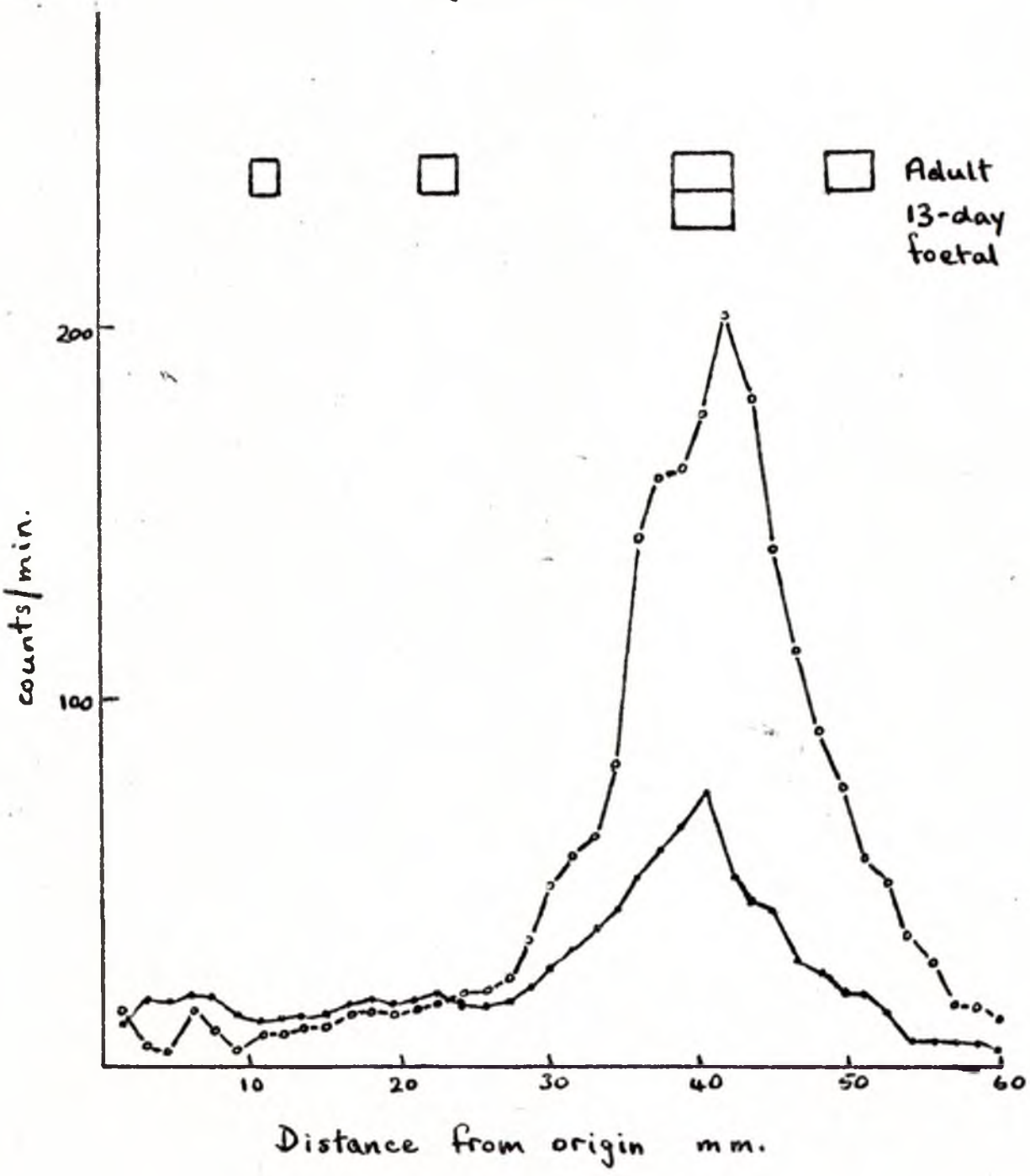

Adult
13-day
foetal
200
100
counts/min.
10
20
30
40
50
60
Distance from origin mm.

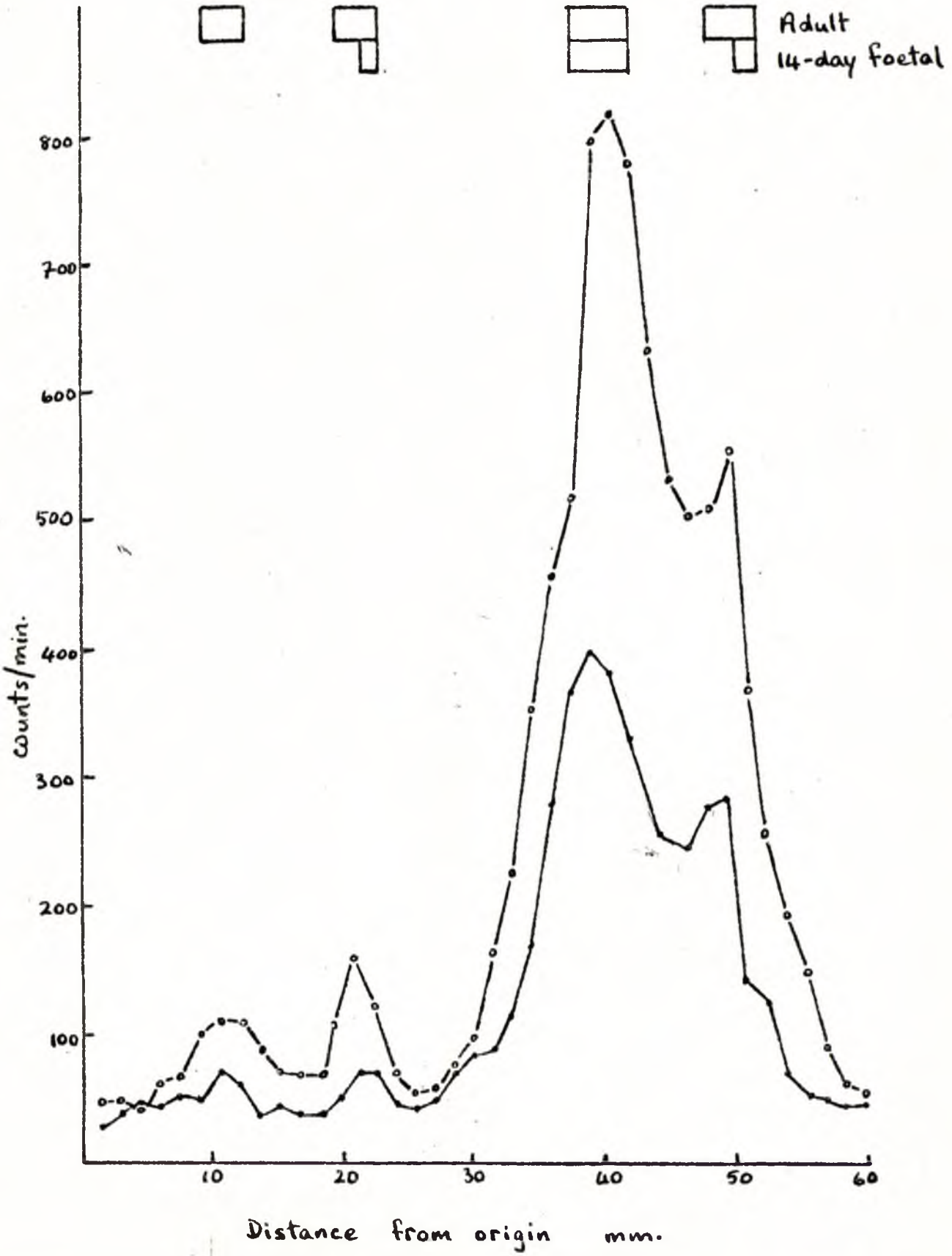
Figure 44

The pattern of haemoglobin synthesis in 14-day rat foetal liver cells, after treatment with step 2 erythropoietin, at 0.5 u./10^6 cells.

The cells were pulsed with ^{59}Fe between 0 and 14 hr. incubation.

Legend as Figure 43.

Figure 44


Adult
14-day foetal
800
700
600
500
400
300
200
100
counts/min.
10
20
30
40
50
60
Distance from origin mm.

Foetal liver cells. During day 14 and day 15, when the erythropoietic activity of the foetal liver was increasing, additional varieties of haemoglobin were appearing in the circulation; this may be due to recruitment of fresh cohorts of cells or to an increase in the number of haemoglobins synthesised by erythroid cells already active. In either case, treatment with erythropoietin may induce the precocious appearance of the additional haemoglobins.

Preliminary studies were carried out using buffer A. 13-day cells, grown in medium with and without erythropoietin, were labelled with ^{59}Fe for 21 hr.; adult haemoglobin was added to the cell extracts as marker. The resulting electrophoretic pattern is shown in Figure 43. The only component detected was haemoglobin '2', but the activity recovered was so low that components being made only in small amounts might be undetectable. Although erythropoietin caused an increase of 81% in the amount of haemoglobin synthesised, there was no discernable change in the variety of haemoglobin produced.

14-day cells were next studied; adult haemoglobin was added as marker. The activity recovered after 14 hr. labelling with ^{59}Fe was much higher (Figure 44) and was associated with all four components whether or not erythropoietin was present. The overall increase in haemoglobin synthesis after erythropoietin treatment was 211%; the synthesis of each component was increased by the same factor. In this case, as in the previous experiment, although erythropoietin was able to increase haemoglobin synthesis, it did not alter the relative amounts of the haemoglobin components

Figure 45

The pattern of haemoglobin synthesis in 12-day rat foetal liver cells, after treatment with step 1 erythropoietin (Lot 117/1), at 0.25 u./10^6 cells, after precipitation of impurities at pH 4.3.

a. pulsed with ^{59}Fe between 0 and 6 hr. incubation.

b. pulsed with ^{59}Fe between 24 and 30 hr. incubation.

The haemoglobin components were separated by starch gel electrophoresis in buffer C; adult haemoglobin was added as marker.

.——. - counts/min. in 1.5 mm. slices of gel after electrophoresis of haemoglobin from untreated cultures.

.——. - counts/min. in 1.5 mm. slices of gel after electrophoresis of haemoglobin from erythropoietin-treated cultures.

 - position on the gel of the visible bands separated in the haemoglobin samples shown.

Figure 45

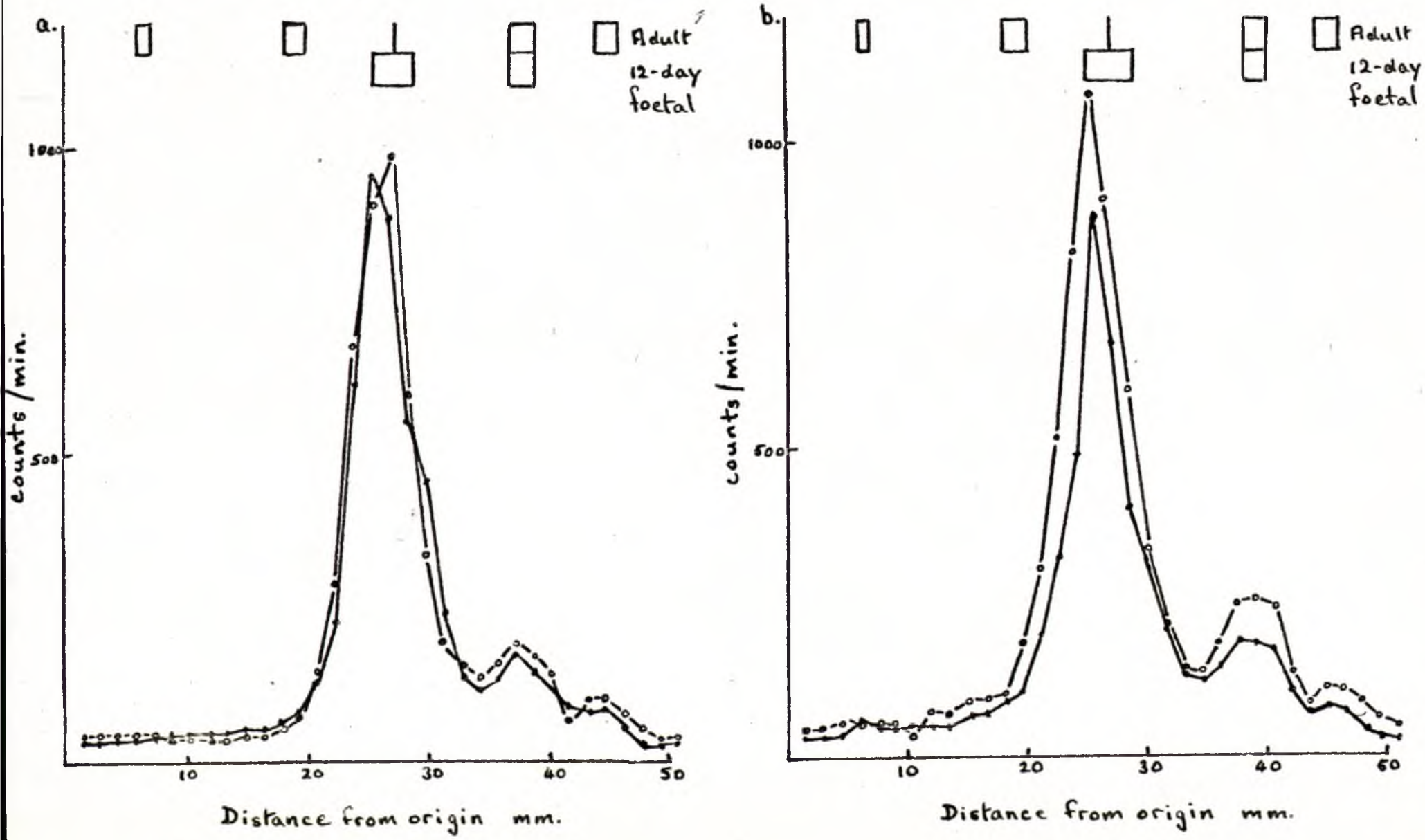

a.
Adult
12-day
foetal
1000
500
counts/min.
10
20
30
40
50
Distance from origin mm.
b.
Adult
12-day
foetal
1000
500
counts/min.
10
20
30
40
60
Distance from origin mm.

Figure 46

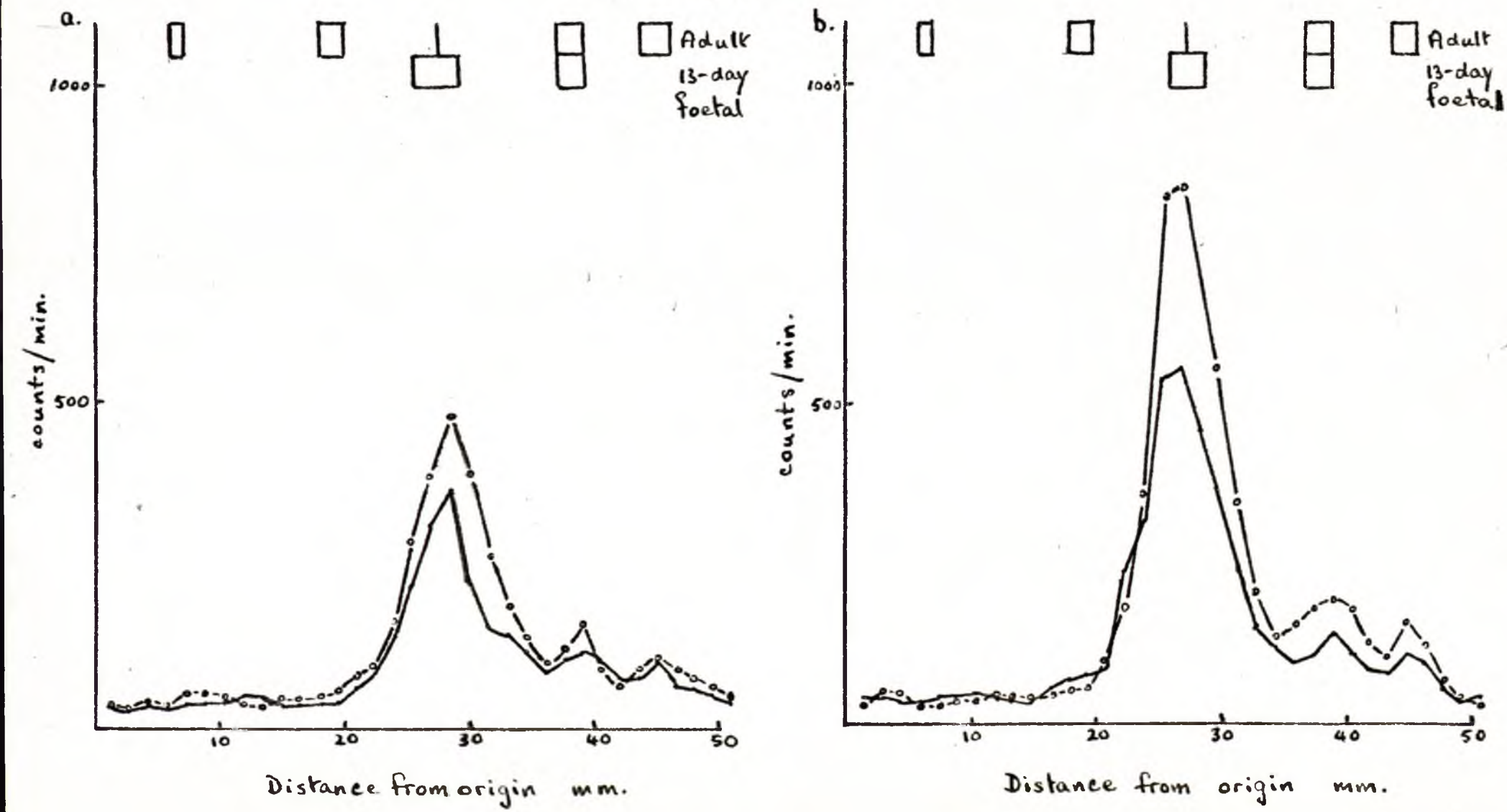
The pattern of haemoglobin synthesis in 13-day rat foetal liver cells, after treatment with step 1 erythropoietin (Lot 117/1), at 0.25 u./10^6 cells, after precipitation of impurities at pH 4.3.

a. pulsed with ^{59}Fe between 0 and 5 hr. incubation.

b. pulsed with ^{59}Fe between 24 and 32 hr. incubation.

Legend as Figure 45.

Figure 46


a.
Adult
13-day foetal
1000
500
counts/min.
10
20
30
40
50
Distance from origin mm.
b.
Adult
13-day foetal
1000
500
counts/min.
10
20
30
40
50
Distance from origin mm.

synthesised by the cells. It appeared also, that erythropoietin was unable, by itself, to initiate the synthesis of haemoglobins '1', '3' and '4' in cells which did not already make these components.

A more complete investigation was carried out, using buffer C to separate the components. To determine whether the relative rates of syntheses of the components altered during cell maturation, the cells were labelled with ^{59}Fe at two separate times after erythropoietin treatment.

The effect of erythropoietin upon the haemoglobins made in 12-day foetal liver cell cultures is shown in Figure 45. Most of the activity recovered was associated with haemoglobin 'c'; haemoglobins 'b' and 'a' were also detected, but no other components were present. Erythropoietin produced an overall increase in haemoglobin synthesis of 27% during the first pulse and of 36% during the second; each component present was increased by a similar amount at both times. Since the maximum response to erythropoietin by 12-day cells occurred after 47 hr. (Figure 9) a longer period of culture in this experiment might well have enabled a larger response to take place.

A similar pattern was found in 13-day foetal liver cell cultures, only haemoglobins 'a', 'b' and 'c' being detected; most of the activity was associated with haemoglobin 'c' (Figure 46). The increased activity in extracts of untreated cells during the second pulse was probably due to its greater length. Erythropoietin increased haemoglobin synthesis by 29% during the first pulse and by 50% during the second. During both pulses the relative rate of synthesis of each component was the

Figure 47

The pattern of haemoglobin synthesis in 14-day rat foetal liver cells after treatment with step 1 erythropoietin (Lot 1/4), at 0.2 u./10^6 cells.

a. pulsed with ^{59}Fe between 0 and 6.5 hr. incubation.

b. pulsed with ^{59}Fe between 23 and 29.5 hr. incubation.

Legend as Figure 45.

Figure 47

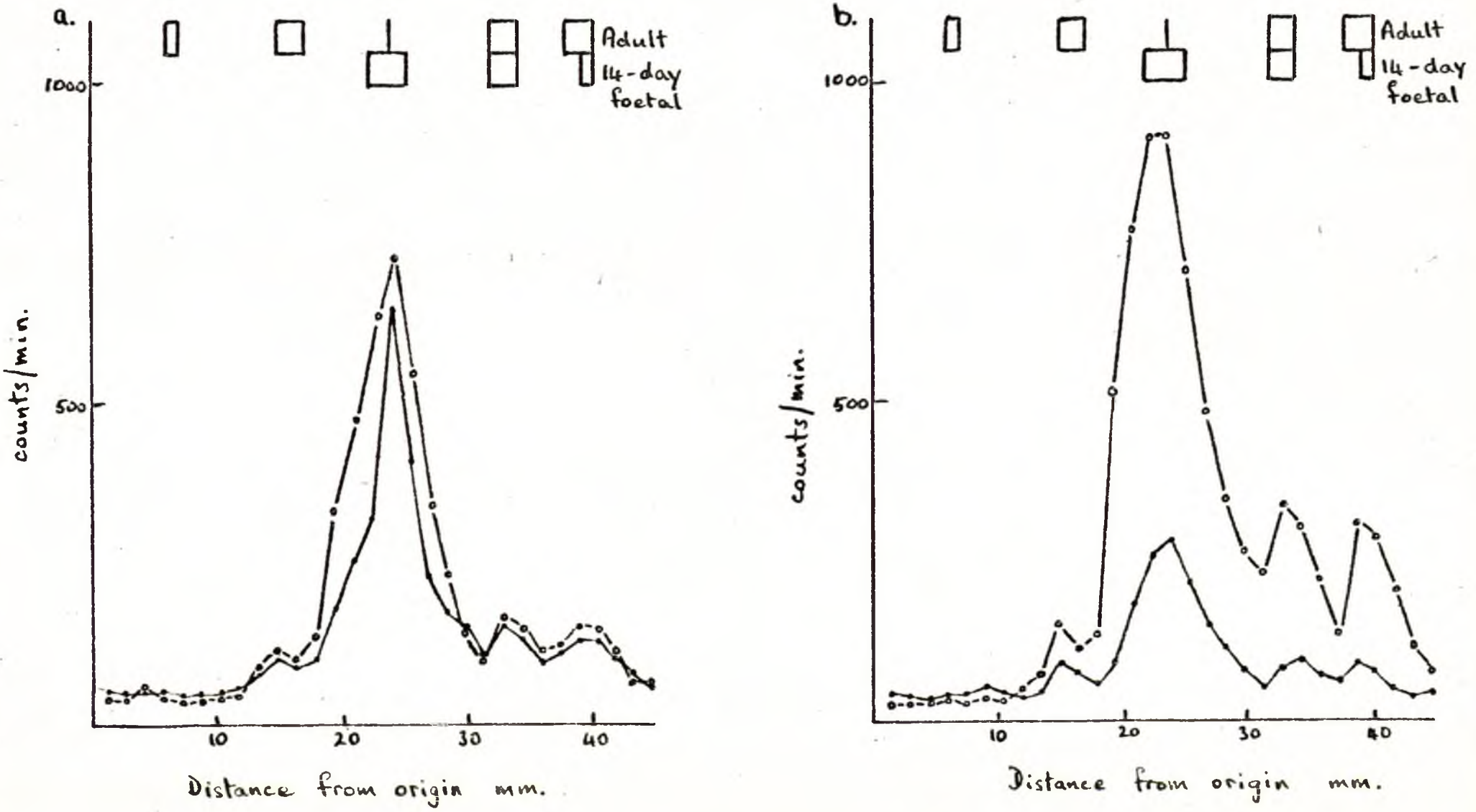

a.
Adult
14-day foetal
1000
500
counts/min.
10
20
30
40
Distance from origin mm.
b.
Adult
14-day foetal
1000
500
counts/min.
10
20
30
40
Distance from origin mm.

Figure 48

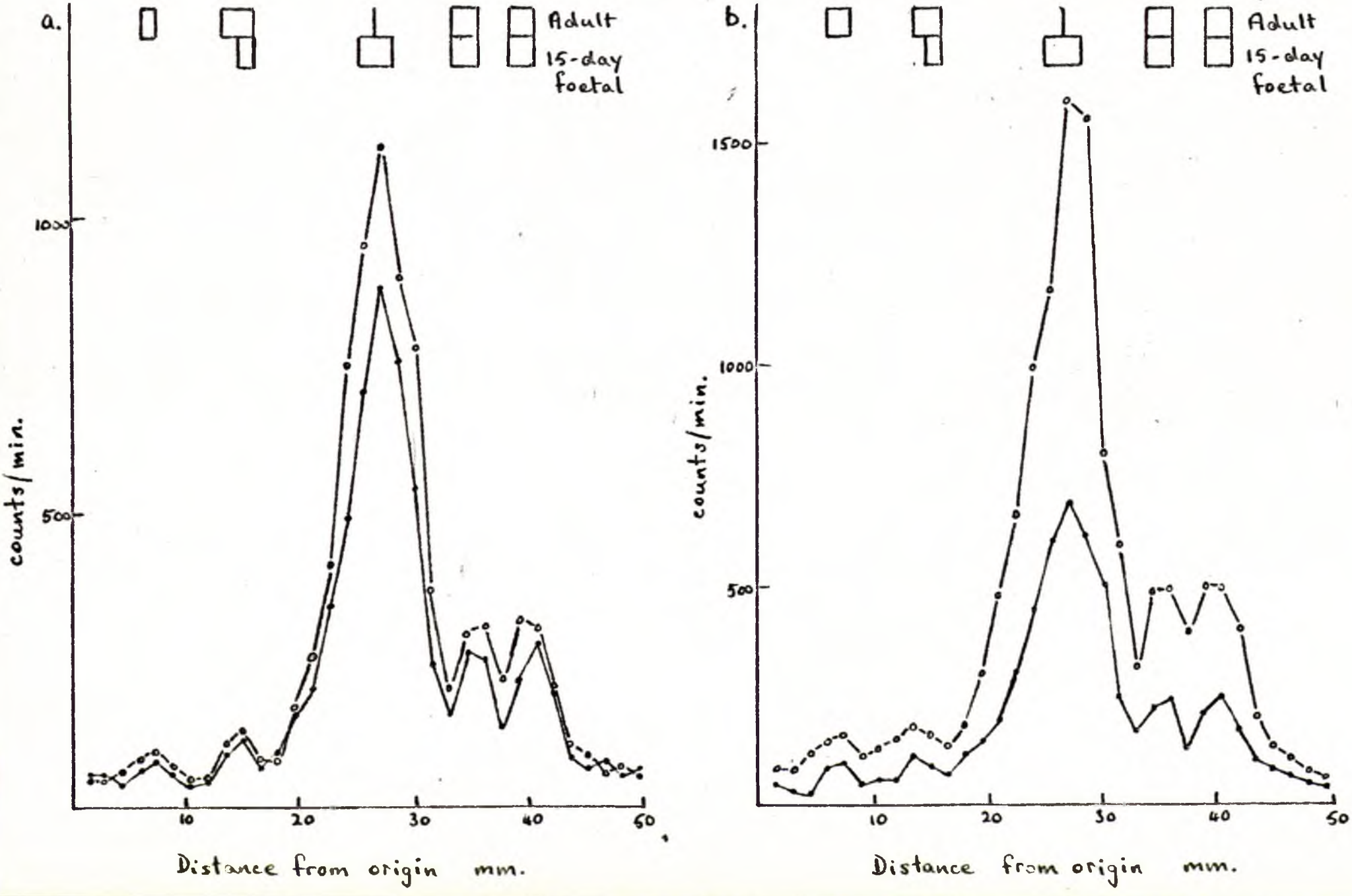
The pattern of haemoglobin synthesis in 15-day rat foetal liver cells after treatment with step 2 erythropoietin, at 0.5 u./10^6 cells.

a. pulsed with ^{59}Fe between 0 and 6 hr. incubation.

b. pulsed with ^{59}Fe between 24 and 30 hr. incubation.

Legend as Figure 45.

Figure 48


a.
Adult
15-day foetal
counts/min.
1000
500
10
20
30
40
50
Distance from origin mm.
b.
Adult
15-day foetal
1500
1000
500
counts/min.
10
20
30
40
50
Distance from origin mm.

same, irrespective of erythropoietin treatment. The relatively small effect of erythropoietin in this and in the previous experiment may be due to the use of the crude erythropoietin preparation, step 1, Lot 117/1.

Haemoglobin 'd' was first detected in 14-day cell cultures, with or without erythropoietin treatment; haemoglobins 'a', 'b' and 'c' were also labelled, most of the activity being associated with haemoglobin 'c' (Figure 47). Erythropoietin stimulated haemoglobin synthesis by 30% during the first pulse and by 244% during the second pulse; the proportion of the total activity associated with each component was the same during both pulses, irrespective of erythropoietin treatment.

All five components were detected in 15-day cell cultures whether or not erythropoietin was present; most of the activity was associated with haemoglobin 'c'. Erythropoietin caused an increase in haemoglobin synthesis of 37% during the first pulse and of 165% during the second; again the relative amount of each component was the same during both pulses, with or without erythropoietin treatment (Figure 48).

In none of these experiments did erythropoietin treatment alter the relative rates of synthesis of the haemoglobin components. Haemoglobin 'd' which was found in the blood late in day 14 was not detected in 12-day or 13-day liver cell cultures at any time, with or without erythropoietin treatment. Similarly, haemoglobin 'e' which appeared in the circulation on day 15 was not found in 14-day liver cell cultures at any time whether or not erythropoietin was present.

Figure 49

The pattern of haemoglobin synthesis in 18-day rat foetal liver cells after treatment with step 2 erythropoietin, at 0.5 u./10^6 cells.

a. pulsed with ^{59}Fe between 0 and 6 hr. incubation.

b. pulsed with ^{59}Fe between 22 and 28 hr. incubation.

The haemoglobin components were separated by starch gel electrophoresis in buffer C; 18-day foetal haemoglobin was added as marker.

.——. - counts/min. in 1.5 mm. slices of gel after electrophoresis of haemoglobin from cultures incubated with or without erythropoietin.

□ - position on the gel of the visible bands separated in the haemoglobin samples shown.

Figure 49

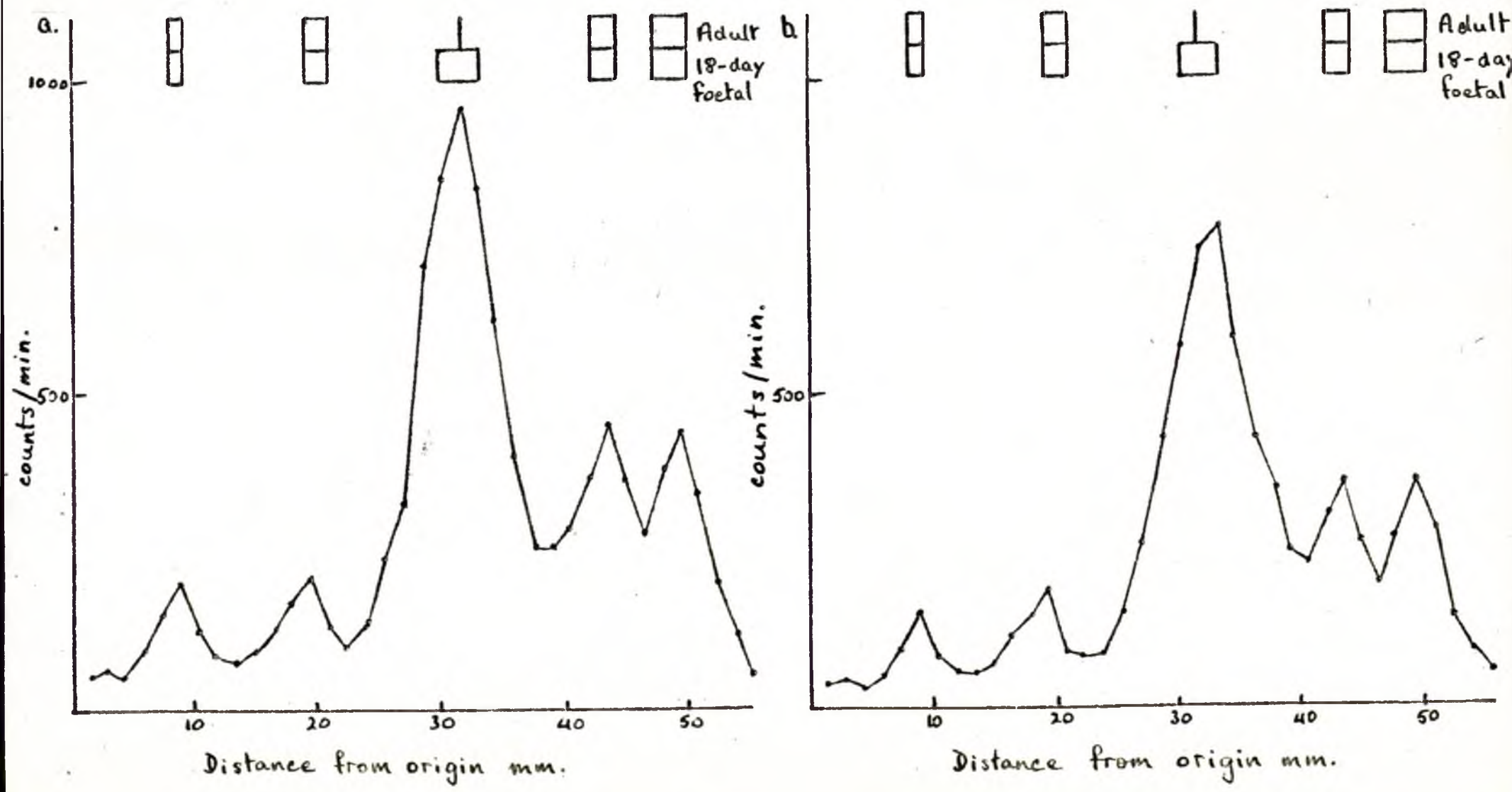

a.
Adult
18-day foetal
1000
500
counts/min.
10
20
30
40
50
Distance from origin mm.
b.
Adult
18-day foetal
500
counts/min.
10
20
30
40
50
Distance from origin mm.

Figure 50

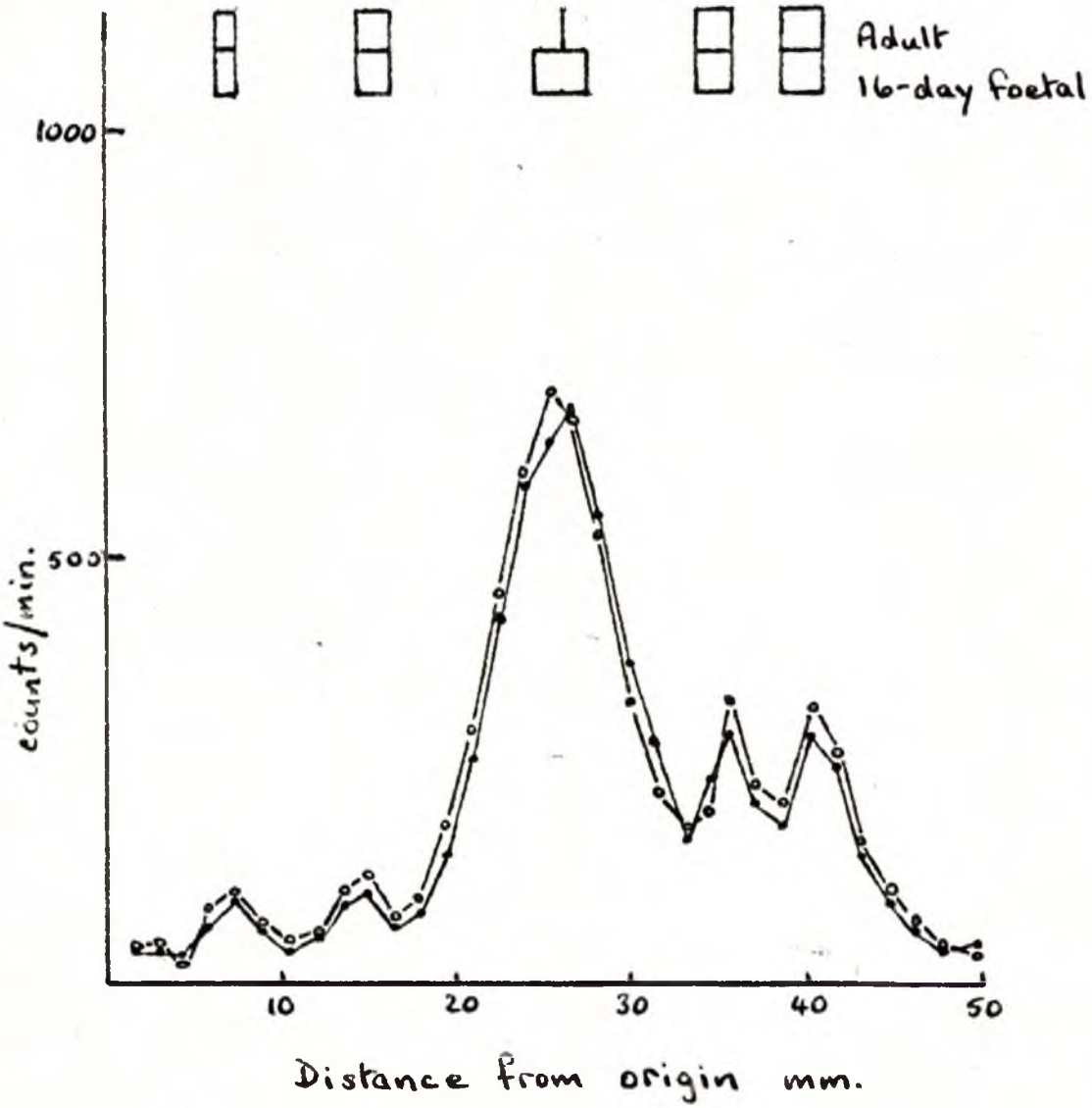
The pattern of haemoglobin synthesis in 16-day rat foetal blood cells, after treatment with step 2 erythropoietin, at 0.5 u./10^6 cells.

The cells were pulsed with ^{59}Fe between 0 and 10 hr. incubation.

The haemoglobin components were separated by starch gel electrophoresis in buffer C; no marker haemoglobin was added.

Legend as Figure 45.

Figure 50


Adult
16-day foetal
1000
500
counts/min.
10
20
30
40
50
Distance from origin mm.

Thus, foetal liver cells growing *in vivo* became able to synthesise haemoglobins 'd' and 'e' while foetal liver cells growing *in vitro* did not; *in vitro* treatment with erythropoietin did not alter this pattern. This suggested that erythropoietin acted by increasing the number of cells entering maturation or by increasing the activity of those cells already maturing; it had no effect on the pattern of haemoglobin synthesis characteristic of the cells at a given developmental stage.

Cells from older livers showed no quantitative response to erythropoietin (Figure 20); to exclude the possibility that a qualitative response might occur, the effect of erythropoietin on haemoglobin synthesis by 18-day cells was examined. As shown in Figure 49, all five components were present; the relative proportions of the components differed from the pattern observed in younger livers. Erythropoietin had no discernible effect on the cells; the relative rates of synthesis of the components were the same during both pulses. This finding further suggested that erythropoietin had no effect on the pattern of haemoglobin synthesis characteristic of a given foetal age; it seemed that the hormone did not affect haemoglobin synthesis directly.

The effect of erythropoietin on haemoglobin synthesis in cultures of 16-day foetal blood cells was examined. The pattern observed (Figure 50) was similar to that found in 15-day liver cells (Figure 48); erythropoietin treatment provoked an increase in haemoglobin synthesis of less than 10%. Since many of the cells were non-nucleated, a greater increase would probably have been observed if erythropoietin

Figure 51

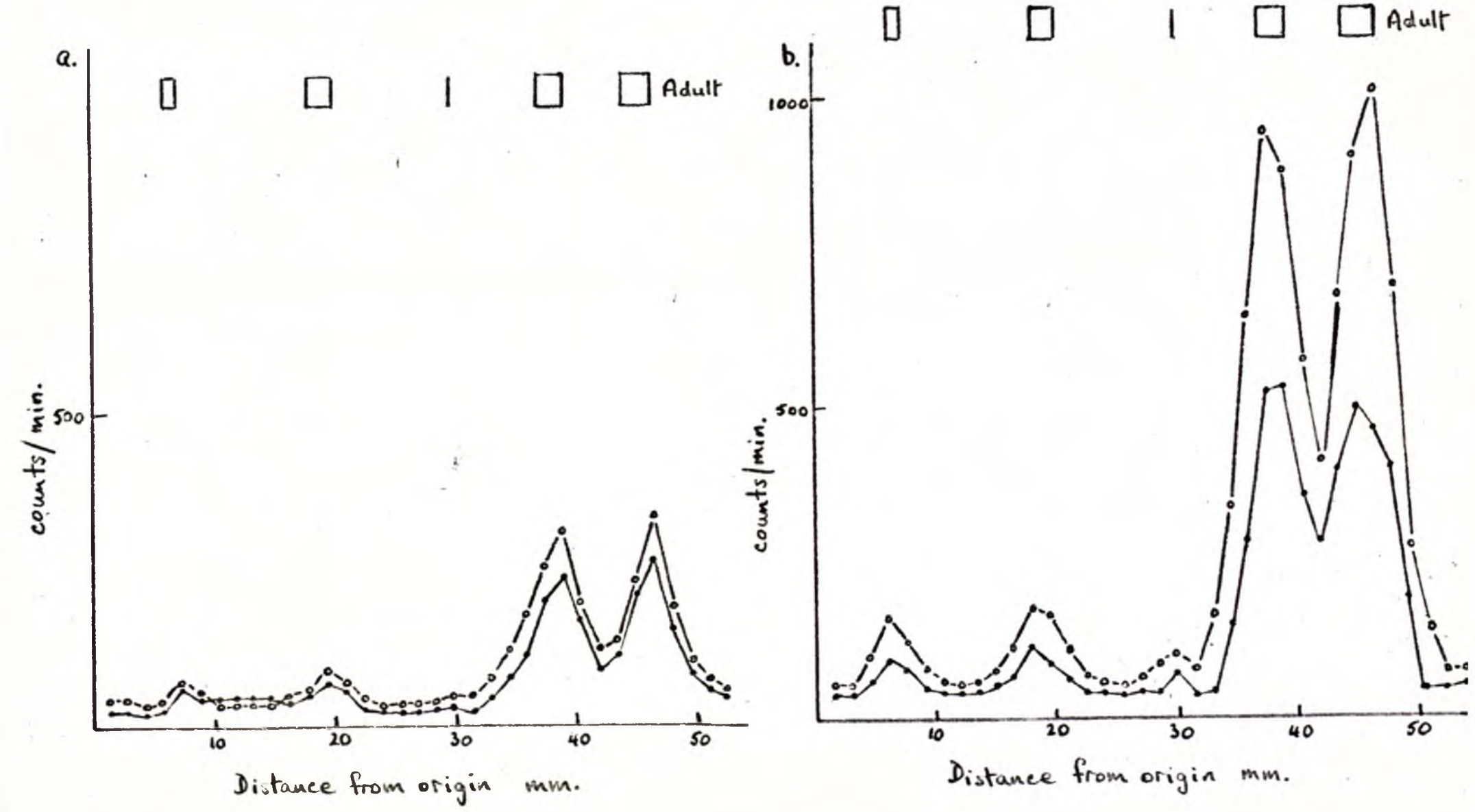
The pattern of haemoglobin synthesis in adult rat marrow cells, after treatment with step 1 erythropoietin (Lot 117/1), at 0.25 u./10^6 cells, after precipitation of impurities at pH 4.3.

a. pulsed with ^{59}Fe between 0 and 4 hr. incubation.

b. pulsed with ^{59}Fe between 22 and 28 hr. incubation.

Legend as Figure 45.

Figure 51


a.
Adult
counts/min.
500
10
20
30
40
50
Distance from origin mm.
b.
Adult
1000
500
counts/min.
10
20
30
40
50
Distance from origin mm.

affected haemoglobin synthesis directly, at the translation level. Immature cells were also present in the blood; the response may be due to stimulation of these, or of any ESC present in the blood. Again, the proportion of the total activity associated with each component was unchanged by erythropoietin treatment.

Thus, erythropoietin appeared to stimulate foetal erythroid tissue by increasing the number or the activity of maturing cells; it did not appear to affect haemoglobin synthesis directly. It has been shown that erythropoietin had no effect on haemoglobin synthesis in adult rabbit reticulocytes (Eralev, 1962); this suggests that the hormone affects haemoglobin synthesis only indirectly in both foetal and adult erythroid tissue. It was possible, however, that the maintenance after erythropoietin stimulation of the pattern of haemoglobin synthesis characteristic of the cells at explantation might be a property of foetal erythroid cells only. The effect of erythropoietin on haemoglobin synthesis by adult rat marrow cells was therefore examined.

Adult marrow cells. Rats were starved for three days to suppress endogenous erythropoietin production (Reissmann, 1964); the marrow was removed from the hind limbs and the cells suspended in medium by gentle pipetting. A single-cell suspension was obtained and the effect of erythropoietin on haemoglobin synthesis was determined (Figure 51). All five components were detected during both pulses although the amount of haemoglobin 'c' synthesised was very small

indeed. Erythropoietin treatment stimulated an overall increase in haemoglobin synthesis of 13% during the first pulse and of 78% during the second; at both times all of the components responded to erythropoietin to the same extent. This result indicated that the proportion of each haemoglobin synthesised was unchanged, even when the overall rate of haemoglobin synthesis was stimulated by erythropoietin; in both adult and foetal erythroid tissue erythropoietin appeared to stimulate erythroid cells without affecting their characteristic pattern of haemoglobin synthesis.

6. <u>The pattern of haemoglobin synthesis in foetal rat erythroid tissues</u>

The experiments in the previous section indicated that erythroid cells from rat yolk sac and foetal liver possessed a pattern of haemoglobin synthesis characteristic of the stage of development. The results also suggested that the relative rates of synthesis of the haemoglobin components were unchanged during at least 30 hr. of cell maturation. It was necessary to ensure that the pattern of haemoglobin synthesis was unaffected by trypsinisation and that the distribution of radioactivity observed was a true reflection of the cells' <u>in vivo</u> activity.

Figure 52

The pattern of haemoglobin synthesis in 20-day rat foetal liver cells.

a. The cells were disaggregated by gentle pipetting in medium and incubation was begun immediately.

b. The cells were treated overnight with cold trypsin and incubation was begun on the day following explantation.

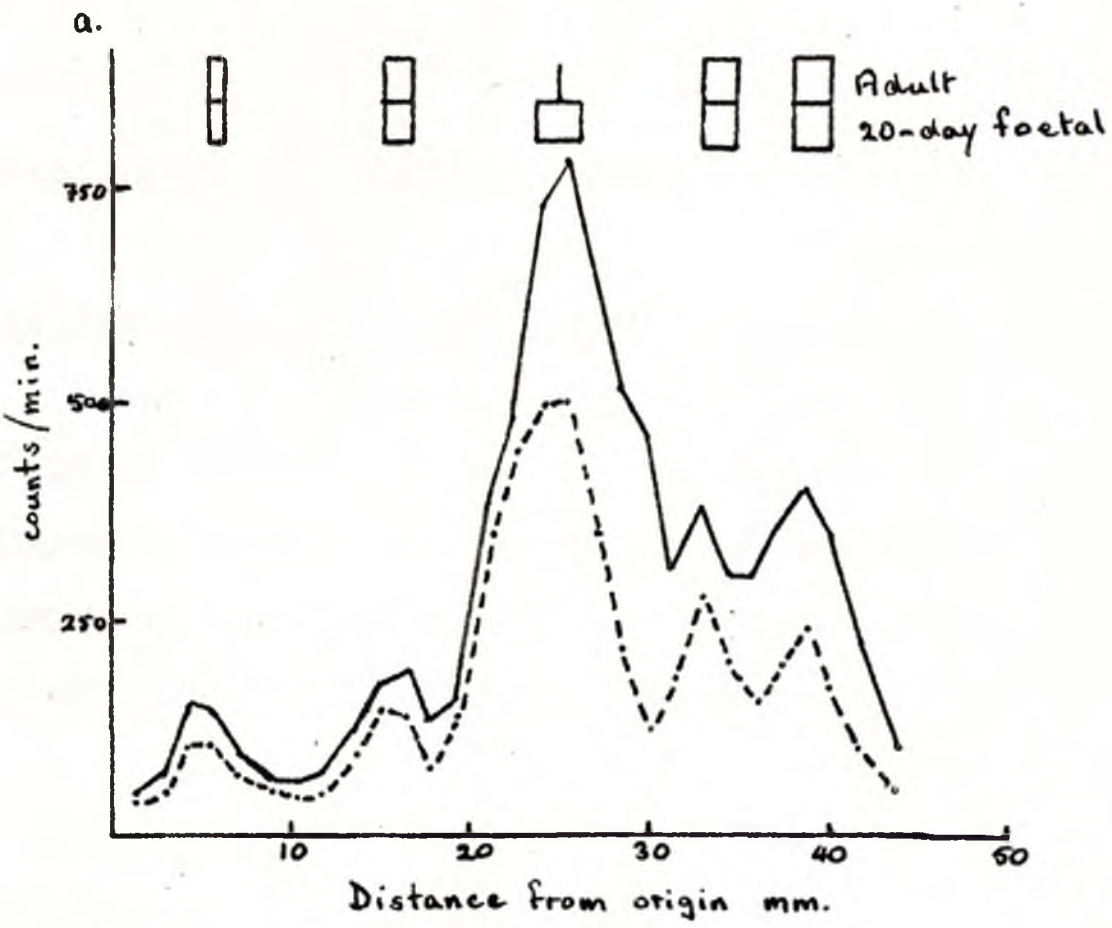
The haemoglobin components were separated by starch gel electrophoresis in buffer C; 20-day foetal haemoglobin was added as marker.

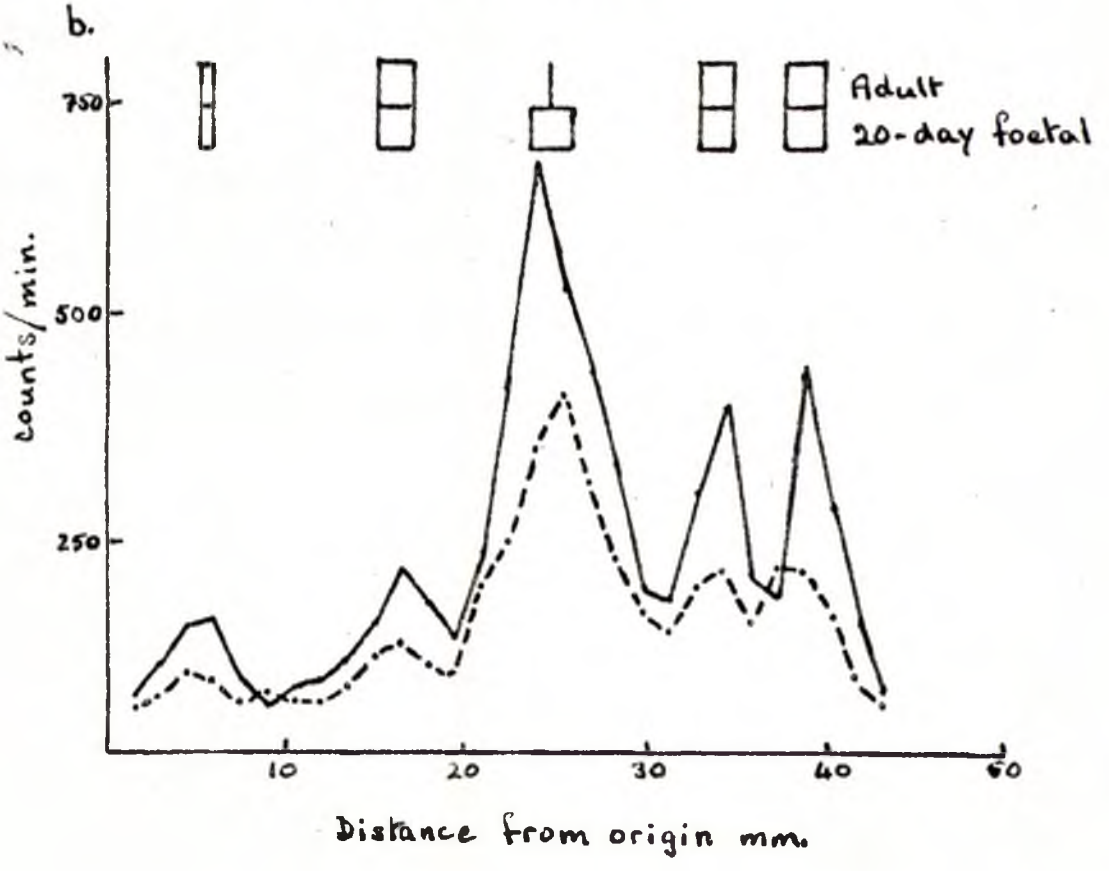
——— - counts/min. in 1.5 mm. slices of gel after electrophoresis of haemoglobin from cultures pulsed with ^{59}Fe between 0 and 5 hr. incubation.

·---· - counts/min. in 1.5 mm. slices of gel after electrophoresis of haemoglobin from cultures pulsed with ^{59}Fe between 23 and 28 hr. incubation.

 - position on the gel of the visible bands separated in the haemoglobin samples shown.

Figure 52


a.
Adult
20-day foetal
750
500
250
counts/min.
10
20
30
40
50
Distance from origin mm.


b.
Adult
20-day foetal
750
500
250
counts/min.
10
20
30
40
50
Distance from origin mm.

The effect of trypsinisation on the pattern of haemoglobin synthesis

Pooled 20-day foetal livers were finely chopped and divided into two aliquots. One aliquot was treated with cold trypsin overnight and incubation was begun on the following day; the second aliquot was disaggregated by gentle pipetting in medium and incubation was started immediately. The latter method produced a cell suspension containing many clumps so that the cell counts were probably inaccurate. Both sets of cultures were pulsed twice with ^{59}Fe during incubation; the pattern of haemoglobin synthesis was determined and is shown in Figure 52. It was found that trypsinisation had no effect on the relative amounts of the components synthesised during each pulse; the proportion of the activity associated with each component was the same during both pulses. Thus, the pattern of haemoglobin synthesis in these culture conditions may be regarded as a true reflection of the *in vivo* activity of the cells. That the response to erythropoietin was also unaffected by trypsinisation has already been demonstrated (Figure 19).

Proportions of haemoglobin components produced during yolk sac and foetal liver erythropoiesis. The relative amounts of the haemoglobin components synthesised during the initial period of culture without erythropoietin treatment was found to vary as the liver developed; this suggested that the proportions of the components produced may be characteristic of the age of the foetus. The observations were extended

Figure 53

The pattern of haemoglobin synthesis in 16-day rat foetal liver cells.

The cells were pulsed with ^{59}Fe between 0 and 9 hr. incubation.

The haemoglobin components were separated by starch gel electrophoresis in buffer C; 16-day foetal haemoglobin was added as marker.

•——• - counts/min. in 1.5 mm. slices of gel.

 - position on the gel of the visible bands separated in the haemoglobin samples shown.

Figure 53

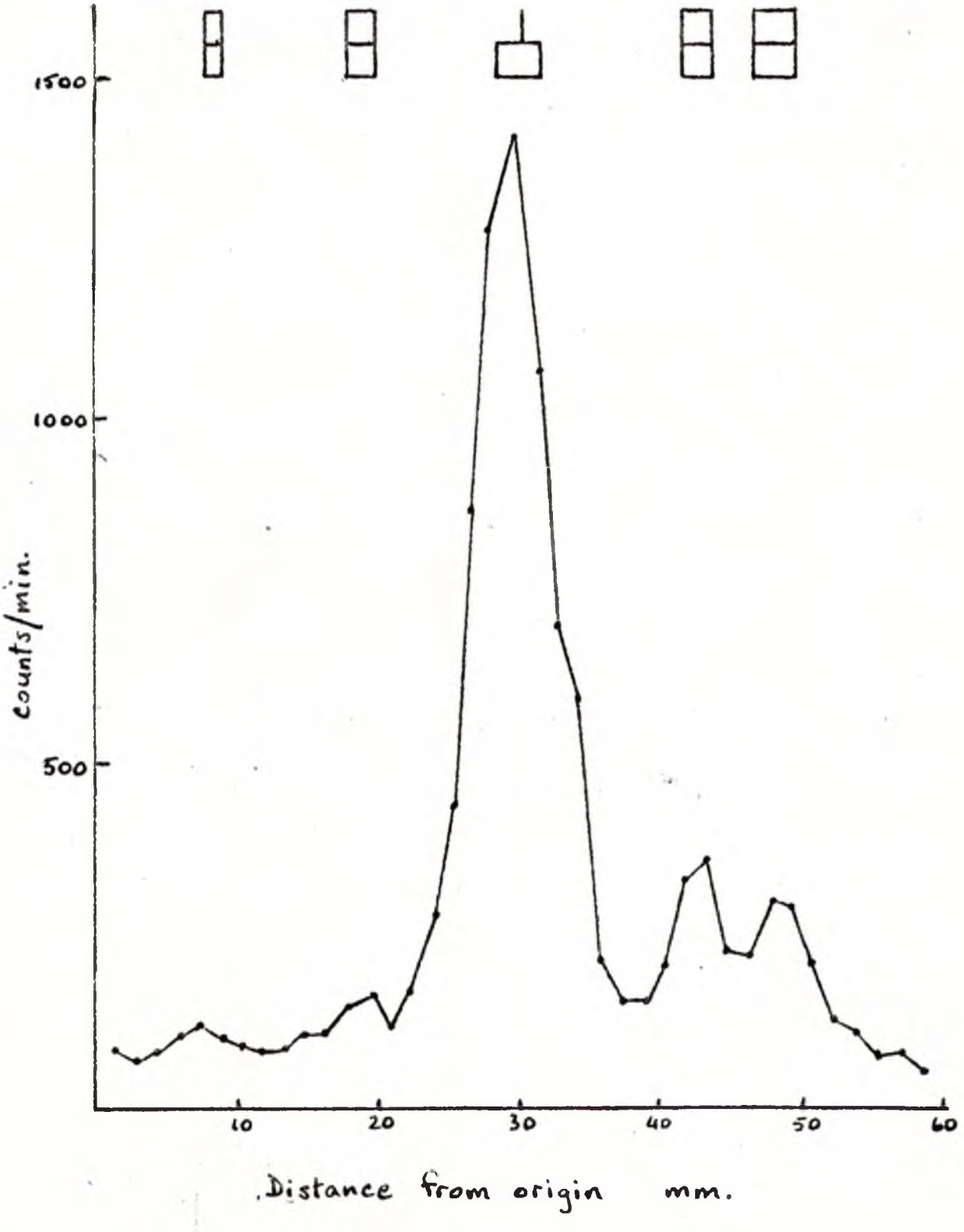

1500
1000
500
counts/min.
10
20
30
40
50
60
Distance from origin mm.

Figure 54

The pattern of haemoglobin synthesis in neonatal rat liver cells.

The haemoglobin components were separated by starch gel electrophoresis in buffer C; 20-day foetal haemoglobin was added as marker.

•——• - counts/min. in 1.5 mm. slices of gel after electrophoresis of haemoglobin from cultures pulsed with ^{59}Fe between 0 and 6 hr. incubation.

•– – –• - counts/min. in 1.5 mm. slices of gel after electrophoresis of haemoglobin from cultures pulsed with ^{59}Fe between 22 and 28 hr. incubation.

 - position on the gel of the visible bands separated in the haemoglobin samples shown.

Figure 54

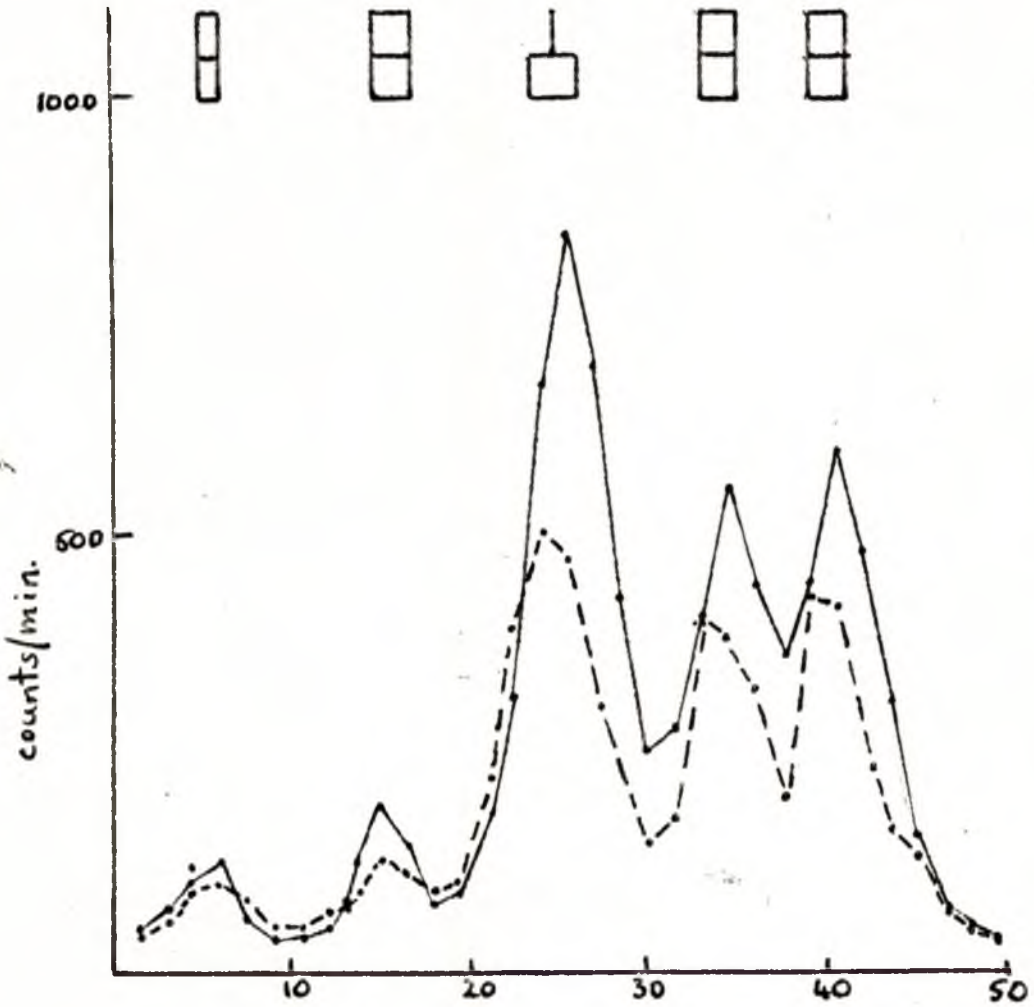


Figure 55

The relative amounts of the components of rat haemoglobin synthesised by yolk sac and foetal liver cells of different ages.

•——• - proportion of haemoglobin 'a'.

○- - -○ - proportion of haemoglobin 'b'.

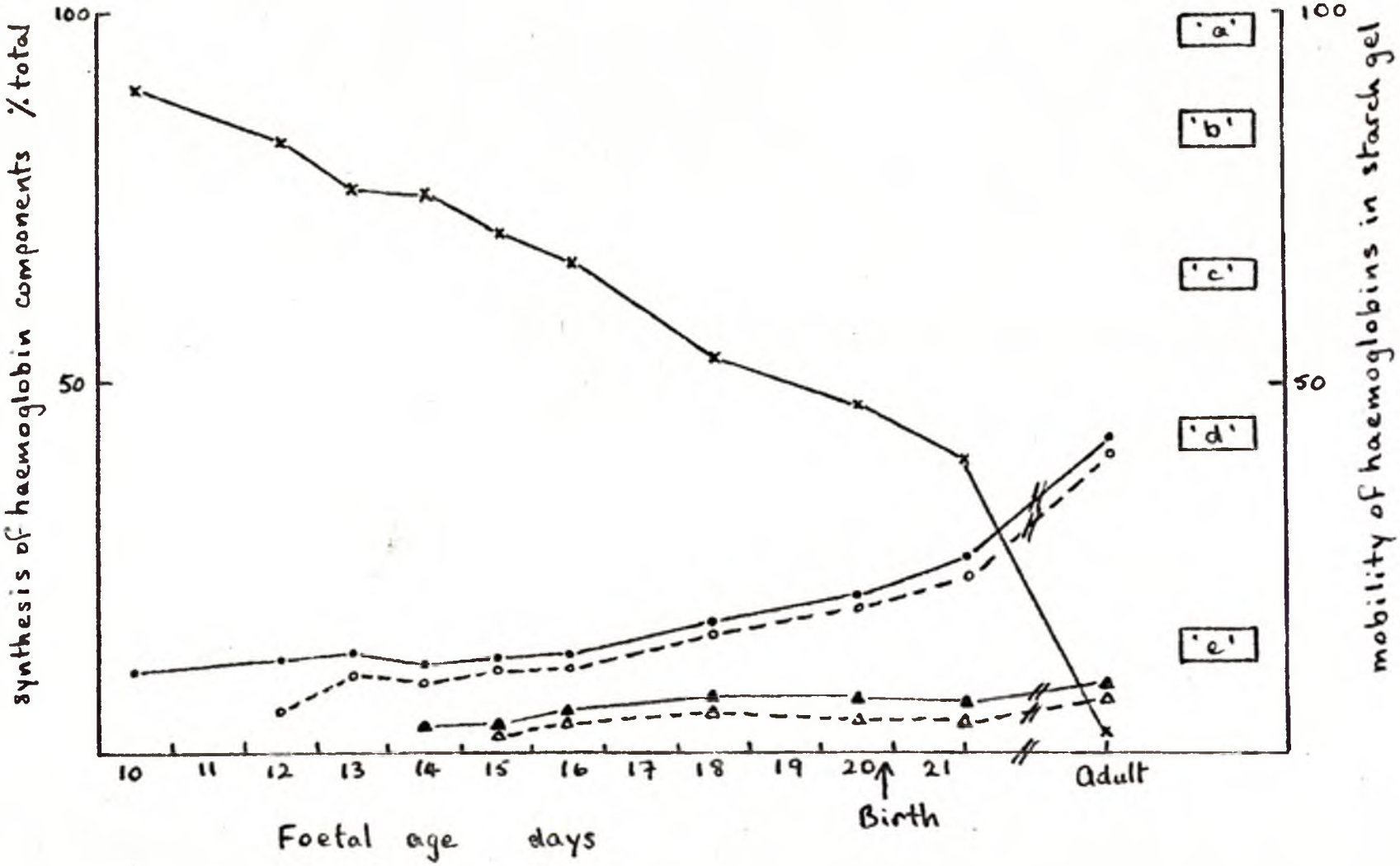
×——× - proportion of haemoglobin 'c'.

▲——▲ - proportion of haemoglobin 'd'.

△- - -△ - proportion of haemoglobin 'e'.

 - mobility of each component during starch gel electrophoresis in buffer C, expressed as a percentage of the mobility of haemoglobin 'a'.

Figure 55


synthesis of haemoglobin components % total
100
50
'a'
'b'
'c'
'd'
'e'
100
50
mobility of haemoglobins in starch gel
10
11
12
13
14
15
16
17
18
19
20
21
Adult
Birth
Foetal age days

to 16-day foetal liver cells (Figure 53); all five components were detected. Neonatal liver cells were also studied (Figure 54); again all five components were found, in proportions differing from the pattern observed in the cell cultures of the younger livers. The proportion of each component was the same during both pulses with ^{59}Fe.

The changes in the proportion of each haemoglobin component synthesised during rat yolk sac and foetal liver erythropoiesis are shown in Figure 55; this is based on data from most of the preceding experiments and the adult pattern is included for comparison. There was a continual decrease in the proportion of haemoglobin 'c'; the rate at which it decreased appeared to be unaffected by the appearance of the other components or by birth. The proportions of haemoglobins 'a' and 'b' remained relatively constant until day 16; thereafter they increased at similar rates. The proportions of haemoglobins 'd' and 'e' increased slightly until day 18; thereafter they remained relatively constant until after birth. This pattern agrees quite well with the pattern of visible bands observed in foetal blood samples during gestation.

<u>The pattern of haemoglobin synthesis in foetal spleen cells</u>. The pattern of haemoglobin synthesis shown in Figure 55 may be determined by the development of the foetal liver or by the development of the whole foetus. To determine whether the pattern of haemoglobin synthesis

Figure 56

The pattern of haemoglobin synthesis in 20-day foetal spleen cells.

The haemoglobin components were separated by starch gel electrophoresis in buffer C; 20-day foetal haemoglobin was added as marker.

.——. -counts/min. in 1.5 mm. slices of gel after electrophoresis of haemoglobin from cultures pulsed with ^{59}Fe between 0 and 5 hr. incubation.

.— —. -counts/min. in 1.5 mm. slices of gel after electrophoresis of haemoglobin from cultures pulsed with ^{59}Fe between 24 and 29 hr. incubation.

▭ -position on the gel of the visible bands separated in the haemoglobin samples shown.

Figure 57

The pattern of haemoglobin synthesis in neonatal rat spleen cells.

The haemoglobin components were separated by starch gel electrophoresis in buffer C; 20-day foetal haemoglobin was added as marker.

.——. - counts/min. in 1.5 mm. slices of gel after electrophoresis of haemoglobin from cultures pulsed with ^{59}Fe between 0 and 6 hr. incubation.

.- - -. - counts/min. in 1.5 mm. slices of gel after electrophoresis of haemoglobin from cultures pulsed with ^{59}Fe between 22 and 28 hr. incubation.

▭ - position on the gel of the visible bands separated in the haemoglobin samples shown.

Figure 56

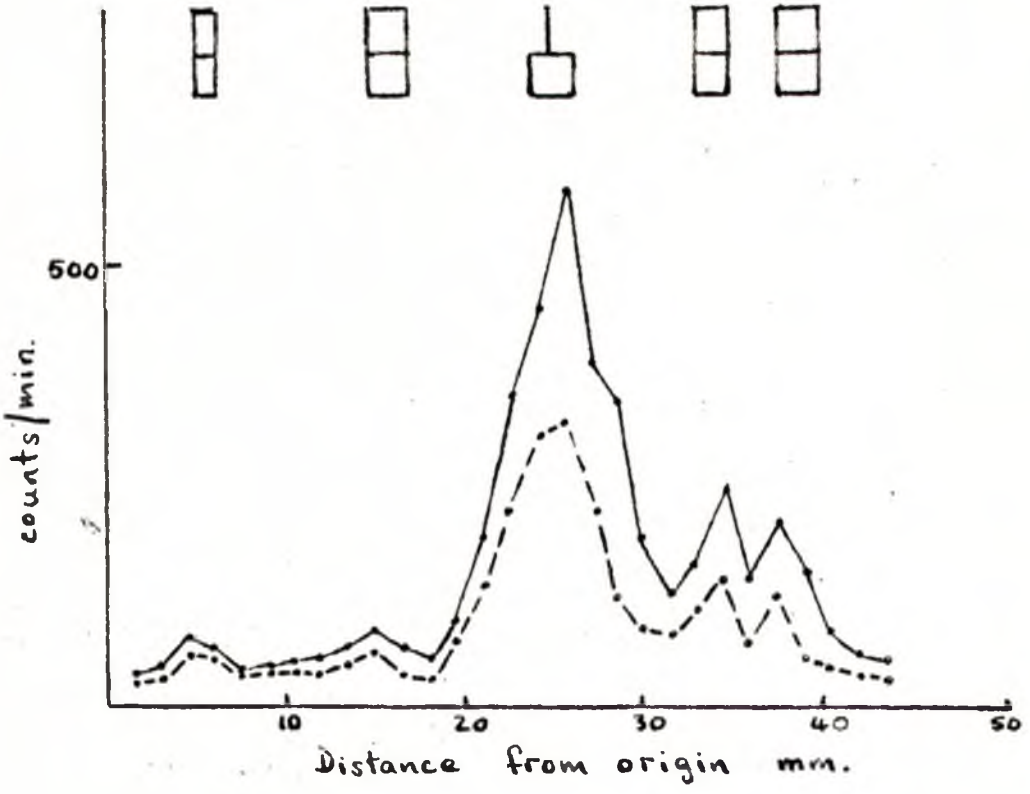


Figure 57

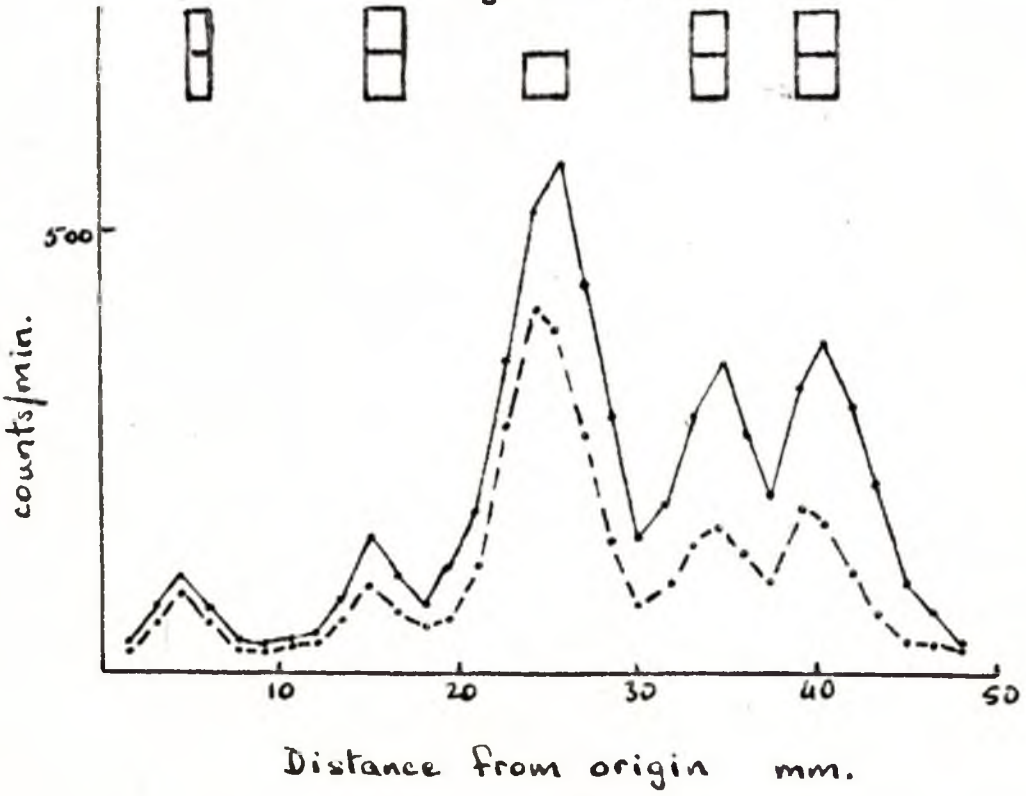


Table 17

The relative rates of syntheses of rat haemoglobin components during incubation of rat erythroid cells from different erythroid sites.

Tissue of origin	Incubation time hr.	Haemoglobin component. % total				
		'a'	'b'	'c'	'd'	'e'
12-day foetal liver	6	4.3	12.5	83.1		
	30	5.2	14.2	80.6		
13-day foetal liver	6	10.4	13.1	76.5		
	32	8.1	11.7	80.2		
14-day foetal liver	6.5	9.1	11.9	76.2	2.8	
	29.5	9.7	12.7	72.7	2.5	
15-day foetal liver	6	12.4	12.1	68.2	4.1	3.3
	30	12.7	12.7	66.0	4.3	4.2
18-day foetal liver	6	16.4	17.2	53.6	7.2	5.5
	28	16.1	16.5	55.2	7.2	5.0
20-day foetal liver	5	19.8	21.0	47.4	5.9	5.8
	28	18.7	19.1	50.1	5.8	6.3
neonatal liver	6	23.8	26.3	39.2	6.6	3.9
	28	22.1	27.7	40.1	4.7	5.4
20-day foetal spleen	5	17.6	21.3	50.3	4.8	6.0
	29	19.7	22.5	46.4	5.7	5.7
neonatal spleen	6	24.6	25.0	38.6	6.7	5.1
	28	22.8	23.9	40.3	6.4	6.6
adult bone marrow	4	41.8	40.4	2.7	8.0	7.1
	28	40.1	41.9	2.5	8.6	6.9

observed was characteristic only of foetal liver cells, the studies were extended to spleen cells.

Cultures of 20-day foetal spleen cells synthesised the five components in proportions (Figure 56) very similar to those observed in 20-day foetal liver cells (Figure 52); the proportion of each component was the same during both pulses. In cultures of neonatal spleen cells (Figure 57) the pattern found was similar to that found in neonatal liver cell cultures (Figure 54); again, the relative rates of syntheses of the components were the same during both pulses. These results suggest that the proportion of each component synthesised is characteristic of the developmental stage of the foetus and not of the development of the individual erythroid sites.

Relative rates of synthesis of haemoglobin components during maturation of rat erythroid cells. There was, in general, a decrease in the total amount of haemoglobin synthesis in control cultures with time; this is similar to the decrease in haem synthesis observed during incubation of control cultures (see section 1). The relative amounts of each component synthesised, however, appeared quite constant at both periods of incubation studied (Table 17). This suggested that each component was synthesised at a characteristic rate during at least 30 hr. of maturation. In addition, cells from younger livers which synthesised only three or four of the components did not begin to synthesise the remaining components during incubation, despite the

fact that cells *in vivo* began synthesis of the remaining components during the same period of time. The rate of synthesis of each component appeared to be determined by the progenitor cells, either stem cells or ESC; once the cells had begun to mature the rates appeared to be unalterable, at least *in vitro*. This phenomenon was observed in cultures of foetal liver and spleen cells and in adult bone marrow cells; it thus appeared to be characteristic of rat erythropoiesis.

Since the entire period of cell maturation might not have been included in the incubation periods studied, it was possible that the relative rates of synthesis might change late in maturation, when total haemoglobin synthesis was decreasing. This possibility was investigated in cultures of foetal blood cells.

The pattern of haemoglobin synthesis in 12-day blood cells resembled that of 10-day yolk sac cells, some 42 hr. earlier (Figures 41 and 42); this suggested that the relative rate of synthesis of each component was unchanged during yolk sac cell maturation also. The pattern observed in 16-day foetal blood cells (Figure 50) resembled that of 15-day liver cells (Figure 48), some 28 hr. earlier in gestation, but not that of 14-day liver cells (Figure 47), some 50 hr. earlier. This may indicate that erythroid cells were released from the liver between 28 and 50 hr. after the beginning of maturation; it is also possible that, *in vivo*, the cells may begin to synthesise new varieties of haemoglobin in the course of maturation. Cultures of neonatal blood cells possessed a pattern of haemoglobin synthesis

Figure 58

The pattern of haemoglobin synthesis in neonatal rat blood cells. The cells were pulsed with ^{59}Fe between 0 and 6 hr. incubation.

The haemoglobin components were separated by starch gel electrophoresis in buffer C; no marker haemoglobin was added.

.——. - counts/min. in 1.5 mm. slices of gel.

□ - position on the gel of the visible bands separated in the haemoglobin samples shown.

Figure 58

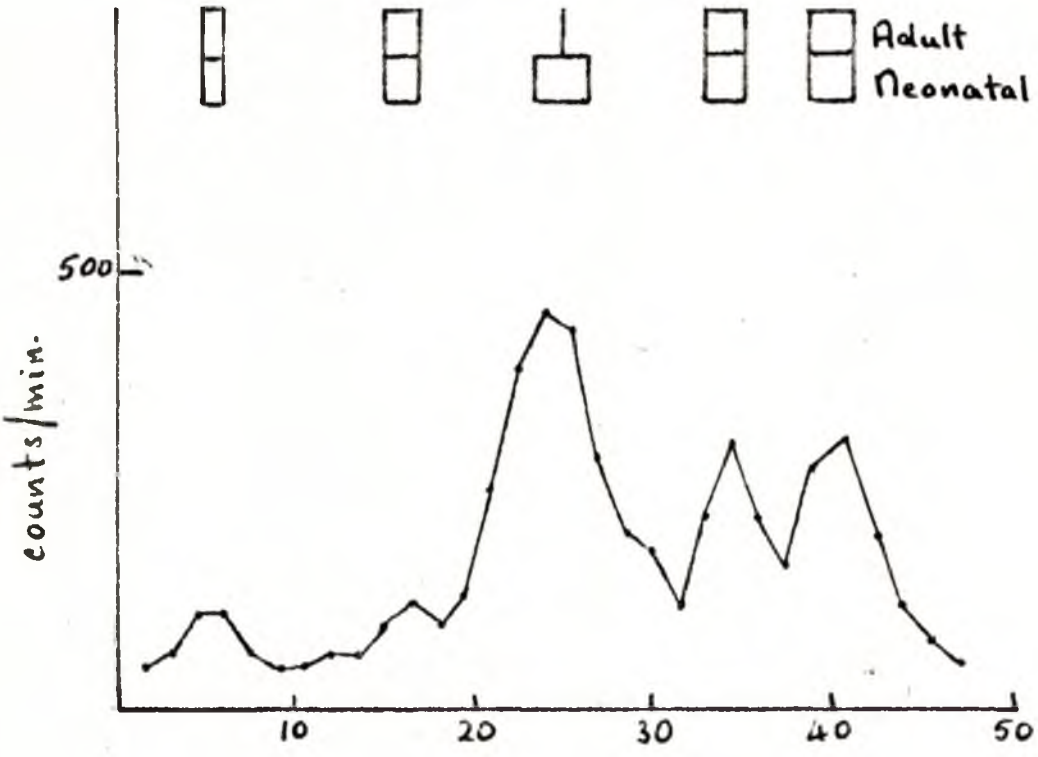

Adult
Neonatal
500
counts/min.
10
20
30
40
50
Distance from origin mm.

Table 18

The relative rates of syntheses of rat haemoglobin components by circulating blood cells in culture.

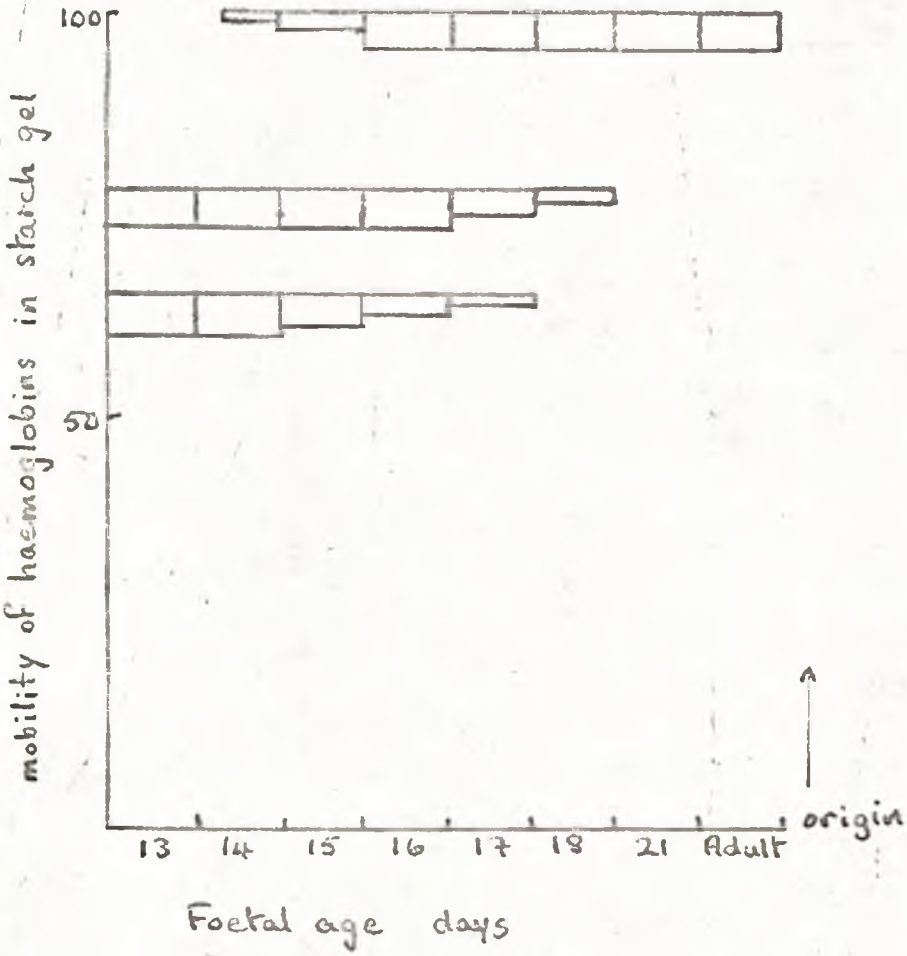
Foetal age dys.	Incubation time hr.	Haemoglobin component. % total				
		'a'	'b'	'c'	'd'	'e'
12	7		13.9	86.1		
16	9	12.6	13.0	65.4	4.8	4.1
21½ (neonatal)	6	20.2	19.6	48.0	6.5	6.1

Figure 59

Haemoglobins in foetal rabbit blood during gestation, separated by starch gel electrophoresis in buffer C.

 - position on the gel of the visible bands in haemoglobin samples from foetuses of the ages shown; the mobility of each haemoglobin is expressed as a percentage of the leading haemoglobin, adult haemoglobin.

Figure 59


100
50
mobility of haemoglobins in starch gel
origin
13
14
15
16
17
18
21
Adult
Foetal age days

(Figure 58) similar to that of 20-day liver and spleen cells, some 36 hr. earlier (Figures 52 and 56). The proportions of each component made in cultures of circulating cells of different ages are shown in Table 18. If the cells are released into the circulation between 30 and 50 hr. after maturation begins, these observations suggest that *in vivo*, as *in vitro*, the relative rate of synthesis of each component remains the same during much, if not all, of cell maturation.

7. Rabbit foetal haemoglobins

Samples of rabbit haemoglobin were separated in both buffer A and buffer C; the number of components resolved was the same in each case. The results described were obtained with buffer C.

Adult rabbit blood contained a single haemoglobin component which was taken as a standard. 13-day foetal blood contained two components, having mobilities 78% and 65% of that of the adult haemoglobin. Small amounts of adult haemoglobin were found on day 14 and its concentration increased rapidly thereafter; the amounts of the slower bands were unaltered on day 14. The slowest component became fainter on day 15, decreased steadily in density thereafter and was no longer detectable in 18-day foetal blood. The intermediate component decreased in density on day 17; it was still visible on day 18 but had vanished by day 21. These results are represented in Figure 59.

This pattern is basically similar to that found in mouse foetal blood during gestation, and it may be inferred that the slower com-

Figure 60

The effect of actinomycin D on the response of 14-day rat foetal liver cells to step 1 erythropoietin (Lot 117/1), at 0.25 u./10^6 cells, after precipitation of impurities at pH 4.3

•——• - rate of haem synthesis in untreated cultures

○——○ - rate of haem synthesis in erythropoietin-treated cultures

△- - -△ - rate of haem synthesis in cultures treated with 10 µg./ml. actinomycin D, incubated with or without erythropoietin.

The thickened portions of the abscissa indicate the period of incubation during which the cultures were pulsed with ^{59}Fe; the results are plotted at the mid-points of the pulses with ^{59}Fe.

Figure 61

The effect of FUdR and colchicine on the response of 14-day rat foetal liver cells to step 1 erythropoietin (Lot 117/1), at 0.25 u./10^6 cells, after precipitation of impurities at pH 4.3.

•——• - rate of haem synthesis in untreated cultures.

○——○ - rate of haem synthesis in erythropoietin-treated cultures.

⊗- - -⊗ - rate of haem synthesis in cultures incubated with 10^{-4}M-colchicine.

×- - -× - rate of haem synthesis in erythropoietin-treated cultures incubated with 10^{-4}M-colchicine.

□- · - · -□ - rate of haem synthesis in cultures treated with 10^{-4}M-FUdR, incubated with or without erythropoietin.

Pulses with ^{59}Fe indicated as in Figure 60.

Figure 60

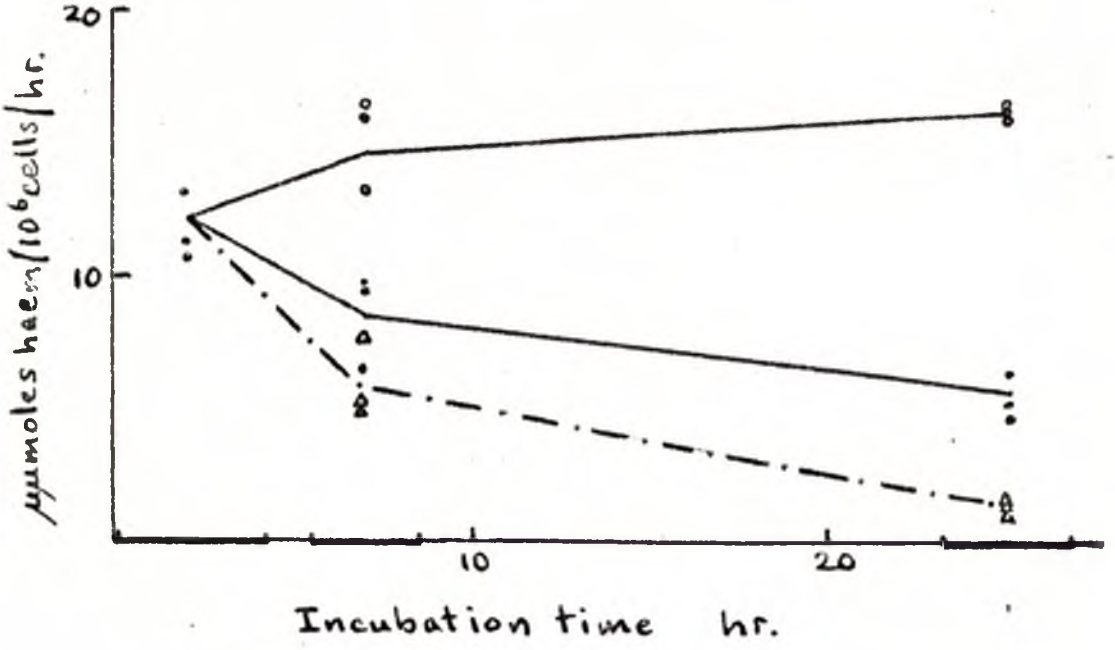
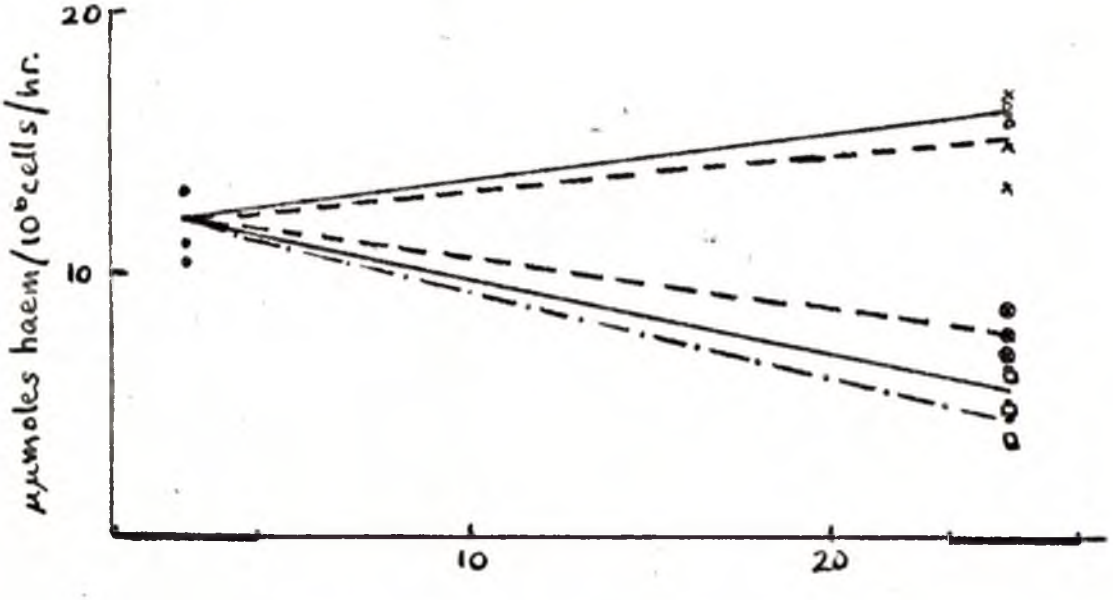


Figure 61



ponents were synthesised by yolk sac cells. The timing of the appearance and increase in quantity of the adult haemoglobin in foetal blood corresponded to the timing of the increase in the erythropoietic activity of the foetal liver (section 2), suggesting that, as in mouse, adult haemoglobin is synthesised by foetal liver cells.

8. Mode of action of erythropoietin on rat foetal liver cells

Although erythropoietin was able to increase the amount of haemoglobin synthesised by foetal liver cells of certain ages, it did not appear to affect haemoglobin synthesis directly. The manner in which the hormone exerted its effect on sensitive erythroid cells was therefore studied in more detail. Preliminary studies were carried out on 14-day rat foetal liver cells which showed a marked response to erythropoietin.

Haemoglobin synthesis begins some time after the onset of erythroid maturation (section I, 2) and is preceded by DNA and RNA syntheses, cell division and synthesis of other proteins. To determine which of these were essential to the increase in haem synthesis after erythropoietin treatment, the effect of colchicine, FUdR and actinomycin D on the response of foetal liver cells to erythropoietin was determined. Haem synthesis was measured at the times shown in Figure 60 and in Figure 61. It was found that FUdR and actinomycin D abolished the response to erythropoietin; actinomycin D also depressed haem synthesis in cultures without erythropoietin. Colchicine, on the other hand, had no visible effect on the response during 24 hr..

Figure 62

The response of 14-day rat foetal liver cells to step 2 erythropoietin, at 0.5 u./10^6 cells.

a. thymidine incorporation. The thickened portions of the abscissa indicate the periods of incubation during which the cells were pulsed with [^{3}H] thymidine; the results are plotted at the mid-points of the pulses.

•——• - rate of thymidine incorporation in untreated cultures.

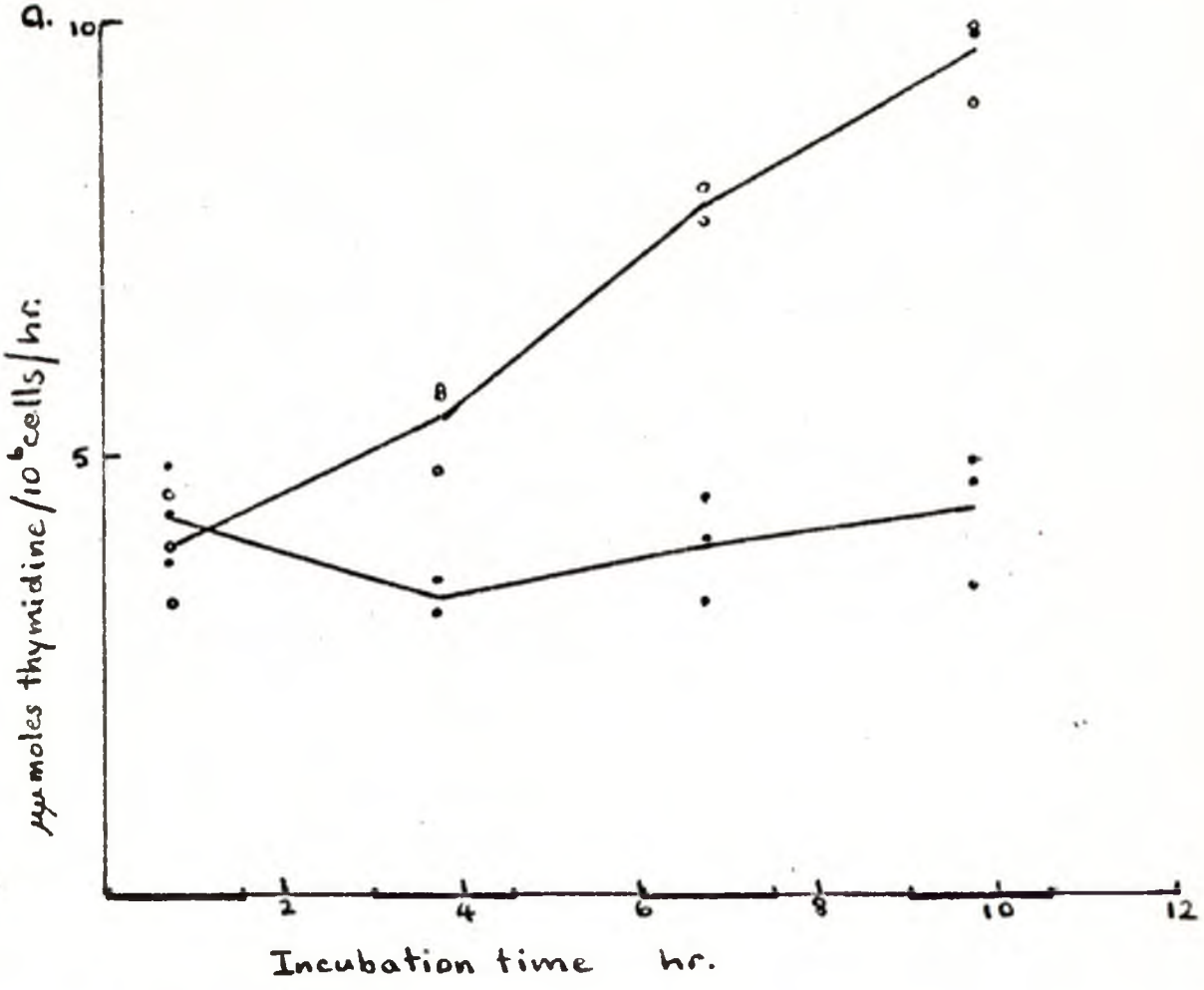
o——o - rate of thymidine incorporation in erythropoietin-treated cultures.

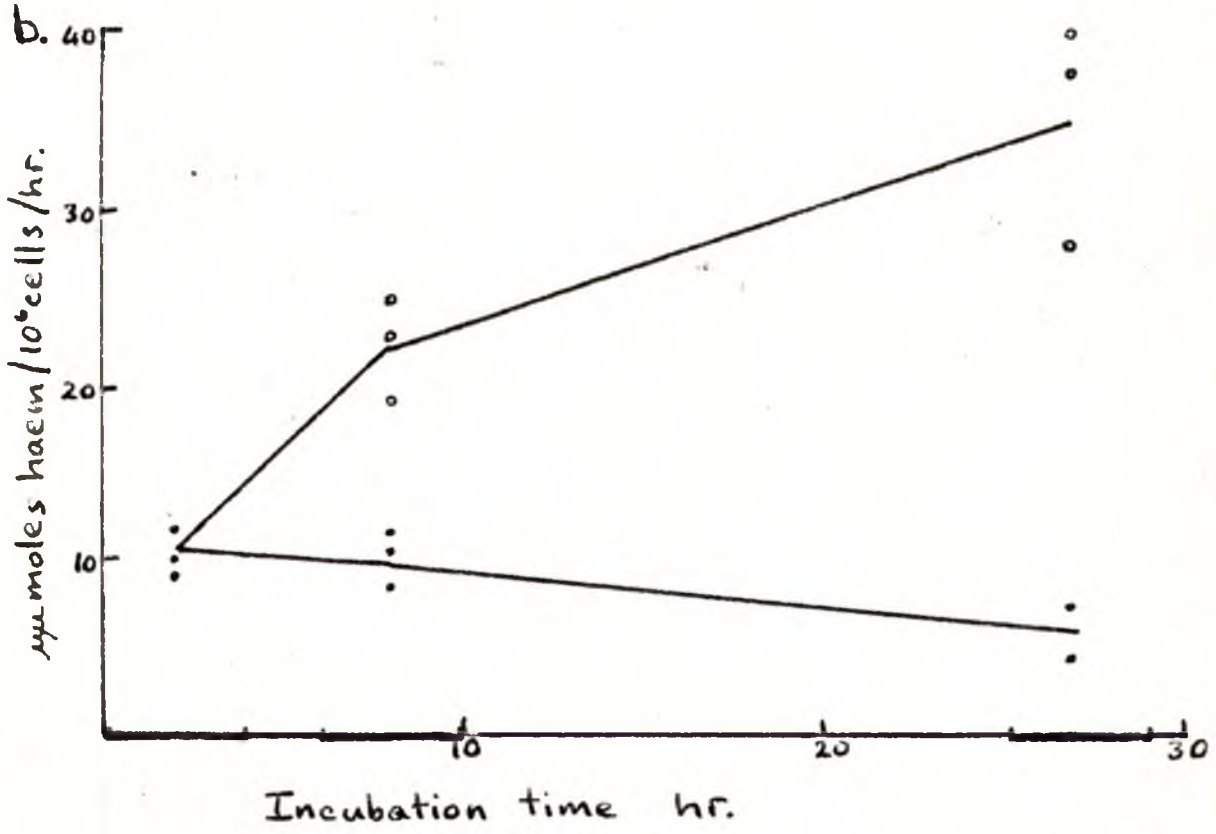
b. haem synthesis. Pulses with ^{59}Fe indicated as in Figure 60.

•——• - rate of haem synthesis in untreated cultures.

o——o - rate of haem synthesis in erythropoietin-treated cultures.

Figure 62


a.
10
5
μμ moles thymidine/10⁶cells/hr.
2
4
6
8
10
12
Incubation time hr.


b.
40
30
20
10
μμ moles haem/10⁶cells/hr.
10
20
30
Incubation time hr.

These results suggest that DNA and RNA syntheses were necessary for the increase in haem synthesis after erythropoietin stimulation; cell division, however, appeared to be unnecessary, at least for 24 hr. after hormone treatment. Actinomycin D reduced the rate of haem synthesis in control cultures, suggesting that it prevented detection of the response to erythropoietin, at least in part, by directly inhibiting haem synthesis; erythropoietin may have produced cellular alterations which were not detected because of the direct effect of actinomycin D on haem synthesis. FUdR, on the other hand, had little or no effect on haem synthesis by control cultures, indicating that haem synthesis, once initiated, could continue for some time without further DNA synthesis. Nevertheless, the effect of FUdR on the erythropoietin-treated cells demonstrated the importance of DNA synthesis in increasing the rate of haem synthesis.

The effect of erythropoietin on DNA synthesis itself was next examined; the effect of erythropoietin on haem synthesis in cultures from the same cell suspension was also observed. $[^3H]$ thymidine incorporation and haem synthesis were measured at the times shown in Figure 62. It was found that the rate of haem synthesis increased after erythropoietin treatment; in control cultures the rate decreased continually. The rate of thymidine incorporation in control cultures first decreased and then increased slowly until 10½ hr. incubation. In erythropoietin-treated cultures the rate of thymidine incorporation increased continually, more rapidly than in the controls, and had

Table 19

Experiment designed to determine the effect of haem synthesis of removing erythropoietin from foetal liver cell cultures after a limited period of incubation.

Groups of cultures	Period of incubation hr.		
	0 - 8	Spun at 800 g for 5 min.	8 - 26
	Erythropoietin		Erythropoietin
1	-	+	-
2	+	-	+
3	+	+	+
4	+	+	-

doubled after 10½ hr. incubation.

The presence of non-erythroid cells in liver cell suspensions forbids inferences as to the amount of thymidine incorporation due to erythroid cells only. However, unless erythropoietin, or the impurities present in the hormone preparation used, stimulated DNA synthesis in non-erythroid cells, it appeared that erythropoietin caused a rapid increase in DNA synthesis after treatment. Stimulation by erythropoietin of non-erythroid cells has not been reported, but it has not been excluded.

The continual increase in DNA synthesis after erythropoietin treatment may suggest that more cells began DNA synthesis as time passed; if this were so, it would indicate that, in these cultures, not all cells capable of responding to erythropoietin were stimulated immediately. Since the rate of DNA synthesis may not be constant throughout the cells' S period (Howard and Pelc, 1953) it is possible that the increase in the rate of DNA synthesis was due, at least in part, to this type of variation. These data are not sufficient to separate the two possibilities.

If the number of cells stimulated by erythropoietin increased with time, the magnitude of the response should depend upon the length of the exposure to the hormone. This was tested by the experiment shown in Table 19; as in subsequent experiments of this type, the medium was withdrawn after centrifugation and replaced with the least possible disturbance of the cells. Haem synthesis was measured at the

FIGURE 63.

The response of 14-day rat foetal liver cells to varying periods of treatment with step 1 erythropoietin (Lot 1/4), at 0.2 u./10^6 cells. The experiment was carried out as shown in Table 19.

•——• rate of haem synthesis in untreated cultures.

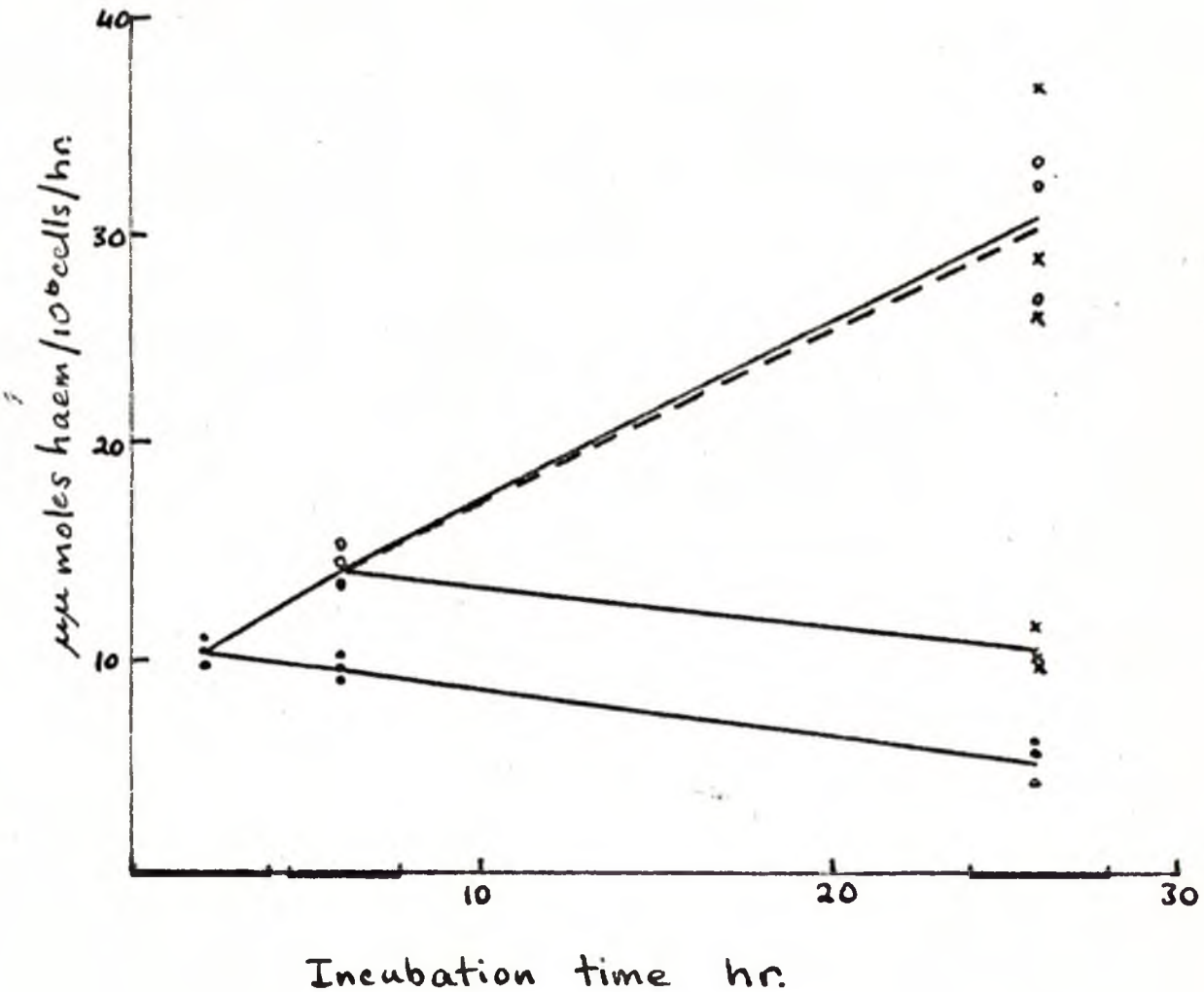
o——o rate of haem synthesis in erythropoietin-treated cultures, undisturbed during incubation.

x- - -x rate of haem synthesis in erythropoietin-treated cultures, returned to medium containing erythropoietin after centrifugation.

x——x rate of haem synthesis in erythropoietin-treated cultures returned to medium without erythropoietin after centrifugation.

Pulses with ^{59}Fe indicated as in Figure 60.

Figure 63


40
30
20
10
μμ moles haem/10⁹cells/hr.
10
20
30
Incubation time hr.

times shown in Figure 63. It was found that centrifugation had no effect on the response provided that erythropoietin was present during both periods of incubation; the rate of haem synthesis after 25 hr. treatment with erythropoietin, irrespective of centrifugation, was 6 times the rate in untreated cultures at the same time. After 26 hr. incubation the rate of haem synthesis in cultures exposed to erythropoietin for only 8 hr. was twice the rate in untreated cultures at the same time.

It appeared that only a partial response had occurred when erythropoietin was withdrawn after 8 hr.. If all ESC were immediately affected by erythropoietin, this suggested that the continued presence of the hormone was necessary to enable these cells to continue maturation. It is also possible that some sensitive cells were not stimulated within 8 hr.; these sensitive cells may be ESC derived during incubation from insensitive cells. They may also be maturing cells derived from hormone-stimulated ESC and retaining their sensitivity to erythropoietin as they mature; both these possibilities may combine to produce the result observed.

These preliminary experiments indicated not only that the effect of erythropoietin on individual cells might be very complex, but that the picture might be further confused by the possibility that the individual cells were responding to erythropoietin at different times after adding the hormone to the cultures. Despite the observed uniformity of the effect of erythropoietin on the synthesis of the different

FIGURE 64.

The effect of actinomycin D, puromycin and colchicine on the response of 13-day mouse foetal liver cells to step 1 erythropoietin (Lot 117/1), at 0.75 u./10^6 cells, after precipitation of impurities at pH 4.3; the ability of thymidine and of uridine to reverse the effect of FUdR.

●——● untreated cultures.

○——○ erythropoietin-treated cultures.

×-·--·-× cultures treated with 10^{-4}M-FUdR.

×-·--·-× cultures treated with erythropoietin and 10^{-4}M-FUdR.

▲---▲ cultures treated with 10^{-4}M-FUdR and 10^{-4}M-thymidine.

△---△ cultures treated with erythropoietin, 10^{-4}M-FUdR and 10^{-4}M-thymidine.

□·····□ cultures treated with 10^{-4}M-FUdR and 10^{-4}M-uridine.

□·····□ cultures treated with erythropoietin, 10^{-4}M-FUdR and 10^{-4}M-uridine.

Pulses with ^{59}Fe indicated as in Figure 60.

Figure 64

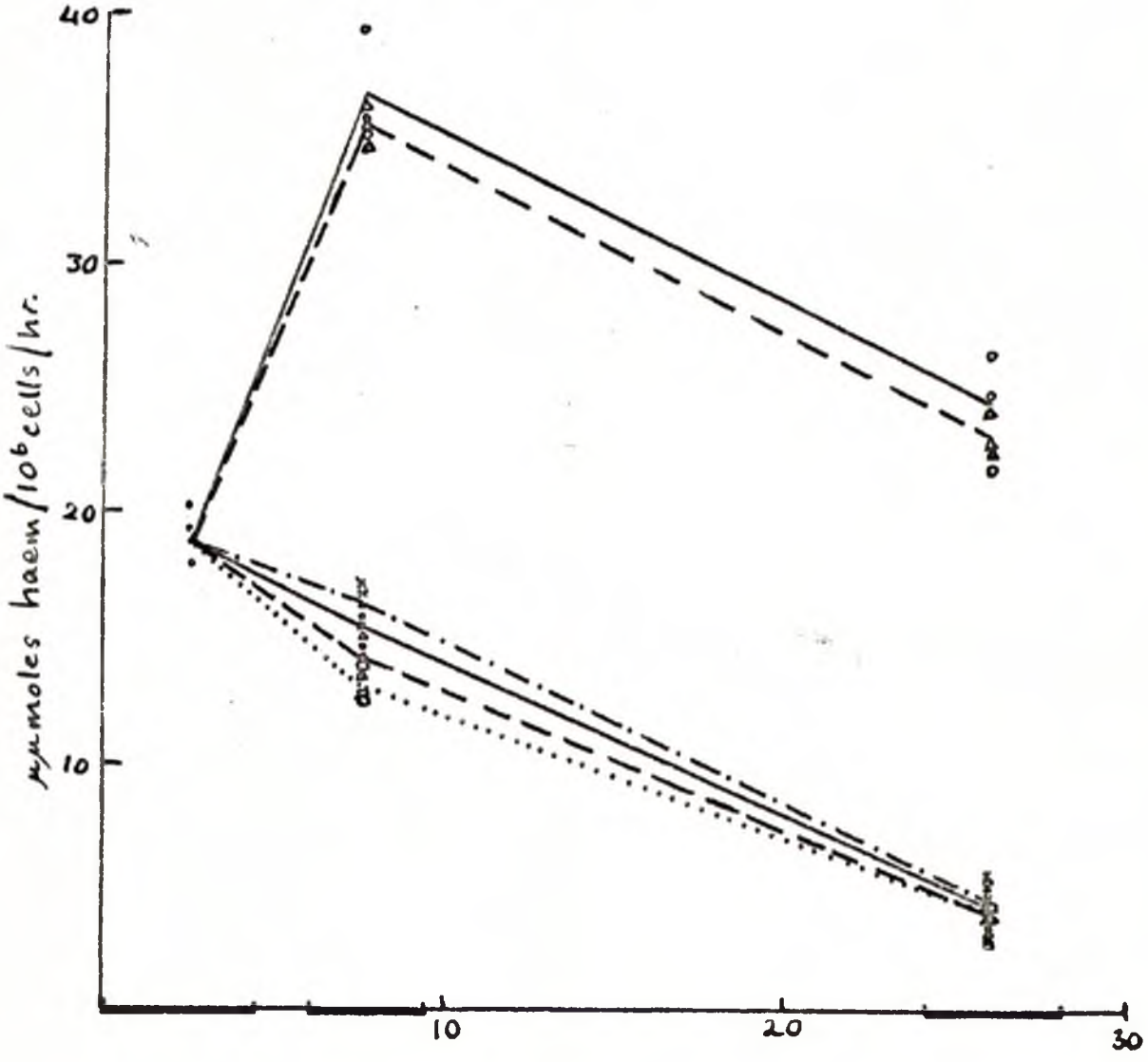

40
30
20
10
μmoles haem/10⁶cells/hr.
10
20
30
Incubation time hr.

FIGURE 65.

The effect of actinomycin D, puromycin and colchicine on the response of 13-day mouse foetal liver cells to step 1 erythropoietin (Lot 117/1), at 0.75 u./10^6 cells, after precipitation of impurities at pH 4.3

•——• untreated cultures.

○——○ erythropoietin-treated cultures.

×— - —·—× cultures treated with 10 µg./ml. actinomycin D.

×—·—·—× cultures treated with erythropoietin and 10 µg./ml. actinomycin D.

△·····△ cultures treated with 10^{-4}M-puromycin.

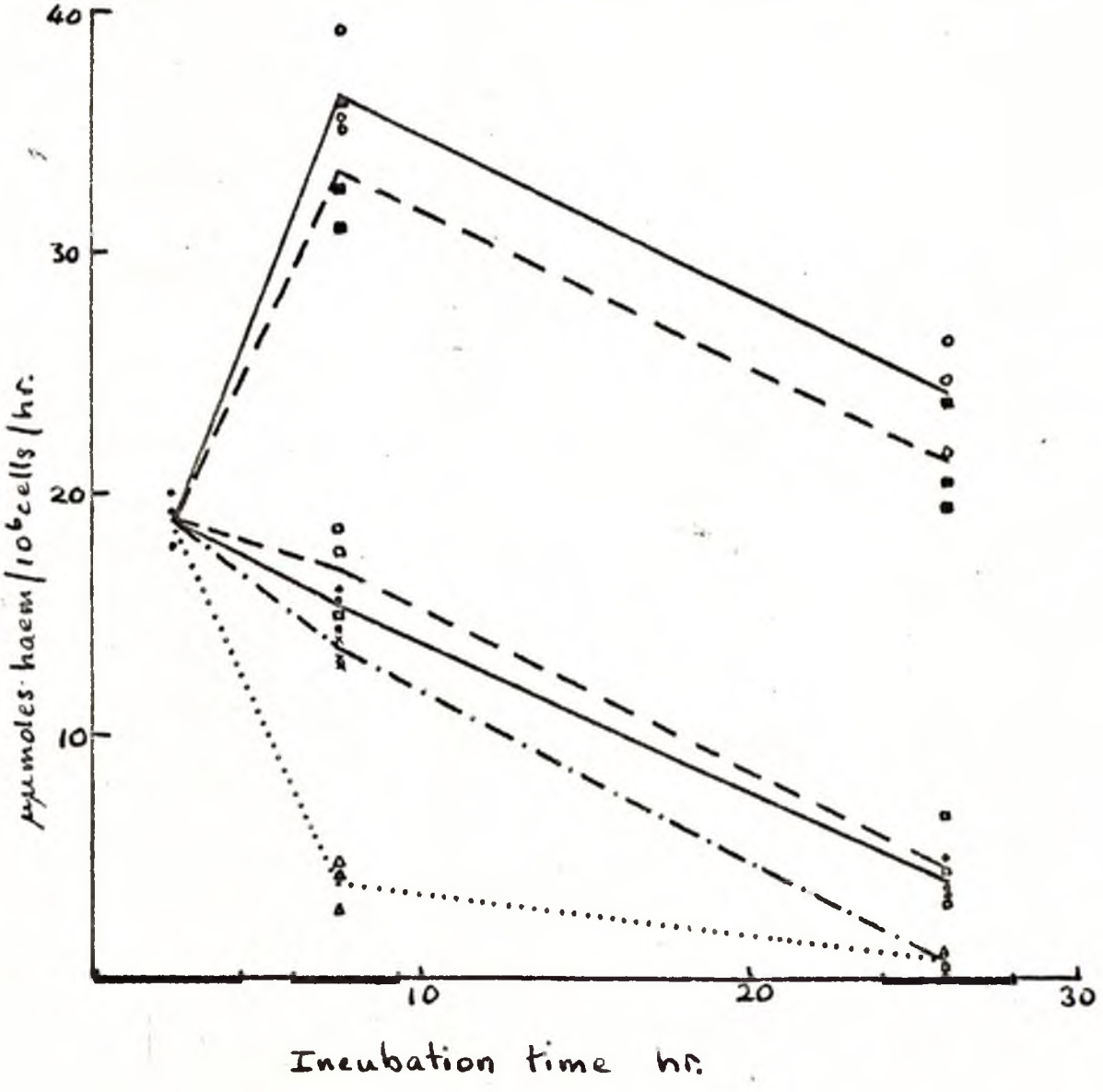
▲·····△ cultures treated with erythropoietin and 10^{-4}M-puromycin.

□ - - - -□ cultures treated with 10^{-4}M-colchicine.

■ - - - -■ cultures treated with erythropoietin and 10^{-4}M-colchicine.

Pulses with ^{59}Fe indicated as in Figure 60.

Figure 65


40
30
20
10
μμmoles haem/10⁶cells/hr.
10
20
30
Incubation time hr.

components of rat haemoglobin during 24 hr. incubation (section 5), it is possible that the effect of erythropoietin on other aspects of maturation may be less uniform. There is, for instance, no evidence that all of the haemoglobins are made by the same cells. It was felt that this possible complication should be eliminated if possible.

In the mouse only one type of haemoglobin is made by foetal liver cells, which suggests more strongly that only one cell series is involved. In addition, there is more information about the structure of mouse haemoglobin and the genetic control of its production; this may be of use to further studies of this nature. Accordingly, the subsequent experiments were performed on 13-day mouse foetal liver cells.

9. Mode of action of erythropoietin on mouse foetal liver cells

Effect of inhibitors on haem synthesis in response to erythropoietin.

The effects of FUdR, actinomycin D, puromycin and colchicine on the response of haem synthesis to erythropoietin were investigated; the ability of uridine and of thymidine to reverse the action of FUdR was also studied. Haem synthesis was measured at the times shown in Figure 64 and in Figure 65.

A picture emerged very similar to that observed in 14-day rat cells. FUdR abolished the response to erythropoietin but had little effect on the rate of haem synthesis in control cultures. Thymidine at a concentration equal to that of FUdR permitted the response to

proceed normally, while uridine at the same concentration did not. This suggests that FUdR had little or no effect on RNA synthesis; it appeared to prevent the response by inhibiting DNA synthesis. As in rat cells DNA synthesis thus appeared to be essential for the response to erythropoietin.

Actinomycin D and puromycin also abolished the response to erythropoietin; in addition these inhibitors depressed the rate of haem synthesis in control cultures, puromycin having the more rapid effect. This indicated that protein synthesis and RNA transcription were taking place in the control cultures. RNA synthesis and protein synthesis thus appeared to be necessary both for the initiation of haem synthesis in response to erythropoietin and for its continuation in the absence of erythropoietin. Colchicine did not prevent the response nor did it have any effect on the rate of haem synthesis in control cultures; although the mean rate of haem synthesis was lower in colchicine/erythropoietin-treated cultures than in erythropoietin-treated cultures, the disparity was small and the individual results from both groups of cultures overlapped.

Time-course of the response to erythropoietin. Studies with rat cells had suggested that an early step in the cells' response to erythropoietin was increased synthesis of DNA (Figure 62); also, FUdR had been bound to abolish the response of mouse cells to erythropoietin. The pattern of $[^3H]$ thymidine incorporation by mouse cells after erythro-

FIGURE 66.

DNA synthesis in 13-day mouse foetal liver cells treated with step 3 erythropoietin, at 0.2 u./10^6 cells.

•——• untreated cultures.

○——○ erythropoietin-treated cultures.

The thickened portions of the abscissa indicate the periods of incubation during which the cells were pulsed with [^{3}H] thymidine; the results are plotted at the mid-points of the pulses.

Figure 66

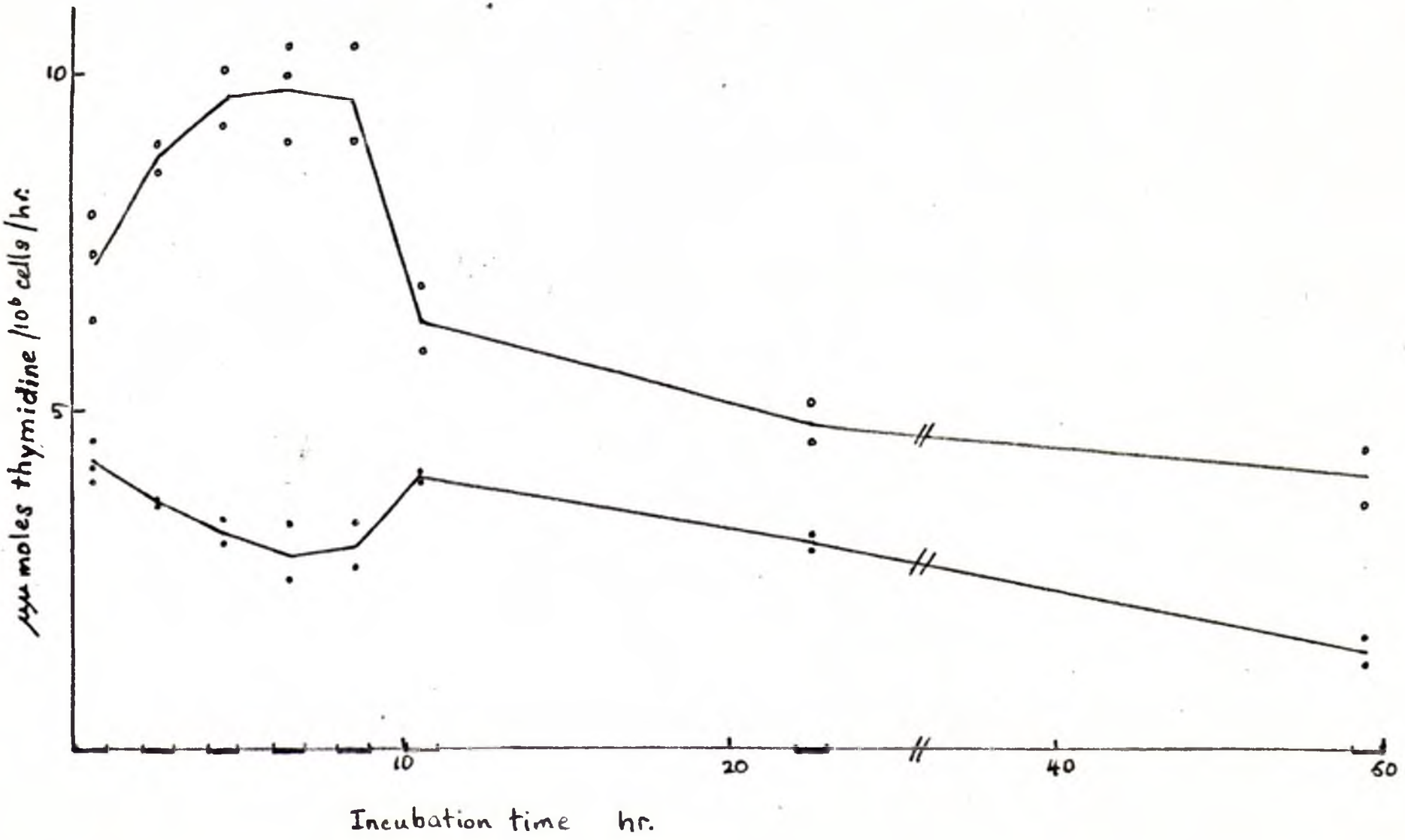

μμ moles thymidine /10⁶ cells /hr.
10
5
10
20
40
60
Incubation time hr.

FIGURE 67.

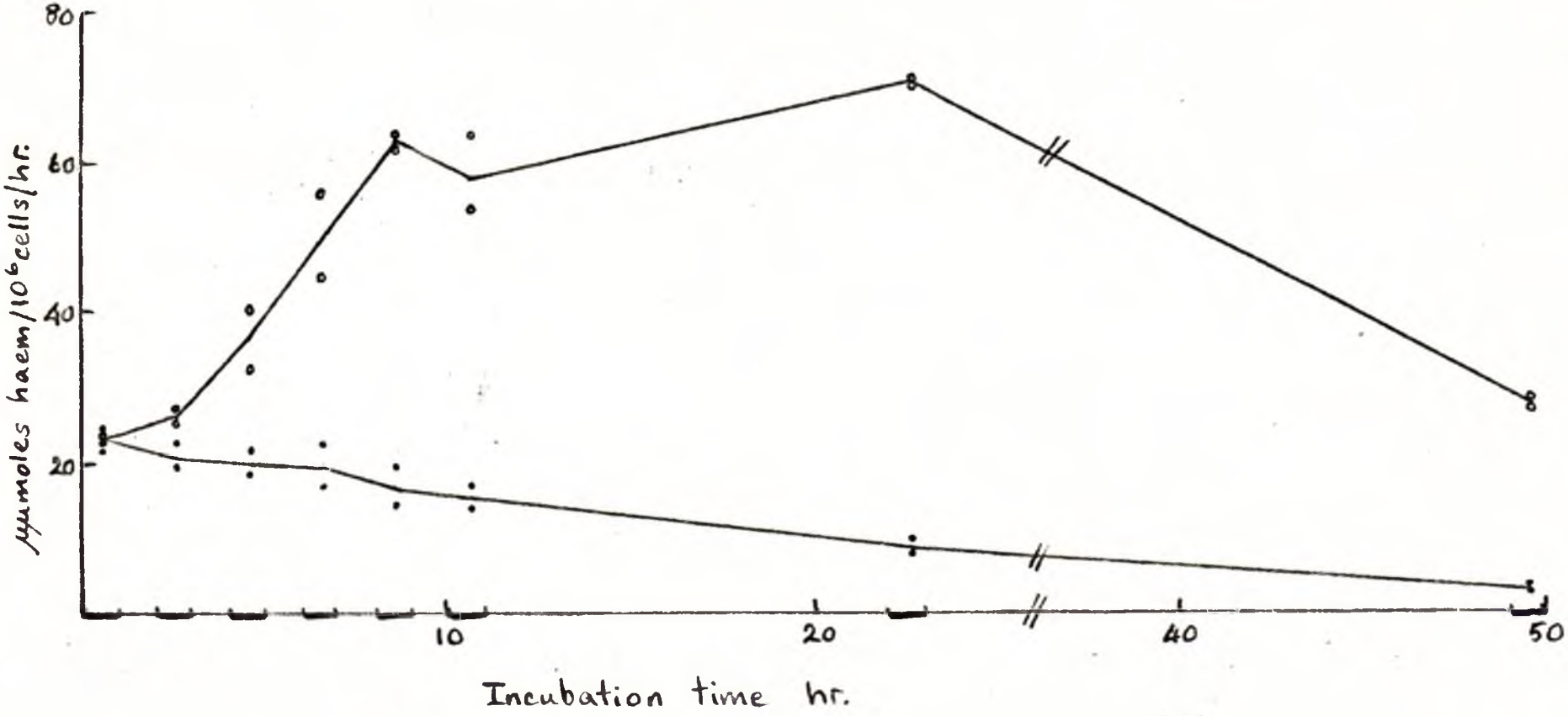
Haem synthesis in 13-day mouse foetal liver cells treated with step 3 erythropoietin, at 0.2 u./10^6 cells.

●——● untreated cultures.

○——○ erythropoietin-treated cultures.

Pulses with ^{59}Fe indicated as in Figure 60.

Figure 67


80
60
40
20
μmoles haem/10⁶cells/hr.
10
20
40
50
Incubation time hr.

poietin treatment was next examined; haem synthesis by the cells was measured during the same period. To time haem synthesis more precisely, the pulse with ^{59}Fe was reduced to 1 hr.; the incorporation into haem during this time was easily detectable by scintillation counting. The response of DNA synthesis to erythropoietin is shown in Figure 66; the response of haem synthesis to erythropoietin is shown in Figure 67.

DNA synthesis in control cultures decreased until 7 hr., rose until 10 hr. and then decreased again quite slowly. It is not known what proportion of the DNA synthesis was due to erythroid cells. In erythropoietin-treated cultures the rate of DNA synthesis during the first hour was double the rate in control cultures; the rate continued to increase until 7 hr., remained at this level until 9 hr. and then decreased. DNA synthesis in erythropoietin-treated cultures remained at a higher level than in control cultures throughout incubation; again it is not known how much of the observed incorporation was due to erythroid cells. Haem synthesis in control cultures decreased throughout incubation. In erythropoietin-treated cultures an increase in the rate of haem synthesis was perceptible after 3 hr.; the increase continued until, after 9 hr., the rate was 3 times the initial rate. There was a slight decrease at 11 hr., but after 23 hr. incubation the rate of haem synthesis was still 3.2 times the initial rate. It is not known how long the dip in haem synthesis observed at 11 hr. persisted nor whether the maximum observed at 23 hr. is the true maximum response of the cells. Similarly, it is not known whether

there was a second increase in DNA synthesis in erythropoietin-treated cells between 11 and 23 hr. incubation. The difference between the pattern of DNA synthesis observed here and that found in rat cells (Figure 62) may be due to a species difference or to the use of different erythropoietin preparations.

It can be seen that haem synthesis increased more slowly after erythropoietin treatment than did DNA synthesis; haem synthesis did not double in rate until some 6 hr. after the rate of DNA synthesis had doubled. This was probably a reflection of the later onset of haemoglobin synthesis in maturing cells (section I,2). A small increase in the rate of haem synthesis did, however, occur quite quickly after erythropoietin stimulation; it may be that cell maturation was somewhat accelerated in these culture conditions or that the histochemical techniques employed to study in vivo maturation were less sensitive than the present techniques.

The very rapid increase in DNA synthesis suggested that the cultures contained a large pool of ESC many of which responded immediately to erythropoietin; several explanations are possible for the subsequent alterations in the rate of synthesis. It may be that there occurred near-simultaneous stimulation of all available ESC to begin DNA synthesis; the pattern observed may be attributed to the stimulated ESC entering an S phase 7-9 hr. in length with a non-uniform rate of DNA synthesis. The persistently higher rate of DNA synthesis in erythropoietin-treated cultures may then be attributed to an increase

FIGURE 68.

The response of 13-day mouse foetal liver cells to varying periods of treatment with step 2 erythropoietin at 0.5 u./10^6 cells. The experiment was designed as shown in Table 19.

•——• untreated cultures.

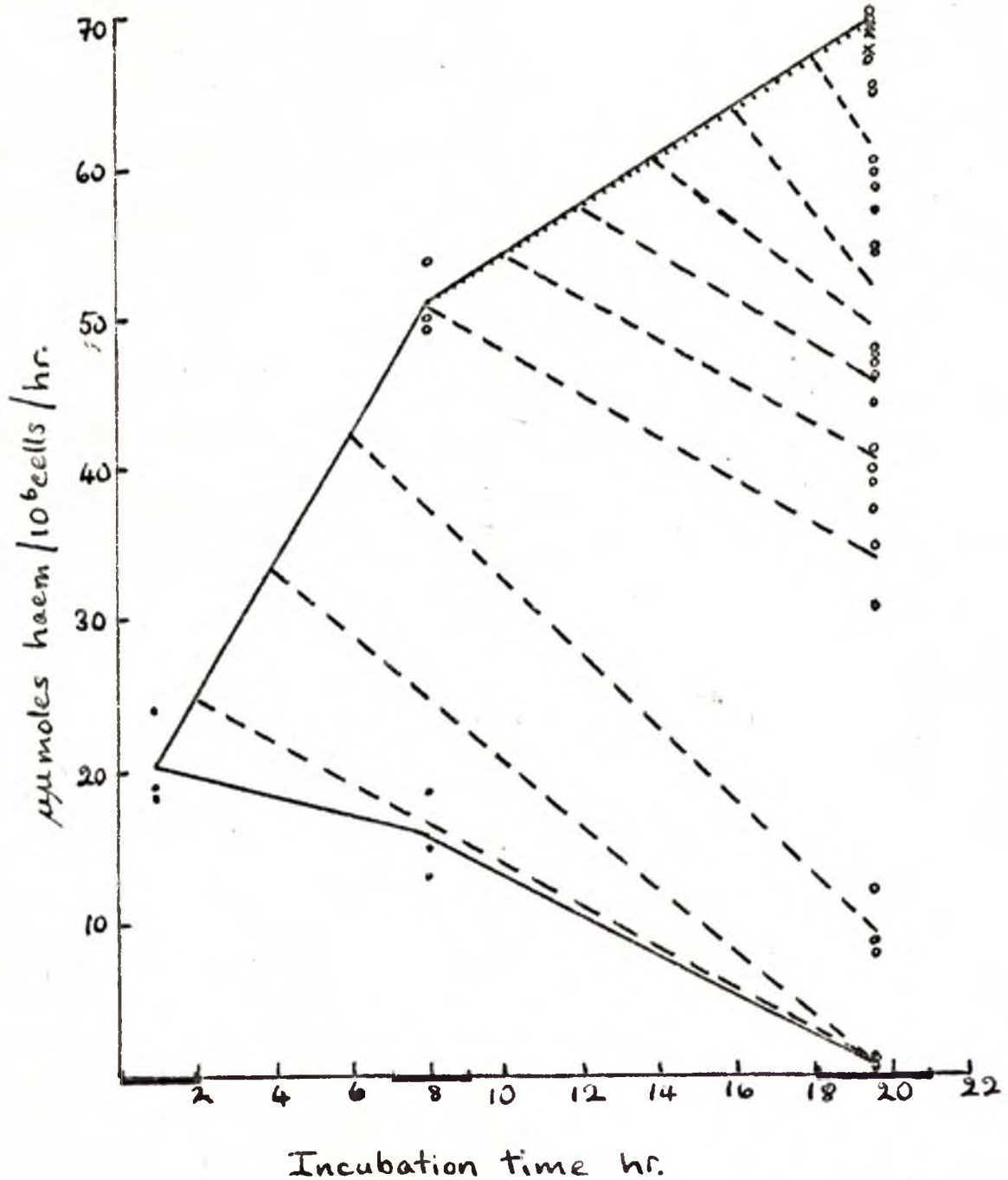
o——o erythropoietin-treated cultures, undisturbed during incubation.

x.....x erythropoietin-treated cultures, returned to medium containing erythropoietin after centrifugation at the end of 8 hours incubation.

o - - - - o erythropoietin-treated cultures, returned to medium without erythropoietin after centrifugation at the end of the incubation periods shown.

Pulses with ^{59}Fe indicated as in Figure 60.

Figure 68


70
60
50
40
30
20
10
μμ moles haem / 10⁶ cells / hr.
2
4
6
8
10
12
14
16
18
20
22
Incubation time hr.

in the number of maturing cells as a result of the early stimulation of ESC. It is also possible that not all ESC or potential ESC were immediately stimulated by erythropoietin and that the persistently higher rate of DNA synthesis was due to the stimulation of further ESC with time. In this case it would appear that many of the available ESC were stimulated after 9-10 hr. and that relatively fewer ESC were stimulated after this time. The pattern may also result from a combination of these possibilities. The high rate of haem synthesis reached after 9 hr. was maintained and even slightly increased after 23 hr.; this may indicate an increase in the rate of haem synthesis as the cells mature or the initiation of haem synthesis in a greater number of cells.

Continual stimulation of cells by erythropoietin during incubation.

It appeared that, in cultures of rat foetal liver cells, the number of cells stimulated by erythropoietin increased with time (Figure 63); the time-course of the response to erythropoietin of mouse cells was also open to this interpretation. To determine the effect on the response of different periods of exposure to erythropoietin, an experiment of the type shown in Table 19 was performed. The effect on haem synthesis of periods of erythropoietin treatment ranging from 2-21 hr. was examined. Haem synthesis was measured at the times shown in Figure 68.

It was found that centrifugation of the cells after 8 hr. incubation did not affect the magnitude of the response after 21 hr.

FIGURE 69.

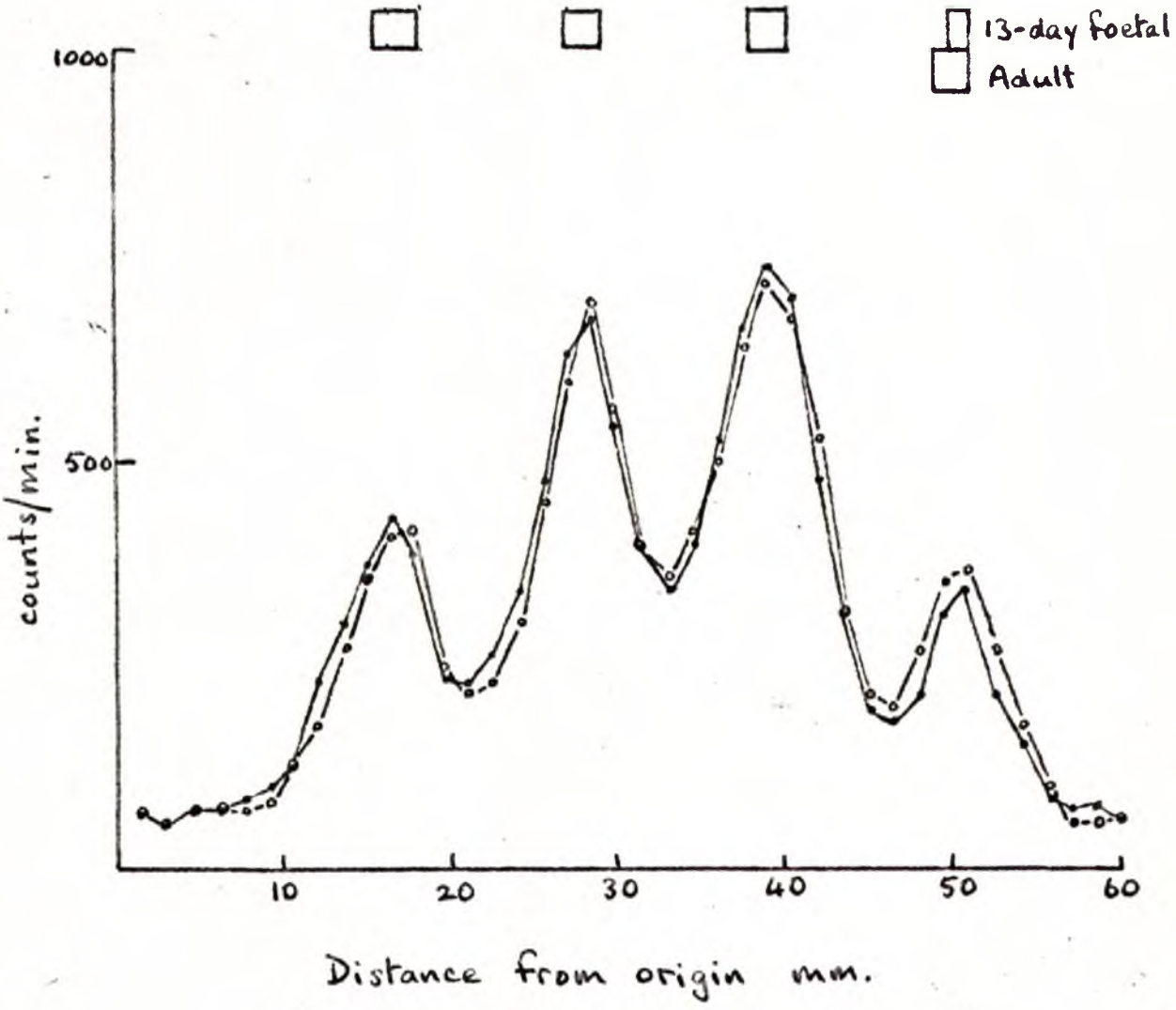
The pattern of haemoglobin synthesis in 13-day foetal mouse blood cells treated with step 1 erythropoietin (Lot 117/1), at 0.75 u./10^6 cells, after precipitation of impurities at pH 4.3.
The cells were pulsed with ^{59}Fe between 0 and 9 hours incubation.
The haemoglobins were separated by starch gel electrophoresis in buffer A containing 1.0% v/v mercaptoethanol, no marker haemoglobin was added.

•——• counts/min. in 1.5 mm. slices of gel after electrophoresis of haemoglobin from untreated cultures.

o——o counts/min. in 1.5 mm. slices of gel after electrophoresis of haemoglobin from erythropoietin-treated cultures.

□ position on the gel of the visible bands separated in the haemoglobin samples shown.

Figure 69


13-day foetal
Adult
1000
500
counts/min.
10
20
30
40
50
60
Distance from origin mm.

incubation, provided that erythropoietin was present during both periods of incubation. The effect of exposure to erythropoietin for only 2-4 hr. was not detectable after 21 hr. incubation; longer periods of exposure produced a progressively greater effect. It was possible that erythropoietin had no effect on the cells during 2-4 hr. treatment; since it had previously been found that the cells were affected within this time (Figures 66 and 67), it appeared more likely that the effect produced did not persist for 17-18 hr. in the absence of erythropoietin. The increased response after longer treatment with erythropoietin may be due to stimulation of further ESC by erythropoietin during incubation or to an effect of erythropoietin upon already maturing cells. It had been found that the response of circulating cells from 16-day rat foetuses was so small that it suggested that erythropoietin did not stimulate haemoglobin synthesis directly (Figure 50). The effect of erythropoietin on 13-day mouse blood cells (Figure 69) was also very small; just over 10%. This suggested that, in mouse cells also, erythropoietin had little effect on the non-nucleated cells; the response was probably due to stimulation of ESC or immature cells in the circulation. Together with the need for DNA synthesis before erythropoietin treatment could increase the rate of haem synthesis in liver cell cultures (Figure 64), this indicated that erythropoietin did not directly affect haemoglobin synthesis; if erythropoietin provoked a response from cells other than ESC (section I,3), these cells must have been capable of DNA synthesis, i.e. cells which had not matured beyond the polychromatic normoblast stage (section I,2).

FIGURE 70.

The response of 13-day mouse foetal liver cells to varying periods of treatment with step 3 erythropoietin at 0.2 u./10^6 cells. The experiment was designed as shown in Table 19.

•——• untreated cultures.

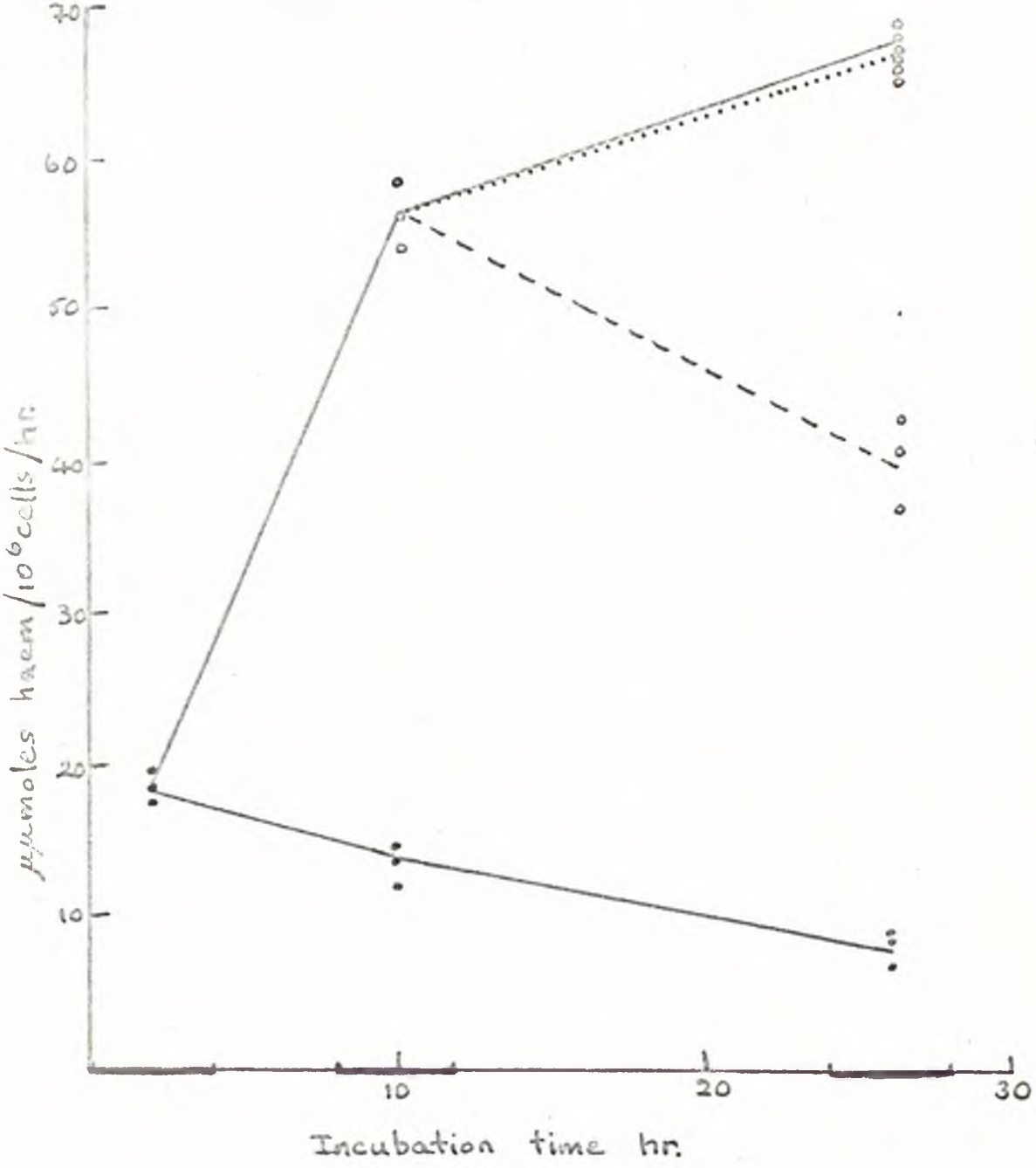
o——o erythropoietin-treated cultures.

o.....o erythropoietin-treated cultures, spun at 800 g for 5 min. after 12 hours incubation and returned to medium containing erythropoietin.

o - - - o erythropoietin-treated cultures, spun at 800 g for 5 min. after 12 hours incubation and returned to medium without erythropoietin.

Pulses with ^{59}Fe indicated as in Figure 60.

Figure 70


70
60
50
40
30
20
10
μμmoles haem/10⁶cells/hr.
10
20
30
Incubation time hr.

If, however, sensitivity to erythropoietin is restricted to ESC, the question arises as to how some of these cells remained unaffected by the hormone for up to 18 hr. in culture. Since a relatively crude erythropoietin preparation was used, it was possible that there might be some delay in its effect on some cells. The experiment was repeated, exposing the cells for 12 hr. to the step 3 preparation which had been found to increase haem synthesis rapidly (Figure 67); the response was measured after 26 hr. and compared to that produced in cultures exposed to erythropoietin throughout this period. As shown in Figure 70, the rate of haem synthesis after 26 hr. exposure to erythropoietin was 8.4 times the rate in untreated cultures at the same time; the rate in cultures exposed to erythropoietin for only 12 hr. was 5 times the rate in untreated cultures. The dependence of the magnitude of the response upon the length of treatment with erythropoietin had now been observed in cells from two species treated with three different hormone preparations (Figures 63, 68 and 70); it seemed likely that it was characteristic of the effect of erythropoietin itself on the cell cultures and not dependent on the degree of purification of the hormone.

It appeared that ESC might persist in the cultures for some time even in the presence of erythropoietin. It may be that the hormone concentration is limiting and that those cells first affected removed so much erythropoietin from the medium that there was too little to stimulate all sensitive cells present until the affected cells released

Table 20

Experiment designed to determine the survival in culture of cells capable of responding to erythropoietin.

Groups of cultures	Time at which erythropoietin was added. hr.	Period during which haem synthesis was observed. hr.
1	-	0 - 24
2	0	0 - 24
3	-	24 - 48
4	24	24 - 48
5	-	48 - 72
6	48	48 - 72
7	-	72 - 96
8	72	72 - 96

FIGURE 71.

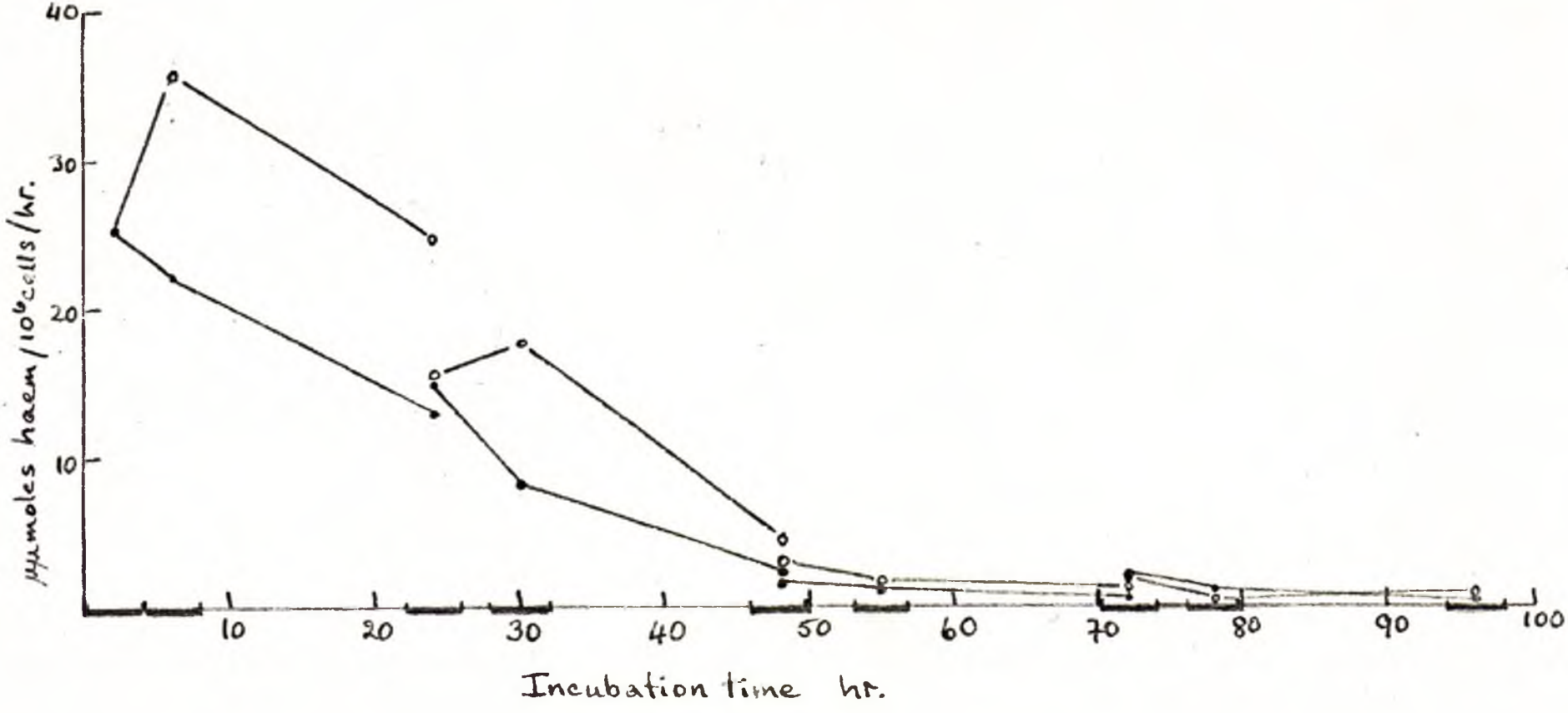
The survival of erythropoietin-sensitive cells *in vitro*. The experiment was carried out as shown in Table 20; step 1 erythropoietin, (Lot 117/1), was added at 0.75 u./10^6 cells, after precipitation of impurities at pH 4.3.

•——• untreated cultures.

o——o erythropoietin-treated cultures.

Pulses with ^{59}Fe indicated as in Figure 60.

Figure 71


40
30
20
10
μμmoles haem/10⁶cells/hr.
10
20
30
40
50
60
70
80
90
100
Incubation time hr.

it some hours later. It is also possible that ESC may be produced in culture from progenitor cells. The results may be attributed to either of these suggestions; however, evidence supporting either, or both, might not exclude the possibility of a stimulatory effect of erythropoietin on immature, dividing normoblasts also.

These results implied that the culture medium was able to maintain erythropoietin-sensitive cells for up to 18 hr. To determine whether a response could be elicited from cells after longer periods of incubation the experiment shown in Table 20 was performed. After 48 hr. all of the remaining cultures were spun at 800 g for 5 min., their medium was removed and replaced with fresh medium. Haem synthesis was measured at the times shown in Figure 71. The cells responded to erythropoietin added at 0 or 24 hr., but not later; since the response had been shown to be unaffected by centrifugation (Figure 68), this was probably not responsible for the disappearance of sensitive cells between 24 and 48 hr. incubation. In addition, the response was smaller after 24 hr. than at 0 hr., suggesting that the number of sensitive cells was decreasing throughout incubation without erythropoietin. The response observed in this experiment was small and of short duration; this was probably due to loss of activity of the step 1, Lot 117/1 erythropoietin preparation during storage.

Although this result indicated that sensitive cells may persist in culture for up to 24 hr., it provided no information as to their identity; nonetheless, it showed that, if cells were not immediately

Table 21

Experiment designed to determine the effect on haem synthesis by foetal liver cell cultures of inhibiting DNA synthesis after an initial period of treatment with erythropoietin.

Groups of cultures	Period of incubation hr.				
	0 - 8		Spun at 800 g for 5 min.	8 - 26	
	erythropoietin	FUdR		erythropoietin	FUdR
1	-	-	+	-	-
2	+	-	-	+	-
3	+	-	+	+	-
4	+	-	+	-	-
5	-	-	+	-	+
6	+	-	+	+	+

FIGURE 72.

The effect on the response of 13-day mouse foetal liver cells to step 3 erythropoietin at 0.2 u./10^6 cells, of FUdR added 8 hours after erythropoietin. The experiment was carried out as shown in Table 21.

●——● untreated cultures.

○——○ erythropoietin-treated cultures, undisturbed during incubation.

○-·-·-○ erythropoietin-treated cultures, returned to medium containing erythropoietin after centrifugation.

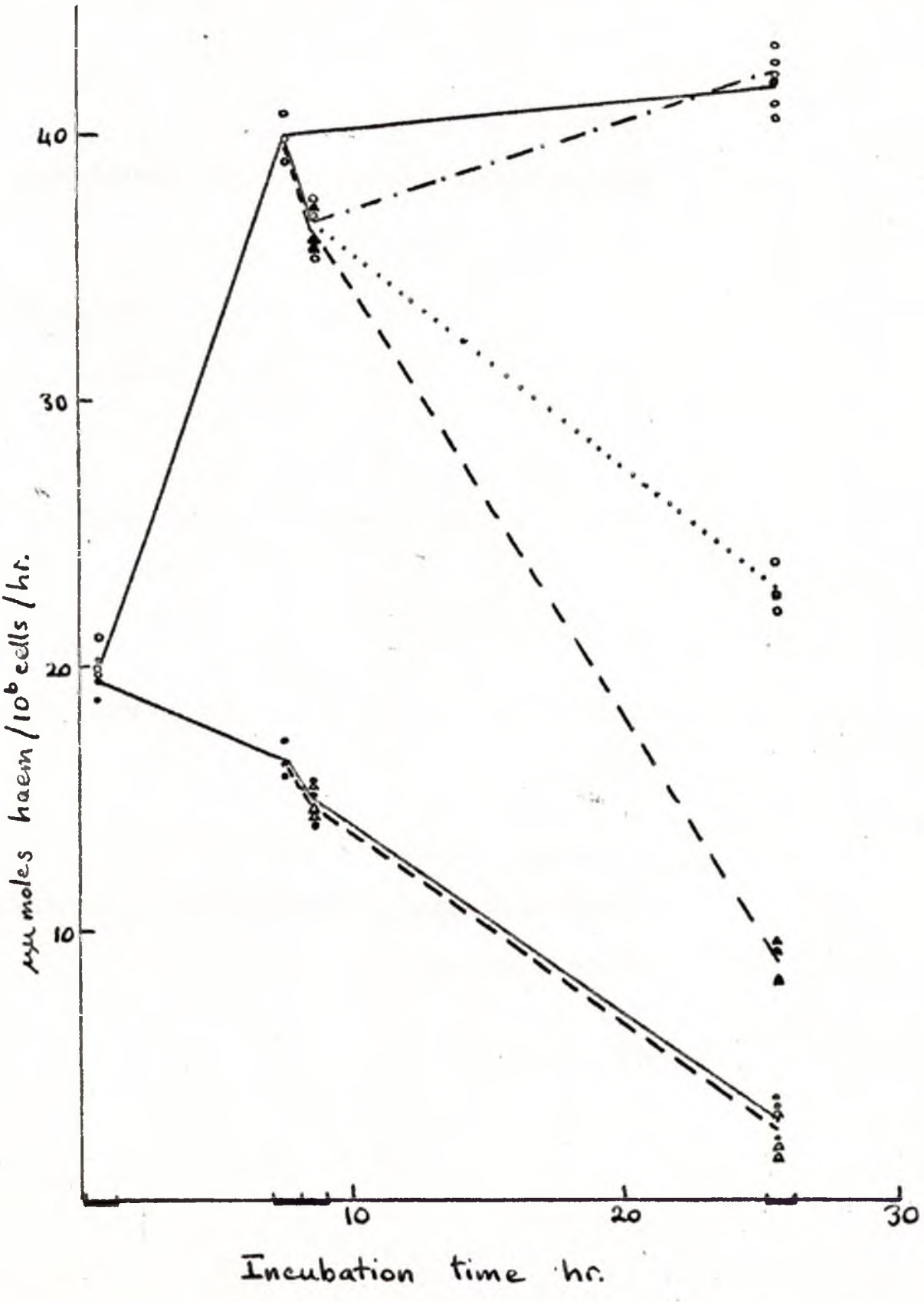
○·····○ erythropoietin-treated cultures, returned to medium without erythropoietin after centrifugation.

△ – – – △ untreated cultures, returned to medium containing 10^4M-FUdR after centrifugation.

▲ – – – ▲ erythropoietin-treated cultures returned to medium containing erythropoietin and 10^4M-FUdR after centrifugation.

Pulses with ^{59}Fe indicated as in Figure 60.

Figure 72


40
30
20
10
μμ moles haem / 10^b cells / hr.
10
20
30
Incubation time hr.

stimulated by erythropoietin, some at least would remain viable for a period at the end of which they could still respond to erythropoietin. The decrease in the magnitude of the response as the period of incubation lengthened was probably due to the inability of the culture medium to support complete survival of sensitive cells.

The experiments involving limited periods of treatment with erythropoietin indicated that the hormone continued to increase the rate of haem synthesis for at least 18 hr.. DNA synthesis increased very rapidly after treating the cells with erythropoietin (Figure 66) and was essential for the increase in haem synthesis (Figure 64). To determine whether the increased rate of haem synthesis continued to depend on DNA synthesis, the experiment shown in Table 21 was performed; haem synthesis was measured at the times shown in Figure 72.

It was found that the rate of haem synthesis decreased immediately after centrifugation; this was only temporary and, after 26 hr., the rate was similar in all cultures treated with erythropoietin throughout incubation, irrespective of centrifugation. After 26 hr. erythropoietin treatment the rate was 14 times the control rate at the same time. In cultures treated with erythropoietin for only 8 hr. the rate was 7.7 times the control rate; in those cultures to which FUdR was added after 8 hr. the rate of haem synthesis was only three times the control rate at the same time. There was no visible effect of FUdR on the cells immediately after it was added; it is not known

whether the rate of haem synthesis in erythropoietin/FUdR-treated cultures fell continuously between 9 and 26 hr. incubation or whether it recovered to some extent after centrifugation, before decreasing further. It was evident, however, that the effect on haem synthesis by erythropoietin-stimulated cells of adding FUdR during incubation was greater than that of removing the erythropoietin after the same length of time.

This result indicated that DNA synthesis, needed to maintain a high rate of haem synthesis after erythropoietin treatment, continued for at least 8 hr.; it also suggested that any cells stimulated by erythropoietin after some time in culture, responded in a manner similar to those which responded immediately after the hormone was added. It did not, however, indicate whether the cells stimulated after some hours were ESC, or whether they were early, dividing normoblasts, perhaps derived from ESC rapidly affected by erythropoietin. If such normoblasts were sensitive to erythropoietin, then some of the earliest effects of erythropoietin on foetal liver cell cultures might also be due to stimulation of maturing cells initially present in the liver cell suspension.

The addition of FUdR after 8 hr. incubation reduced haem synthesis more than did the removal of erythropoietin at the same time. It may be that some cells became insensitive to erythropoietin while still synthesising DNA needed for haem synthesis. It is also possible that not all of the erythropoietin was removed after centrifugation and

that the remaining hormone continued to affect some of the cells; the total lack of response to erythropoietin by FUdR-treated cultures (Figure 64) suggests that this could not occur after adding FUdR.

These results suggested that in mouse, as in rat, cells, DNA synthesis was an early and important feature of the response to erythropoietin. They also suggested that cells were responding to erythropoietin at different times after hormone treatment; if this were the case, the activity of cells at different stages of the response was being measured at any given time. Studies extending over long periods of incubation were thus difficult to interpret in terms of the immediate effects of erythropoietin on the cells. A more suitable system for such studies would be cloned cultures of stem cells or ESC; it may also be desirable, if not necessary, to induce synchrony in these cells so as to measure only one stage of the response to erythropoietin at a time.

In the mouse foetal liver cell cultures the very rapid increase in DNA synthesis suggested that many cells were responding quickly; it was possible that overnight treatment in cold trypsin had induced some degree of cell synchrony. In the absence of a more suitable system, short-term studies on 13-day mouse foetal liver cells might produce some indication of the earliest results of erythropoietin treatment of sensitive cells.

Specificity to erythroid cells of the parameters measured. The foetal liver cell cultures contained cells other than erythroid cells; since most haem synthesis occurs in erythroid cells the results obtained by this means were almost certainly a true reflection of the activity of erythroid cells. Other parameters are not so specific; experiments measuring incorporation of precursors into DNA, RNA or total protein may be complicated by the activity of non-erythroid cells. In addition there is no information about the effect of erythropoietin, or its impurities, on non-erythroid foetal liver cells.

The rate of incorporation into DNA, RNA or protein by cultures not treated with erythropoietin is very likely to be the result of the activity of all the types of cell present and the activity of erythroid cells cannot be judged on these measurements alone. In the following experiments, only the difference between control and erythropoietin-treated cultures is considered; even this, however, may possibly be affected by the presence of non-erythroid cells. The effect of metabolic inhibitors upon the response of haem synthesis to erythropoietin suggested that the changes observed in DNA, RNA and protein synthesis following treatment with erythropoietin, may be attributed, at least in part, to changes taking place in erythroid cells. The effect of treatment with erythropoietin and inhibitors on haem synthesis was always checked, and the results are, for simplicity, discussed in terms of an effect of erythropoietin itself on erythroid cells.

FIGURE 73.

The effect of actinomycin D and puromycin on the response of 13-day mouse foetal liver cells to step 3 erythropoietin at 0.2 u./10^6 cells.

a) thymidine incorporation

●——● untreated cultures.

○——○ erythropoietin-treated cultures

b) thymidine incorporation

●——● cultures treated with 10 µg./ml. actinomycin D.

○——○ cultures treated with erythropoietin and 10 µg./ml. actinomycin D.

Pulses with [^{3}H] thymidine indicated as in Figure 66.

Figure 73

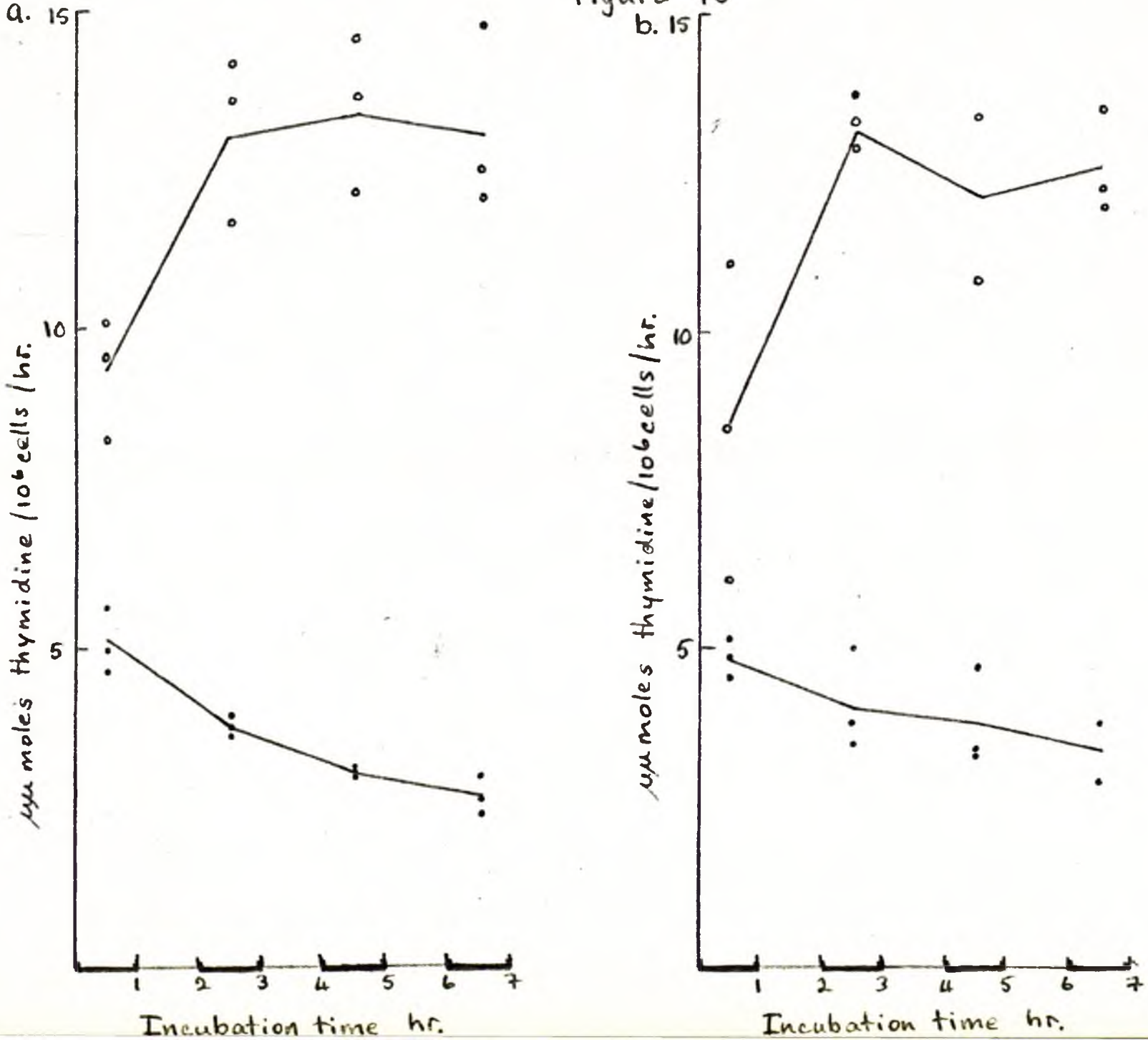

a.
b.
15
10
5
μμ moles thymidine / 10⁶ cells / hr.
μμ moles thymidine / 10⁶ cells / hr.
1
2
3
4
5
6
7
Incubation time hr.
Incubation time hr.

FIGURE 73 (cont'd.)

c) thymidine incorporation

●——● cultures treated with 10^{-4}M-puromycin

○——○ cultures treated with erythropoietin and 10^{-4}M-puromycin

Pulses with (^{3}H) thymidine indicated as in Figure 66.

d) haem synthesis

●——● untreated cultures

○——○ erythropoietin-treated cultures

×——× cultures treated with 10 µg./ml. actinomycin D ± erythropoietin

△——△ cultures treated with 10^{-4}M-puromycin ± erythropoietin.

Pulses with ^{59}Fe indicated as in Figure 60.

Figure 73 (cont.)

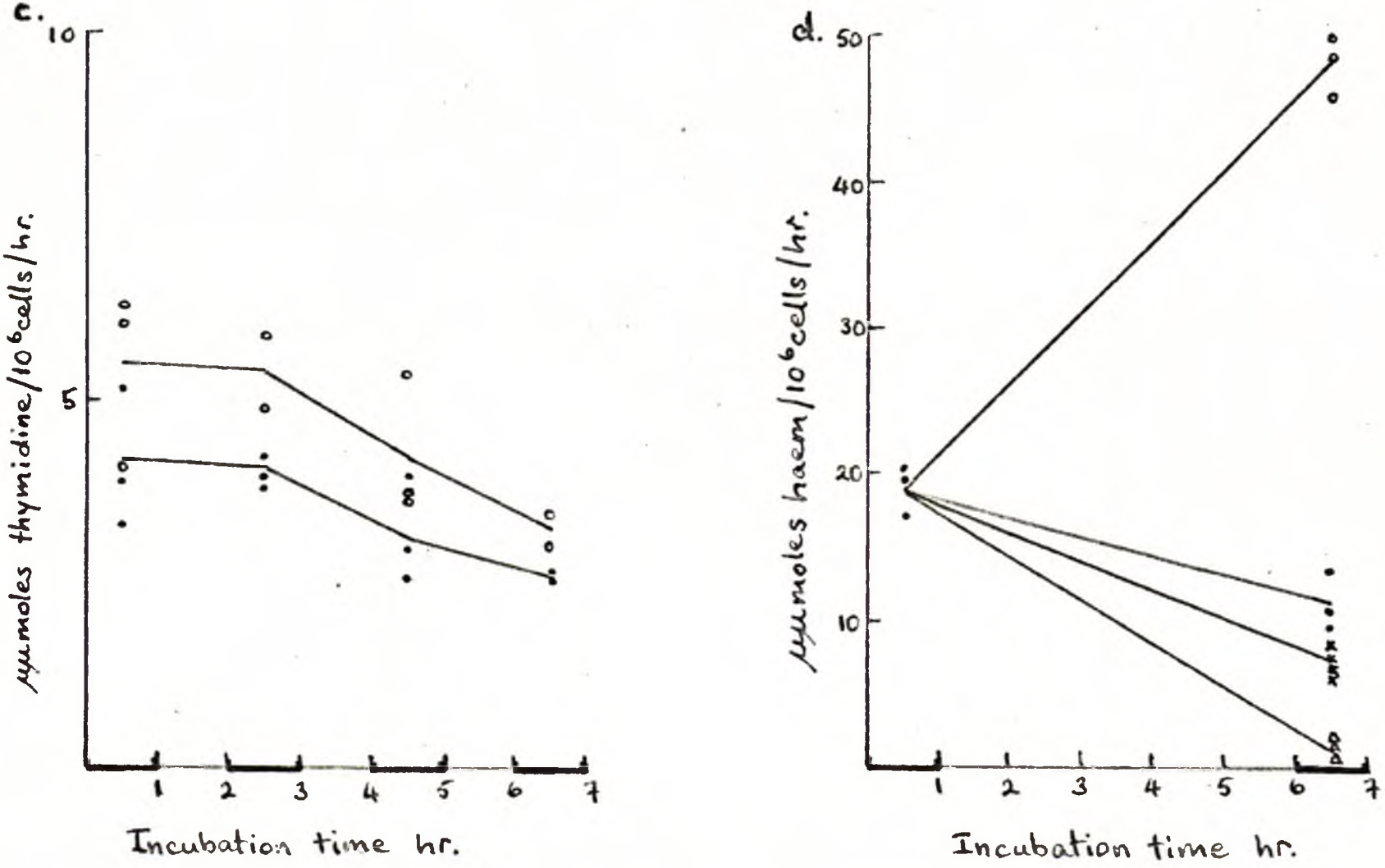


FIGURE 74.

The effect of actinomycin D and puromycin on the response of 13-day mouse foetal liver cells to step 3 erythropoietin at 0.2 u./10^6 cells.

a) thymidine incorporation

●——● untreated cultures.

●——○ erythropoietin-treated cultures

b) thymidine incorporation

●——● cultures treated with 10 µg./ml. actinomycin D

○——● cultures treated with erythropoietin and 10 µg./ml. actinomycin D.

Pulses with [^{3}H] thymidine indicated as in Figure 66.

Figure 74

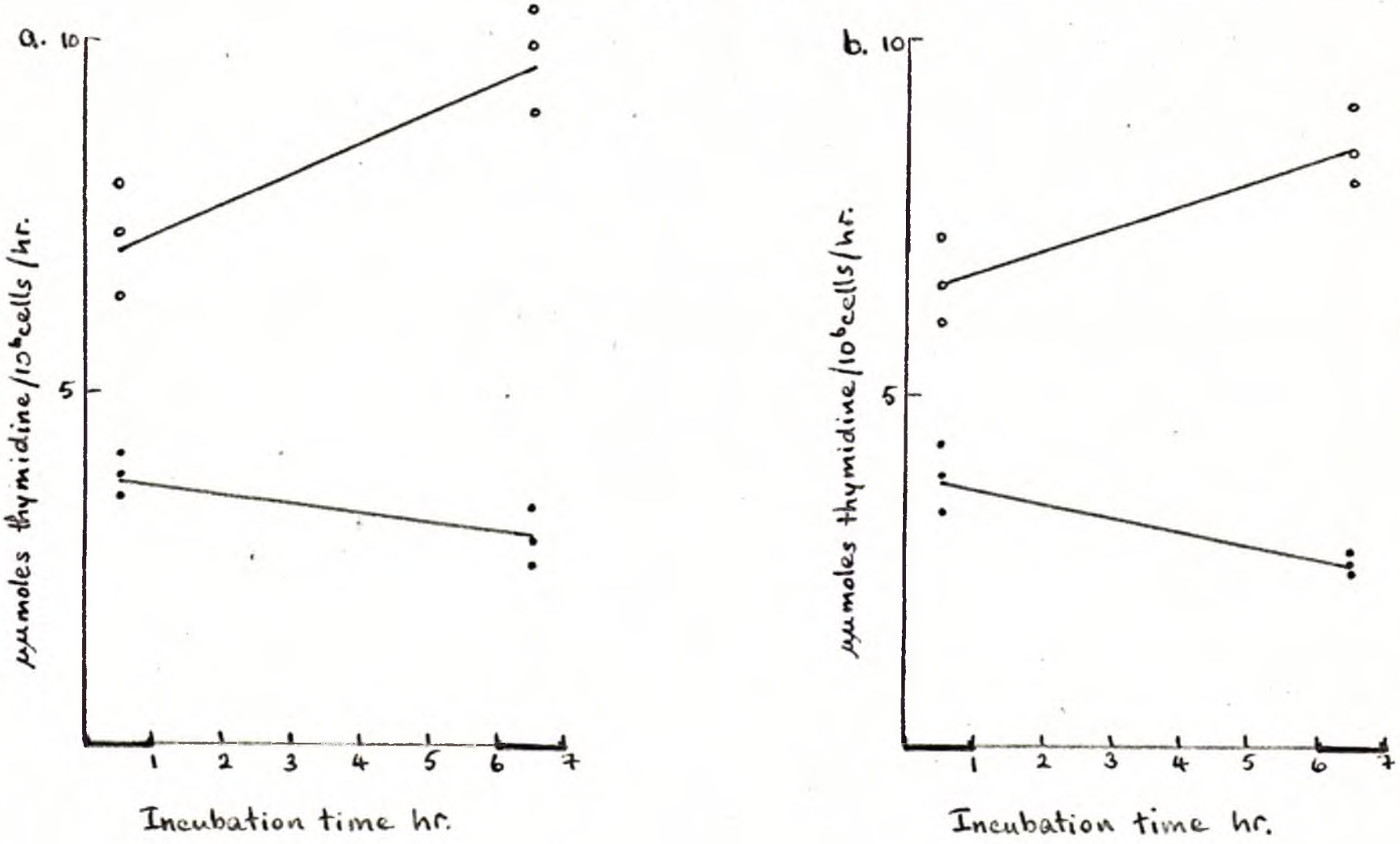

a.
b.
10
5
μμmoles thymidine/10⁶cells/hr.
1
2
3
4
5
6
7
Incubation time hr.

FIGURE 74 (Cont'd.)

c) thymidine incorporation

•——• cultures treated with 10^{-4}M-puromycin.

•——o cultures treated with erythropoietin and 10^{-4}M-puromycin.

Pulses with [^{3}H] thymidine indicated as in Figure 66.

d) haem synthesis

•——• untreated cultures.

o——o erythropoietin-treated cultures.

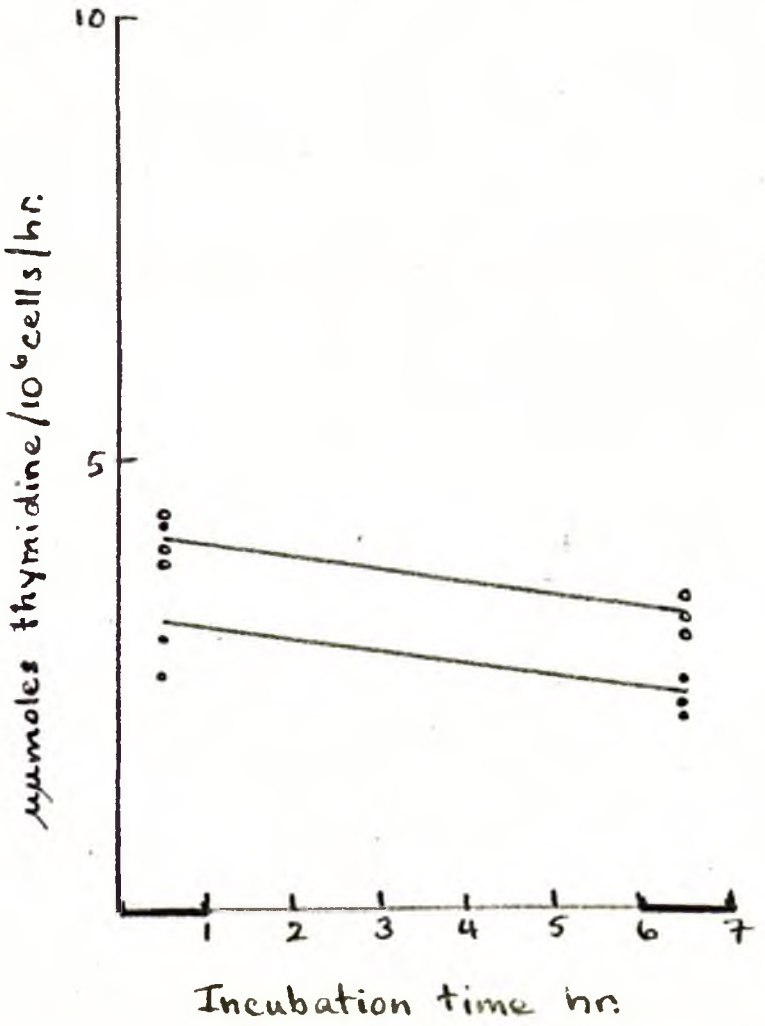
×——× cultures treated with 10 µg./ml. actinomycin D, ± erythropoietin.

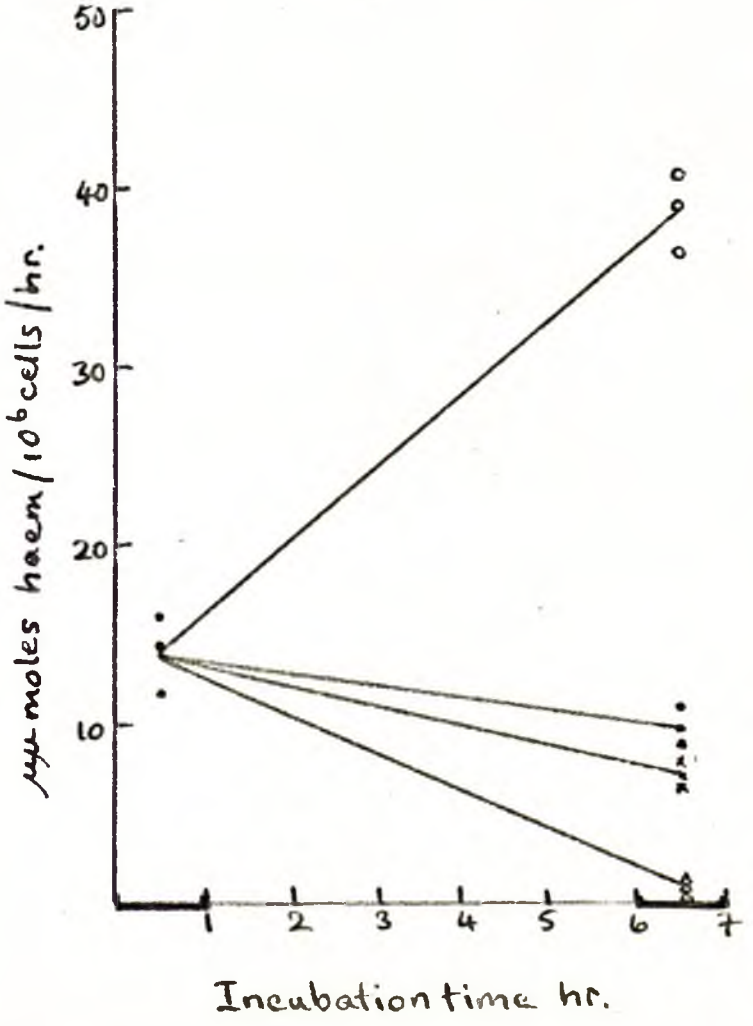
Δ——Δ cultures treated with 10^{-4}M-puromycin ± erythropoietin.

Pulses with ^{59}Fe indicated as in Figure 60.

Figure 74(cont.)

c.





Effect of erythropoietin on DNA synthesis. In 13-day mouse foetal liver cells, increased DNA synthesis is evidently important in the early stages of the response to erythropoietin; it may even be the initial step in the response. To determine whether this was the case, the effect of actinomycin D and of puromycin upon the early increase in DNA synthesis was examined; the incorporation of [^{3}H] thymidine and haem synthesis were measured at the times shown in Figure 73.

Again, both actinomycin D and puromycin abolished the effect of erythropoietin on haem synthesis; they also reduced the rate of synthesis in control cultures, puromycin having the greater effect. Treatment with erythropoietin caused an immediate increase in DNA synthesis; the rate continued to rise for 3 hr. and remained at this high rate for the next 4 hr. Puromycin abolished the response of DNA synthesis to erythropoietin; although the rate of DNA synthesis was higher in the puromycin/erythropoietin-treated cultures than in the puromycin-treated cultures, the difference between the two was small, and the individual results from both groups of cultures overlapped. In this experiment, the response of DNA synthesis to erythropoietin was not prevented by actinomycin D. The results suggested that protein synthesis, but not RNA synthesis, was necessary for the increase in DNA synthesis after erythropoietin treatment.

The experiment was repeated, sampling at fewer times. As shown in Figure 74 a similar pattern was found. Puromycin prevented any increase in DNA synthesis after treatment with erythropoietin; again

FIGURE 75.

The effect of actinomycin D on [^{3}H] uridine incorporation by 13-day mouse foetal liver cells.

●——● untreated cultures

○——○ cultures treated with 10 µg./ml. actinomycin D.

The thickened portions of the abscissa indicate the periods of incubation during which the cells were pulsed with [^{3}H] uridine; the results are plotted at the mid-points of the pulses.

FIGURE 76.

The effect of actinomycin D on DNA synthesis in 13-day mouse foetal liver cells treated with step 3 erythropoietin at 0.2 u./10^6 cells.

The cells were incubated with 10 µg./ml. actinomycin D for 1 hour prior to adding erythropoietin; they were pulsed for 1 hour with [^{3}H] thymidine between 5 and 6 hours after erythropoietin treatment.

A. untreated cultures

B. erythropoietin-treated cultures

C. cultures treated with actinomycin D

D. cultures treated with erythropoietin and actinomycin D.

Figure 75

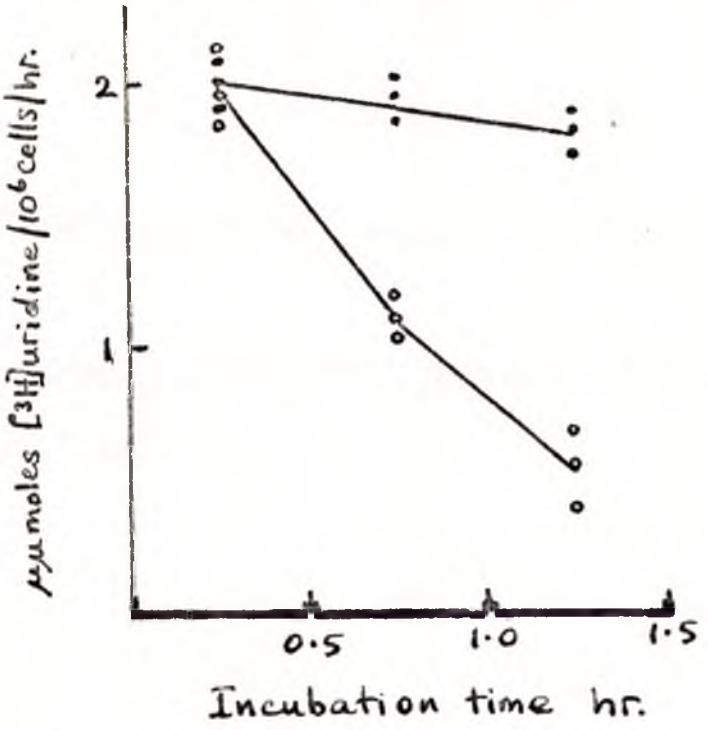
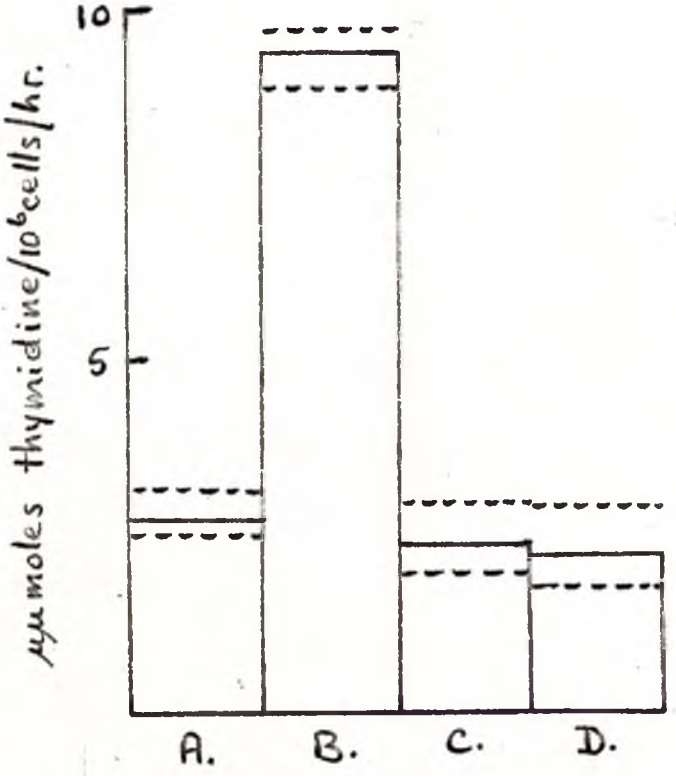


Figure 76



a small, but consistent, difference was observed between the rates of DNA synthesis in puromycin/erythropoietin-treated and puromycin-treated cultures. It may be that the cells are affected more quickly by erythropoietin than by puromycin. Actinomycin D permitted DNA synthesis to increase after erythropoietin treatment, although the rate of DNA synthesis in actinomycin D/erythropoietin-treated cultures was slightly lower than that in erythropoietin-treated cultures.

Although the results apparently indicated that RNA synthesis was not essential for the increase in DNA synthesis in response to erythropoietin, it was also possible that the effect on the cells of actinomycin D was less rapid than that of erythropoietin. The latter possibility was tested by measuring the effect of actinomycin D on [^{3}H] uridine incorporation by the cells. As shown in Figure 75, actinomycin D had little visible effect on [^{3}H] uridine incorporation during the first ½ hr. in culture; thereafter its effect was progressively greater, but the rate of incorporation after 1½ hr. was still some 25% of the initial rate. Some of this incorporation may have been due to turnover of the terminal cytidylyl-cytidylyl-adenylate (CCA) sequence of tRNA. It is evident that the effect of actinomycin D on RNA synthesis was delayed for a period which might well have been sufficient to permit stimulation of the cells by erythropoietin.

A further test was performed on this cell suspension. Cultures were incubated with actinomycin D for 1 hr.; erythropoietin was added after this time and [^{3}H] thymidine incorporation was measured after a further 5 hr. incubation. It was found that no response to erythro-

FIGURE 77.

The effect of actinomycin D on the response of 13-day mouse foetal liver cells to step 3 erythropoietin at 0.2 u./10^6 cells; the cells were incubated with actinomycin D for 1 hour prior to adding erythropoietin.

a) thymidine incorporation

•——• untreated cultures.

o——o erythropoietin-treated cultures.

x·····x cultures treated with 10 µg./ml. actinomycin D ± erythropoietin.

Pulses with [^{3}H] thymidine indicated as in Figure 66.

b) haem synthesis

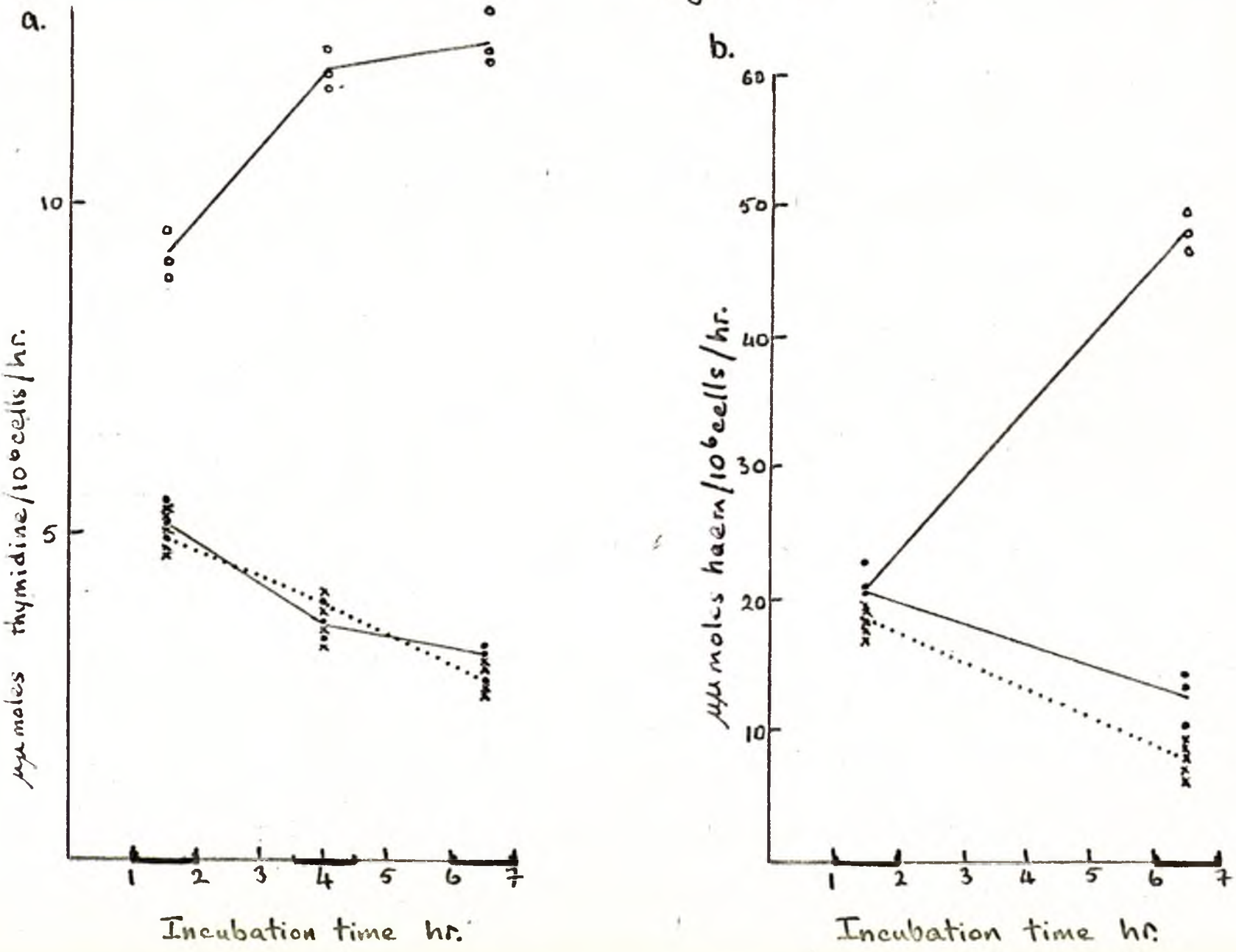
•——• untreated cultures.

o——o erythropoietin-treated cultures.

x·····x cultures treated with 10 µg./ml. actinomycin D ± erythropoietin.

Pulses with ^{59}Fe indicated as in Figure 60.

Figure 77


a.
10
5
μμ moles thymidine/10⁶ cells/hr.
1
2
3
4
5
6
7
Incubation time hr.
b.
60
50
40
30
20
10
μμ moles haem/10⁶ cells/hr.
1
2
3
4
5
6
7
Incubation time hr.

poietin occurred in the presence of actinomycin D (Figure 76), suggesting that the increase in DNA synthesis was dependent on RNA synthesis.

The effect of treatment with actinomycin D for 1 hr. prior to adding erythropoietin upon [^{3}H] thymidine incorporation was studied in more detail. As shown in Figure 77, actinomycin D had no effect upon the rate of DNA synthesis in control cultures. There was no perceptible response to erythropoietin at any time in cultures also containing actinomycin D; it thus seems likely that RNA synthesis as well as protein synthesis is essential to the erythropoietin-induced increase in DNA synthesis.

Since the rate of DNA synthesis doubled during the first hour of erythropoietin treatment, events which precede it must have taken place very quickly indeed. Incubation of the cells with actinomycin D for 1 hr. prior to adding erythropoietin completely prevented an increase in DNA synthesis; treatment of the cells with actinomycin D and erythropoietin simultaneously produced not only an initial increase in DNA synthesis but also allowed it to continue unchecked for at least 6 hr. This suggests that the RNA synthesis which appeared to precede DNA synthesis would indeed occur very rapidly; the RNA produced may also have a lifetime of 6 hr. or more. It is also possible that unstable mRNA was produced and that the protein(s) synthesised thereby enabled DNA synthesis to continue, after inhibition of further RNA synthesis by actinomycin D.

Thus, although DNA synthesis increased very rapidly in response

Table 22

Experiment designed to determine the effect of erythropoietin on the incorporation of [^{3}H] uridine or [^{3}H]lysine by foetal liver cell cultures, while DNA synthesis was blocked with 10^{-4}M-FUdR

Groups of cultures	FUdR added at 0 hr.	Erythropoietin added after 1 hr. incubation
1	-	-
2	-	+
3	+	-
4	+	+

FIGURE 76.

RNA synthesis in 13-day mouse foetal liver cells treated with step 3 erythropoietin at 0.2 u./10^6 cells, while DNA synthesis was blocked by FUdR. The experiment was carried out as shown in Table 22.

a) RNA synthesis

●——● untreated cultures.

○——○ erythropoietin-treated cultures.

△——△ cultures treated with 10^{-4}M FUdR.

▲——▲ cultures treated with erythropoietin and 10^{-4}M-FUdR.

Pulses with [^{3}H] uridine indicated as in Figure 75.

b) Haem synthesis

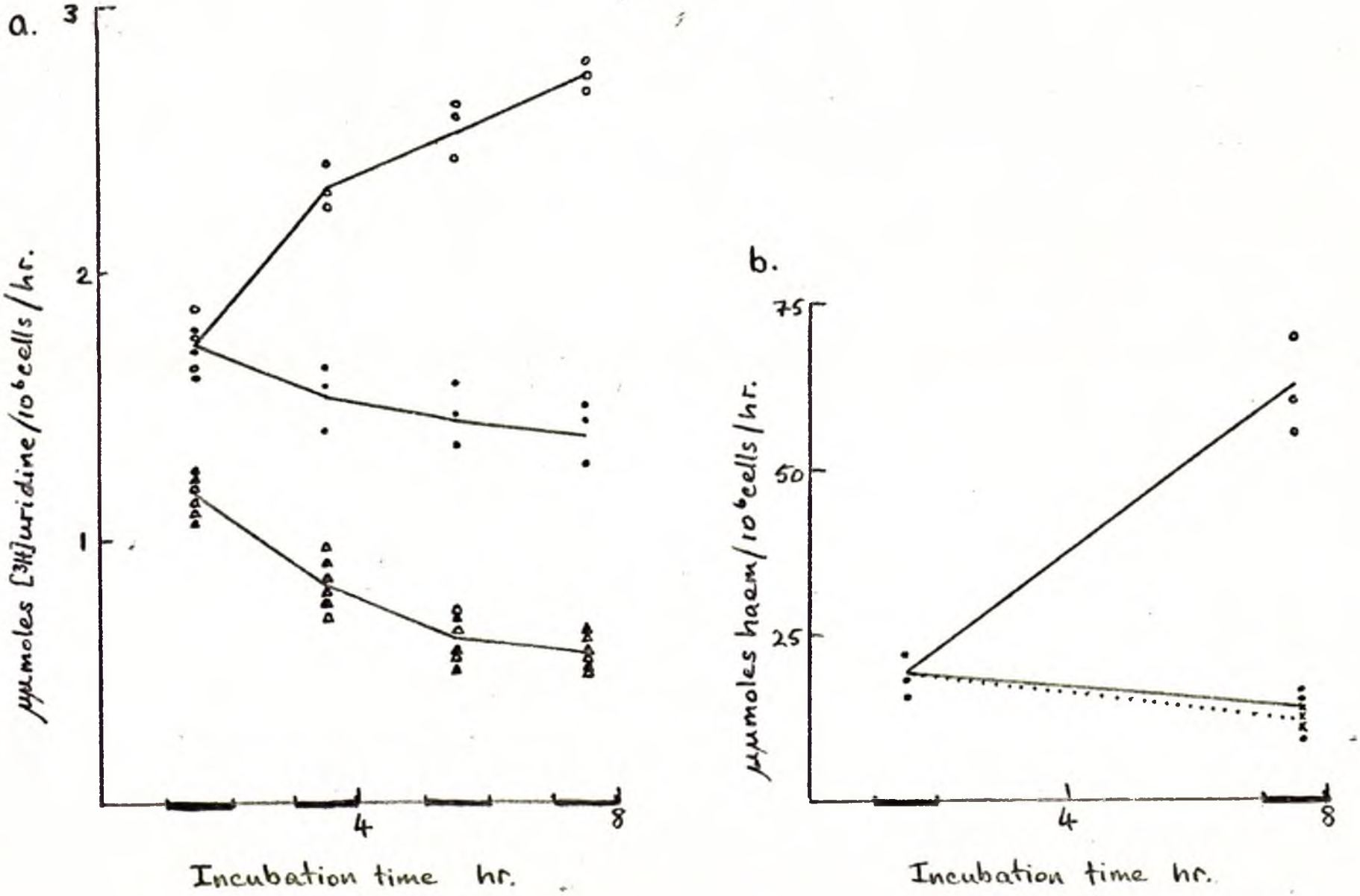
●——● untreated cultures.

○——○ erythropoietin-treated cultures.

x·····x cultures treated with 10^{-4}M-FUdR ± erythropoietin.

Pulses with ^{59}Fe indicated as in Figure 60.

Figure 78


a.
3
2
1
μμmoles [3H]uridine/10⁶cells/hr.
4
8
Incubation time hr.
b.
75
50
25
μμmoles haem/10⁶cells/hr.
4
8
Incubation time hr.

to erythropoietin, the effect of actinomycin D and of puromycin indicated that synthesis of RNA and of protein preceded DNA synthesis.

Effect of erythropoietin on RNA synthesis. The postulated RNA synthesis preceding most of the erythropoietin-induced DNA synthesis might itself depend on very rapid synthesis of some DNA. To study this possibility the effect of erythropoietin on [^{3}H] uridine incorporation while DNA synthesis was blocked by FUdR was determined. The experiment was planned as shown in Table 22; [^{3}H] uridine incorporation and haem synthesis were measured at the times shown in Figure 78.

The effect of erythropoietin on haem synthesis was abolished by FUdR. RNA synthesis in control cultures decreased slowly during incubation. Although erythropoietin had no visible effect during the first pulse, a continual increase in RNA synthesis was observed during later pulses; after 7 hr. treatment with erythropoietin, the rate of synthesis was 1.6 times the initial rate. Treatment with FUdR for 1 hr. reduced the rate of RNA synthesis during the first pulse to 67% of the control rate; thereafter the rate in all FUdR-treated cultures decreased more rapidly than the rate in untreated cultures. Erythropoietin had no visible effect on RNA synthesis in the presence of FUdR. It would appear that the erythropoietin-induced increase in RNA synthesis was dependent on DNA synthesis which was, in turn, dependent on RNA synthesis. It is impossible at present to distinguish which of the two was initially stimulated.

The fact that erythropoietin treatment caused [^{3}H] thymidine

FIGURE 79.

RNA synthesis in 13-day mouse foetal liver cells, treated with step 3 erythropoietin at 0.2 u./10^6 cells, while DNA synthesis was blocked by FUdR. The experiment was carried out as shown in Table 22.

a) RNA synthesis.

●——● untreated cultures.

○——○ erythropoietin-treated cultures.

▲——▲ cultures treated with 10^{-4}M-FUdR ± erythropoietin.

b) RNA synthesis.

10^{-4}M-cytidine was added to all cultures.

●——● untreated cultures.

○——○ erythropoietin-treated cultures.

▲——▲ cultures treated with 10^{-4}M-FUdR ± erythropoietin.

Pulses with [^{3}H] uridine indicated as in Figure 75.

Figure 79

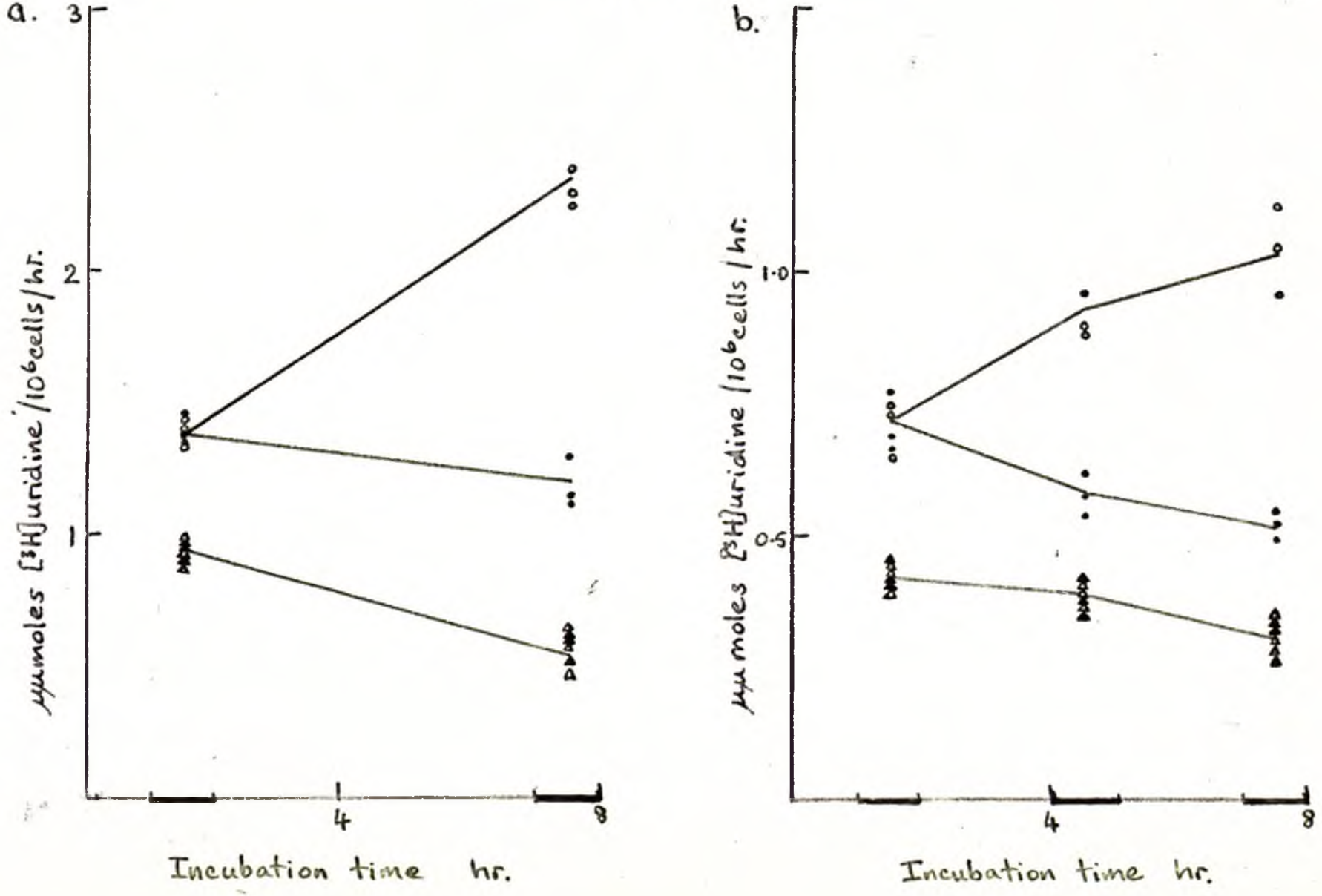

a.
3
2
1
μμmoles [3H]uridine /10^6cells/hr.
4
8
Incubation time hr.
b.
1·0
0·5
μμ moles [3H]uridine /10^6cells / hr.
4
8
Incubation time hr.

FIGURE 79 (Cont'd.)

c) haem synthesis

●——● untreated cultures.

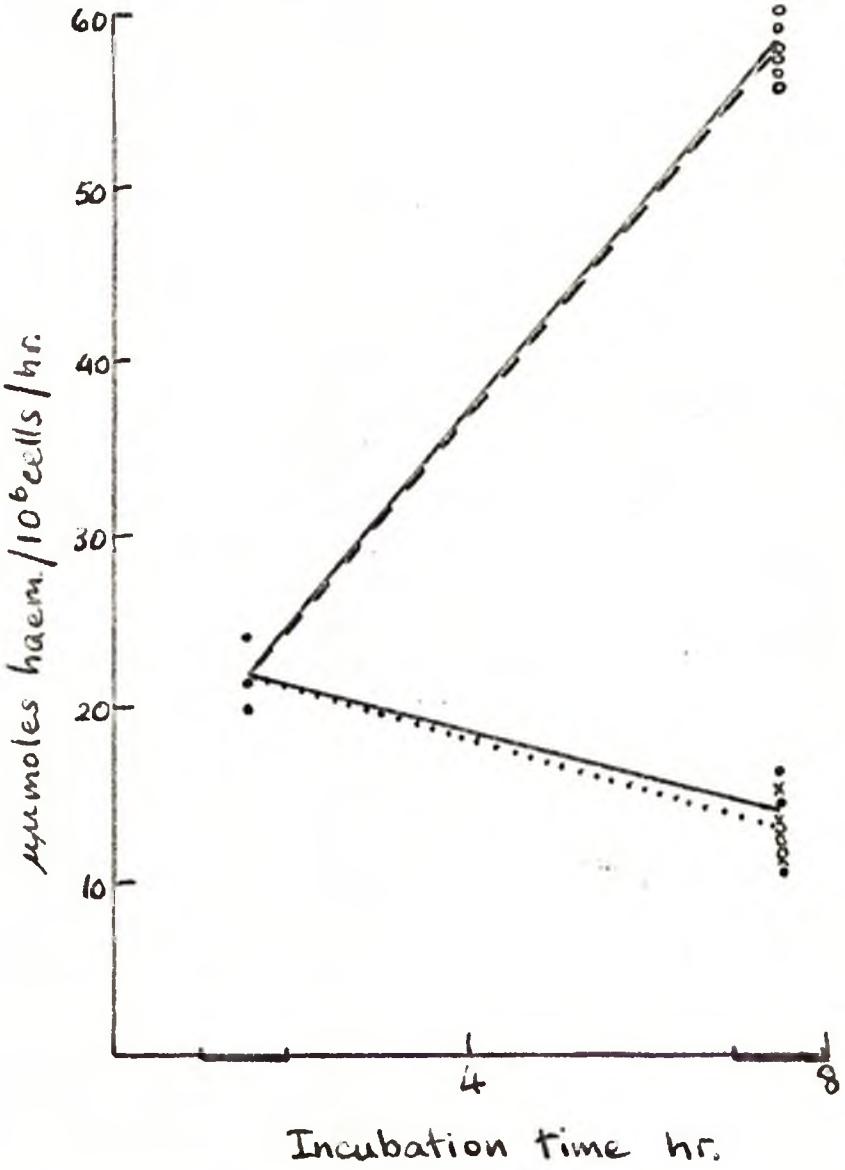
○——○ erythropoietin-treated cultures.

○ – – – ○ cultures treated with erythropoietin and 10^{-4}M-cytidine.

× ..... × cultures treated with 10^{-4}M-FUdR ± erythropoietin.

Pulses with ^{59}Fe indicated as in Figure 60.

Figure 79 (cont.)



incorporation to double at a time when it had no perceptible effect on [^{3}H] uridine incorporation suggests that there was little or no incorporation of [^{3}H]uridine into DNA during a pulse of 1 hr. Some of the [^{3}H] uridine incorporation may reflect turnover of the terminal sequence of tRNA rather than RNA synthesis (Holt et al., 1966). Two of the three terminal bases in tRNA are cytidyl residues; the previous experiment was repeated, using medium containing 10^{-4} M-cytidine. Control cultures containing no cytidine were also included. [^{3}H] uridine incorporation and haem synthesis were measured at the times shown in Figure 79.

The effect of erythropoietin on haem synthesis was again abolished by FUdR. The addition of 10^{-4} M-cytidine to the medium reduced the rate of [^{3}H] uridine incorporation by 50%; the pattern of the response to erythropoietin and the effect of FUdR upon it were unchanged. A reduction of 50% in uridine incorporation is larger than would be anticipated if only tRNA turnover were affected. It has been shown that uridine incorporation by Krebs ascites tumour cells into all varieties of RNA was reduced by 70% in the presence of 10^{-3} M-cytidine (Burdon et al., 1967); the amount of RNA synthesised was not reduced. It seems likely that the addition of 10^{-4} M-cytidine to foetal liver cell cultures may similarly have prevented some of the [^{3}H] uridine incorporation into RNA other than tRNA. The proportion of the activity incorporated owing to tRNA turnover thus remained uncertain.

FIGURE 80

Protein synthesis in 13-day mouse foetal liver cells treated with step 3 erythropoietin, at 0.2 u./10^6 cells, while DNA synthesis was blocked by FUdR. The experiment was carried out as shown in Table 22.

a) protein synthesis

●——● untreated cultures.

○——○ erythropoietin-treated cultures.

▲——▲ cultures treated with 10^{-4}M-FUdR.

△——△ cultures treated with erythropoietin and 10^{-4}M-FUdR.

The thickened portions of the abscissa indicate the periods of incubation during which the cells were pulsed with [^{3}H] lysine; the results are plotted at the mid-points of the pulses.

b) haem synthesis

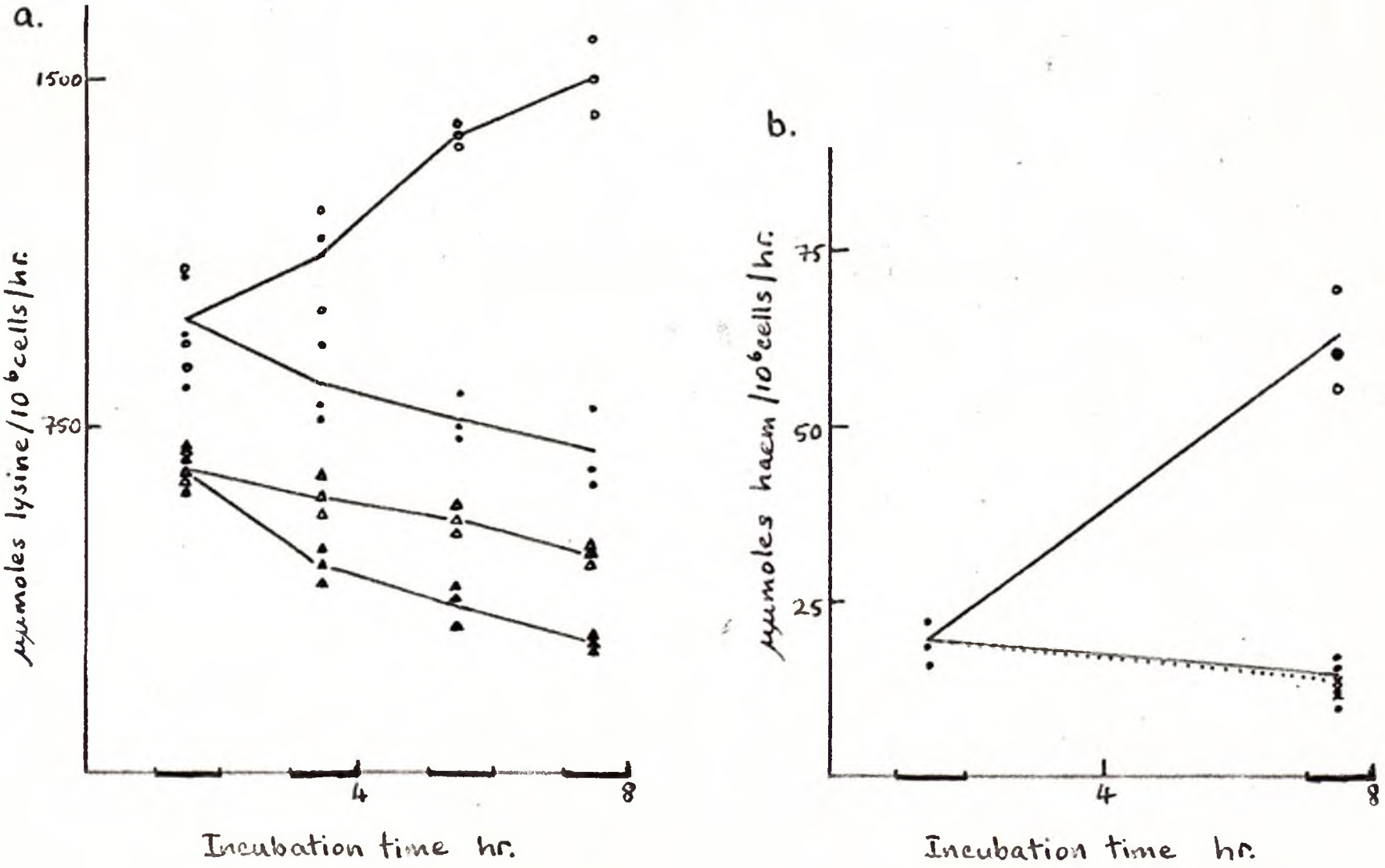
●——● untreated cultures.

○——○ erythropoietin-treated cultures.

x·····x cultures treated with 10^{-4}M-FUdR ± erythropoietin.

Pulses with ^{59}Fe indicated as in Figure 60.

Figure 80


a.
1500
750
μμmoles lysine/10⁶cells/hr.
4
8
Incubation time hr.
b.
75
50
25
μμmoles haem /10⁶cells/hr.
4
8
Incubation time hr.

Effect of erythropoietin on protein synthesis. The early increase in DNA synthesis after erythropoietin treatment was abolished by puromycin, indicating that protein synthesis was necessary. If the protein(s) required were synthesised in complete independence of all DNA synthesis and were stable, it was possible that they might accumulate in detectable amounts while DNA synthesis was inhibited. An experiment was carried out as shown in Table 22; [^{3}H] lysine incorporation and haem synthesis were measured at the times shown in Figure 80.

Again, the response of haem synthesis to erythropoietin was abolished by FUdR. In control cultures, the rate of protein synthesis decreased slowly during incubation; treatment with erythropoietin had no visible effect during the first pulse, but thereafter the rate increased continually. After 7 hr. treatment with erythropoietin, the rate of protein synthesis was 1.5 times the initial rate. Treatment with FUdR reduced the rate of protein synthesis during the first pulse by 30%; thereafter the rate decreased in the absence of erythropoietin. Although treatment with erythropoietin produced no increase in the rate of protein synthesis when FUdR was present, it slowed the decrease in rate throughout incubation. The difference in the rate of protein synthesis between FUdR/erythropoietin-treated cultures and FUdR-treated cultures was not evident during the first pulse but increased continually thereafter. Even after 3 hr. treatment with erythropoietin, the disparity between the two sets of results

Table 23

Experiment designed to determine the effect of erythropoietin on [^{3}H] lysine incorporation in foetal liver cell cultures treated with 10^{-4}M-FUdR and/or 10μg./ml. actinomycin D.

Groups of cultures	FUdR added at 0 hr.	Actinomycin D added at 0 hr.	Erythropoietin added after 1 hr.
1	-	-	-
2	-	-	+
3	+	-	-
4	+	-	+
5	-	+	-
6	-	+	+
7	+	+	-
8	+	+	+

FIGURE 81

The effect of actinomycin D on protein synthesis in 13-day mouse foetal liver cells treated with step 3 erythropoietin at 0.2 u./10^6 cells, while DNA synthesis was blocked with FUdR. The experiment was carried out as shown in Table 23.

a) protein synthesis.

●——● untreated cultures.

○——○ erythropoietin-treated cultures.

▲——▲ cultures treated with 10^{-4}M-FUdR.

△——△ cultures treated with erythropoietin and 10^{-4}M-FUdR.

b) protein synthesis

●——● cultures treated with 10 µg./ml. actinomycin D.

○——○ cultures treated with erythropoietin and 10 µg./ml. actinomycin D.

×– – –× cultures treated with 10 µg./ml. actinomycin D and 10^{-4}M-FUdR.

△– – –△ cultures treated with erythropoietin, 10 µg./ml. actinomycin and 10^{-4}M-FUdR.

Pulses with [^{3}H] lysine indicated as in Figure 80.

Figure 81

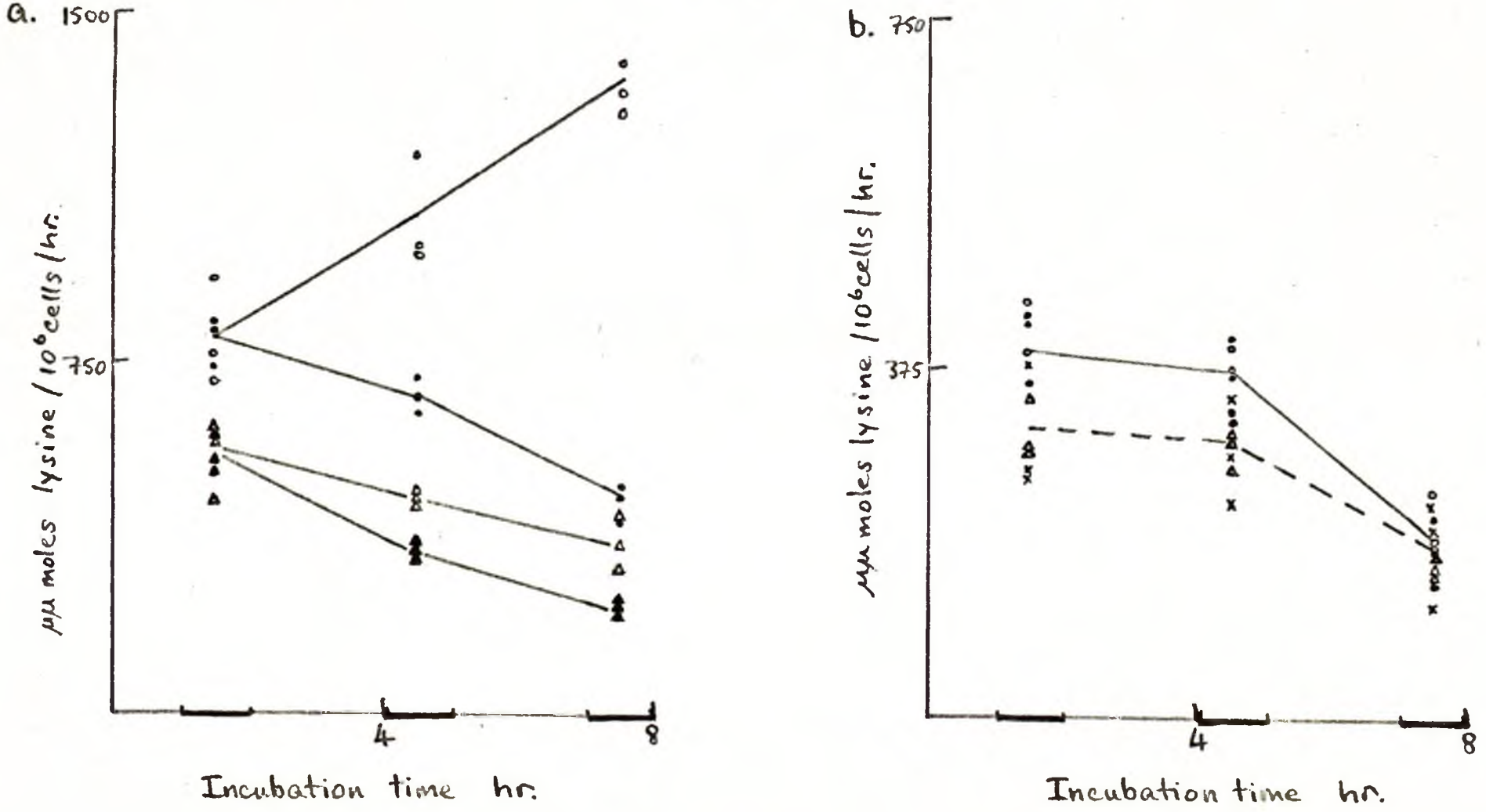

a.
1500
750
μμ moles lysine / 10⁶cells / hr.
4
8
Incubation time hr.
b.
750
375
μμ moles lysine / 10⁶cells / hr.
4
8
Incubation time hr.

FIGURE 81 (Cont'd.)

c) haem synthesis.

A. untreated cultures, pulsed with ^{59}Fe between 1 and 2 hr. incubation.

The remaining cultures were pulsed with ^{59}Fe between 7 and 8 hr. incubation.

B. untreated cultures.

C. erythropoietin-treated cultures.

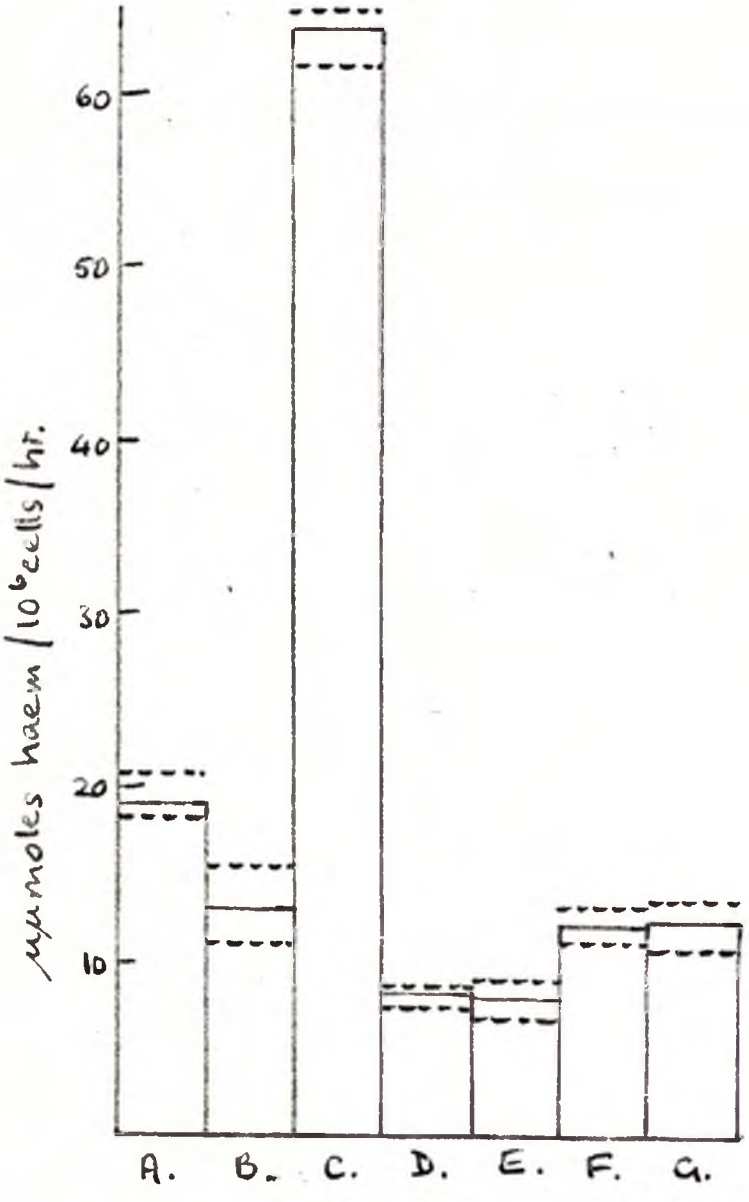
D. cultures treated with 10 µg./ml. actinomycin D.

E. cultures treated with erythropoietin and 10 µg./ml. actinomycin D.

F. cultures treated with 10^{-4}M-FUdR.

G. cultures treated with erythropoietin and 10^{-4}M-FUdR.

Figure 81 (cont.)



was more than twice the standard deviation of the FUdR-treated cultures. This suggests that DNA synthesis was important to the greater part of erythropoietin-stimulated protein synthesis but not to all of it. This is in contrast to dependence of detectable amounts of erythropoietin-stimulated RNA synthesis upon DNA synthesis.

To confirm this result and to determine whether the increase in protein synthesis was dependent on RNA synthesis, the effect of actinomycin D on [^{3}H] lysine incorporation in these conditions was measured. The experiment was designed as shown in Table 23. [^{3}H]-lysine incorporation and haem synthesis were measured at the times shown in Figure 81.

Haem synthesis in FUdR-treated and in FUdR/erythropoietin-treated cultures decreased at the same rate as in control cultures. Actinomycin D, irrespective of the presence of FUdR or of erythropoietin, caused a greater decrease in rate. The effect of FUdR upon the response of [^{3}H]lysine incorporation to erythropoietin was identical to that found in the previous experiment; there was again a small amount of incorporation after erythropoietin treatment which was not prevented by FUdR. Actinomycin D reduced the initial rate of protein synthesis by 50%; thereafter the rate decreased during incubation whether or not erythropoietin was present. FUdR and actinomycin D together reduced the initial rate of protein synthesis by 60% and erythropoietin treatment had no visible effect.

These results suggest that during the early increase in DNA

synthesis after erythropoietin treatment, there was also some protein synthesis which was independent of DNA synthesis but not of RNA synthesis. Some of this protein synthesis appeared to be essential for the DNA synthesis since puromycin inhibited the latter. It is possible then, that erythropoietin first stimulated RNA synthesis so that protein would be made; only then could DNA synthesis occur. There was no detectable increase in RNA synthesis in erythropoietin/FUdR-treated cultures (Figure 78) at the time when erythropoietin treatment gave rise to FUdR-resistant protein synthesis; this suggests that the amount of RNA necessary was very small or that it was very unstable. However, the RNA or the protein synthesised from it appeared to be fairly stable since DNA synthesis was hardly affected by actinomycin D for 6-8 hr. (Figure 73). In the absence of more precise data on the nature and number of the RNA(s) and protein(s) involved, however, the inferences drawn must remain tentative.

If the RNA and/or proteins required for the erythropoietin-stimulated DNA synthesis were stable for 6-8 hr., it was possible that cells treated with erythropoietin in the presence of FUdR would be capable of increasing their rate of haem synthesis without further erythropoietin treatment, when the inhibitory effect of FUdR was reversed. It had been shown that thymidine added together with FUdR permitted haem synthesis to increase after erythropoietin treatment (Figure 64); replacement of FUdR by thymidine after some hours incubation might permit DNA synthesis to proceed. The experiment shown

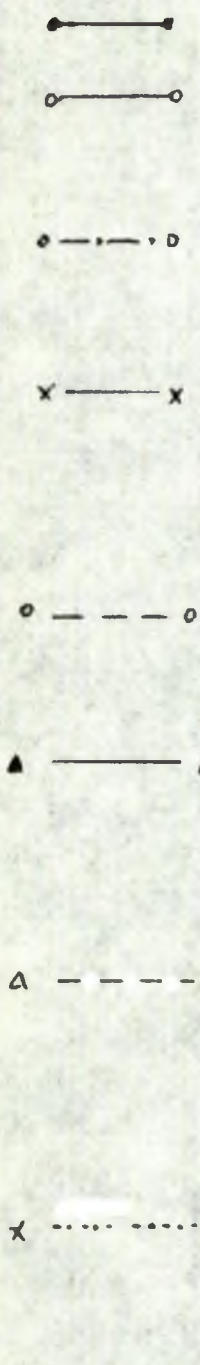
Table 24

Experiment designed to determine the effect on haem synthesis by foetal liver cell cultures treated with erythropoietin and 10^{-4}-FUdR of the removal of both and the addition of 10^{-4}M-thymidine after some hours' incubation.

Groups of Cultures	Period of incubation hr.					
	0 - 8		Spun at 800 g for 5 min.	8 - 26		
	FUdR	Erythro-poietin		FUdR	Erythro-poietin	thymidine
1	-	-	+	-	-	-
2	-	+	-	-	+	-
3	-	+	+	-	+	-
4	-	+	+	-	+	+
5	-	+	+	-	-	-
6	+	-	+	-	-	+
7	+	+	+	-	-	+
8	+	+	+	-	+	+

FIGURE 82.

The effect on the response of 13-day mouse foetal liver cells to step 3 erythropoietin at 0.2 u./10^6 cells, of treatment with FUdR followed after some hours by the replacement of FUdR with thymidine. The experiment was carried out as shown in Table 24.



untreated cultures.

erythropoietin-treated cultures, undisturbed during incubation.

erythropoietin-treated cultures, returned to medium containing erythropoietin after centrifugation.

erythropoietin-treated cultures, returned to medium containing erythropoietin and 10^{-4}M-thymidine after centrifugation.

erythropoietin-treated cultures, returned to medium without erythropoietin after centrifugation.

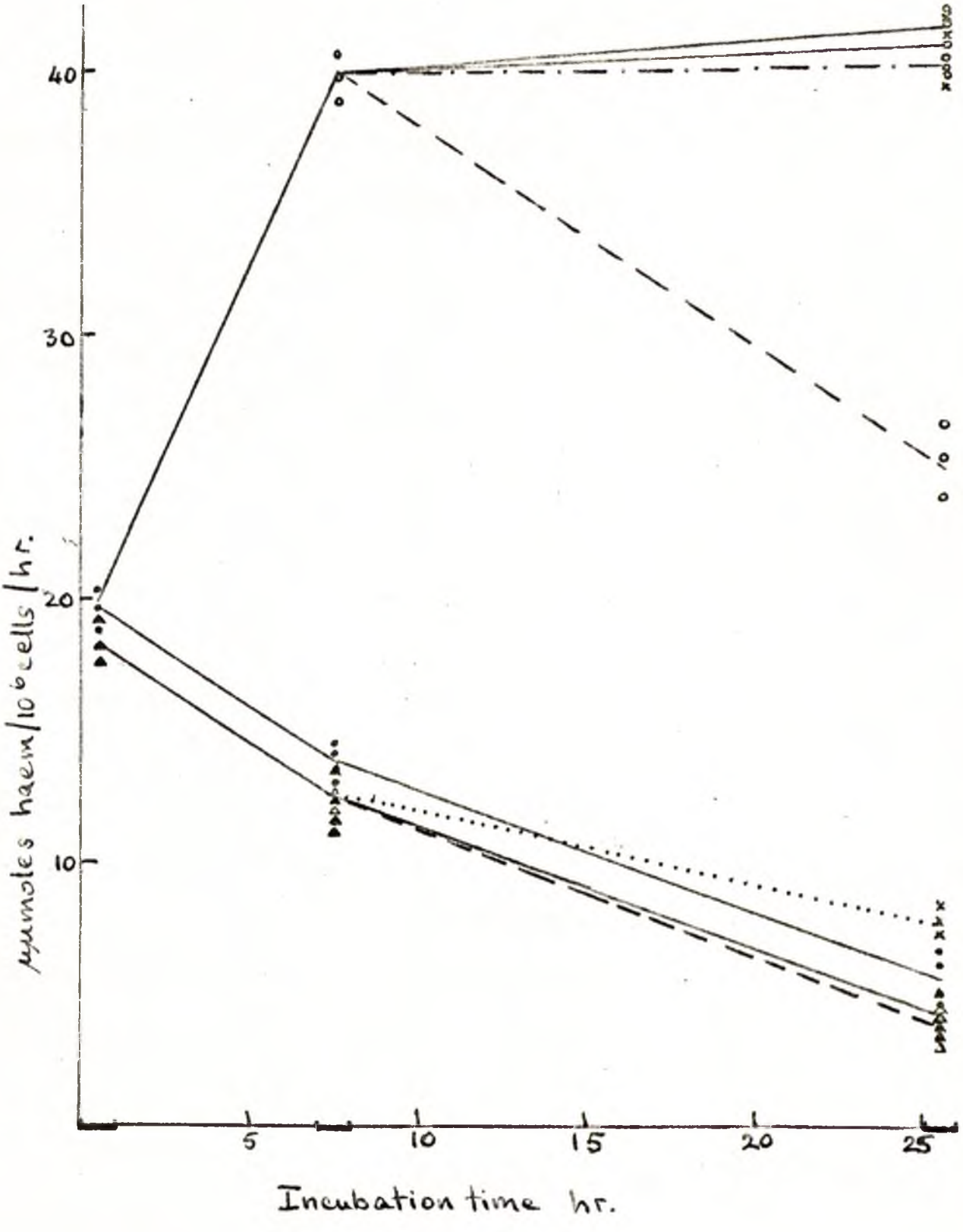
cultures treated with 10^{-4}M-FUdR, returned to medium containing 10^{-4}M-thymidine after centrifugation.

cultures treated with erythropoietin and 10^{-4}M-FUdR returned to medium containing 10^{-4}M-thymidine after centrifugation.

cultures treated with erythropoietin and 10^{-4} FUdR returned to medium containing erythropoietin and 10^{-4}M-thymidine after centrifugation.

Pulses with ^{59}Fe indicated as in Figure 60.

Figure 82


40
30
20
10
μμmoles haem/10⁶cells/hr.
5
10
15
20
25
Incubation time hr.

in Table 24 was performed; haem synthesis was measured at the times shown in Figure 82.

It was found that neither centrifugation nor the addition of thymidine affected the rate of haem synthesis by the foetal liver cells after 26 hr. incubation, provided that their treatment had been identical in all other respects. After 8 hr. incubation the rate of haem synthesis in cultures treated with FUdR and erythropoietin was indistinguishable from that in cultures treated with FUdR alone; after the addition of thymidine and incubation for a further 18 hr., the two groups of cultures were still indistinguishable. It appeared that the cells were unable to proceed with DNA synthesis and haem synthesis in the absence of erythropoietin; this may indicate that the RNA and/or proteins synthesised independently of DNA synthesis were not stable, or that thymidine was unable to reverse the effects of FUdR on the cells. The addition of thymidine and erythropoietin to cultures treated for 8 hr. with FUdR and erythropoietin produced no increase in haem synthesis, but only slowed the rate at which it decreased. This suggested that 8 hr. treatment with FUdR and erythropoietin had made most of the cells incapable of responding to erythropoietin, even in the presence of thymidine. It may be that, in normal circumstances, cell division follows closely upon DNA synthesis after erythropoietin treatment; cells attempting to divide in the presence of FUdR would probably die (Eidinoff and Rich, 1959). The slight response observed when both erythropoietin and thymidine were added

to FUdR/erythropoietin-treated cultures suggested that a small number of erythropoietin-sensitive cells remained viable; these may be cells which responded to erythropoietin more slowly or later than most. As a test for the stability of erythropoietin-induced RNA and/or protein needed for DNA synthesis, this experiment was inconclusive.

IV. DISCUSSION

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1. The role of erythropoietin in foetal life.

Quantitative studies on haem synthesis by foetal erythroid cells. The studies on the effect of erythropoietin upon haem synthesis by foetal rat tissues during development (section III, 1) showed that yolk sac cells were refractory to erythropoietin; foetal liver and spleen cells responded to the hormone in the early stages of organogenesis but became refractory at later stages. It was suggested that yolk sac cells were either inherently unable to respond to erythropoietin or that they were fully stimulated before explantation by small amounts of endogenous erythropoietin. ESC were considered to accumulate in the foetal liver and in the foetal spleen until sufficient endogenous erythropoietin became available to them, after which they were stimulated *in vivo* as soon as they were formed. It was also suggested that some ESC and/or maturing cells were released from the liver and from the spleen near to the time when these organs became insensitive to *in vitro* treatment with erythropoietin, i.e. from the liver between days 15 and 16 and from the spleen between days 19 and 20. If ESC were released at these times they could contribute to the onset of erythropoiesis in the spleen and the bone marrow respectively.

In the foetal rabbit only liver erythropoiesis was studied (section III, 2). The pattern of sensitivity to erythropoietin suggested that, in this species also, ESC accumulated in the foetal liver until sufficient endogenous erythropoietin was produced to

FIGURE 83.

Summary of the erythroid activity and response to erythropoietin of mouse foetal liver cells of different ages (compiled from the data of Cole and Paul (1966), by permission of the authors).

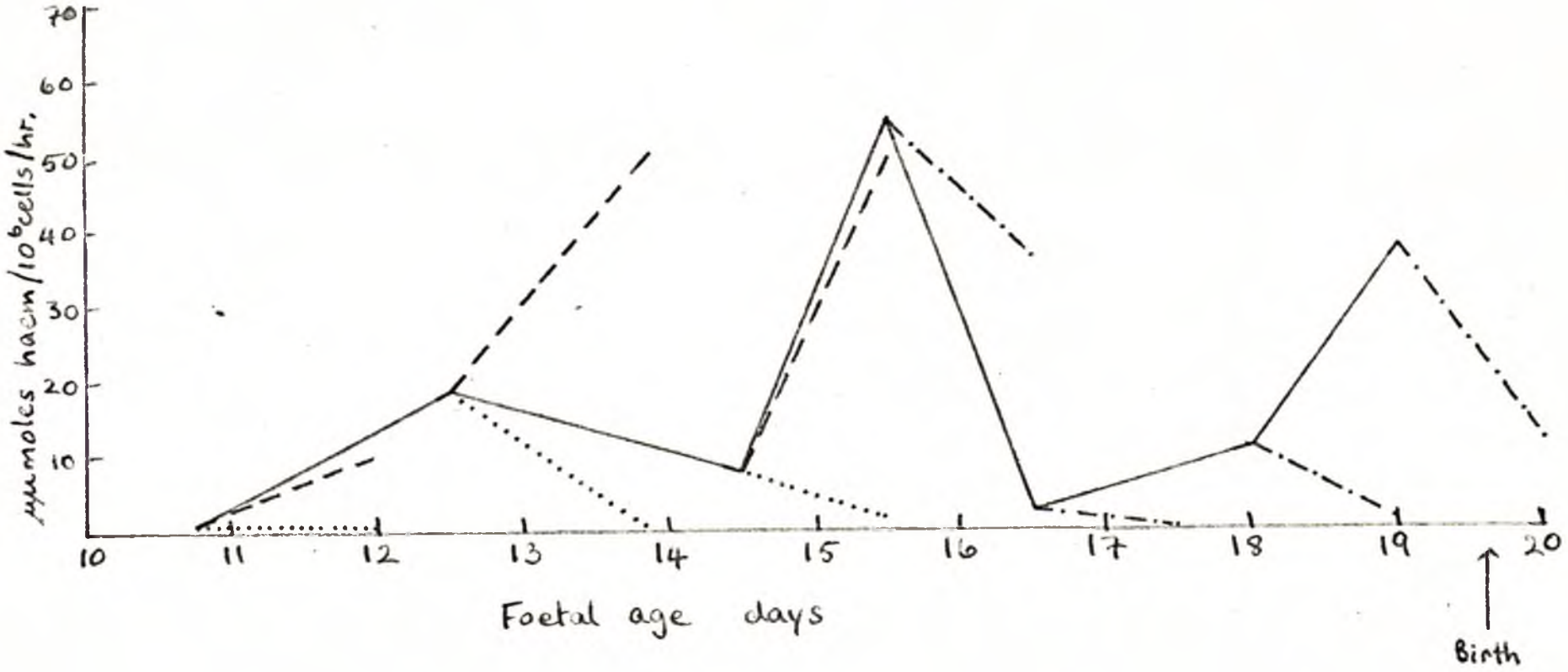
_____ initial rate of haem synthesis in cultures of foetal liver cells explanted at the foetal ages shown. The period during which the cells were treated with cold trypsin is neglected and the results represented as if the cells had been placed in culture immediately following explantation.

_ _ _ _ rate of haem synthesis after treatment with erythropoietin for the times shown.

..... rate of haem synthesis in untreated cultures after incubation for the periods shown.

_ . _ . _ rate of haem synthesis in untreated cultures and in cultures which did not respond to erythropoietin, after incubation for the periods shown.

Figure 83


70
60
50
40
30
20
10
μmoles haem/10⁶cells/hr.
10
11
12
13
14
15
16
17
18
19
20
Foetal age days
Birth

stimulate them; this was followed by the loss of the response to erythropoietin *in vitro*, indicating that ESC were stimulated *in vivo* as soon as they were produced. It appeared to take some days for the postulated endogenous erythropoietin to reach a concentration high enough to stimulate all ESC present in the foetal liver. It was suggested that, as in the rat, ESC and/or maturing cells were released from the liver between day 15 and day 16; ESC released at this time may 'seed' the erythroid foci of the foetal spleen.

Similar studies on mouse foetal erythropoiesis were carried out by Cole and Paul (1966), whose results with liver cells are shown in Figure 83. Yolk sac cells were found to be insensitive to erythropoietin; no erythroid activity or ESC were detected in the spleen. These authors suggested that little or no endogenous erythropoietin was present until day 13 or day 14 and that ESC accumulated in the liver until then. All the accumulated ESC were then stimulated and the *in vivo* activity observed on day 15 was as great as that found after erythropoietin treatment *in vitro* of 12-day and 14-day cells. There appeared to be no release of ESC or of maturing cells from the liver before day 15; it appeared that many maturing cells were released between day 15 and day 16. These data do not determine whether yolk sac cells were truly refractory to erythropoietin or whether they were fully stimulated by endogenous erythropoietin before explantation, as appeared to happen in the foetal liver after day 14; the erythropoietin activity of the 8-9 day mouse yolk sac was so much less than that of the foetal liver that very

low levels of erythropoietin would probably suffice to control it. In the foetal liver, the *in vivo* erythroid activity observed on day 12 was quite high, suggesting that some endogenous erythropoietin was present early in foetal liver erythropoiesis.

Observations of this nature show only that foetal erythroid cells are capable of responding to erythropoietin; they do not prove that erythropoietin is present *in vivo* during foetal development. Although the effect of erythropoietin on foetal erythroid cells *in vitro* appeared to simulate quite closely the subsequent course of *in vivo* foetal erythropoiesis in all three species, it is possible that the resemblance may be fortuitous; the course of foetal erythropoiesis may be regulated by other humoral factors or be determined by the inherent properties of the erythroid cells themselves.

Qualitative studies on haemoglobin synthesis by foetal erythroid cells. In both rat and mouse foetal liver cell cultures erythropoietin caused increased synthesis of haemoglobin components which were already being made by the cells (section III, 4, 5); it did not provoke the appearance of abnormal varieties of haemoglobin, nor initiate the synthesis of rat haemoglobin components earlier than they appeared *in vivo*. In addition, erythropoietin did not alter the relative rates of synthesis of rat haemoglobin components by foetal liver cells which responded to the hormone with an overall increase in haemoglobin synthesis. Haemoglobin synthesis in rat and mouse yolk sac cell cultures was unaffected by erythropoietin treatment (Figures 37 and 41); 18-day

rat foetal liver cells were also unaffected (Figure 49). These findings suggest that the nature of the haemoglobin(s) produced by foetal erythroid cells is not determined by erythropoietin, which appeared to have only a quantitative effect.

Erythropoietin treatment of adult rat marrow cells caused an increase in haemoglobin synthesis without altering the relative rates of synthesis of the components (Figure 51), suggesting that, in adult animals also, erythropoietin had only a quantitative effect. Since erythropoietin is almost certainly concerned in the control of normal adult erythropoiesis (section I, 6), it seems likely that the normal role of erythropoietin is to increase the amount of haemoglobin made without altering the variety of haemoglobin(s) made. The effect of erythropoietin on foetal erythroid cells was entirely in keeping with its effect on adult erythroid cells. Although this does not prove that erythropoietin is active in foetuses in vivo, it lends some support to the hypothesis. Erythropoietin is evidently not the only control factor concerned in foetal, and adult, erythropoiesis; the changing pattern of haemoglobin varieties during foetal development appeared to be independent of erythropoietin in both rat and mouse, as was the pattern of haemoglobin synthesis in adult rat cells.

Time of appearance of foetal erythropoietin. It seems likely that erythropoietin is concerned in the quantitative control of foetal erythropoiesis. The data available suggest three possible times at which erythropoietin may first appear in the foetus:

1. at the beginning of yolk sac erythropoiesis,

2. at the beginning of foetal liver erythropoiesis,

3. part way through foetal liver erythropoiesis, around 13-14 days of gestation in rat, mouse and rabbit. Whether or not erythropoietin is present in earlier foetuses, it seems very likely that it increases sharply in amount at this time.

If erythropoietin is concerned in yolk sac and early foetal liver erythropoiesis, the accumulation of ESC in the foetal liver (Figures 20, 35 and 83) seems unexpected. The amount of erythroid activity in the yolk sac is small compared to that of the liver. (Figure 8 ; Cole and Paul, 1966) and low levels of erythropoietin would probably suffice to control it; such low levels of erythropoietin may permit ESC in the liver to remain unstimulated. Similarly, the erythroid activity in young livers is low compared to that of 15-day livers and may also indicate that only small amounts of erythropoietin are available. The survival of ESC may also be influenced by the extent to which the liver is vascularised in the early stages of its formation. It seems most likely, however, that some erythropoietin is available from the beginning of foetal liver erythropoiesis, since the inherent erythroid activity of the foetal liver cells constantly increased (Figures, 20, 35, and 83). It is perhaps less likely that erythropoietin is concerned in the control of yolk sac erythropoiesis in view of the differences between this and later foetal, and adult,

erythropoiesis; this may imply that erythropoietin is not present in the foetus before the onset of foetal liver erythropoiesis.

Site of production of foetal erythropoietin. Mice made polycythaemic by hypertransfusion throughout pregnancy produced erythropoietically normal offspring (Jacobson et al., 1959), suggesting that maternal erythropoietin is not required by the foetus. In addition, no erythropoiesis was observed in the mothers during gestation; this suggests that any erythropoietin produced by the foetus does not cross the placenta, at least in an active form. Furthermore, rats treated with phenylhydrazine throughout pregnancy produced erythropoietically normal offspring (Block, 1946); this also suggests that active erythropoietin cannot cross the placenta. It might also suggest that foetal rat erythroid cells were unaffected by maternal erythropoietin; the present work, however, has shown that adult erythropoietin can elicit a response from foetal rat erythroid cells. Thus, if erythropoietin is indeed concerned in the regulation of foetal erythropoiesis, the hormone must be produced by the foetus itself.

Analogy with the adult would suggest that foetal erythropoietin is produced mainly by the kidney and reaches its target tissue through the circulation. If synthesis of foetal erythropoietin begins at 13-14 days of gestation in rat, mouse and rabbit, it is possible that the developing metanephros is the site of erythropoietin production. If, however, erythropoietin also controls earlier foetal erythropoiesis it may be produced by the mesonephros during the early stages of liver

erythropoiesis and by the pronephros during yolk sac erythropoiesis. The times of development of the pronephros, mesonephros and metanephros in agreement with these hypotheses (Witschi, 1956). Whether or not the pronephros or mesonephros produce erythropoietin, the postulated increase in erythropoietin production at 13-14 days may be due to the development of the metanephros. Extension of the analogy to adult erythropoietin suggests that the oxygen tension of the production site may influence the amount of erythropoietin produced. The rapid development of the foetus at the time of formation of the mesonephros and metanephros may well lower the oxygen tension sufficiently (Witschi, 1956) to cause increased production of foetal erythropoietin leading to the continual increase in foetal liver erythroid activity.

Erythropoiesis in suckling rats was found to be unaffected by nephrectomy, suggesting that little or no erythropoietin was being made by the kidney (Stohlman et al, 1964). The animals were suckled by nephrectomised or polycythaemic mothers to ensure that no maternal erythropoietin reached them in the milk (Grant, 1955). Nephrectomy of adult animals causes a very rapid decrease in erythropoiesis (Naets and Heuse, 1962) because of the speed with which erythropoietin is removed from circulation (Keighley, 1962); it is not known how fast erythropoietin is cleared from the circulation in neonatal animals, which survived nephrectomy for only 2-3 days. It is possible that no effect would become detectable during this short time. Nevertheless, this work suggests that the kidney may not be so important a site of erythropoietin production in foetal as in adult life.

It has been reported that factors enhancing erythropoiesis are produced by adult liver and spleen (Erslev, 1962; De Franciscis et al., 1965) but their identification with erythropoietin has not been proven. Unspecified extra-renal sites of production of erythropoietin have also been reported (Erslev, 1964a; Jacobson, 1962) and these may be liver and/or spleen. Foetal liver and spleen may also produce erythropoietin; the amount of erythropoietin produced may be regulated by the oxygen tension of the organs and the hormone, after stimulating the erythroid foci in the same organ, may or may not enter the foetal circulation.

The detection of pools of ESC in rat, mouse and rabbit foetal liver and in rat foetal spleen at times when some erythropoiesis is taking place in the same organ or elsewhere, suggests that any erythropoietin produced by the foetus is available to erythroid cells to a varying degree, which may depend upon the location of the cells. This is especially marked in the case of rat spleens where ESC persist although liver erythropoiesis is very active. If erythropoietin is made in sites separate from the erythroid sites it must reach its target cells through the circulation. It may be that the pools of ESC are in parts of the organs not yet vascularised and that the ESC persist until circulating erythropoietin can approach them; it is also possible that the amount of erythropoietin present during the early stages of foetal liver erythropoiesis is limiting, so that ESC may persist even within reach of circulating erythropoietin. This is less likely to occur in the case of rat foetal spleen ESC. If erythropoietin is

produced by the erythroid sites themselves, it may well enter the circulation unless it is utilised and inactivated by the erythroid cells of the same organ; in this case also, shielding of ESC in erythroid foci separate from the site of production of erythropoietin may occur.

In summary, the evidence so far available suggests that erythropoietin, or an erythropoietin-like factor, is concerned in the quantitative regulation of foetal erythropoiesis; this factor may be identical with adult erythropoietin or be a different entity with very similar activity. Yolk sac erythropoiesis appeared to be independent of erythropoietin, but this is not conclusively demonstrated. It seems most likely that erythropoietin controls foetal liver erythropoiesis from its beginning; the amount of erythropoietin produced probably increases shortly before the observed increase in liver erythroid activity. The increase in erythropoietin production may be influenced by the low oxygen tension of the foetal tissues at this time. The erythropoietin is not of maternal origin, but there is no evidence as to the site of its production by the foetus; it may be produced by the developing excretory organs or by other foetal organs.

To prove the existence of foetal erythropoietin it would be necessary to demonstrate erythropoietin activity in foetal extracts or perfusions; the production of polycythaemic foetuses might also be used to substantiate the hypothesis that erythropoietin assists in the control of foetal erythropoiesis. Erythropoietin has been detected in the cord blood of newborn human infants (Halvorsen, 1963), but it has not been reported in other species or at earlier stages of gestation.

This observation suggests, however, that erythropoietin may also be concerned in foetal erythropoiesis in species other than those studied here.

2. Colonisation of foetal erythroid sites by stem cells *in vivo*

Two hypotheses may be framed as to the source of stem cells in the erythroid sites during foetal development. The first is that stem cells migrate to or are formed in all of the presumptive erythroid sites at the time when yolk sac erythropoiesis is established; in those sites not immediately active, the cells remain quiescent until the sites do become active. The second is that stem cells initially occur only in the yolk sac and that they are released to settle in the foetal liver and initiate foetal liver erythropoiesis; this process may then be repeated as erythropoiesis proceeds in the foetal liver and begins in foetal spleen. These are extreme cases and intermediates may occur, as, for instance, that cells released from yolk sac 'seed' all later erythroid sites at the time of their release; only those cells reaching the foetal liver become immediately active, those settling in other sites remaining inactive until later stages of foetal development. There is some evidence consistent with all of these but none is conclusive.

The morphological appearance of some of the cells in erythroid tissue has been interpreted to mean that erythroid cells differentiate *in situ* from reticular cells (Pease, 1956; Marks and Kovach, 1966); if this interpretation were correct such *in situ* differentiation may result from the settlement of stem cells in the presumptive erythroid foci very early in foetal development. Morphological evidence is

difficult to interpret conclusively in this way, however, and haematopoietic cells are notoriously difficult to identify with certainty. Also, such studies provide no information as to when the cells settled in the erythroid foci; even if *in situ* transformation of this nature were proven, it would have to be shown whether the cells undergoing transformation had been present from very early developmental stages or whether they had reached the erythroid sites only a short time before differentiating.

It is known that foetal liver cells can form spleen colonies (McCulloch and Till, 1963; Silini *et al.*, 1967) and that they can also colonise other haematopoietic sites (Taylor, 1965; Witschi, 1956) of the recipients. Colonisation of incipient erythroid foci from previous erythroid sites is thus conceivable, either as the sole source of stem cells or to reinforce those already present. In rabbit foetal liver and in foetal rat liver and spleen a loss of ESC without a corresponding increase in *in vivo* haem synthesis was observed (section III, 1, 2); ESC were found in rat spleens after their disappearance from the liver. In the mouse the loss of ESC was followed by a corresponding increase in the *in vivo* erythroid activity of the liver and no erythroid activity or ESC were found in the spleen (Cole and Paul, 1966). This may also indicate that progenitor cells are released from erythroid foci to colonise other foci becoming erythropoietic as the foetus develops. However, if erythropoietin is present in foetal blood from 13-14 days of gestation it would appear that the released ESC become insensitive

to erythropoietin before being released and recover their sensitivity only upon reaching their new sites of activity. This may indicate that these cells are erythropoietin-sensitive during only a part of their cycle or that the hypothesis is wrong. Since the observed decrease in erythroid activity of rat foetal liver cells coincides with the period at which the number of non-nucleated circulating cells increases most rapidly (Kindred and Corey, 1930), the cells released from the liver may be maturing cells; the ESC detected on day 15 may be stimulated in the liver by endogenous erythropoietin, but the increased haem synthesis as they mature may be masked by the loss of cells to the circulation. A similar explanation may be advanced to explain the decrease in erythroid activity of rabbit foetal liver cells on day 16. It may be that a much higher rate of inherent activity would have been found had the livers been explanted some hours earlier. As evidence for 'seeding' of incipient erythroid foci by cells from earlier foci, this is thus inconclusive.

The establishment of erythroid foci throughout the foetal mesenchyme before they become limited to the liver (Le Douarin , 1966) may indicate a transfer of stem cells from the yolk sac to all future erythroid sites at this stage; after this brief burst of activity the cells persist only in presumptive erythroid sites, whose environment is most favourable to the cells' survival. It may be argued that the yolk sac erythrocytes are so different from future erythrocytes that such a model is very unlikely. On the other hand, rat yolk sac cells make haemoglobins electrophoretically identical with some of the components

made by later cells; this may indicate a closer similarity than is apparent from the cells' morphology. In addition, the cells which may colonise the later sites are progenitor cells, not differentiated cells. It is known that stem cells can give rise to cell lines so different as the erythroid, lymphoid and granuloid cell lines; the difference between yolk sac and later erythroid cells is probably less than the difference between erythroid and other cell lines. It has been suggested that pluripotent cells entering lymphoid tissue are altered by their environment so as to limit them to lymphoid maturation (Taylor, 1965). A similar limitation may be imposed upon yolk sac cells entering future liver or spleen erythroid sites so that only non-nucleated erythrocytes are formed there.

3. Regulation of haemoglobin synthesis by erythropoietin

It was found that the three mouse foetal haemoglobins were synthesised only by yolk sac cells while foetal liver cells made only adult haemoglobin (Figures 37 and 38); these findings are in agreement with previous studies on mice possessing a single adult haemoglobin component, Hb^s/Hb^s mice (Kovach et al., 1967). Only adult haemoglobin synthesis was stimulated by erythropoietin in foetal liver cells (Figure 38) and in circulating blood cells (Figure 69). Foetal haemoglobin synthesis by yolk sac cells was unchanged by erythropoietin treatment (Figure 37); nor were these cells induced to synthesise adult haemoglobin.

The nature of the haemoglobin(s) synthesised by foetal rabbit

erythroid cells was not determined; however, the pattern of replacement of foetal haemoglobins by adult haemoglobin during gestation (Figure 59) was similar to that of the mouse, suggesting that, in rabbit foetuses also, foetal haemoglobins were made by yolk sac cells while adult haemoglobin was made by foetal liver cells. This is supported by the fact that the appearance of adult haemoglobin in the foetal circulation followed closely upon the increase in foetal liver erythroid activity (Figure 35). Also, it has been reported that the haemoglobin contained in nucleated erythrocytes differs from that in non-nucleated foetal erythrocytes (Ackerman, 1962). It may be inferred that in the rabbit, as in the mouse, erythropoietin would affect only liver cells and would stimulate the production of adult haemoglobin only. This, however, requires definite demonstration.

In these species the change from nucleated to non-nucleated erythrocytes is accompanied by a change in the variety of haemoglobin made by the cells. In that erythropoietin appears to begin affecting foetal erythropoiesis at the onset of liver erythroid activity, the change in the nature of the haemoglobin made may be regarded as an indirect result of erythropoietin stimulation; there is no evidence to suggest that erythropoietin itself directly affects the change. Indeed, the results obtained with rat foetal cells suggest that it does not.

In man, α-chains have been found in both yolk sac and later erythroid sites (Huehns et al., 1964 a,b); it is possible that ζ-chain synthesis may be confined to yolk sac cells (Butler et al., 1960)

but later foetal erythroid sites can synthesise α -, β- and γ- chains (Ingram, 1963; Baglioni, 1963). It may be that only the β -like chain is altered in the change from yolk sac to foetal liver erythropoiesis. In sheep, too, it seems that the foetal haemoglobin differs from the adult haemoglobin in the structure of its β-chain (Van Vliet and Huisman, 1964). There is no information about the chain structure of foetal rabbit haemoglobins. In Hb^S/Hb^S mice, it has been shown that two of the foetal haemoglobins contain adult α-chains; their β -chains differ from the adult β-chain and from each other (Fantoni et al., 1967). The third foetal haemoglobin is composed of non-adult α -chains and of β-chains similar to those of one of the other foetal haemoglobins; it was suggested that its α-chains may differ from adult α-chains only in possessing an N-terminal acetyl group, but this is not proven. This suggests however that, as in man and sheep, it is in β-chain(s) synthesis that mouse yolk sac cells differ from foetal liver and later erythroid cells; it may be that it is in a similar way that rabbit yolk sac cells differ from later erythroid cells.

The pattern of haemoglobin synthesis during the development of the foetal rat is very different from that of the mouse or the rabbit. Instead of a switch from several foetal haemoglobins to a single, different, adult haemoglobin, there was a progresive increase in the number of haemoglobins synthesised during foetal liver erythropoiesis. (section III, 6). Two of the haemoglobins were common to all erythroid sites from yolk sac to adult marrow, although one of these, haemoglobin 'c',

decreased considerably in amount as the animals aged, it was detected in a significant number of adults up to $2\frac{1}{2}$ years old. Although erythropoietin markedly increased the total amount of haemoglobin synthesised by adult marrow and some foetal liver cells, it had no effect upon the relative amounts of the different haemoglobin varieties (section III, 5). Also, in yolk sac and early foetal liver cells erythropoietin did not induce the synthesis of those haemoglobin components which were not already being made by the cells. It appeared that the cells were 'programmed' for a certain pattern of haemoglobin synthesis at a given stage of development; while erythropoietin could increase the activity of the cells, it was unable to alter this pattern.

It may be that, while erythropoietin controls the amount of foetal liver erythropoiesis, a further humoral factor determines the nature of the haemoglobin in the mature cells; this would account for the fact that explanted cells retain their original pattern of haemoglobin synthesis while cells in vivo alter their pattern. It is also possible that the different components are made in different cells and that the onset of maturation of the cell cohorts making some components takes place later in gestation; the proportion of each component would then be governed by the number of maturing cells in which it is made, as well as by the rate at which it is synthesised by the cells. The number of maturing cells in each cohort would depend

on the size of the pool of progenitor cells, which would also determine the number of ESC available. Since the pattern of haemoglobin synthesis was the same in liver and spleen cells from foetuses of the same age (section III, 6), it would appear that the relative sizes of the pools of cells making the different components were the same in each erythroid site.

A third possibility may be envisaged, similar to that suggested by Baglioni (1963) for man. In this model, all of the components are synthesised by each cell and the ability to make each component is acquired by the stem cells in the foetus as they replicate. A pool of replicating stem cells thus exists in the erythroid foci, able to synthesise certain haemoglobins at certain rates; this ability changes according to the number of stem cell replications and thus to foetal development. Some of the progeny of each replication are, or become, ESC which mature *in vivo* or are detectable *in vitro*; although ESC may survive *in vitro* (Figure 71), it appears that no replication of stem cells occurs, since the pattern of haemoglobin synthesis remained constant for at least 30 hours incubation. In this model, the function of erythropoietin during foetal erythropoiesis is to stimulate ESC as they are produced, in numbers which depend upon the amount of erythropoietin available, as well as the number of ESC produced; the pattern of haemoglobin synthesis is determined by the cells themselves, no exogenous stimulus being required.

In rat, mouse, and probably rabbit, foetuses erythropoietin appeared to be concerned in the control only of the amount of haemoglobin synthesised; it did not alter the variety of haemoglobin synthesised. Its effect on adult erythroid cells was similar (Figure 51), suggesting that this was characteristic of the mode of action of erythropoietin in normal erythropoiesis.

4. Synthesis of rat haemoglobins

In the present work, four haemoglobins were found in all adult blood samples; a fifth component was found in some and may have been present in undetectable quantities in all of the animals. Seven components have previously been found in random-bred Wistar adults (Brada and Tobiska, 1963); the disparity may be due to the rats being derived from different colonies or to a better resolution of the haemoglobins with CM-cellulose chromatography than with starch gel electrophoresis. Five components were resolved in the blood of some random-bred Wistar rats by starch gel electrophoresis in the discontinuous buffer system of Morton (1962); only 4 were found in other random-bred Wistar animals. It was suggested that the 5-component pattern was present in rats heterozygous for two 4-component patterns observed in two inbred strains, one Wistar and one a Wistar/black hybrid (Marinkovic et al., 1967). At first sight, this suggestion appears to agree well with the pattern observed in the present study. However, the gel with which these authors chose to illustrate their

conclusions casts some doubt upon their interpretation; it was very much overloaded so that the bands were difficult to distinguish. Also, the number of bands present did not always appear to agree with the number said to be present by the authors. If this gel was a representative sample of the results obtained, then the authors' conclusions are ill-founded.

Since inheritance patterns of the haemoglobin components were not studied, no conclusions may be drawn as to the genetic control of rat haemoglobins. It is of interest that the order in which the components appeared in the foetal blood was different in rats from different Wistar colonies (section III, 5), suggesting that this aspect of haemoglobin synthesis may be controlled by genetic factors separate from the genes controlling the structure of the components. It is tempting to speculate that the decrease in haemoglobin 'c' synthesis while the proportions of the other components, especially haemoglobins 'a' and 'b', increased (Figure 55), is indicative of repression of the activity of the haemoglobin 'c' gene by the activity of the genes for the other components; this would imply that several, if not all, of the components are made in each cell and would be analogous to the decrease in haemoglobin F synthesis in human erythroid cells during foetal development (section 1, 5). In the absence of genetic and structural studies on rat haemoglobins, however, such inferences remain tentative.

The observation that the relative rates of synthesis of the components did not change during much of cell maturation (section III, 6) is in agreement with the work of Brada and Tobiska (1963). If cells are released from foetal erythroid sites at all stages of gestation between 24 and 48 hours after maturation begins (section III, 6) then the relative rates of synthesis of the components by circulating cells (Figures 42, 50 and 58) suggests that the same pattern is maintained until haemoglobin synthesis ceases; this requires more conclusive evidence.

Many suggestions may be made to account for the differing rates at which the components are synthesised. If they are made in separate cells, the rate may be determined by the number of maturing cells synthesising the different components. Since foetal liver and spleen possess identical synthetic patterns on the same day of gestation (section III, 6) this would imply that the numbers of each kind of cell were similarly controlled in each organ. If the components are all made by each cell the rates of synthesis may be controlled at many points on the synthetic pathway. There may be multiple genes for the more abundant components, or the more active genes may interfere with the activity of the less active genes. Transcription of mRNA for the less abundant components may be deficient or the mRNA produced may be in some way defective. At the level of translation the rate of chain initiation or assembly may differ, perhaps owing to variable steric hindrance of continuing peptide bond formation as the chains of the different components lengthen. Some of these rate-limiting steps have been demonstrated in cells of other species (section I, 4) but such

studies on rat cells have not been reported.

It is not known whether the possession of multiple components is advantageous to the rat or whether it is merely accidental; haemoglobin heterogeneity has been found in so many species as to suggest that it confers some advantage, the nature of which remains uncertain. It has been suggested that multiple components may serve to stabilise the intracellular haemoglobin solution at a higher concentration than would be possible with only one component (Perutz et al., 1959). Rat haemoglobin in vitro is extremely unstable; although it is evidently more stable in vivo, a less complex haemoglobin solution might well be equally stable within erythrocytes.

5. Nature of the erythropoietin-sensitive cell

ESC were defined (section I, 3) as progenitor cells destined to become erythroid cells after erythropoietin stimulation; they may or may not be capable of limited self-maintenance, and cannot be induced to form other cell lines. Such cells may well exist but it is less certain that they are the only cells capable of responding to erythropoietin. Two hypothesis may be advanced: the first is that only ESC can respond to erythropoietin and that the stimulated cells mature without further exogenous stimulation. The second is that all erythroid cells capable of cell division can also respond to erythropoietin and that the maturation of erythropoietin-stimulated progenitor cells may be accelerated by the effect of erythropoietin upon their progeny.

These are extreme viewpoints and some intermediate situation may be a truer interpretation than either.

It has been clearly demonstrated by *in vivo* studies on polycythaemic animals that erythropoietin can induce maturation of undifferentiated precursor cells, (Filmanowicz and Gurney, 1961; Nakao *et al.*, 1966; Orlic *et al.*, 1965). This is substantiated by the effect of erythropoietin on spleen colonies established in polycythaemic recipients (Curry *et al.*, 1967; Bruce and McCulloch, 1964). Further evidence comes from studies on the effect of the large amounts of endogenous erythropoietin produced after massive bleeding (Alpen *et al.*, 1962). That erythropoietin acts on progenitor cells is well-documented.

Although erythropoietin can facilitate reticulocyte release *in vivo* (Fruhman and Fischer, 1962; Fisher *et al.*, 1964.) it appears to have no direct effect on haemoglobin synthesis by reticulocytes (Eralew, 1962; 1964b). This is borne out by the very small response of foetal rat and mouse blood cells to erythropoietin (Figures 50 and 69). In addition, erythropoietin-induced haem synthesis is dependent on DNA synthesis (Figures 61 and 65), suggesting that the hormone cannot affect acidophilic normoblasts which, although nucleated, do not synthesise DNA. (section I, 2). On this evidence the most mature erythroid cell upon which the hormone may act is the polychromatic normoblast; the effect of the hormone on the release of reticulocytes is irrelevant *in vitro* since the cells must remain in the culture vessel.

High doses of erythropoietin *in vivo* were found to cause the production of macrocytic erythrocytes (Stohlman, 1961; Stohlman *et al.*, 1964); although the haemoglobin concentration of these cells was normal, their large size suggested that one, or more, of the maturation divisions had been missed out. Since the dose of erythropoietin was very high it was not certain whether such cells were produced in response to erythropoietin in normal circumstances. Macrocytes appear to be the end-product of an erythroid cell sub-line which may be distinguished from the normal line by its more rapid acquisition of higher haemoglobin concentration and its larger size at each maturation stage; it has been suggested that these cells arise by missing a division cycle at the basophilic or polychromatic normoblast stage, or by maturing from the polychromatic normoblast directly to the reticulocyte (Suit *et al.*, 1957; Borsook *et al.*, 1962). Such cells may constitute a small proportion of the maturing cells in normal marrow (Borsook *et al.*, 1968; Lord 1968) suggesting that erythropoietin may, in normal conditions, cause some cells to mature in this way. It has recently been observed that erythropoietin treatment increases the proportion of these cells in rabbit marrow cultures (Borsook *et al.*, 1968); the effect was observed so rapidly that it was attributed to direct action of erythropoietin on cells more mature than the ESC. The dose of erythropoietin used was 1 u./10^6 cells of a crude preparation of erythropoietin, a dose comparable to those used in the present work. However, a serum factor which increased the erythropoietin activity 4-fold (Van Dyke *et al.*, 1968) was also added to the cultures, so that the effective dose was 4 u./10^6 cells. This is 8 - 20 fold greater

than the doses used in this study; it is possible that the results reported were influenced by the use of so high a dose of hormone. Nevertheless, this work does suggest that erythropoietin can affect maturing cells as well as ESC.

The results obtained in the present study are consistent with the hypothesis that erythropoietin affects not only ESC, but also the early normoblast stages capable of DNA synthesis. It was clearly established that the magnitude of the response depended upon the length of exposure to the hormone (Figures 63, 68 and 70), suggesting that erythropoietin continued to act upon the cells for some time. This could indicate that further ESC were produced in culture to replace those immediately affected by erythropoietin, or that normoblasts formed as erythropoietin-stimulated ESC matured were in turn stimulated by erythropoietin. Although the cells remained sensitive for at least 24 hours in culture (Figure 71), it is not known whether precursor cells can become ESC in these conditions. CFC increase in number in mouse foetal liver throughout gestation (Silini et al., 1967), although ESC were not found later than day 14 (Cole and Paul, 1966); the loss of ESC from the liver after this time was attributed to stimulation of all sensitive cells by endogenous erythropoietin before explantation (section 1). However, if CFC could give rise to ESC in culture, a response of 15-18-day foetal liver cells to erythropoietin might be expected to occur in vitro, perhaps after quite lengthy incubation; this was not observed (Cole and Paul, 1966), suggesting that precursor

cells do not produce ESC in these culture conditions. If ESC are capable of self-maintenance and are insensitive to erythropoietin during cell division, as has been suggested for stem cells (Lajtha, 1964), ESC dividing when incubation started would not be able to respond immediately to erythropoietin. Whether the proportion of foetal liver ESC dividing at this time would be large enough to account for these results is questionable; this hypothesis also implies that division of ESC may take 18 hours or longer. ESC might also persist in erythropoietin-treated cultures if those cells first stimulated deprived the remaining ESC of erythropoietin for some time. In view of the rapidity with which erythropoietin acts (Figures 66 and 67), it would appear that the cells have no need to sequester it for any length of time; yet the inductive effect of the hormone could be seen after 18 hours incubation (Figure 68), suggesting that some ESC remained even then. While the cells may temporarily sequester erythropoietin, it appears that this alone would be insufficient to explain these results; the two latter hypotheses might together be sufficient.

The increase in haem synthesis stimulated by erythropoietin in cultures of young rat and rabbit foetal liver cells accelerated after about 24 hours incubation (Figures 9 and 28). If it is assumed that, as in mouse cell cultures, ESC are unlikely to arise from stem cells in vitro, two other interpretations of these results are possible. The first is that normoblasts formed by erythropoietin-stimulated ESC

are themselves further stimulated by erythropoietin; the second is that only ESC are stimulated and that the results reflect the variation in haemoglobin synthesis as the cells mature, without further stimulation. The latter may well account for part of the acceleration in rabbit cells, in which the rate of haem synthesis increased somewhat in the absence of erythropoietin especially in cultures of young foetal liver cells (Figure 35), suggesting that there may be an increase in haemoglobin synthesis in maturing cells without further stimulation. This is not the case in rat foetal liver cells, however, (Figure 20), and it may not be sufficient to explain all of the acceleration in haem synthesis observed in erythropoietin-stimulated rabbit foetal liver cells.

Although the results just discussed are consistent with the hypothesis that erythropoietin acts on cells other than ESC, they are not conclusive. Some other observations are more conclusive. As rat and rabbit foetal livers developed, the maximum response to erythropoietin was achieved progressively earlier in incubation (Figures 20 and 35); this suggests that the hormone was acting on the increased number of maturing cells present in the developing foetal liver, to increase haem synthesis more rapidly than stimulation only of ESC would achieve. Also, haem synthesis increased detectably 3 hours after erythropoietin treatment (Figure 67), much more quickly than haemoglobin synthesis begins in cells maturing from progenitor cells (Grasso et al., 1963). This may also suggest that erythropoietin affects maturing normoblasts.

The response observed thus probably consisted of the effects of erythropoietin on ESC and on maturing cells at various times during incubation. It is not known at what stage of maturation the cells become independent of erythropoietin, save that DNA synthesis is necessary for the response; thus, any cell may respond to erythropoietin until the polychromatic normoblast stage. Until the different erythroid cells and their progenitor cells can be identified and isolated, the stage of maturation at which sensitivity to erythropoietin is lost cannot be demonstrated with certainty.

Effect of erythropoietin on the cell cycle of sensitive cells. In mouse foetal liver cells DNA synthesis was greatly increased immediately after erythropoietin treatment (Figure 66); in rat cells the increase, although of the same magnitude, was less rapid (Figure 62). The difference may be a species difference or due to the use of different erythropoietin preparations. It appeared that many cells were stimulated almost simultaneously in mouse liver cell cultures; the rapid onset of DNA synthesis suggests that the cells were nearing the end of G_1 before stimulation or were accelerated into the S period by erythropoietin. It has been suggested that the G_1 period of progenitor cells may be of variable length (Kretchmar, 1966) and that its length depends on the requirement for cell division; cells may remain in G_1 for quite long periods if the pool is depleted only slowly. It has also been suggested that cells are sensitive to erythropoietin during only one phase of

their cycle (Goldwasser, 1966); in mouse cells the sensitive phase appeared to be G_1. It may be that the cells are erythropoietin-sensitive throughout G_1 and that erythropoietin curtails G_1 by precipitating the cells into DNA synthesis. It is not known whether maturing cells may also remain in G_1 for varying times; if they can, it is possible that erythropoietin, by cutting short this period, would accelerate the replication of proerythroblasts and basophilic normoblasts. Since increased haemoglobin synthesis is dependent on DNA synthesis (Figure 64) it may be that accelerated DNA synthesis increases the concentration of haemoglobin to a level at which it can affect the integrity of the nucleus as suggested by Granick and Levere (1964); such a mechanism is consistent with the production by large doses of erythropoietin of macrocytes which are not markedly hypochromic (Stohlman, 1961). This may imply more rapid synthesis of DNA concerned in haemoglobin synthesis than of other regions of DNA, in the manner discussed later, in section 6.

Thus, three sites of erythropoietin action on cell maturation may be envisaged:

1. On ESC to induce maturation by curtailing G_1 and initiating S; the pool of ESC would be depleted to an extent depending on the concentration of erythropoietin acting on it. The pool would then be replenished from the stem cell pool, whose members are held to be insensitive to erythropoietin.

2. On early normoblasts to accelerate their replication, perhaps also by curtailing G_1

3. On polychromatic normoblasts by increasing DNA synthesis leading to the synthesis of haemoglobin, which then prevents the final cell divisions and accelerates nuclear breakdown.

As discussed in this section, there is evidence consistent with all of these, 1 and 2 being much more strongly supported than 3.

It also appears that the dose of erythropoietin required to effect these changes increases as the cells mature. If the sensitivity of cells to erythropoietin is determined primarily by the attachment of the hormone to the cell membrane, prior to its entry into the cell or to the production of an intracellular 'activator' distinct from erythropoietin itself, the number of receptors on the cell surface may dictate the degree of sensitivity of the cells. If the ESC possesses a certain number of receptors, the number may decrease at each maturation division so that a higher hormone concentration is required to achieve an effective intracellular concentration. It is recognised that the postulated receptors may also be intracellular, but decrease in number in the same way. The continuing effect of exogenous erythropoietin for 18 hours or more (Figure 68) suggests that the hormone, or its active intracellular product, does not have a long intracellular lifetime, so that it must be constantly replenished to maintain the inductive effect. A brief period of intracellular survival of erythropoietin was suggested by Kretchmar (1966), but studies on the

entry of erythropoietin into the cells and its survival there have not been reported.

6. Mode of action of erythropoietin on erythroid cells

Many of the experiments depended upon the effect of inhibitors; the results might also be influenced by changes in the size of the intracellular pools of nucleic acid and protein precursors. Before discussing the results, these factors must be considered.

Puromycin has been reported to affect only polypeptide chain assembly (Williamson, and Schweet, 1965; Goldberg, 1965); its effect on other cellular activities has been found to depend upon this primary effect (Studzinski and Ellem, 1966). It may be taken, therefore, that the inhibitory effect of puromycin on erythroid cells is due to its effect on peptide chain synthesis.

The binding of actinomycin D to DNA markedly inhibits RNA polymerase (EC. 2.7.7.6); it also causes physical changes in the DNA, which may inhibit DNA synthesis and cell division after some time (Reich and Goldberg, 1964). At 10 µg./ml., the secondary effects may become significant when the inhibitor is present throughout incubation; only the early inhibitory effects may be considered as being due solely to inhibition of DNA - primed RNA synthesis. It was found also, that there was some delay in the effect of actinomycin D, perhaps because it took some time to enter the cells. Its effect on RNA synthesis became evident between ½ - 1 hour after treatment (Figure 75) but only 75%

inhibition was achieved after 1½ hours; how much of the remaining 25% uridine incorporation was due to CCA-turnover in tRNA is not known. Both DNA synthesis (Figure 73) and, to a lesser extent, haem synthesis (Figure 65) could continue in the presence of actinomycin D for some time; this suggests that any later effect of actinomycin D on these pathways was indirect and depended on its inhibition of RNA synthesis.

FUdR has been shown to inhibit DNA synthesis but not RNA synthesis in mammalian cells (Cheong et al., 1960); its effect could be reversed by subsequent addition of thymidine until the cells entered division (Eidinoff & Rich, 1959). It was found that thymidine added at the same time as FUdR by-passed the action of the inhibitor, while uridine did not (Figure 64); this suggests that only DNA synthesis was affected by FUdR in foetal liver cells.

Detailed studies on the effect of colchicine on foetal liver cells have not yet been performed. Previous in vitro studies on erythroid cells utilised colchicine at lower concentrations than that used here: 2.5×10^{-6}M (Eralev, 1962) and 10^{-8}M (Gallien - Lartigue and Goldwasser, 1965). In both instances only partial inhibition of erythropoietin-stimulated haem synthesis was observed; it was suggested that the maturation steps leading to haemoglobin synthesis could occur without cell division, which served only to magnify the results.

The observed rates of incorporation of labelled precursors into nucleic acids and protein will depend not only on the rates of syntheses but also upon the sizes of the intracellular pools of unlabelled

precursors. The pool sizes in foetal liver cells are unknown; nor is it known whether they alter during maturation. If erythropoietin caused a change in the permeability of the cells leading to reduction of the pool sizes, an artificially high rate of isotope incorporation might occur. This seems unlikely, since the pools appear to increase in size as cells become more active rather than to decrease; this has been found for the pool of DNA precursors in adult marrow cells (Alpen and Johnston, 1967), and for the pool of RNA precursors in lymphocytes (Forsdyke, 1968) and in liver cells (Ove et al., 1966). In the absence of precise data on the precursor pools, the results will be discussed on the assumption that the size of the pools and the rate at which labelled precursors equilibrate with them are the same in control and in erythropoietin-treated foetal liver cells.

Studies on haem synthesis in response to erythropoietin. In both rat and mouse foetal liver cells, syntheses of DNA, RNA and protein were required before erythropoietin could provoke an increase in haem synthesis, while cell division was not (Figures 60, 61, 64 and 65). Since all of these steps are known to precede haemoglobin synthesis during maturation (section I, 2), the former might be anticipated, but the latter would not; it is possible that haem synthesis may increase only to a certain level without cell division, and that, over a longer period of incubation, colchicine may have exerted an inhibitory effect on the response to the hormone.

Puromycin reduced haem synthesis in control cultures quite quickly (Figure 64), suggesting that the haem synthesising enzymes had a short half-life. A similar suggestion was made on the basis of the effect of puromycin on adult marrow cells (Gallien - Lartigue and Goldwasser 1965). It is also possible that globin synthesis was arrested by puromycin and that the subsequent accumulation of haem inhibited further haem synthesis (Karibian and London, 1965; Grayzel et al, 1967). A fairly long life span of the haem synthesising system, 8 hours or more, has been estimated (Wilt, 1965; Goldwasser, 1966), suggesting that most of the inhibition of haem synthesis by puromycin after 7 hours incubation (Figure 64) is more likely to be due to its effect on globin synthesis, rather than a direct effect on the formation of haem synthesising enzymes. Actinomycin D also reduced the rate of haem synthesis in control cultures (Figures 60 and 65), although more slowly than puromycin. This indicates that some mRNA synthesis for globin or haem synthesising enzymes was taking place in these cells. The complete inhibition of the erythropoietin-stimulated increase in haem synthesis by actinomycin D agrees with previous studies carried out in vivo and in vitro (Keighley and Lowy , 1966; Gurney and Hofstra, 1963; Dukes and Goldwasser, 1965; Gallien - Lartigue and Goldwasser, 1965). Although FUdR completely prevented an increase in haem synthesis in response to erythropoietin it had little or no effect on haem synthesis in cultures without erythropoietin (Figures 61 and 64). This indicates that, in all erythropoietin-stimulated rat and mouse foetal liver cells, DNA synthesis is a necessary prelude to haem synthesis; it may also suggest that

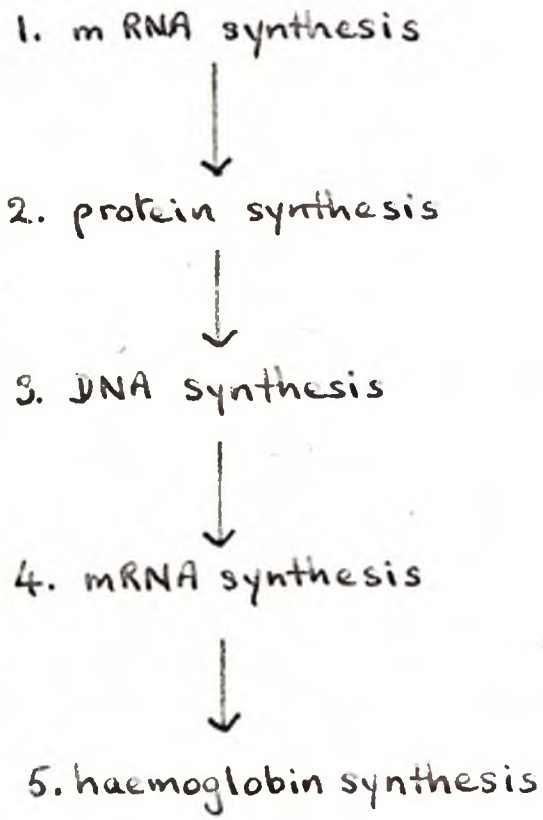
haem synthesis by maturing cells reaches its maximum rate only after DNA synthesis is complete. Since control cultures contain cells at all stages of maturation, including early, dividing normoblasts, it might be argued that haem synthesis by these cells would be reduced by FUdR treatment. That this was not observed may suggest that erythropoietin must be present to ensure that maturation of rat and mouse foetal liver cells proceeds normally (section 6). The increase in the rate of haem synthesis in some cultures of rabbit foetal liver cells without erythropoietin treatment (Figure 35), may suggest that this is not the case in all species; in the absence of more detailed studies on rabbit, and other cells, this question remains open.

It seems likely that DNA synthesis is followed in normal circumstances by cell division. This is also suggested by the fact that after 8 hours incubation, FUdR had rendered erythropoietin-treated cells unable to respond to further erythropoietin treatment in the presence of thymidine (Figure 82). Since thymidine could, if present throughout incubation, reverse the effect of FUdR, this result suggests that the cells, after erythropoietin treatment, attempted to divide, but could not while FUdR was present, and were killed. If correct, this argument would imply that most erythropoietin-treated cells enter division within 8 hours of erythropoietin treatment. The studies with colchicine, however, indicate that cell division is not mandatory for haem synthesis, at least in the early stages of the <u>in vitro</u> response to erythropoietin (Figures 61 and 65).

Studies on the steps preceding haem synthesis in response to erythropoietin. Since actinomycin D and puromycin affected the pathway of haem synthesis directly, these results give little information as to the effects of erythropoietin on the cells which lead to the formation or increase the formation of the haem synthesising system. FUdR, on the other hand, had little or no direct effect on haem synthesis but completely prevented it from increasing after erythropoietin treatment. It appeared that DNA synthesis must precede the onset of haem synthesis but was not essential to its continuation. The importance of DNA synthesis to the response of mouse foetal liver cells to erythropoietin was further demonstrated by the observation that erythropoietin caused an immediate and large increase in DNA synthesis (Figure 66). Although autoradiographic studies have shown that there is an increase in the number of cells synthesising DNA some time after erythropoietin treatment (Powsner and Berman, 1965), no increase in [^{14}C] formate incorporation into DNA was observed 4 hours after erythropoietin treatment (Pieber-Perretta, et al., 1965); nor was thymidine incorporation increased by 9 hours after erythropoietin treatment (Goldwasser, 1966). The medium used by the latter two groups contained quite large amounts of unlabelled deoxyribonucleosides; in addition de novo synthesis of unlabelled DNA precursors was in no way inhibited. It is possible that the labelled precursors were so diluted after addition to the cultures that increased DNA synthesis would not be matched by increased incorporation of the labels.

Figure 84

Postulated mode of action of erythropoietin on foetal mouse erythroid cells.



The studies on erythropoietin-stimulated syntheses of nucleic acids and protein, and the affects of inhibitors upon them showed that the earliest detectable effect of erythropoietin was on mRNA synthesis (section 111, 9), and suggested that mouse foetal liver cells responded to erythropoietin as outlined in Figure 84..

This scheme is only skeletal; the cells evidently produce RNA other than mRNA and proteins other than haemoglobin. It represents only the central pathway by which erythropoietin appeared to stimulate the cells to synthesise haemoglobin. It is assumed that the effect of erythropoietin on all sensitive cells, ESC and/or normoblasts, proceeds along similar lines.

That erythropoietin treatment stimulated increased syntheses of DNA, RNA, haemoglobin and other proteins was directly demonstrated by incorporation of [^{3}H] thymidine (Figure 66), [^{3}H] uridine (Figure 78), ^{59}Fe (Figure 67) and [^{3}H] lysine (Figure 80) respectively. That these took place in the sequence shown in Figure 84, was suggested by the effects of inhibitors on the response to erythropoietin.

It was shown that syntheses of RNA and protein were essential for increased DNA synthesis after erythropoietin treatment (Figures 73 and 77). It was evident that, although DNA synthesis increased very quickly, it was not the first effect of erythropoietin on the cells. It was also found that a detectable increase in RNA synthesis after erythropoietin treatment was dependent on DNA synthesis (Figure 78) whereas a small amount of erythropoietin-stimulated protein synthesis

could continue in the presence of FUdR (Figure 80). The latter confirmed directly that not all protein synthesis in erythropoietin-stimulated cells followed DNA synthesis. It was then found that the erythropoietin-stimulated protein synthesis preceding DNA synthesis was prevented by actinomycin D (Figure 81), indicating that RNA synthesis preceded this protein synthesis. These results and the inhibition of increased DNA synthesis by puromycin indicate that the response of the cells to erythropoietin leading to increased DNA synthesis occurred as shown in Figure 84.

Steps 1 and 2 must occur very quickly since DNA synthesis doubled within 1 hour of erythropoietin-treatment. Treatment with actinomycin D for 1 hour completely prevented erythropoietin from stimulating DNA synthesis (Figure 77), while erythropoietin added together with actinomycin D was able to stimulate DNA synthesis, which continued for 6 - 8 hours unaffected by the inhibitor (Figures 73 and 74); this suggests that all of the mRNA required for the increased DNA synthesis, during 6 - 8 hours at least, was made during the first hour after erythropoietin treatment and that the mRNA or the protein synthesised from it was stable for these 6 - 8 hours. FUdR prevented a detectable increase in RNA synthesis after erythropoietin treatment while, in the same circumstances, protein synthesis, sensitive to actinomycin D, could continue. This suggests that the amount of mRNA synthesis preceding DNA synthesis is very small indeed. This agrees with the work of Krantz and Goldwasser (1965b) who found no detectable increase in total RNA synthesis

15 minutes after erythropoietin treatment of adult marrow cells; after sucrose gradient centrifugation of the cells' RNA, they observed increased activity in one part of the gradient relative to other parts. The more active RNA(s) sedimented in the manner postulated for mRNA (Singer and Leder, 1966).

That DNA synthesis precedes most RNA synthesis after erythropoietin treatment is indicated by a comparison of the time-course of DNA synthesis (Figure 66) with that of RNA synthesis (Figure 78); DNA synthesis increased much more rapidly than RNA synthesis. This is corroborated by the fact that FUdR prevented any detectable increase in RNA synthesis after erythropoietin treatment (Figure 78).

Some, at least of this RNA synthesis is directed towards haemoglobin synthesis. That increased haem synthesis after erythropoietin treatment depends on DNA synthesis is shown by the fact that FUdR inhibited the increase and that the inhibition was reversed by thymidine (Figure 64). The inhibition by actinomycin D of erythropoietin induced haem synthesis may be due to the inhibition of DNA synthesis as well as to the inhibition of haem synthesis itself. However, there is evidence that RNA synthesis intervenes between DNA synthesis and haem synthesis: this is that actinomycin D reduced haem synthesis in control cultures while FUdR did not. Thus, the RNA synthesised in this instance was directed not toward DNA synthesis but toward haem synthesis. It may be inferred that some of the DNA-dependent RNA synthesis after erythropoietin treatment is required for haemoglobin synthesis, for the haem synthesising

enzymes and for globin; this is also in accord with the current model of protein synthesis (section 1, 4).

It should be noted that the conclusions drawn are tentative and must be confirmed by more precise studies. In particular, it must be ascertained what proportion of the syntheses of nucleic acids and protein is due to erythroid cells; it should also be investigated in pure cultures of erythroid cells whether similar results would follow upon erythropoietin treatment.

If this model is correct some questions arise as to the steps involved. It is not known whether all of the protein synthesis preceding DNA synthesis in erythropoietin-treated cells is necessary for DNA synthesis, although it appeared that some of it, at least, was necessary. Since DNA synthesis reached its maximum rate while this protein synthesis was still increasing (cf. Figures 66 and 80), it seems possible that not all of the protein(s) were directly concerned with DNA synthesis. The protein necessary for DNA synthesis may be DNA polymerase (E.C.2.7.7.7.) itself. An increase in DNA polymerase synthesis in parallel with, though slightly earlier than, increased DNA synthesis has been observed following treatment of mammary gland epithelium with insulin (Lockwood et al., 1967a) If DNA sequences required for maturation are inactive by virtue of their complex with chromatin constituents, enzymes to remove these constituents may also be formed prior to DNA replication.

Increased RNA, protein and haem syntheses could be detected within 3 hours of erythropoietin treatment and were all dependent on DNA synthesis ;

if the S period lasts 4 - 6 hours, as has been suggested for erythroid cells (Cooper, 1967; Alpen and Johnston, 1967), this suggests that the newly synthesised DNA may be transcribed before the end of the S period. This suggests that in erythroid cells, cell proliferation does not preclude the synthesis of specialised products. This was suggested by the fact that myoblasts after fusion synthesise myosin but not DNA, whereas no myosin is formed during the pre-fusion cell divisions (Stockdale and Holtzer, 1961). In the mammary gland epithelium, too, the formation of milk proteins follows cell division (Lockwood et al., 1967b); it has been reported that this is also the case during lens regeneration from iris cells (Yamada, 1967). Foetal liver cells are also unusual in the rapidity with which they begin DNA synthesis after erythropoietin treatment; the increased DNA synthesis following treatment of mammary gland epithelium with insulin is not great until 24 hours later (Lockwood et al., 1967a)

The synthesis of specialised products before the cells have completed DNA synthesis may imply that different regions of the DNA replicate earlier than others. It has been reported that the DNA of different chromosomes in marrow cells replicates at different times during the S period (Alpen and Johnston, 1967); the present work implies that the DNA directing haem synthesis replicates early in the S period. A similar distinction between the functioning of DNA responsible for differentiation and that responsible for cell proliferation is reported in myoblasts; incorporation of bromodeoxyuracil into myoblast

DNA permitted cell division to continue but prevented fusion and subsequent myosin synthesis (Stockdale et al., 1964).

The increased RNA synthesis (Figure 78) probably comprises all forms of cellular RNA; sucrose gradient centrifugation 9 hours after erythropoietin treatment of marrow cells showed that labelled uridine had been incorporated into all RNA species (Krantz and Goldwasser, 1965b). Stimulation of ESC may result in a much larger amount of rRNA synthesis than stimulation of early maturing cells, since the earliest maturation stages contain a higher concentration of ribosomes than later stages (Marks and Kovach, 1966) and many of the ribosomes are made at, or shortly before, the procrythroblast stage (De Bellis et al., 1964). Even if the ribosomes turn over rapidly during maturation, the rate of replacement would seem to be lower than the rate of destruction. Many new mRNA species will be required for maturation; this work provides partial identification of some of these. One, or more, is required to increase DNA synthesis; mRNA for haem synthesising enzymes and presumably for globin also, is produced. Other species of mRNA produced following erythropoietin treatment have also been pinpointed, those responsible for synthesis of the cell stroma (Dukes and Goldwasser, 1965) and for transport of iron across the cell membrane (Hrinda and Goldwasser, 1966).

The changes observed in the activity of foetal liver cells after erythropoietin treatment resemble, in general terms, those occurring in other differentiating cells. There are differences, however, some of

which have been mentioned; it is possible that there may also be differences of detail in the biochemistry of maturation of erythroid cells of different species. Nevertheless, some light has been shed on the way in which erythropoietin affects its target cells; it is hoped that this will be of use in further studies on these and other differentiating systems.

V. SUMMARY

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1. This work was based on techniques developed to assay the effect of erythropoietin *in vitro* on adult rat marrow cells (Krantz *et al.*, 1963) and foetal mouse liver cells (Cole and Paul, 1966); the activity of the hormone was measured by estimating the incorporation of ^{59}Fe into protein-bound haem.

2. The sensitivity to erythropoietin of rat yolk sac, liver and spleen cells during foetal development was determined; a similar study was carried out on foetal rabbit liver cells of different ages. The results from the two species were compared with each other and with those from foetal mice (Cole and Paul, 1966). It was concluded that erythropoietin was probably concerned in the regulation of foetal erythropoiesis, from the onset of liver erythropoiesis.

3. Three haemoglobins were detected in the blood of young mouse foetuses by starch gel electrophoresis; they were replaced by adult haemoglobin during gestation. The technique was adapted so as to identify, by starch gel electrophoresis, the haemoglobins synthesised by erythroid cells *in vitro*. It was found that mouse yolk sac cells made only foetal haemoglobins, while foetal liver cells made adult haemoglobin; the results agree with previous studies on Hb^s/Hb^s mice (Craig and Russell, 1964; Kovach *et al.*, 1967). Erythropoietin, to which yolk sac cells did not respond, stimulated only adult haemoglobin synthesis by foetal liver cells.

4. Two haemoglobins were found in the blood of young rabbit foetuses; they were replaced by adult haemoglobin during gestation.

5. Two haemoglobins were found in the blood of young rat foetuses; three further haemoglobins appeared in the blood during gestation. Treatment of sensitive foetal liver cells with erythropoietin did not induce synthesis of different haemoglobin components; although total haemoglobin synthesis was increased by erythropoietin, the relative amount of each component synthesised was unaltered. Erythropoietin stimulated adult rat marrow cells in the same way. It was concluded that, in normal circumstances, erythropoietin had only a quantitative effect on haemoglobin synthesis and did not control the nature of the haemoglobin made. In all foetal, and adult, cells the relative rates of synthesis of the components of rat haemoglobin appeared to be constant during most, if not all, stages of cell maturation.

6. The mode of action of erythropoietin on foetal erythroid cells was studied in more detail. The effect of inhibitors on erythropoietin-treated cells indicated that erythropoietin-induced haem synthesis was dependent on syntheses of DNA, RNA and protein, but not on cell division. The assay technique was modified to measure the incorporation of [^{3}H] thymidine, [^{3}H] uridine and [^{3}H] lysine. Although these parameters are not specific to erythroid cells, the results indicated that syntheses of DNA, RNA and protein increased after erythropoietin-treatment; DNA synthesis increased most rapidly. The effect of

inhibitors on erythropoietin-treated cells indicated that the earliest detectable result of erythropoietin treatment was mRNA synthesis; this was followed by synthesis of protein required for DNA synthesis. Only after synthesis of DNA did haem synthesis increase. The significance of the results was discussed and compared with *in vitro* studies on other differentiating systems.

7. The time-course of the increase in haem synthesis in erythropoietin-treated foetal liver cell cultures was also studied. The results, in conjunction with those quoted in 6., suggested that erythropoietin may affect not only progenitor cells but also maturing cells, capable of DNA synthesis.

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