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# STUDIES ON ERYTHROID CELL MATURATION.

Hugo Burgos.

Summary of thesis presented for the degree of Doctor of Philosophy, University of Glasgow, June 1971.

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## SUMMARY.

Studies on erythroid cell maturation were carried out by means of two different experimental approaches leading to the characterization of (a) the haemoglobin type synthesized by spleen colonies produced by transplantation of rat haemopoietic tissue into heavily irradiated mice, and (b) the daughter cells derived from mitoses of earlier haemopoietic precursors, and factors influencing their maturation and proliferation.

Optimal experimental conditions for spleen recolonization by heterologous (rat) transplants were investigated and established through a series of experiments, and methods for preparation and analysis of the type of haemoglobin synthesized by the recolonizing cells were developed.

Investigation of the behaviour of normal and experimental haemoglobins in starch gel electrophoresis and development of assay for their differential characterization were undertaken.

Supralethally irradiated mice (Porton strain) were transplanted with foetal liver and adult bone marrow cells from Wistar rats. After a suitable post-transplantation period, the exogenously recolonized spleens were obtained. The confluent spleen colonies

were isolated and disaggregated, and cell cultures prepared and incubated with $^{59}FeCl_3$. Haemoglobins were extracted, purified by CMC column chromatography, and fractionated by starch gel electrophoresis. The incorporation of $^{59}Fe$ into haemoglobin was determined by cutting the gel strip containing the haemoglobin components into 1mm or $1\frac{1}{2}$ mm thick slices, which were then individually hydrolysed and counted by liquid scintillation system.

It was possible to demonstrate the heterogeneity of rat haemoglobin, and the presence of haemoglobin aggregates. Synthesis of catalase and other non-haemoglobin, haem-containing proteins, along with synthesis of haemoglobin was also demonstrated. This makes the interpretation of assays based on iron incorporation difficult for haemoglobin synthesis studies.

Although recolonization of mouse spleens by rat haemopoietic tissues was achieved in spite of the antigenic disparity between donor and host, the analysis of the type of haemoglobin synthesized by the spleen recolonizing cells was obscured by the presence of endogenous haemoglobin and aggregates. However, there was some indication that the recolonizing cells continue to produce the same type of haemoglobin.

For the <u>in vitro</u> study of behavioural and morpho-

logical characteristics of erythroid cells, and factors influencing their maturation and proliferation, 13-day foetal mouse livers were isolated and disaggregated. Cell cultures were prepared in plasma and fibrin clots, and observed with time-lapse cinemicrography during mitosis. The events recorded on the film were analysed and correlated with the fixed and stained cells.

The effects of different plasma, and erythropoietin stimulation on mitosis of erythroid cells were studied by measuring differences in frequency distribution of mitoses and mitotic indices, and also by autoradiography. The results demonstrated an increased number of mitoses in erythropoietin-stimulated cultures. Erythropoietin appeared to permit the continuation of the cell cycle and to maintain mitotic activity throughout the period of incubation. The results also suggested that erythropoietin produced shortening of G2 and G1, i.e. precipitat the initiation of mitosis as well as synthesis.

By the use of plasma from mice made polycythaemic by hypertransfusion, and stimulation with erythropoietin, the presence of an erythropoietic mitotic inhibitor was demonstrated.

# STUDIES ON

# ERYTHROID CELL MATURATION

A Thesis submitted for the Degree of Doctor of Philosophy in the Faculty of Medicine,

by

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# INTRODUCTION.

## I. The problem of identification of the haemopoietic stem cell.

The classical haematological theories (Maximow and Bloom, 1934; Ferrata, 1935) postulate that there is a stem cell group in the haemopoietic organs, which is capable of indefinite division to renew the stem cell line, and also of differentiation into mature blood cell elements. The fact that the erythrocyte compartment is constantly being replaced calls for a compartment of stem cells with both differentiating and self-maintaining characteristics (Lajtha, 1964). Without the stem cell compartment, erythropoiesis cannot continue despite the proliferation of the erythroblast (Astaldi, 1959).

At the present time there is no direct method of identifying the stem cells by cytological or biochemical methods (Marks and Kovach, 1966). Reasonably established criteria for cell characterization exist in general, as pointed out by Yoffey (1960) from the more mature cell stages back to the so-called haemocytoblast, beyond which the problem is intractable.

As pointed out by Marks and Kovach (1966), the best available method for evaluating the presence of stem cells in a particular cell population is the ability to give rise

to haemopoietic cell colonies by the spleen recolonization technique described by Till and McCulloch (1961) as an assay for haemopoietic stem cells. Although the characteristics of the stem cell are still poorly defined (Jenkins, Upton and Odell, 1969), the spleen colony forming cells seem to satisfy two basic criteria for being considered at least part of the stem cell pool (Marks and Kovach, 1966), that is, they are capable of self-replication, and they are capable of multipotent differentiation into the erythrocytic, granulocytic and megakaryocytic series (Till and McCulloch, 1961; McCulloch and Till, 1962; Siminovitch, McCulloch and Till, 1963; Lewis and Trobaugh, 1964; Juráskova, Tkadlečeck and Drásil, 1964; Curry and Trentin, 1967; Curry, Trentin and Wolf, 1967; Wu et al., 1967, 1968; Chen and Schooley, 1968).

This sort of physiological approach permits the use of the concept of the stem cell without having to refer to its morphological characteristics, and has been responsible for valuable information about the stem cell problem; but sooner or later, the necessity arises to refer functional events to a defined morphological entity, and here again the problem emerges: What morphological stage corresponds to a stem cell?

As pointed out by Turner et al. (1967), haemopoietic

cell suspensions have been fractionated by density gradients to obtain cell populations with an enriched content of colony-forming cells but only one attempt has been made to correlate a definite morphological cell type with the production of spleen colonies (Niewisch, Vogel and Matioli, 1967).

Identification of stem cells by the capacity to form spleen colonies has limitations which include the possibility that not all stem cells may be capable of dividing under the experimental conditions (Marks and Kovach, 1966). Moreover, multipotent differentiation and self-renewal, as essential characteristics of the colony-forming cells, have also been put into doubt by the studies of Bennett and Cudkowicz (1968)[6] and Bennett et al., (1968), who stated that "the contention that multipotent colony-forming cells exist, does not necessarily conflict with the concept that unipotent colony-forming cells also exist". In other words, a problem of different nature has arisen: although the colony-forming units have the general characteristics of the stem cells in that they either differentiate or reproduce themselves (Till, McCulloch and Siminovitch, 1964). Some of these characteristics may also be typical not only of the stem cells, but of their immediate descendants, as well (Bennett and Cudkowicz, 1968)[6].

## II. Spleen Colonies.

Growth and differentiation.

Many years ago it was known that lethally irradiated mice could be restored to health by injections of marrow cell suspensions (Loutit, 1968). Specific markers on the donor's cells - immunological (Mitchinson, 1956), histochemical (Nowell et al., 1956), or chromosomal (Ford et al. 1956) - demonstrated that such restoration was due to repopulation of the aplastic haemopoietic tissues of the host by donor cells (Loutit, 1968).

The existence of a pluripotent stem cell in mouse marrow was suggested by the presence of the same radiation induced chromosome aberrations in a great majority of marrow and lymphoid cells (Ford, Micklem and Gray, 1959) or cells of different organs (Barnes et al., 1959) in endogenously repopulated mice.

Murray (1948) and later Stroud et al. (1955) observed that haemopoietic recovery in sublethally irradiated mice occurred as clusters of haemopoietic cells in the spleen. Till and McCulloch (1961) described the spleen recolonization technique as an assay for haemopoietic cells, based on the fact that the intravenous injection of an appropriate number of marrow cells into isologous hosts previously exposed to supralethal total body irradiation leads to

the formation of colonies of proliferating cells in the spleens of these animals (Marks and Kovach, 1966).

The status of the clonal nature of the spleen colonies.

Becker, McCulloch and Till (1963) found that an overwhelming majority of cells in a colony contained the same markers, i.e. chromosome aberrations induced by irradiation of donor cells, and concluded that each spleen colony represented a cell clone derived from a single progenitor cell. This suggestion has been supported by other experiments using marked chromosomes (Wolshons, 1964; Wu et al., 1967; Chen and Schooley, 1968).

However, the clonal nature of the spleen colonies has been put into doubt by cytogenetic studies made with marked chromosomes as well, which suggested that most spleen colonies were not clones (Lewis et al., 1967). In addition, observations of multiple colonies from a single cell (Barnes et al., 1968) suggested that colony-forming cells (C.F.Cs) and their immediate descendants can migrate in the spleen from one site to another. The same set of radiation-induced chromosome markers was identified in up to 3 to 4 colonies within the same spleen. Since such migration appeared to have occurred shortly after injection, there is no compelling reason to suggest that intrasplenic migration of colony-forming units (C.F.Us) and/or other

cells could not occur at later times (Bennett and Cudkowicz, 1968). The possibility of multiple colonies from a single cell also raises the possibility of single colonies from multiple cells.

It appears, therefore, that at least part of the spleen colonies may not be clones, but the number and distribution of them is unknown.

Cytological composition of the spleen colonies.

During the development of colonies, and after a lag period of 24 hours (McCulloch and Till, 1964) or 48 hours (Matioli, Vogel and Niewisch, 1968) the colony-forming cells have a capacity for extensive proliferation; from the 2nd to the 9th day an exponential multiplication phase with a doubling time of 20 to 30 hours is established, followed by a saturation phase beginning at the 9th day (Matioli, Vogel and Niewisch, 1968). C.F.Cs can give rise to colonies containing an excess of one million cells within 10 days (Till, McCulloch and Siminovitch, 1964).

Numerous reports have appeared on the cytological composition of the colonies. The colonies themselves may be "pure", i.e. predominantly erythropoietic, or granulopoietic, or megakaryopoietic, or they may be "mixed" (McCulloch, 1963; Duplan, 1963; Davis and Kay, 1964; Lewis and Trobaugh, 1964; Trobaugh and Lewis, 1964;

Juraskova, Tkadlečeck and Drásil, 1964; Schooley, 1964; Curry, Trentin and Wolf, 1964; Mekori and Feldman, 1965; Liron and Feldman, 1965; Juraskova and Tkadlečeck, 1965; Silini, Pons and Pozzi, 1968). Lymphocytic colonies have been claimed (Mekori, Chieco-Bianci and Feldman, 1965) but not confirmed (Micklem, 1966; Curry, Trentin and Chong, 1967; Curry and Trentin, 1967). In addition to the bulk of differentiated cells, most spleen colonies contain a variable number of C.F.Cs, i.e. cells capable of giving rise to colonies upon transplantation into new hosts (Siminovitch, McCulloch and Till, 1963). They can be found in practically all other colonies, but they also form undifferentiated or primitive cell colonies. The number of these small foci diminishes with time, presumably because they are obscured by the growth of much larger colonies around them, or because they differentiate (Curry and Trentin, 1967).

The frequency of each spleen colony type has been variously reported in the literature (Curry and Trentin, 1967). Differences in technique, time of harvest, and criteria of evaluation probably account for at least some of the variations noted, but certain features are common: (1) the predominant colony is erythroid; (2) granulocytic and megakaryocytic colonies occur in about equal frequencies, and are each around a half or a third as

common as erythroid colonies; (3) discrete lymphoid colonies have not been observed (Curry and Trentin, 1967).

Mixed colonies have been considered by some (Wu et al., 1967) to be derived from random differentiation of multipotent dividing stem cells within one colony. However, Curry and Trentin (1967) have pointed out that, although colonies appear pure during the first week post-transplantation, it is uncertain if the other cell lines of differentiation arise by (a) further endogenous differentiation of multipotent primitive cells that were growing along one line of differentiation, determined by one type of microenvironment but, because of the increasing size, the colony has encroached on adjacent microenvironments, determining a different type of differentiation; (b) transmigration of mono- or multipotent cells from other colonies; or (c) confluence of two colonies of distinct cell type of differentiation.

A random association of two or more C.F.Cs at the time of seeding has been considered unlikely by Silini, Pons and Pozzi (1968).

Factors responsible for the heterogeneity of the spleen colonies.

Individual colonies are very heterogeneous, not only in their differentiated cell content, both

quantitative and qualitative (Lewis and Trobaugh, 1964; Juraskova, Kadleček and Drásil, 1964; Fowler et al., 1967; Curry and Trentin, 1967), but also in their content of stem cells (Siminovitch, McCulloch and Till, 1963).

Various factors have been proposed to explain these differences: a stochastic growth of the colony-forming unit (Till, McCulloch and Siminovitch, 1964), a haemopoietic inductive microenvironment (Curry and Trentin, 1967) and intrinsic differences in the colony-forming units (Worton, McCulloch and Till, 1969).

1. Stochastic model of stem cell proliferation. On the basis of the number and distribution of C.F.Cs experimentally examined by Siminovitch, McCulloch and Till (1963), who found that while most colonies contained these cells, their distribution among colonies was very heterogeneous with many colonies containing few C.F.Cs and few colonies containing very many C.F.Cs, a theoretical model of stem cell proliferation was proposed.(*)

It is of interest to consider here the three kinds of cell division which the stem cells may undergo: the stem cell can divide to produce either two new stem cells, or two differentiated cells, or one new stem cell and one differentiated cell (Osgood, 1957; Lajtha, 1964). Besides this of course, the stem cell can differentiate into a more mature cell (Vogel, Niewisch and Matioli, 1969).

(*)Till, McCulloch and Siminovitch, 1964.

Briefly, according to the stochastic (probabilistic) model, each C.F.U. may undergo either a "birth" process, i.e. division to give rise to two new stem cells, or a "death" process, i.e. differentiation to a more mature descendant. These events would occur at random in a single cell, but there would be definite probabilities of "birth" and "death" over the whole population in each generation. Numerical values for these probabilities were worked out by Till et al., using Monte Carlo calculations. Changes in probabilities may act as control mechanisms at the population level (Silini, Pons and Pozzi, 1968).

The case of a stem cell dividing to give rise to one new stem cell and one differentiated cell has called for an adjustment in probabilities, since there is no way of distinguishing a "birth" followed by a "death", unless individual cell divisions were observed (Till, McCulloch and Siminovitch, 1964).

This model was originally developed to account for differences in the number of new stem cells from colony to colony (Siminovitch, McCulloch and Till, 1963) but it can be extended to account for differences in numbers of differentiated cells as well (Worton, McCulloch and Till, 1969).

In this model, all stem cells are supposed to undergo

transformation at a fixed time after the previous transformation (Vogel, Niewisch and Matioli, 1969). However, the haemopoietic tissue of a healthy adult animal differs from this, mainly by having time-independent numbers of cells of various types (Vogel, Niewisch and Matioli, 1969).

2. Haemopoietic inductive microenvironment. Curry and Trentin (1967) considered the following information:

(1) Ford, Micklem and Gray (1959) suggested that endogenous subpopulations of cells in "spontaneous reversion" arise from single cells.

(2) Becker, McCulloch and Till (1963) obtained evidence to suggest that the spleen colonies were clones derived from a single C.F.C.

(3) Lewis and Trobaugh (1964) showed that pure erythroid colonies, when transplanted into a second irradiated host, produced new colonies with all cell types of differentiation, and suggested therefore the multipotency of the C.F.U.

(4) Curry and Trentin (1967) observed that spleen colonies were pure at least during the first week post-transplantation, but became mixed later on, supporting the multipotency of the C.F.U., although the formation mechanism of mixed colonies is uncertain; but the following questions still remain unanswered:

Why do most of such colonies differentiate first into one or other single cell line, rather than into two or more lines simultaneously? and to what extent does mixing also occur by transmigration? (Curry and Trentin, 1967).

(5) Savage (1964), Brecher and Smith (1965) and Wolf and Trentin (1967) found that in spleen the colonies were predominantly erythroid, while regenerating haemopoiesis in the marrow was predominantly neutrophilic, suggesting either the influence of environment on the pathway of differentiation of a pluripotent cell, or a differential migration of a monopotent erythroid or granuloid C.F.U. to a different locale (process of "homing").

(6) Curry and Trentin (1967) observed that even in the recolonized spleen, different types of colonies tended to occupy different topographical sites, suggesting the influence of a microenvironment within the spleen either for differentiation of a multipotent C.F.U. or for "homing" of monopotent C.F.Us.

(7) Curry, Trentin and Wolf (1964), Schooley (1964), Liron and Feldman (1965)[a,b], Bleiberg, Liron and Feldman (1965) observed that in hypertransfused mice

erythroid colonies remained small and did not develop unless erythropoietin was administered. All these experiments suggested that multipotent C.F.Us. were limited in their pathways of differentiation not only by systemic erythropoietin, but by their particular microenvironment within the spleen (Curry and Trentin, 1967).

On the basis of these observations, Curry and Trentin (1967) proposed that "most, if not all C.F.Us. are single pluripotent stem cells in nature whose commitment to a certain line of differentiation is influenced by microenvironmental influences as well as systemic humoral factors".

Accordingly, they suggested that the initial progeny of the pluripotent C.F.U. are undifferentiated. The majority of these colonies undergo first erythroid differentiation as a result of contact with the predominant type of splenic haemopoietic inductive microenvironment, i.e. erythroid, but only if erythropoietin is present. As the undifferentiated stem cells of the expanding colony encroach on an adjacent microenvironment determinative for a second line of differentiation, the colony becomes mixed in cell type.

Such a mechanism does not rule out the origin of some mixed colonies by transmigration of cells from one

colony to another, or by confluence of two colonies of distinct cell types. Nor does it necessarily rule out homing phenomena on the part of haemopoietic stem cells (Curry and Trentin, 1967).

3. Intrinsic differences in the colony-forming cells. The two previous explanations (stochastic model and haemopoietic inductive microenvironment) are not mutually exclusive, as both of them may play a role in determing the composition of the colonies (Worton, McCulloch and Till, 1969). However, they both assume that the stem cells have equally homogeneous intrinsic potentialities for differentiation and self-renewal (Worton, McCulloch and Till, 1969).

Such an assumption is incorrect (Worton, McCulloch and Till, 1969). By utilizing a velocity sedimentation technique to fractionate populations of haemopoietic "stem cells" into subpopulations and finding their capacity for self-renewal by injecting them into a second host, it was found that C.F.Us. in fractions containing cells of low sedimentation velocity had a larger capacity for self-renewal than C.F.Us. in fractions containing cells of high sedimentation velocity. Worton et al. concluded that intrinsic differences among the stem cells themselves, besides stochastic and micro-environmental factors, were responsible for the

heterogeneity observed in the C.F.U. content of individual spleen colonies.

Other observations which also provide evidence for the existence of intrinsic differences among the colony-forming population will be discussed in the following paragraph.

for the existence of intrinsic differences among the

The problems of the uni- or multipotentiality of the haemopoietic C.F.C. and the heterogeneity of the haemopoietic C.F.C. population.

Since Becker, McCulloch and Till (1963) suggested that each spleen colony results from the proliferation of a single cell, cytological studies of the spleen colonies have led to controversy rather than agreement about the potential for differentiation of the C.F.U. (Fowler et al., 1967).

It has been suggested that most or all C.F.Us. may be committed to a single pathway of differentiation (Juraskova and Tkadleček, 1965; Curry, Trentin and Wolf, 1964); but there is good evidence to believe that the C.F.Us. are pluripotent, as judged by the interpretations of karyotypic and haematological analyses, although the majority may become committed to a single pathway of differentiation soon after transplantation (Juraskova, Tkadleček and Drásil, 1964; Lewis and Trobaugh 1964; Schooley, 1964; Curry, Trentin and Wolf, 1964; Liron and Feldman, 1965).

However, restrictions in the potential for differentiation would not disqualify haemopoietic cells from being regarded as stem cells, provided that the capacities for self-replication and differentiation were maintained (Bennett and Cudkowicz, 1968). Curry and Trentin (1967) suggested that C.F.Cs. are pluripotent but depend on microenvironmental and humoral factors to differentiate along one line or another.

"Suicide" experimental observations by means of $^{3}H$ thymidine (Bruce and Meeker, 1965) or chemical anticancer drugs (Bruce, Meeker and Valeriote, 1966) showed two functionally distinct classes of C.F.Cs. (Silini, Pons and Pozzi, 1968). Fowler et al.(1967) and Wu et al. (1967), by karyotypic, cytochemical and autoradiographic analyses of spleen colony cells, and Silini, Pons and Pozzi (1968) by quantitative histological examinations of spleen colonies, reached the conclusion that at least some C.F.Cs. were multipotent, and were able to differentiate along more than one pathway.

A further step towards a solution of the problem was brought about by the studies of Bennett and Cudkowicz (1968)[a,b] and Bennett et al.,(1968) which provided evidence for the heterogeneity of the haemopoietic C.F.C. population, as far as cell type

is concerned. This consequently gives rise to differences in the potential for differentiation, which could account, at least in part, for the existence of mono- and multipotent C.F.Us. Their studies comprised transplantations of marrow cells which generated new undifferentiated and differentiated cells, and spleen cells which generated only differentiated cells, as judged in terms of $^{125}$IUdR and $^{59}FeCl_3$ *in vivo* incorporation into the recolonized spleen (differentiative function) and production of new colonies by replantation in secondary hosts (self-replicative function). They distinguished two types of haemopoietic C.F.Cs., or repopulating cells, or progenitor cells, as they called them; stem cells and early differentiated precursors.

They proposed that the primordial stem cells are pluripotent, although unipotent stem cells may also exist, and both are capable of self-replication and colony formation. The early differentiated precursor cells are unipotent and incapable of self-replication but they are capable of colony formation. Since the latter cells have lost their capacity for self-replication, they would no longer be considered stem cells, although still capable of colony formation. Moreover, in spleen colony studies using marrow cell

suspensions filtered through columns of glass wool, Bennett and Cudkowicz (1968) suggested that separate progenitor cells for erythropoiesis and leukopoiesis are present in bone marrow of adult mice.

Other factors influencing spleen colony formation.

Schooley (1966) studied the effect of erythropoietin on the growth and development of C.F.Us. and concluded that erythropoietin does not act on the C.F.C. itself, but on a cell derived from it. This is in accord with the suggestion made by Bruce and McCulloch (1964) that an erythropoietin-sensitive cell is not capable of colony formation.

In hypertransfused irradiated mice it has been shown that erythroid colonies do not develop unless erythropoietin is administered (Curry, Trentin and Wolf, 1964; Liron and Feldman, 1965a,b; Bleiberg, Liron and Feldman, 1965). The erythroid colonies remain small and undifferentiated while the number of neutrophilic colonies is normal or not significantly increased (Curry and Trentin, 1967).

Estradiol administered during repopulation of mouse spleens by endogenous cells increased the number of erythroid and undifferentiated colonies, and reduced the numbers of myelocytic colonies (Jenkins, Upton and Odell, 1969). Treatment of bone marrow donors with

estradiol reduced the ability of such marrow to form all types of colonies, although the effect was more marked in myelocytic colonies (Jenkins, Upton and Odell, 1969). There is also evidence that genetically controlled factors play a role in spleen colony formation. Macrocytic anaemic mice of $W/W^v$ genotype have defective C.F.Us. in that they are unable to form macroscopic colonies (McCulloch, Siminovitch and Till, 1964). However, exogenous transplants do form spleen colonies in these animals (McCulloch, Siminovitch and Till, 1964), and if they have radiation-induced chromosomal markers (Wu et al., 1967). The C.F.Us. of $Sl/Sl^d$ mice are normal but the tissues of mice of this genotype are unable to provide sufficient support for proliferation and differentiation of C.F.Cs. (McCulloch et al., 1965).

Administration of endotoxin has been reported to induce rapid accumulation of C.F.Us. in experimental spleens (Smith et al., 1966). The same effect has been found with the administration of heat-killed corynebacterium parvum organisms (Bennett and Cudkowicz, 1968).

## III. Erythroid cell differentiation.

### Structural and biochemical aspects.

Differentiation of the erythroid cell from the proerythroblast proceeds through a series of recognizable morphological changes (Marks and Kovach, 1966). These

structural changes are associated with biochemical alterations the characteristics of which are broadly similar in foetal liver, adult bone marrow and peripheral blood (Marks and Kovach, 1966). The yolk sac erythroid differentiation possesses certain differences (Marks and Kovach, 1966), which will be pointed out if they become relevant to the present work.

Following the review of Marks and Kovach (1966) the proerythroblast has the highest nucleocytoplasmic ratio (Thorell, 1947). Each successive erythroblast has normally a reduced diameter, and the nucleocytoplasmic ratio decreases progressively (Thorell, 1947). However, in anaemia, or following administration of erythropoietin, the reticulocytes develop a larger size and appear to have increased rates of haemoglobin synthesis, suggesting that they may develop directly from polychromatic erythroblasts and skipping the other stage (Lajtha and Suit, 1955; Suit et al., 1957; Lajtha and Oliver, 1961; Goldman and Huntsman, 1961; Boecker and Stohlman, 1961; Borsook, 1964).

DNA synthesis is active in proerythroblasts and basophilic erythroblasts in human bone marrow and foetal rat liver (Marks and Kovach, 1966). There are probably at least three cell divisions after the stem cell stage (Marks and Kovach, 1966). Cell division does not occur

after the polychromatic erythroblast (Bond et al., 1959; Cronkite et al., 1959; Grasso, Woodard and Swift, 1963; Lajtha, 1964; Cronkite, 1964; Borsook, 1964). The DNA content is progressively decreased, and the nucleus is lost predominantly at the orthochromatic erythroblast stage, mostly by extrusion (Albrecht, 1951; Bessis, 1955; Bro-Rasmussen and Henriksen, 1964). However, under conditions of anaemic stress and, occasionally, in normal conditions, the nucleus can be expelled earlier in the polychromatic erythroblast (Borsook et al., 1962; Brecher and Stohlman, 1959).

RNA synthesis occurs primarily during and prior to the basophilic erythroblast stage in foetal rat liver and rabbit bone marrow (Grasso, Woodard and Swift, 1963; Borsook et al., 1962; De Bellis, Gluck and Marks, 1964). There is little RNA synthesis in the polychromatic erythroblast in the foetal rat or mouse (Grasso, Woodard and Swift, 1963; Kovach et al., 1966). Reticulocytes and possibly even orthochromatic erythroblasts of normal or anaemic humans and rabbits do not synthesize RNA (Marks, Burka and Schlessinger, 1962; Marks et al., 1962; Nathans et al., 1962; Borsook et al., 1962). Hence RNA concentration decreases progressively as the pro-erythroblast differentiates to the polychromatic erythroblast and then there is little change through the

orthochromatic erythroblast stage (Thorell, 1947). Presumably mRNA is synthesized primarily during and prior to the basophilic erythroblast stage and remains stable through to the reticulocyte (Marks et al., 1962). There are no specific studies on synthesis of tRNA during erythroid cell differentiation (Marks and Kovach, 1966). RNA is lost from erythroid cells by degradation, the mechanism of which is unknown (Bertles and Beck, 1962; Burka, De Bellis and Marks, 1964).

The rate of protein synthesis and concentration of protein are subject to changes as erythroid cells develop (Marks and Kovach, 1966). Haemoglobin synthesis does not appear to begin until the polychromatic erythroblast stage (Thorell, 1947; Sondhaus and Thorell, 1960; Ackerman, 1962; Grasso, Woodard and Swift, 1963). The total cytoplasmic protein decreases between the pro-erythroblast and the early polychromatic erythroblast stages, increasing sharply thereafter to the reticulocyte stage as haemoglobin is formed (Thorell, 1947; Ackerman, 1962; Grasso, Woodard and Swift, 1963). The decline in total protein in the early stages of development is associated with a decrease in the cytoplasmic area and, probably, does not reflect the relative rates of protein synthesis. It appears that rates of protein synthesis change little from the early erythroblast to the poly-

chromatic erythroblast (Grasso, Woodard and Swift, 1963; Borsook, 1964). As the reticulocyte matures to erythrocyte, there is a loss of capacity to synthesize protein (Marks et al., 1962; Mathias et al., 1964; Glowacki and Millette, 1965), loss of mitochondria, and loss of activity of some enzymes (Rubinstein et al., 1956; Marks et al., 1958; London, 1960, 1961; Marks, 1962; Marks and Kovach, 1966). There is no loss of haemoglobin as the erythrocyte ages (London, 1960, 1961; Marks, 1962; Marks and Kovach, 1966).

## IV. Haemoglobins.

### Ontogenesis of haemoglobins and the switch from foetal to adult haemoglobin.

In the embryo, erythropoiesis takes place first in the blood islands of the yolk sac (Bloom and Bartelmez, 1940). The exact duration of this mesoblastic period of erythropoiesis in the human is not known, although blood islands are demonstrable in the 2.25 mm. embryo and disappear at the 5 mm. stage (Wintrobe, 1961). This haemopoietic activity occurs in the mouse from the 8th to the 12th day of development (de Aberle, 1927; Russel and Bernstein, 1966; Fantoni, Bank and Marks, 1967) and from the 8th to the 12th or 13th day in the rat (Nagel, 1968). Coincidental with the loss of erythropoietic activity in the yolk sac, the liver replaces it

as the haemopoietic organ in the 5 mm. to 7 mm. ~~embryo~~, that is, around the 1½ month human foetus (Wintrobe, 1961), on the 12th day and continuing with full activity to the 16th or 17th day in the mouse (Russel and Bernstein, 1966; Fantoni, Bank and Marks, 1967), and from the 12th or 13th to the 17th day in the rat (Brdička, 1966; Nagel, 1968). At the end of pregnancy the liver progressively loses its haemopoietic elements, which disappear around 10 months of age in the infant (Wintrobe, 1961), 11 days after birth in the mouse (Jezequel, 1965) and 6 days in the rat (Brdička, 1966). Some erythropoiesis occurs in the spleen from about the 3rd to the 5th month of foetal life in the human (Wintrobe, 1961), the 15th to 17th day in the mouse (Russel and Bernstein, 1966) and from the 17th day before birth to 14 to 21 days after birth in the rat (Brdička, 1966; Nagel, 1968). Finally, haemopoiesis shifts into the bone marrow about the 5th month of foetal life in humans (Wintrobe, 1961), the 16th day in the mouse (Russel and Bernstein, 1966; Fantoni, Bank and Marks, 1967) and between the 18th and 20th day in the rat (Brdička, 1966; Nagel, 1968).

Attempts have been made to identify the types of haemoglobin with the different sites of erythropoiesis during development of the embryo and foetus (Wintrobe, 1961); but the problem is not simple. Adult haemoglobin

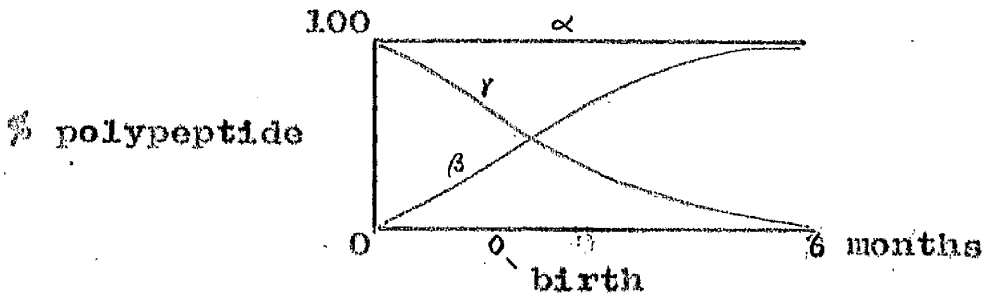
has been observed even as early as the 13-week human foetus (Walker and Turnbull, 1955). Even in adult rats in which no haemopoietic activity is present in the liver, 20% show characteristics of foetal haemoglobin (Brdička, 1966). The three types of human haemoglobins, embryonic, foetal and adult, differ only in one of two pairs of globin chains (Hb Gower II: $\alpha_2$, $\varepsilon_2$; Hb F: $\alpha_2$ $\gamma_2$; Hb A: $\alpha_2$ $\beta_2$; $HbA_2$: $\alpha_2$ $\delta_2$), but there is no information as to whether, in human foetuses, embryonic haemoglobin is formed in the same or different erythroid cells as foetal and adult haemoglobins (Fantoni, Bank and Marks, 1967). Human foetal spleen, liver and bone marrow have been shown to synthesize both Hb A and F in different relative amounts (Thomas et al., 1960) but there is in general little correlation between site of production and nature of haemoglobin produced (Cole, Hunter and Paul, 1968).

In the mouse the foetal haemoglobins are made by the yolk sac cells, while adult haemoglobin is made by the foetal liver (Kovach et al., 1967; Fantoni, Bank and Marks, 1967). Cells of the yolk sac contain haemoglobin components not found in any later stage (Craig and Russel, 1963, 1964). The three yolk sac haemoglobins in the mouse are $Hb_I$: $X_2$ $Y_2$; $Hb_{II}$: $\alpha_2$ $Y_2$; $Hb_{III}$: $\alpha_2$ $Z_2$; but they still need further characterization

(Fantoni, Bank and Marks, 1967). The Hb A made by the mouse foetal liver is indistinguishable from the Hb A of the bone marrow (Fantoni, Bank and Marks, 1967).

In the rat, two haemoglobins, electrophoretically identical to those of the adult, persist from yolk sac to adult marrow erythropoiesis, and further components are added during foetal liver erythropoiesis. Even when two erythropoietic sites are active simultaneously the pattern of synthesis is characteristic of the stage of gestation (Hunter and Paul, 1969).

What happens during the switch from Hb F to A? The following figure, taken from Ingram (1963) illustrates the relative proportion of $\alpha$, $\beta$ and $\gamma$ peptide chains made in the red cells of a developing human foetus and in the newborn:




It must be remembered that the $\alpha$ chain forms part of both Hb F and A, and the $\gamma$ chain is part of Hb F, while the $\beta$ chain is part of Hb A.

The production of $\alpha$ chains remains constant, the

$\gamma$ chains decrease and eventually go down to almost zero, around the 6th month of extra-uterine life; while the $\beta$ chains increase in production more or less synchronously (Ingram, 1963).

In man, at birth, the level of Hb F is normally variable around 85% of the total haemoglobin present (White and Beaven, 1959; Armstrong et al., 1963; Marks and Kovach, 1966) and erythroid cell synthesis of Hb A, relative to that of Hb F, is 3 to 4 times greater than the ratio of Hb A to Hb F in the peripheral blood (Burka and Marks, 1964). This is compatible with the concept that erythroid cell synthesis of Hb F decreases during late foetal development while the capacity for Hb A synthesis increases, so that in normal human adults Hb F synthesis becomes very limited (Marks and Kovach, 1966). The level of Hb F in normal adults is less than 2% of the total haemoglobin (Marks and Kovach, 1966). However, in adults with certain types of thalassaemia, Hb F may comprise as much as 90% of the total haemaglobin (Rich, 1952). The thalassaemia syndromes are a group of inherited diseases which are characterized by a marked decrease in the capacity of erythroid cells to synthesize Hb A (Marks and Burka, 1964). The hereditary persistence of Hb F, which is characterized by persistence of high levels of Hb F into adult life (Edington and Lehmann,

1955; Jacob and Raper, 1958; Weatherall, 1965) is another condition in which there is a failure of the mechanisms regulating conversion from the synthesis of Hb F to that of Hb A during the course of erythroid cell differentiation in the developing foetus (Marks and Kovach, 1966). No Hb A or A2 is synthesized in the homozygote, and there is no detectable Hb A in the heterozygote (Jacob and Raper, 1958; Went and MacIver, 1958). The amount of Hb F, however, is not related to that of Hb A (Marks and Kovach, 1966).

It has been suggested that in early human foetal life (10-20 weeks) the embryonic haemoglobin is formed under separate genetic control (Halbrecht and Klibanski, 1956, 1957, 1959) and that production of Hb F in humans is determined by genes non-allelic to those regulating formation of adult Hb (Wintrobe, 1961).

In the mouse, all the different haemoglobins examined are formed by similar polypeptide chains to those in man. They are called α chains and β chains or, in the case of a minor component, β-like chains (Russel and Bernstein, 1966).

Different characteristics (structure, solubility, electrophoretic patterns) of mouse haemoglobins are controlled by allelic genes at two independent loci; a locus Hb-a, which appears to control the structure of

α chains, and a complex locus Hb-b, which appears to control the structure of β and β-like chains (Russel and Bernstein, 1966).

Several mouse haemoglobins produce a homogenous electrophoretic pattern (single band), but other haemoglobins show a diffuse pattern (several bands). Chromatography on Amberlite CG-50 (Hutton et al., 1962) revealed that the single haemoglobin (Hb-s) was formed by one stable component and the diffuse haemoglobin (Hb-d) was formed by two components, a major component in the same region as the Hb-s forming 80% of the total haemoglobin, and a minor component forming 20%, one of them at least being somewhat unstable (Russel and Bernstein, 1966).

The basis for the diffuse haemoglobin electrophoretic patterns appears to be the presence of the two dissimilar haemoglobins, controlled by two alleles (Hb-s and Hb-d). The allele at the locus Hb-b appears to be compound, probably consisting of two adjacent cistrons. One or both of them may be homologous to the single cistron of the allele at locus Hb-b of the single haemoglobin (Glucksohn-Waelsch, 1960).

These several electrophoretic variants, controlled by the presence of the two alleles, result in a single haemoglobin ($Hb^s$ $Hb^s$) pattern, homozygous diffuse haemoglobin ($Hb^d$ $Hb^d$) pattern, and heterozygous diffuse

haemoglobin ($Hb^s$ $Hb^d$) pattern (Biddle and Petras, 1967).

Morton (1962, 1966) reported an electrophoretic variant which appeared to be controlled by a third allele in a strain of polydactylous mice.

The three alleles appear to be co-dominant (Biddle and Petras, 1967).

Different alleles at the loci Hb-a and Hb-b have been detected. They are responsible for variations in structure of α and β chains (amino-acid substitutions) from strain to strain. The variations in structure, solubility and electrophoretic pattern represent normal variations, genetically controlled; none has been associated with pathological conditions. Thus in the mouse no haemoglobinopathies have been discovered (Russel and Bernstein, 1966).

In the foetal blood of 15-day old mice of both $Hb^s$ and $Hb^d$, four haemoglobin fractions have been found on electrophoresis. The concentration of the foetal haemoglobins decreases steadily throughout foetal life, and are entirely absent at birth (Craig and Russel, 1963, 1966). The adult haemoglobin pattern replaces the foetal pattern in late intra-uterine life (Barrowman and Craig, 1961). The switch from foetal to adult haemoglobin in the mouse is coincident with the time at which the proportion of nucleated red cells in foetal peripheral blood falls

rapidly (Barrowman and Craig, 1961). In fact, from the 14th to the 16th day the nucleated erythrocytes, i.e. cells from the yolk sac, fall from 21% to 1% (Astaldi, 1951).

Little information is available in regard to rat haemoglobins. Rosa (1959) showed five haemoglobin components by starch gel electrophoresis in six strains of rats. More recent starch gel electrophoretic studies have revealed the heterogeneity of adult rat haemoglobin within the same strain. The different electrophoretic patterns appeared to be genetically controlled (French and Roberts, 1965; Brdička and Šulc, 1965; Brdička, 1966; Marinković, Martinović and Kanazir, 1967). Ion exchange column chromatography fractionation results also support these findings (Brada and Tobiška, 1964; Mádlo and Šulc, 1964; Tobiška and Brada, 1965; Trávníček, Trávníčková and Šulc, 1965; Brdička, 1966).

With regard to the ontogenesis of rat haemoglobins the situation is even poorer. The study of the switch from foetal to adult haemoglobin is complicated in the rat due to the fact that there is no visible dependence between morphological and biochemical (in terms of haemoglobin type) erythroid differentiation as there is in man (Brdička, 1966). In fact, while in humans morphological differentiation comes first, and biochemical

(i.e. type of haemoglobin) follows later, in the rat biochemical transformation is established in the course of early morphological differentiation. The haemoglobin type in the rat is well determined by the 14th day of foetal life and only quantitative shifts among the haemoglobin fractional components take place later (Brdička, 1966). The circulating blood in the 15-day rat foetus is formed predominently by cells of prehepatic generation (Last and Hays, 1941). Non-nucleated cells increase rapidly between the 14th and 18th day (Last and Hays, 1941). This would correspond to the activity of the foetal liver as a haemopoietic organ. It should be remembered that erythropoietin activity in the foetal liver occurs from the 12th or 13th to the 17th day in rats (Brdička, 1966; Nagel, 1968). During ontogenesis the chromatographic profile of rat haemoglobin is much more constant than in human haemoglobin or in haemoglobins of other animals (Tobiška and Brada, 1965).

Two haemoglobins electrophoretically identical to two adult haemoglobins persist from yolk sac to adult marrow erythropoiesis, and other components are added during foetal liver erythropoiesis (Hunter and Paul, 1969). In the mouse, adult haemoglobin arises as a result of sequential replacement of the foetal haemo-

haemoglobin; but rat haemoglobin synthesis results in the sequential appearance of adult haemoglobin (Cole, Hunter and Paul, 1968).

It has been suggested that, at least in rats, the cells of the erythrogenic series have all the genetic information for building the whole haemoglobin complex, and this is accomplished gradually (Brdička, 1966). It has also been suggested that the stem cells of the foetal rat are initially programmed for a given pattern of haemoglobin synthesis, and the evolution of the programme may depend, according to Baglioni's ideas (1962) either on the number of stem cell divisions, and hence on the age of the foetus, or on changes in the environment (Hunter and Paul, 1969).

Chromosomal and cytoplasmic regulation of haemoglobin synthesis.

In spite of the great progress made in the knowledge of protein synthesis, understanding of the control of haemoglobin synthesis is far from complete (Baglioni, 1968). Limited knowledge of the complex regulatory mechanisms acting at chromosomal level has been acquired from the study of mutations of haemoglobin genes. Studies on reticulocytes and cell-free systems prepared from reticulocytes have given some information on the regulatory mechanisms operating at translational or

cytoplasmic levels (Baglioni, 1968).

1. Chromosomal control of haemoglobin synthesis.

Haemoglobin depends on the transcription and translation of mRNA for synthesis of haemoglobin peptide chains and other enzymes for the production of haem. The factors inducing transcription of haemoglobin genes and differentiation of erythroid cells are not known (Baglioni, 1968). Although haemoglobin synthesis accompanies differentiation of the erythroid cell, it is not clear if one is a result of the other, or if one is controlled by the other, or if both are under the same mechanism of control.

It seems likely that transcription of haemoglobin genes takes place sometime before the actual initiation of haemoglobin synthesis (Baglioni, 1968). In fact, in cultures of blood islands in chick embryo blastoderms at an earlier stage of initiation of haemoglobin synthesis, the addition of RNA synthesis inhibitors did not prevent haemoglobin synthesis (Wilt, 1965), i.e. the presence of actinomycin does not block haemoglobin synthesis because of a template already present in the blood islands, unless it is administered before the head-fold stage in chick embryos (Davidson, 1968). The existence of cells which are committed to erythroid differentiation and which are morphologically indistinguishable from non-committed cells

seems, therefore, plausible (Baglioni, 1968), in which case selective transcription of haemoglobin genes may occur very early in differentiation and these cells may be committed to synthesize certain haemoglobin types even before initiation of haemoglobin synthesis (Baglioni, 1968), the mRNA remaining "masked" (Spirin, 1966).

It is believed that once the genes for haemoglobin synthesis become functional, the maturation sequence ending in an erythrocyte follows automatically, i.e. the commencement of haemoglobin synthesis irreversibly commits a cell to differentiate into an erythrocyte (Lajtha, 1966).

Finally, when does the erythrocyte stop synthesizing haemoglobin? In other words, what controls the amount of haemoglobin present in the erythrocyte? Since it has been suggested that the amount and life of mRNA for globin synthesis determines the amount of haemoglobin (Krantz and Goldwasser, 1965), so the erythrocyte would stop synthesizing haemoglobin when no more mRNA is available (Schulman, 1968). This would be in conflict with the hypothesis that template is present in the blood islands some time before initiation of haemoglobin synthesis (Wilt, 1965; Davidson, 1968); unless initiation of haemoglobin synthesis is controlled by a different mechanism than that which terminates haemoglobin synthesis.

Whether the latter is the amount of mRNA present (which conforms with the suggestion of Krantz and Goldwasser), or a different factor, is not clear. Since haemoglobin synthesis experimentally decays more rapidly than mRNA for globin synthesis and the ability to synthesize protein (Schulman, 1968) it has been suggested that a decay in the haem synthesis pathway may be the factor that causes the erythrocyte to stop synthesizing haemoglobin (Schulman, 1968). Here again, is erythroid differentiation cause or effect?

Coming back to the discussion of the control mechanisms of haemoglobin synthesis at chromosomal level, in the light of the concepts of Jacob and Monod (1961), the genetic elements capable of being altered by mutations would be a unit of synthesis (operon) of haemoglobin chains. The operon would include genetic elements which may interact with regulatory substances to determine patterns of activity of haemoglobin genes (Zuckerkaudl, 1964). However, one of these hypothetical operons would contain β and δ genes and, indeed, genetic analysis shows that the "high Hb F" and β-δ thalassaemia genes are closely linked to the β-δ-chain locus (Motuslky, 1964). On the other hand, the structure of the Lepore peptide chain, made partly of β and partly of δ chains (Hb Lepore: α2 (δβ)2), shows that the δ chain fragment is contained

in the first part of the chain (N-terminal) and the β chain fragment brings up the rear (Baglioni, 1962). Thus, if β and δ chains are translated from a single genetic message, the δ chain must be synthesized first (Baglioni, 1962); but the rate of synthesis of the δ chain is considerably slower than that of the β and α chains (Winslow and Ingram, 1966), and the rate of synthesis of Hb A2 ($\alpha_2 \delta_2$) relative to that of Hb A ($\alpha_2 \beta_2$) decreases with maturation of the erythroid cells (Rieder and Weatherall, 1965; Winslow and Ingram, 1966). Such completely different rates of synthesis of β and δ chains would be an indication that translation occurs independently (Baglioni, 1968). Moreover, the operon model of Jacob and Monod (1961) represents a system for the coordinate control of polycistronic loci (Davidson, 1968); but in higher organisms no polycistronic messengers have so far been found (Davidson, 1968). On the contrary, polysomes of a size comparable to monocistronic messengers have been observed in haemoglobin synthesizing reticulocytes (Warner, Knopf and Rich, 1963) and haemoglobin template of molecular weight around 150,000 size, predicted for a monocistronic messenger, has been isolated (Burney and Marbaix, 1965). The basic design of Jacob and Monod's model, however, seems correct: linked structural genes undergo coordinate activation and repression

mediated by the products of other genes possessing specific regulatory functions. The products of regulatory genes are able to react specifically with external inducer molecules (Davidson, 1968).

Baglioni (1968) has suggested the existence of mechanisms involving different genetic elements:

(a) In conditions of increased Hb F, not associated with anaemia, a mutation interfering with the regulation of the switch mechanism from Hb F to A (i.e. a failure to "switch off" the synthesis of the Hb F) would occur -

i) in all erythroid cells, in the case of hereditary persistence of Hb F (high F homozygote and heterozygotes), as Hb F is evenly distributed in the erythrocytes;

ii) in a few erythroid clones in the thalassaemic heterozygotes with high levels of Hb F (heterozygotes $\beta$- $\delta$ thalassaemia and Van Leest thalassaemia), as Hb F distribution in the erythrocytes is uneven.

(b) In conditions of increased Hb F associated with anaemia, such as other thalassaemias, sickle cell anaemia and homozygotes of $\beta$- $\delta$ thalassaemia, Van Leest thalassaemia and Lepore conditions, the anaemia would lead to a "foetal" differentiation and proliferation of erythroid clones synthesizing high levels of Hb F, which is suggested by the uneven distribution of Hb F in the erythrocyte population.

2. Cytoplasmic control of haemoglobin synthesis.

After the early haematoblast stage, the chromatin becomes clumped and the nucleoli disappear; events which denote cessation of RNA synthesis and which anticipate the actual extrusion of the nucleus (Davidson, 1968). In the reticulocyte RNA synthesis is practically zero, but haemoglobin synthesis continues long after the elimination of the nucleus (Davidson, 1968; Baglioni, 1968). The presence of a long-lived haemoglobin template remaining active in the polysomes of the enucleated erythrocyte has been established (Davidson, 1968). This also indicates that haemoglobin synthesis is controlled by cytoplasmic mechanisms.

Following Baglioni (1968), the different control mechanisms acting at cytoplasmic level can be grouped into:

(a) a mechanism controlling the rate of synthesis of each type of polypeptide chain;

(b) a mechanism controlling the synthesis of haem, and,

(c) a mechanism coordinating the synthesis of globin to the availability of haem.

(a) Mechanism controlling the rate of synthesis of each type of haemoglobin peptide chains.- Alteration of the rate of synthesis of the different peptide chains occurs in several conditions. In α-thalassaemia there

is a depression of α-chain synthesis. In conditions showing haemoglobins made up of only one type of peptide chain (Hbs β4, γ4, δ4) the β chains (or γ or δ chains) seem to be synthesized in excess of the available α chains; but in β-thalassaemia characterized by a depression in β chain synthesis, there seems to be no overproduction of α chains (Baglioni, 1968). This would suggest that α chain synthesis depends on β chain synthesis while β chain synthesis occurs autonomously (Baglioni, 1968).

The presence of completed α chains on polysomes has been reported (Baglioni and Colombo, 1964; Weatherall, Clegg and Naughton, 1966). Besides, the rate of α and β chain synthesis is uneven: After a short pulse with labelled amino acids the β chain was found to be more labelled than the α chain in haemoglobin isolated from reticulocyte lysate (Colombo and Baglioni, 1966). The reason for this was that α chains present on the polysomes were already completed to the C-terminal amino acid, but there were no completed β chains (Colombo and Baglioni, 1966). Hence the rate of synthesis of α chains seems to exceed potentially that of β chains, but α chains are not released from the polysomes unless they combine with a β chain (Colombo and Baglioni, 1966). If β chains are not available, the progression of ribosomes along

the mRNA strand of polysomes synthesizing α-chains would be blocked (Baglioni, 1968).

Similar principles would apply for γ and δ chains.

(b) Mechanism that controls the rate of haem synthesis.- The control of haem synthesis is explained in terms of feedback inhibition, since addition of haemin to reticulocytes produces a marked inhibition of haem synthesis (Karibean and London, 1965). The main effect of this feedback inhibition is on the reactions concerned with the synthesis of δ-aminolaevulinic acid (Baglioni, 1968). There is also evidence that control of haem synthesis may be exerted early in the metabolic sequence, on fumarate reductase (Kurumada and Labbe, 1966).

(c) Mechanism that coordinates the rate of globin synthesis to haem synthesis.- The haemoglobin molecule is assembled by a combination of two α-peptide chains, two β-peptide chains, and one haem group for each chain. The peptide chains of globin are synthesized at the same rate as protoporphyrin by reticulocytes (Kruh and Borsook, 1956). The iron then combines with protoporphyrin by means of an enzymic reaction (Nevé, 1961) to form haem. The globin chains associate readily with the haem group, folding around it and assuming the proper spatial configuration (Felicitti, Colombo and Baglioni, 1966).

Haem and globin show a balanced rate of synthesis

in reticulocytes (Kruh and Borsook, 1956). It appears that a regulatory mechanism operates to produce this equilibrium (Baglioni, 1968). However, the mechanism of coordination of globin synthesis to the availability of haem is still unknown (Schulman, 1968).

Information collected on the effect of iron on haemoglobin synthesis has indicated that haem prevents the accumulation of free globin (Felicitti, Colombo and Baglioni, 1966). Globin synthesis in intact rabbit reticulocytes decreases in the absence of iron (Morell, Savoie and London, 1958). The addition of iron to reticulocytes treated with iron-chelating agents stimulates the synthesis of globin (Waxman and Rabinowitz, 1965). Addition of haemin inhibits globin synthesis in fresh rabbit reticulocytes *in vitro*, but stimulates globin synthesis in cells which have been incubated for 20 hours (Schulman, 1968).

It has been suggested that haem may combine with the polypeptide chains either before they are completed on the polysomes, or at the moment that the peptide chains are released from the polysomes, and that haem may actually be necessary for such release (Gribble and Schwartz, 1965). However, firstly it has been shown that cobalt inhibits the synthesis of haem and stimulates to some extent the synthesis of globin

(Morell, Savoie and London, 1958). Secondly, haem does not appear to associate with the peptide chains while they are being synthesized on the polysomes. The very small amount of $^{59}Fe$ associated with ribosomes is not bound to haemoglobin chains. When these are released from the polysomes by the action of puromycin, the $^{59}Fe$ continues to be associated with the polysomes (Felicitti, Colombo and Baglioni, 1966). Thirdly, it does not seem likely that haem combines with the peptide chains at the moment that they are released from the polysomes. On the contrary, it seems likely that polypeptide chains are released before combining with haem. In fact, globin present in reticulocyte lysate has been separated by chromatography (Baglioni, 1968).

The most likely pathway for the assembly of the haemoglobin molecule is:

(1) β chains are released as soon as they are completed.

(2) β chains combine with α chains, probably at polysome level (α chains are retained at the polysomes after completion).

(3) α-β subunits formed (maybe predominantly in the dimer form) are set free in the cytoplasm.

(4) Haem combines with α - β subunits to form α2 β2 haemoglobin molecules (Felicitti, Colombo and Baglioni, 1966).

The foregoing data seem to exclude the control of

the rate of synthesis of haemoglobin directly, by combination of haem with peptide chains at polysome level (Felicitti, Colombo and Baglioni, 1966). However, several studies have indicated that the availability of haem exerts a controlling influence on the synthesis of globin, at polysome level (Cole, Hunter and Paul, 1968). Addition of haemin to rabbit reticulocyte preparations increases the incorporation of $^{14}C$ valine into haemoglobin (Bruns and London, 1965). Haemin and other tetrapyrroles stimulate globin synthesis in isolated avian erythrocyte nuclei (Hammel and Bessman, 1965). Addition of $\delta$-aminolaevulinic acid to chick blastoderms increases haemoglobin synthesis (Levere and Granick, 1965, 1967). Prolonged incubation of reticulocytes in the absence of iron produces a decreased rate of haemoglobin synthesis and disaggregation of polysomes (Rabinovitz and Waxman, 1965; Waxman and Rabinovitz, 1965). Apparently all the iron present in reticulocytes is used to synthesize haem, so that when iron is no longer available, haemoglobin synthesis decreases (Baglioni, 1968). If iron or haemin is supplied afterwards, haemoglobin synthesis is resumed and polysomes reform (Waxman and Rabinovitz, 1965). Reticulocytes of rabbits on a low iron diet have less polysomes and a slow rate of haemoglobin synthesis (Graysel, Horelmer

and London, 1966), but iron or haemin stimulates haemoglobin synthesis and polysome formation in these reticulocytes (Grayzel, Horchner and London, 1966; Bruns and London, 1965).

It has been shown that stimulation of haemoglobin synthesis in reticulocytes of rabbits on a low iron diet can be obtained with cobalt, a heavy metal which does not seem necessary for haem synthesis (Grayzel, Horchner and London, 1966). It is known that cobalt inhibits haem synthesis by inhibiting tetrapyrrole synthesis before uroporphyrinogen formation (Eriksen, Eriksen and Haavaldsen, 1961; Waxman, 1970). It is also known that cobalt is effective in protecting polysomes and supporting globin synthesis during prolonged incubation of reticulocytes in the absence of iron or in the presence of iron-chelating agents (Waxman and Rabinovitz, 1968). Moreover, there is **indicatin**that cobalt interferes with $O_2$ transport (Berk, Burchend and Castle, 1949) and stimulates erythropoietin production (Goldwasser et al., 1957, 1958; Kilbridge, Fried and Heller, 1969). However, the mechanism by which cobalt stimulates globin synthesis is not understood (Waxman, 1970). Hence it would appear that haem is not essential for the synthesis of globin. In the absence of iron, globin synthesis can be supported by other metals which do not enter the haem group

(Baglioni, 1968). It might be argued, however, that such globin synthesis was really stimulated by newly formed haem after addition of cobalt, the role of cobalt in this case being to take the place of iron released from some enzyme system requiring heavy metals for activity (Baglioni, 1968). If this hypothesis is correct, then the regulating control of globin synthesis would depend on the amount of haem synthesis (Baglioni, 1968).

The possibility that accumulation of globin causes a feedback inhibition of its own synthesis has been considered (Karibian and London, 1965; Felicitti, Colombo and Baglioni, 1966; Baglioni, 1968). Some data support this point of view. Globin appears to be present in reticulocytes normally as an intermediate in the synthesis of haemoglobin, and it accumulates in reticulocytes incubated with iron-chelating agents such as o-phenanthroline (Felicitti, Colombo and Baglioni, 1966). Whether this feedback inhibition of globin synthesis is caused by the whole globin molecule or by individual chains, or whether it is mediated by the level of some haem precursor such as free iron, is open to further speculation (Felicitti, Colombo and Baglioni, 1966).

Another piece of information in support of free globin as an intermediate in haemoglobin synthesis, which may regulate the synthesis of haem, is that free globin

is present in small amount in mature human erythrocytes (Winterhalter and Huehns, 1963; Winterhalter et al., 1969). The cellular capacity to synthesize globin seems, therefore, to exceed the capacity to synthesize haem (Felicitti, Colombo and Baglioni, 1966).

V. Culture of haemopoietic tissues, and time-lapse cinemamicrography.

To obtain specific information on the nature and properties of the blood-forming cells, cultures of haemopoietic tissues, mainly cultures of bone marrow and foetal liver were used. They allow both proliferation and differentiation to be followed by investigating different parameters (Lajtha, 1965), the value of which depends on the correlations transferred from studies made in vivo (Lala, Malloney and Patt, 1965).

For direct observation and time-lapse cinemamicrography the agar method devised by Pulvertaft and his collaborators (Pulvertaft and Jayne, 1953; Pulvertaft and Humble, 1956; Pulvertaft, Haynes and Groves, 1956) has given the best preparations (Lajtha, 1965). Plasma clot cultures have also been used for this purpose from the early days of tissue culture, although media of this kind have the disadvantage of inducing fibroblastic transformation (Lajtha, 1965).

Morphological, behavioural and locomotory character-

istics of blood and blood-forming cells have been investigated by direct methods. The reviews of the literature by Woodliff (1964) on bone marrow, by Trowell (1965) on lymphocytes, and, studies made by cinemicrography (Rich, Wintrobe and Lewis, 1939; Bessis, 1955[a,b]; Hiraki and Ofugi, 1956; Rind, 1956; Humble, Jayne and Pulvertaft, 1956; Klein, 1958[a,b], 1959; Albrecht, 1959; Pulvertaft, 1959) are good accounts of the enormous amount of research performed along these lines

Despite advances in culture techniques, long-term maintenance of normal mammalian haemopoietic cells in vitro continues to be a challenging problem (Smith and McKinley, 1965). Although there is evidence that certain metabolic pathways (haem and antibody synthesis) remain active for several days, satisfactory maintenance of all cell types, in terms of morphological or reproductive integrity, has not been achieved. The literature reveals that the number of cells in cultures of haemopoietic tissues decreases rapidly during the first 2 or 3 days, although maturation and differentiation continue for a time, and depletion of the blast compartment indicates the inability of the stem cell pool to maintain and replenish itself (Smith and McKinley, 1965).

Since all the available systems of bone marrow culture in vitro run down from the moment of starting,

it is more correct to speak of short-term survival of bone marrow in vitro than bone marrow in culture (Lajtha, 1965). Apparently the conditions of culture do not favour normal stem cell kinetics (Lajtha, Oliver and Gurney, 1962).

Cultures of erythropoietic liver exhibit a similar picture. Cultures of foetal liver cells have been widely employed for diverse erythropoietic studies, mostly in short-term culture (Salvatorelli, Gulinati and Del Grande, 1969). In experiments with histotypic culture (Cole and Paul, 1966) the duration and maintenance of erythropoiesis in in vitro cell suspensions did not exceed 4 hours, as judged by haem synthesis, unless erythropoietin was added, when the peak of haem synthesis reached its maximum at 28 hours.

Organ cultures of human embryonic liver have been claimed to maintain erythropoiesis for 2 or 3 weeks (Benevolenskaja, 1930). Cultures of human embryonic liver showed the presence of haemopoietic cells during the first week of culture (Hillis and Bang, 1962). Bang and Warwick (1965) have also reported persistence of haemopoiesis for several weeks in organ cultures of human embryonic liver in chicken plasma clots, although in agar medium the blood cells disintegrate by the 4th day. Gallien-Lartigue (1966, 1967) cultivated explants

of mouse foetal liver for about 10 days, using the organotypic technique of culture of Wolff and Haffen (1952) but differentiation of stem cells into erythroblasts stopped after the 5th day whereas the maturation of erythroblasts into erythrocytes continued up to the 7th day, as judged by the relative number of morphologically recognizable cells. Salvatorelli, Culinati and Del Grande (1969) also reported the disappearance of stem cell proliferation after the 4th day of culture of guinea pig foetal liver using similar culture techniques and analytical criteria. They observed, however, that using anaemic serum, instead of normal serum, the early stages of morphologically recognizable erythroid cells actually showed an increased number during the 14 days that the cultures lasted.

Valuable information has been obtained from studies using *in vitro* culture methods on different phases of the problem of erythropoiesis. Moreover, proliferation and differentiation of myeloid precursors have been investigated by stathmokinetics (Astaldi and Mauri, 1949), by autoradiography (Lajtha, Oliver and Ellis, 1954; Salera, Tamburino and Manganelli, 1957; Myhre, 1964; Schmid *et al.*, 1966; Kesse-Elias, Harris and Gyftaki, 1967; Wickramasinghe, Cooper and Chalmers, 1968; Boll and Nersch, 1968; Plotkin and Sechter, 1968; Killmann,

1970; Klein et al., 1970) and by cinemicrographic techniques (Boll and Fuchs, 1961; Boll, 1965, 1966; Rondanelli et al., 1964, 1966). However, cell culture techniques have not yet clarified the position of the haemocytoblast, either as to its origin or its potentialities. Hence in comparison with the rich field of literature in morphology and cytochemistry, comparatively little is known about the growth and differentiation of haemopoietic cells in normal and pathological conditions (Klein et al., 1970). What Woodliff (1964) said can still be quoted today: with advances in technique it is conceivable that identification, separation and culture of haemocytoblasts might lead to information as to the factors influencing their multiplication and maturation.

VI. Experimental approach.

The difficulties of bringing forward conclusive evidence on the problem of haemopoietic stem cells are very considerable, as pointed out by Garven (1957).

(a) All forms of the most primitive cells are present in relatively small numbers.

(b) The less differentiated a cell is, the less characteristic its structure tends to be and the more difficult it is to identify it with certainty.

(c) The evidence must be for the cell's potentialities, which is a very different thing from its

structural features at any definite point in time.

Two different experimental approaches were chosen, both of them with the aim of identifying a resulting product. The first approach is concerned with the identification of one of the ultimate products of the erythrocytic cell line, that is the haemoglobin synthesized by the C.F.U. of repopulated spleens in supralethally irradiated mice. The second approach aims at identifying an immediate and recent product, the daughters of a cell of haemopoietic tissue undergoing mitosis, with constant recording on a time-lapse cinemicrographic film, by means of morphocytological techniques.

Rationale for the approach and tentative methods.

Rationale for the approach and tentative methods.Since the similarity or difference between the C.F.U. and the haemopoietic stem cell is still controversial, any study along these lines may clarify the situation or help in the understanding of the problem. Hunter and Paul (1969) showed the feasibility of distinguishing between adult and foetal haemoglobins of Wistar rats by means of their different $^{59}Fe$ uptake ~~in~~ electrophoretic profiles (Fig.1). These haemoglobin profiles both differ from the single band of haemoglobin characteristic of the adult Porton

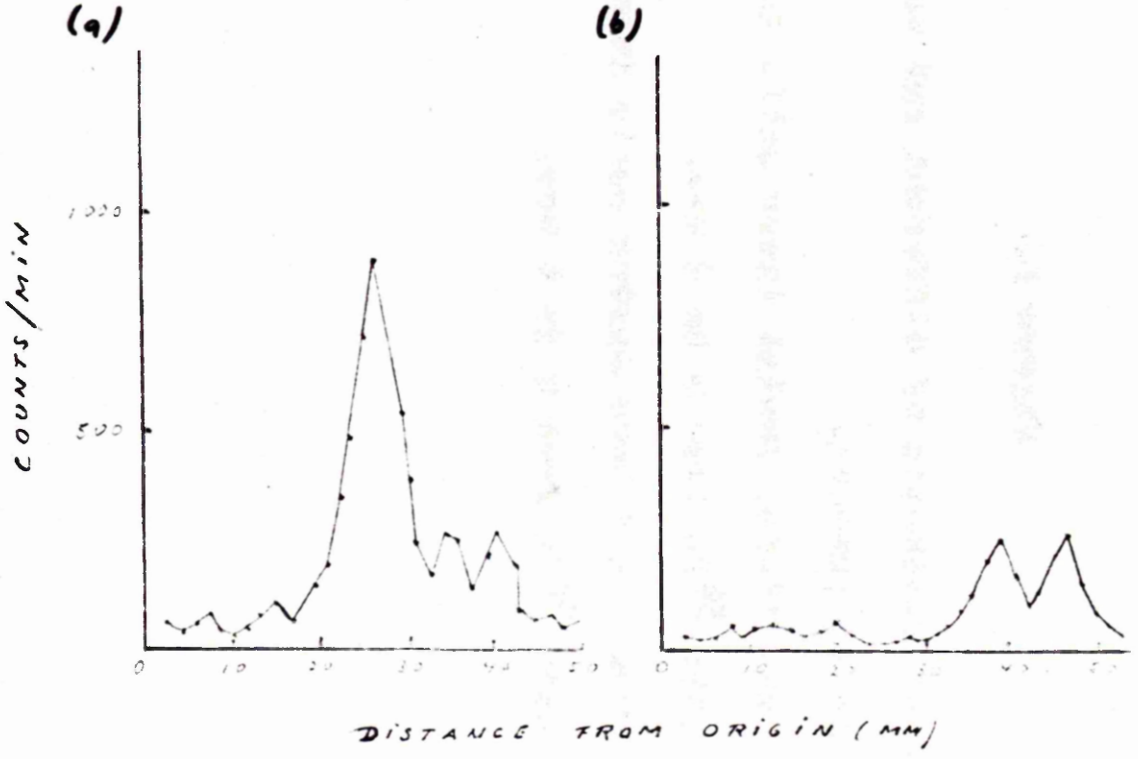
Figure 1.

Rate of synthesis of different rat haemoglobin compononts.

(a) From 15-day foetal liver cells incubated with $^{59}Fe$ from 0 to 6 hrs.

(b) From adult bone marrow cells incubated with $^{59}Fe$ from 0 to 4 hrs.

FIGURE 1




FROM: HUNTER & PAUL (1969)
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(Swiss) mouse (Cole, Hunter and Paul, 1968). It should be possible to transplant adult rat haemopoietic tissue (bone marrow) and foetal (liver) or embryonic (yolk sac) rat haemopoietic tissue into supralethally irradiated mice, so that the rat haemopoietic cells would recolonize the spleens of the experimental mice. The spleen colonies could then be isolated and cultured and the synthesized haemoglobin labelled by the incorporation of radio-iron. The different fractions of labelled haemoglobin could then be separated by electrophoresis and their radioactivity profiles obtained by methods described by Hunter and Paul (1969).

By analysing the type of haemoglobin synthesized by the recolonizing cells, it would be possible to determine whether it was of adult, foetal or embryonic type, or of a different type.

2. Haemopoietic cell culture and time-lapse cinemicrographic studies. Morphological and staining characteristics of the haemopoietic cell series are quite well established from the more mature stages back to the so-called haemocytoblast (Bessis, 1956; Maximow and Bloom, 1957; Garven, 1957; Wintrobe, 1961; McDonald, Dodds and Cruickshank, 1965) and there are quite good criteria for morphological identification by phase contrast microscopy (Bessis, 1949, 1954, 1955; Feissly and

Lädin, 1949; Moeschlin, 1949; Discombe, 1950; Lädin, 1952; Ackerman and Bellios, 1955[a,b]).

By culturing erythropoietic tissue cells in perfusion chambers and recording the events on film, it should be possible to follow one of the immature cells of unrecognizable morphology to a stage at which, due to differentiation, it becomes recognizable, either on the film or after fixation and staining. By correlating the recognized cell with its previous stages on the film, some of the morphological and physiological characteristics of the precursors, or of the stem cell itself, could be established. By adding the hormone erythropoietin and labelling with isotopes, valuable information could be obtained towards the understanding of their kinetics, and by studying the effect of different sera on measurable parameters, additional factors in the haemopoietic physiology and regulatory mechanisms might be identified.

# E X P E R I M E N T A L  I:

## SPLEEN COLONY - HAEMOGLOBIN SYNTHESIS STUDIES.

A. Spleen recolonization:

(1) Materials and Techniques.

(2) Establishment of optimal experimental conditions:

Irradiation and survival time.
Homologous transplantation.
Heterologous transplantation.

B. Electrophoretic studies on normal haemoglobins:

(1) Materials and Techniques.

(2) Development of assays and methods.

(3) Results.

C. Studies on experimentally recolonized spleen haemoglobins:

(1) Experiments.

(2) Results.

D. Discussion and Conclusions.

## A. Spleen Recolonization.

### (1) Materials and Techniques.

Irradiation. Whole body irradiations were performed with a Siemens X-ray unit provided with a filter Thoraeus I of 2,6 mm. Cu, operated at 250 kV and 15 mA, at a F.S.D. of 50 cm, and a central axis surface dose rate of 83 R/min (exposure rate in air), and under full backscatter conditions. Recipient mice were placed in a radially compartmented perspex circular box (12-mouse capacity) (Figure 2).

Under these experimental conditions the actual exposure rate absorbed by the mice was 63 R/min., as measured with a Victoreen roentgen meter placed inside a phantom animal prepared with bolus in one of the compartments of the cage. Irradiation doses presented in the present experiments refer to the absorbed dose rate.

Animals. Irradiated experimental hosts were Swiss albino mice, Porton strain. During the first stage of experimentation hosts were obtained from different sources and kept in the animal house at the Beatson Institute for periods varying from 2 to 20 weeks. Sex, age and weight varied (mostly males, 2½ to 7 months old, 25 to 55 gms). Table 1 shows these characteristics. Once a colony of animals was built up at the Institute, 3-month old males

FIGURE 2

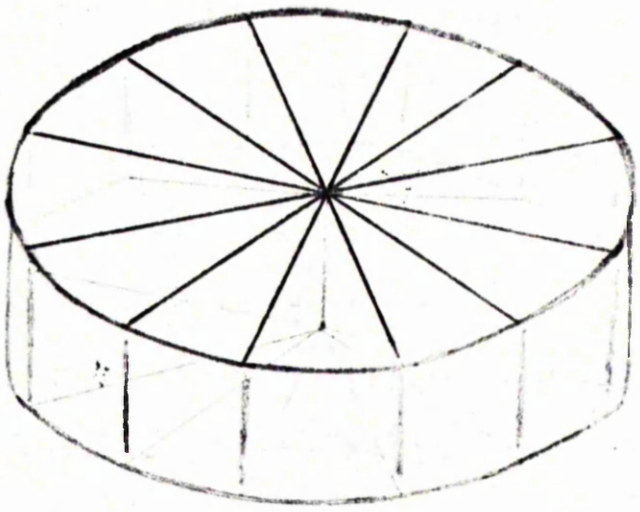


Table 1.

Sex, age and weight of irradiated host mice (Porton) in experiments designed to determine optimal animal conditions.

| Age (months) | Weight (g) ♀ | ♂ |
|---|---|---|
| 2½ | 25-30 | 30-40 |
| 3 | 25-30 | 30-40 |
| 3½ | | 35-40 |
| 4 | 30-35 | 30-40 |
| 5 | | 35-45 |
| 6½ | | 45-55 |
| 7 | | 40-50 |

only were used, 35 to 40 gms' weight. Irradiated animals were kept in groups of 6 to 12 to a plastic cage provided with bedding of humus sphagnum moss garden peat. Food (Diet Oxoid 41) and water were given ad libitum.

Preparation of cell suspensions for transplantation. (a) Adult bone marrow cell suspensions. Marrow cells for homologous transplantation were obtained from adult Porton mice male or female, 2½ to 7 months old, and heterologous marrow cells from adult Wistar rats, male or female, 6 to 12 months old. Femurs and tibias were dissected out aseptically, the heads cut off the shaft and the marrow obtained by flushing the cavity with ice-cold Hank's BSS by means of a syringe and needle. The marrow was disaggregated to a single cell suspension by passing it through hypodermic needles of decreasing gauge and finally through a stainless steel mesh. The number of nucleated cells was counted in a haemocytometer chamber, after lysing a cell suspension sample by adding an aliquot of a 2% acetic acid solution. The concentration of cells was adjusted accordingly for injection in 0.5 to 1 ml. of Hank's BSS via lateral tail vein or dorsal vein of the penis. Transplantation was carried out between 4 and 20 hours post-irradiation. Viability tests were performed in some cell suspensions by the dye

exclusion technique with 0.1% naphthalene black or nigrosin (Paul, 1965). Viability varied around 85 to 90%.

(b) Foetal liver and yolk sac cell suspensions. Foetal liver cell suspensions were obtained from randomly mated Porton mice or Wistar rats following the procedure described by Cole and Paul (1966). Livers were removed aseptically in ice-cold tris-citrate Hank's BSS, pH.7.4, and disaggregated by exposing them to Difco trypsin 1:250 in isotonic sodium chloride/sodium citrate, pH.7.8 containing 0.3% sodium carboxymethyl cellulose (to protect the cell membranes) at 4°C overnight. The supernatant trypsin was then removed and the livers incubated at 37°C for 5 minutes. Hank's BSS was added and disaggregation to single cell suspension completed by pipetting. Counting of nucleated cells and transplantation were performed as for marrow cells. Viability tests were also performed in a similar way on some cell suspension samples. Viability varied between 85 and 90%.

Viability tests were not carried out systematically because of a lack of correlation of the staining data with the direct viability assay by formation of spleen colonies (Silini, Pozzi and Pons, 1967).

Following similar procedures cell suspensions from

yolk sac were also prepared.

In vivo labelling and extraction procedures for haemoglobin. In vivo labelling of recolonized spleens was performed in the first experiments to find the optimal dose of heterologous transplants. Surviving animals were injected intraperitoneally with 20 μc of $^{59}FeCl_3$ (Department of Clinical Physics and Bio-engineering, Western Regional Hospital Board) on the 10th to the 16th days post-transplantation and killed by cervical dislocation 6 hours later. The recolonized spleen was collected, freed from other tissues, cut into small pieces with a scalpel and homogenized in 0.5 ml deionized distilled water in a glass homogenizing tube equipped with a motor-driven Teflon pestle. The homogenate was mixed with 2 vols. of chloroform (Weiss, 1960; Hunter, 1968) and centrifuged at 27,000 g for 30 mins., and the supernatant haemoglobin collected. These procedures were carried out in the cold.

In vitro labelling of haemoglobin. In vitro labelling was performed in the following experiments once the optimal dose of heterologous transplantation was established. After killing the surviving experimental mouse by cervical dislocation, the recolonized spleen was removed aseptically in Hank's BSS, cleared from other tissues and transferred to a glass

Petri dish containing Waymouth's culture medium MB 725/1 (Waymouth, 1959) supplemented with 10% foetal bovine serum, equilibrated overnight at 37°C in 5% $CO_2$ in air. The pH was adjusted to 7.2 - 7.4 by adding a 6.6% sodium bicarbonate solution.

Individual and confluent colonies were excised from the spleen and pooled in another Petri dish containing a small amount of culture medium, just sufficient to cover the colonies, to facilitate the next step. Using fine scalpels the colonies were cut into very small pieces and disaggregated by passing through a stainless steel mesh. All procedures were carried out at ice-cold temperature.

Cell suspensions were prepared in this way and cultures set up in Roux flasks containing 50 ml of culture medium. $^{59}FeCl_3$ (previously equilibrated with 50% rat serum in Hank's BSS in air at 37°C overnight) was added at a level of 0.5 μc/ml, and the cultures incubated at 37°C in 5% $CO_2$ in air.

Preparation of haemoglobin. After incubation, the medium was removed and the cells washed four times with cold normal saline or Hank's BSS. The pellet of cells obtained was lysed by adding 0.5 ml of deionized distilled water and Vortex mixing alternating with freezing and thawing four times. The haemolysate was

mixed with 2 vols. of chloroform to precipitate non-haem proteins, and other cell components (Weiss, 1960; Hunter, 1968). The chloroform was removed by centrifuging at 27,000 g for 20 mins. (The use of chloroform as a clearing agent was abandoned later, for reasons which will become apparent in the course of this work; and the haemoglobin was purified by carboxymethyl cellulose column chromatography).

Electrophoresis of haemoglobin and radioactivity counting. Fractionation of haemoglobins was carried out by horizontal starch gel electrophoresis, following the procedures of Smithies (1955) with a continuous buffer system, and of Poulik (1957) with a discontinuous buffer system. An aliquot of Hb solution was absorbed in a small strip of Whatman No. 31 chromatography paper, placed in a 12% starch gel, and the electrophoretic run performed with an electric potential of 8 to 10 V/cm at 4°C. At the end of each run, the migratory distances of visible bands were measured. The strip of starch containing the Hb fractions was cut out and transversely sliced into 1.5 mm. slices with a wired mechanical cutter, or into 1 mm. slices with an automatic gel slicer (The Mickle Laboratory Engineering Co., Gromshall, Surrey). Each slice was hydrolysed in 0.1 ml of "analar" HCl at 95°C and the whole hydrolysate dried on a Whatman GF/C,

2.5 cm fibre glass disc at 80°C for about 2 hours. Each disc was submerged in 5 ml of toluene-based scintillator fluid and the radioactivity decay counted in a Beckman LS-100 counter.

(2) Establishment of optimal experimental conditions.

Preliminary tests had to be performed to find optimal conditions before the experimental approach could be put into practice. These conditions should permit the formation of rat (heterologous) colonies in the spleens of irradiated mice, with a minimum of endogenous colonies, within a survival period suitable for carrying out the subsequent technical procedures.

Three stages could be distinguished in the performance of these experiments: (a) irradiation and survival; (b) homologous transplantation, and (c) heterologous transplantation.

Finding the optimal X-ray dose:

Experiments to find the survival time of irradiated mice without transplantation, and the time of the first appearance of endogenous colonies. In order to choose the optimal X-ray dose, experimental groups were given increasing single doses of whole body irradiation. On the death of the animals, the spleens were excised, fixed in Bouin's fluid and examined for the presence of endogenous colonies by the naked eye (Silini, Pozzi and

Pons, 1967). Tables 2 and 3 summarize the results of these experiments. In the range of 650 and 750 R the survival rate was more than 80% at 10 days after irradiation and around 60% at 20 days, but the presence of macroscopically visible endogenous colonies was noted at the 7th and 8th day. In the range of 850 R the survival rate was around 40% at 10 days and 34% at 20 days, and the first appearance of macroscopically visible endogenous colonies around the 9th and 12th day. With 950 R no macroscopically visible endogenous colonies were found in the dead animals and the mortality rate increased to give less than 40% survival at 10 days and 24% at 20 days. A dose of 1,000 R, on the other hand, yielded total mortality within the first 6 days after irradiation, due to intestinal post-irradiation syndrome.

These results showed the presence of endogenous colonies in dead animals, since the experiments were set up mainly with the purpose of finding the survival period after irradiation. This does not mean that the surviving animals did not develop endogenous colonies. On the contrary, survival was directly influenced by endogenous spleen repopulation: colonies were present as discrete nodules between the 7th and 12th day; after that, most of them became confluent and by the 20th day the spleens were repopulated to such an extent that they had returned

Table 2.

Mortality of Porton mice (♀, 4 months old, 30-35 g.) irradiated with a single X-ray dose (whole body irradiation).

| X-ray dose (R) | No. of mice. | 10 days post-irradiation | | | | 20 days post-irradiation | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Deaths | | Survivals | | Deaths | | Survivals | |
| | | No. | % | No. | % | No. | % | No. | % |
| 650 | 48 | 6 | 12.5 | 42 | 87.5 | 20 | 41.5 | 28 | 58.5 |
| 750 | 12 | 2 | 16.6 | 10 | 83.4 | 5 | 42.0 | 7 | 58.0 |
| 850 | 12 | 7 | 58.3 | 5 | 41.7 | 8 | 66.0 | 4 | 34.0 |
| 950 | 21 | 13 | 61.9 | 8 | 38.1 | 16 | 76.0 | 5 | 24.0 |
| 1000 | 9 | 9 | 100.0 | 0 | 0 | - | - | - | - |

Table 3.

Mortality of Porton mice (♀, 4 months old, 30-35 g.) irradiated with a single X-ray dose (whole body irradiation).

| X-ray dose (R) | No.of mice. | Number of Deaths. | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 |
| 650 | 48 | | | | | | 1+ | 1 | 4 | 1+ | 5 | 1 | 4 | 1 | | | 2 | | |
| 750 | 12 | | | | | 1+ | 1+ | | | 1+ | 1+ | | | | | | | | |
| 850 | 12 | | | | 1 | 1 | 1 | 2 | 2+ | | 1+ | | | | | | | | |
| 950 | 21 | | 2 | 1 | 4 | | | 1 | 5 | 1 | 2 | | | | | | | | |
| 1000 | 9 | 4 | 3 | 1 | 1 | | | | | | | | | | | | | | |

Days after irradiation

+ Presence of macroscopic endogenous colonies in spleens of dead animals.

to an almost normal state and the former colonies were not readily recognizable. It was concluded that irradiation should be as high as possible, but less than 1,000 R, in spite of the high increment in mortality.

To verify this conclusion an experimental group was irradiated with 950 R. Figure 3 shows the distribution of mortality on the 20 days following irradiation. Two waves of mortality occurred, the first between the 4th and 6th day after irradiation, and a second, and higher, one between the 9th and 12th day, giving a mortality rate of 76.20%. The absence of deaths after the 12th day indicated that endogenous colonies were proliferating in surviving animals.

Finding optimal conditions in the host animal:

Effect of sex, age and weight on the formation of endogenous and exogenous homologous colonies. These experiments were originally planned to find the proportion of exogenous colonies by transplanting foetal mouse liver (F.M.L.) and mouse yolk sac (M.Y. S.) at different ages. Unfortunately the experiments had to be performed on animals available at the time, and thus sex, age and body weight varied over a wide range.

From the results obtained in 7 experimental groups (Table 4) it was clear that no conclusive evidence could be drawn on the average number of exogenous colonies,

Figure 3.

Mortality of Porton mice (♀, 4 months old, 30-35 g) irradiated with a single dose of 950 R (whole body irradiation).

| | |
|---|---|
| Number of mice: | 21. |
| Number of deaths: | 16. |
| Mortality rate: | 76.20% in 20 days post-irradiation. |

FIGURE 3

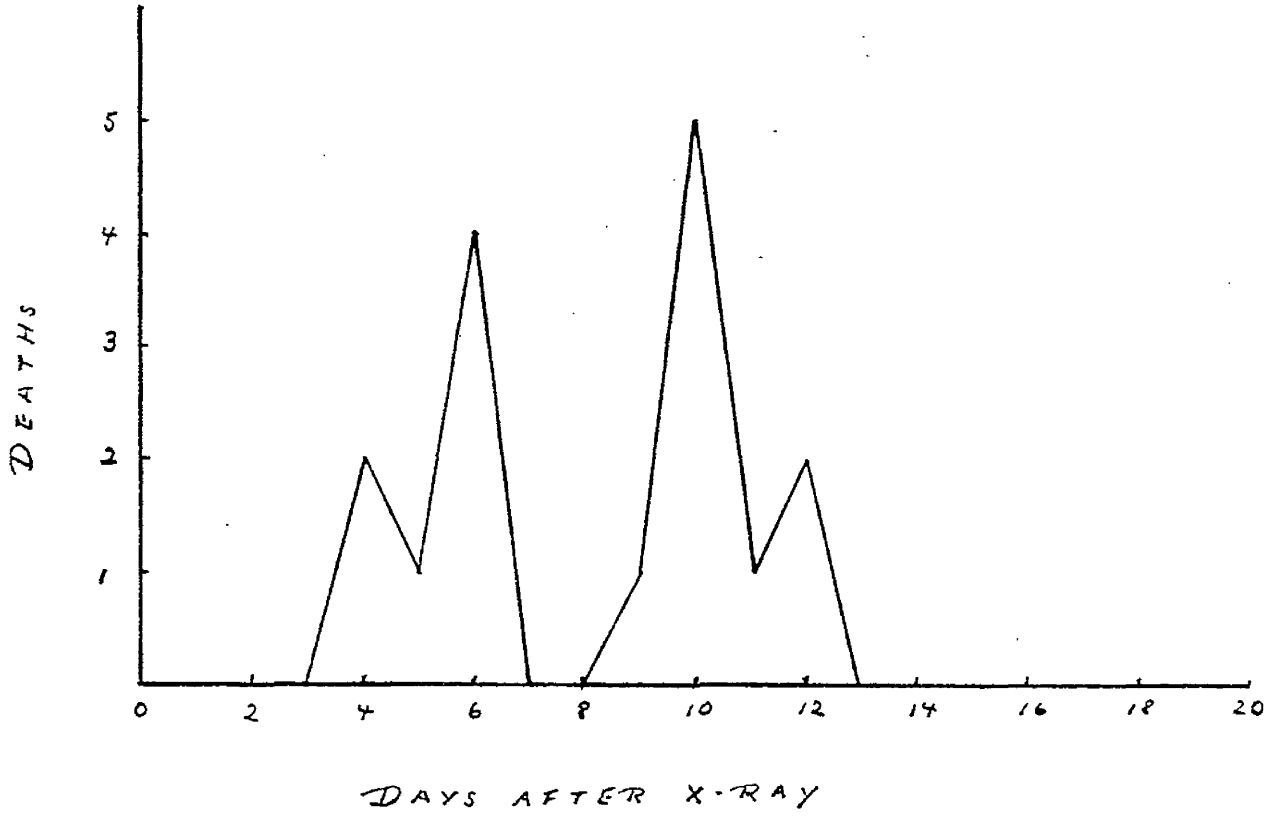


Table 4.

Number of spleen colonies in Porton mice irradiated with 850 R and transplanted with $10^5$ cells of foetal mouse liver and mouse yolk sac.

| Group | Sex | Age (mths) | Weight (g) | Type of Transplant | Number of mice. | | Survivals on the 10th day. | | Average No. of colonies per spleen. | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | E | C | E | C | E | C |
| 1 | ♀ | 3 | 25 - 30 | 10½ d, FML | 10 | 8 | 8 | 7 | 1.2 | 0.8 |
| 2 | ♂ | 3 | 25 - 35 | 11½ d. FML | 10 | 11 | 2 | 0 | 12.0 | |
| 3 | ♂ | 7 | 40 - 50 | 12½ d. FML | 9 | 6 | 7 | 5 | 4.1 | 6.8 |
| 4 | ♂ | 6½ | 45 - 55 | 13½ d. FML | 10 | 6 | 9 | 6 | 13.5 | 12.1 |
| 5 | ♀ | 4-7 | 30 - 50 | 7½ d. MYS | 4 | 20 | 4 | 19 | 1.0 | 3.3 |
| 6 | ♂ | 7 | 45 - 50 | 8½ d. MYS | 13 | 6 | 11 | 6 | 13.0 | 12.0 |
| 7 | ♀ | 4-6 | 30 - 50 | 9½ d. MYS | 13 | 11 | 9 | 9 | 3.2 | 3.7 |

FML = foetal mouse liver.
MYS = mouse yolk sac.

E = experimental mice (given transplant).
C = control mice (without transplant).

because of extreme deviations. However, by analysing the experimental conditions it was seen that the differential parameters were sex, age and weight. Hence some helpful information was obtained to establish the optimal experimental conditions directly concerned with the present research: variations in body weight concomitantly with age, influenced the appearance of endogenous colonies at the same irradiation dosage. Sex also had a certain influence on radiation survival, which was somewhat greater in females.

On the other hand, variations in individual spleens within the same experimental group were very extensive. Some spleens showed no endogenous colonies while others, in contrast, had many colonies. The average number of colonies does not show this variation, which was a point of concern in the present experimental work. If the experimental spleen to be analysed for haemoglobin type corresponded to one of those with many endogenous colonies, the probability of isolating exogenous colonies would be extremely low. In addition, previous results indicated that post-irradiation survival was directly influenced by endogenous colony formation (in absence of transplant). In other words, failure to survive was a failure to form spleen colonies.

Finally, it was felt that there was a need for a

standardized animal, in weight, age and sex. A suitable animal would be 2½ - 3 months old, 35-45 g (male) or 25-35 g (female), and the absorbed radiation dose possibly between 850 and 950 R. It was decided to build up a colony of suitable animals by inbreeding our own stock, thus maintaining standard characteristics, perpetuating the haemoglobin trait, and obtaining familiarization of the experimental animals to the habitat.

Finding the optimal dose of heterologous (rat) transplant for the formation of discrete exogenous colonies.

Experimental groups were transplanted with different amounts of adult rat bone marrow (A.R.B.M.) cells with the aim of obtaining discrete exogenous (rat) colonies of suitable size for isolation. Table 5 shows a summary of the experimental conditions.

Spleens were collected after the death of the mouse host, either as a result of experimental treatment, or by cervical dislocation, and fixed in Bouin's fluid for examination. On the 6th day post-transplantation, spleens in groups 3 and 4 showed no visible colonies. Between the 7th and 9th day, spleens in groups 2 and 5 showed minute dots, which were also noted in group 1 on the 8th and 9th days. Between the 10th and 14th day small colonies of ill-defined and loose appearance were observed. None of the spleens showed the large, discrete, solid, prominent

Table 5.

Conditions of experiments designed to determine the dose of adult rat bone marrow cells for spleen re-colonization in irradiated (950 R) mice.

| Exptl. Group | Sex | Age (mths) | Weight (g) | Transplant Dose. (No. of cells) | Period of observation (days) |
|---|---|---|---|---|---|
| 1 | ♀ | 4-5 | 30 - 45 | 75,000 | 7 - 9 |
| 2 | ♀ | 4-5 | 30 - 45 | 750,000 | 7 - 9 |
| 3 | ♀ | 5-6 | 35 - 55 | 30,000 | 6 - 10 |
| 4 | ♀ | 5-6 | 35 - 55 | 1,000,000 | 6 - 10 |
| 5 | ♂ | 6½ | 45 - 55 | 300,000 | 8 - 10 |
| 6 | ♂ | 4 | 30 - 35 | 1,000,000 | 8 - 14 |

## Plate 1.

(a) Experimentally recolonized mouse spleens:

Left: homologous transplantation.

Right: Heterologous (rat) transplantation.

(b) Size of mouse spleens:

Left: spleen from a normal mouse.

Middle: recolonized spleen.

Right: spleen from an irradiated mouse.

PLATE 1

a)

b)

Table 6.

Conditions and results of experiments designed to determine the optimal dose of rat transplant, and *in vivo* labelled Hb type in recolonised spleens of irradiated (850 R) mice.

| Exptl. Group | Sex | Age (mths) | Weight (g) | Transplant: Type | Transplant: No. of cells | Spleen Collection (days) | Hb Type | Presence of Peak 'X' |
|---|---|---|---|---|---|---|---|---|
| 1 | ♂ | 4 | 35 - 40 | 10½ d. M.embryo | $5 \times 10^6$ | 10 | M | + |
| 2 | ♂ | 4 | 35 - 40 | ARBM | $5 \times 10^6$ | 10 | M | + |
| 3 | ♂ | 3 | 35 - 40 | ARBM | $10 \times 10^6$ | 12 | M | + |
| 4 | ♂ | 4 | 35 - 40 | 15¼d. FML | $5 \times 10^6$ | 12 | M | + |
| 5 | ♂ | 3 | 35 - 40 | 16½ d. FML | $10 \times 10^6$ | 12 | M | - |

ARMB = adult rat bone marrow. FML = foetal mouse liver.
M = mouse.

nodule with well-defined and regular borders produced by homologous transplantation. The attached photographs illustrate the difference between colonies of homologous (mouse) and heterologous (rat) transplants.

It seems that rat C.F.U. located in the mouse spleen has a period of competition and adjustment to the new microenvironment before becoming established and proliferating. Heterologous colonies seem to grow more slowly than the homologous colonies.

Finding the optimal dose of heterologous (rat) transplant to obtain exogenous Hb type in recolonized spleens.

(a) In vivo labelling experiments. Since it appeared that the formation of individual discrete heterologous colonies of suitable size for isolation was not feasible under these experimental conditions, an alternative method had to be chosen: the production of confluent colonies which would permit their excision and isolation. For this purpose a higher dose of transplanted cells had to be administered.

Experimental groups were injected with increasing doses of rat transplant. The haemoglobins synthesized in the recolonized spleens were analysed after a period, following the procedures described in the Methods section. Table 6 is a summary of the conditions and results in

these experiments. Transplantations of $5 \times 10^6$ nucleated cells of $10\frac{1}{2}$-day whole mouse embryo, and $5 \times 10^6$ and $10 \times 10^6$ nucleated cells of A.R.B.M. and F.M.L. yielded a haemoglobin radioactivity pattern similar to that of adult mouse haemoglobin. Also a radioactive peak, here called X, which did not correspond to any visible fraction of haemoglobin, was present in all but one (group 5) of the experiments. Figure 4 is a typical example of these results.

Confirmation that spleen recolonization was taking place. Some additional information obtained during experiments both with homologous and heterologous transplantation showed that repopulation of the experimental spleens was, indeed, occurring. However, the efficiency of repopulation in the case of heterologous transplants was very much less than with homologous transplants. Tables 7 and 8, and the previous photographs, illustrate these results. The size and weight of the experimental spleens (of mice given a transplant after irradiation) were much greater than those of control spleens (of mice irradiated but with no transplantation), but less than those of normal spleens. Haem was extracted by the method of Teale (1959) in one-third of the *in vivo* labelled spleens, and, as seen in Table 8, the $^{59}Fe$ uptake by experimental spleens was very much higher than

Figure 4.

Incorporation of $^{59}Fe$ into experimentally recolonized spleen haemoglobin.

Mice were irradiated with 850 R, and transplanted with 10 x $10^6$ cells of adult rat bone marrow. Experimental conditions in Table 6.

On the 12th day post-transplantation, a mouse was injected with 20 µc of $^{59}FeCl_3$ intraperitoneally and 6 hours later the spleen was collected and haemoglobin prepared by chloroform extraction. The haemoglobin components were separated by starch gel electrophoresis (Smithies, 1955) and radioactivity incorporation determined as described in the text.

The radioactivity peak 'X' did not correspond to any haemoglobin component.

FIGURE 4

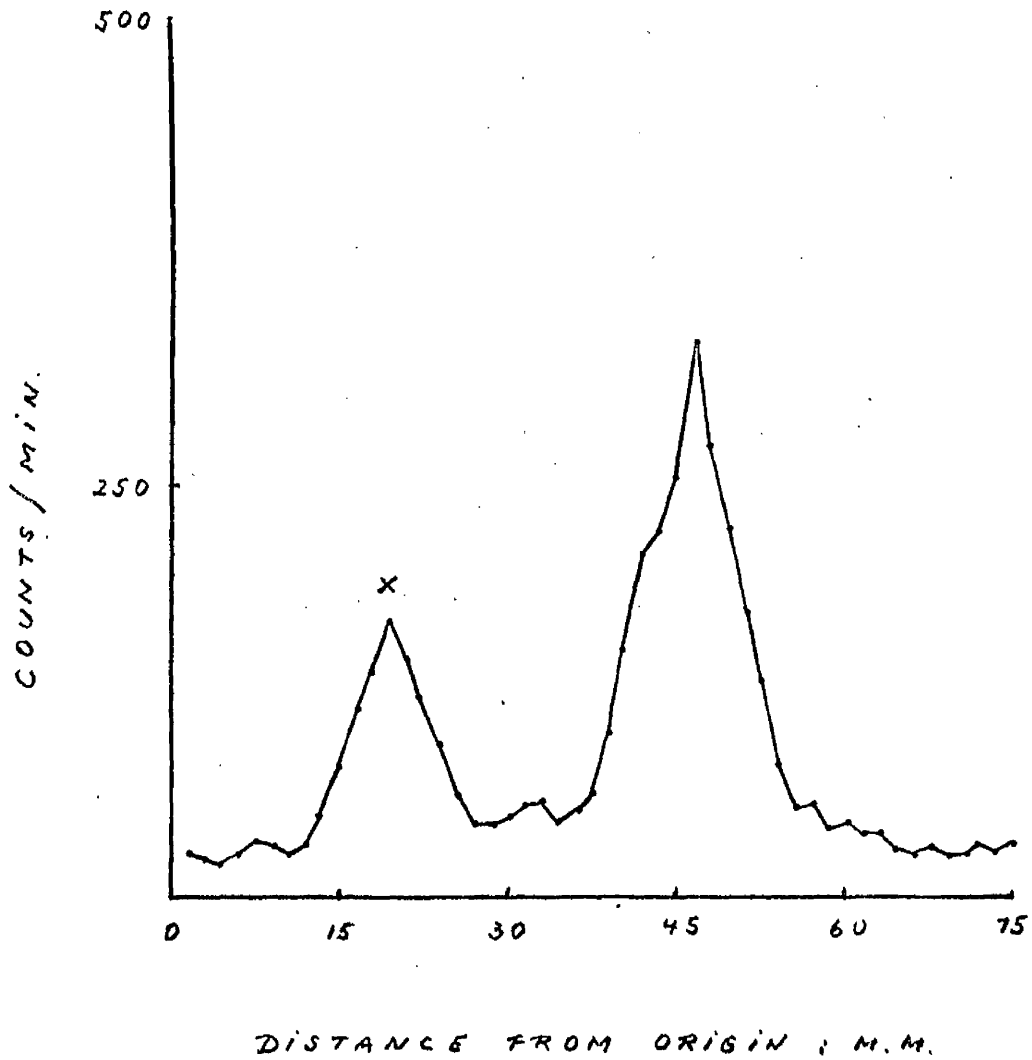


Table 7.

Experimental conditions, and mean weight of spleens of irradiated mice, with or without transplantation.

| Exptl. Group | X-ray R | Sex | Age (mths) | Weight (g) | Transplant (No. of cells) | Spleen Collection (days) | No. of Spleens | | | Spleen mean weight (g) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | C | E | N | C | E | N |
| 1 | 540 | ♀ | 4 | 30 - 35 | 10,000 AMBM | 10 | 2 | 19 | 6 | 0.11 | 0.21 | 0.62 |
| 2 | 850 | ♂ | 4 | 35 - 40 | 1,000,000 AMBM | 10 | 4 | 8 | 4 | 0.11 | 0.22 | 0.32 |
| 3 | 850 | ♂ | 4 | 35 - 40 | 5,000,000 AMBM | 10 | 5 | 5 | 2 | 0.09 | 0.15 | 0.20 |
| 4 | 850 | ♂ | 4 | 35 - 40 | 5,000,000 $10\frac{1}{2}$ d.F. emb. | 10 | 6 | 6 | 4 | 0.05 | 0.16 | 0.21 |
| 5 | 850 | ♂ | 3 | 35 - 40 | 10,000,000 ARBM | 12 | 4 | 5 | 5 | 0.06 | 0.18 | 0.35 |
| 6 | 850 | ♂ | 3 | 35 - 40 | 10,000,000 $16\frac{1}{2}$ d.FML | 14 | 5 | 5 | 5 | 0.03 | 0.16 | 0.49 |

N = normal spleens.
E = Spleens of irradiated mice given transplantation.
C = " " " " without "

AMBM = adult mouse bone marrow.
ARBM = adult rat bone marrow.
FML = foetal mouse liver.

Table 8.

Experimental conditions, radioactivity and optical density values of haem extracted from recolonized and non-recolonized spleens of irradiated mice.

| Exptl. Group | X-ray (R) | Sex | Age (mths) | Weight (g) | Transplant (No. of cells) | HAEM | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Counts/min. | | Optical Density Absorbance | |
| | | | | | | C | E | C | E |
| 1 | 800 | ♂ | 6½ | 45 - 55 | 300,000 ARBM | 10,150 | 19,942 | | |
| 2 | 850 | ♂ | 4 | 35 - 40 | 5,000,000 ARBM | 4,220 | 8,416 | 0.048 | 0.096 |
| 3 | 850 | ♂ | 4 | 35 - 40 | 1,000,000 ARBM | 4,576 | 46,456 | 0.037 | 0.405 |

E = experimental spleen (from mice given transplantation).
C = control spleen (from mice without transplantation).

in controls. Haem concentration, measured by optical density, followed the same trend. Microscopically the recolonized spleens showed repopulation by haemopoietic precursor cells at different stages of maturation. Some haemorrhagic foci were often observed.

Transplanting massive doses of heterologous haemopoietic tissue. Having in mind the data obtained from previous experiments, massive doses of A.R.B.M. cells were tested. Table 9 summarizes the conditions and results of these experiments. A dose of 20 x $10^6$ nucleated cells yielded a radioactivity pattern corresponding to an exogenous (rat) type of haemoglobin in a spleen collected on the 16th day post-transplantation. Figure 5 shows this result. The experiment was repeated with a dose of 40 x $10^6$ nucleated cells, and again the radioactivity pattern obtained corresponded to an exogenous type of haemoglobin in spleens collected on the 12th and 14th days after transplantation. Figure 6 shows the results of one of these experiments. The radioactivity profiles obtained were different from the profiles of adult and foetal rat haemoglobins described by Hunter and Paul (1969). However, normal peripheral blood haemoglobins co-electrophoresed as controls, showed a different pattern (this will be discussed later). A massive dose of 20 to 40 x $10^6$ cells of A.R.B.M. seemed

Table 9.

Conditions and results of experiments designed to determine the optimal dose of rat transplant, and *in vivo* labelled Hb type in recolonized spleens of irradiated mice.

| Exptl. Group | Sex | Age (mths) | Weight (g) | X-ray dose (R) | Transplant | | Spleen Collection (days) | Hb Type | Presence of Peak 'X' |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | Type | No.of cells | | | |
| 1 | ♂ | 4 | 35 - 40 | 850 | ARBM | $20 \times 10^6$ | 16. | R | - |
| 2 | ♂ | 4 | 30 - 40 | 850 | ARBM | $40 \times 10^6$ | 12-14 | R | - |

ARBM = adult rat bone marrow.
R = rat.

Figure 5.

Incorporation of $^{59}Fe$ into experimentally recolonized spleen haemoglobin.

Technical procedures as in Figure 4.

Experimental conditions in Table 9.

Irradiation: 850 R.

Transplant: $20 \times 10^6$ adult rat bone marrow cells.

Spleen collection: 16th day post-transplantation.

FIGURE 5

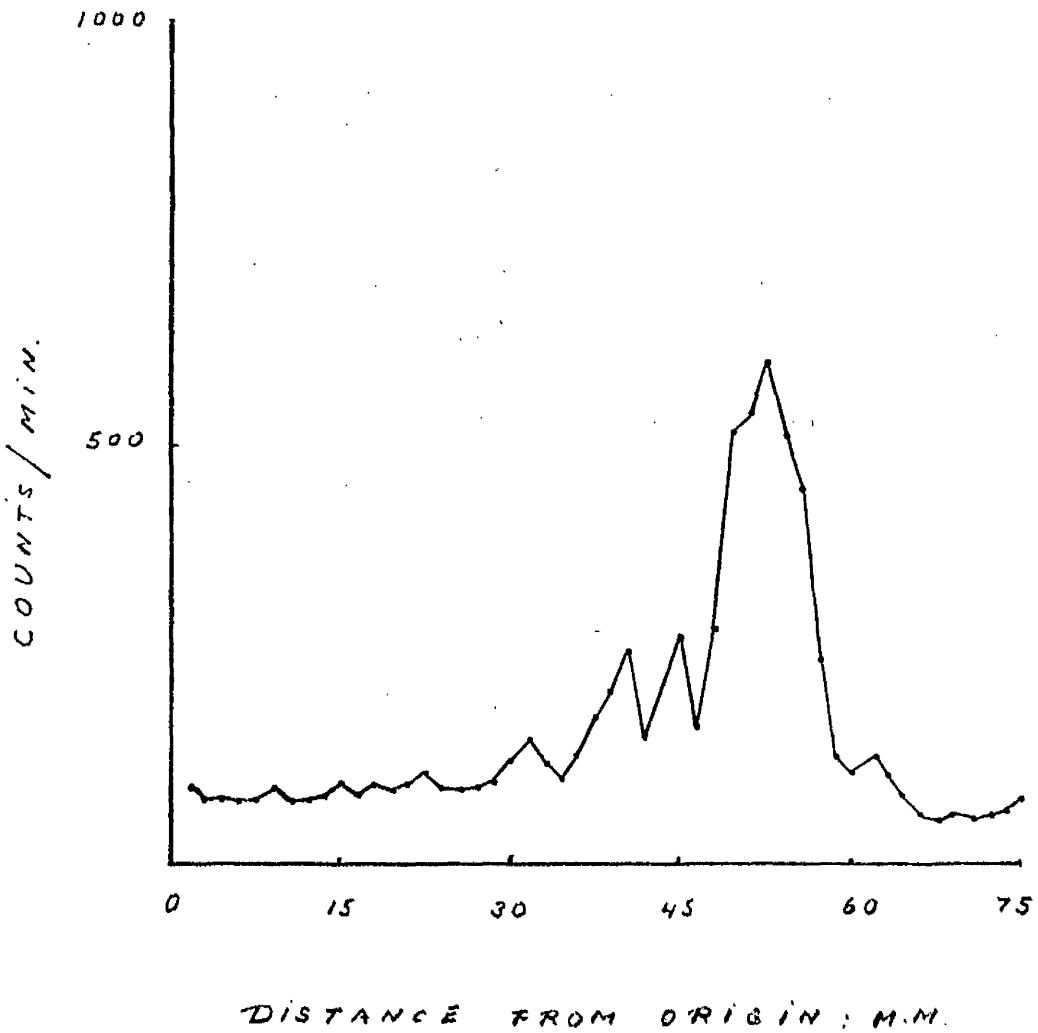


Figure 6.

Incorporation of $^{59}Fe$ into experimentally recolonized spleen haemoglobin.

Technical procedures as in Figure 4.

Experimental conditions in Table 9.

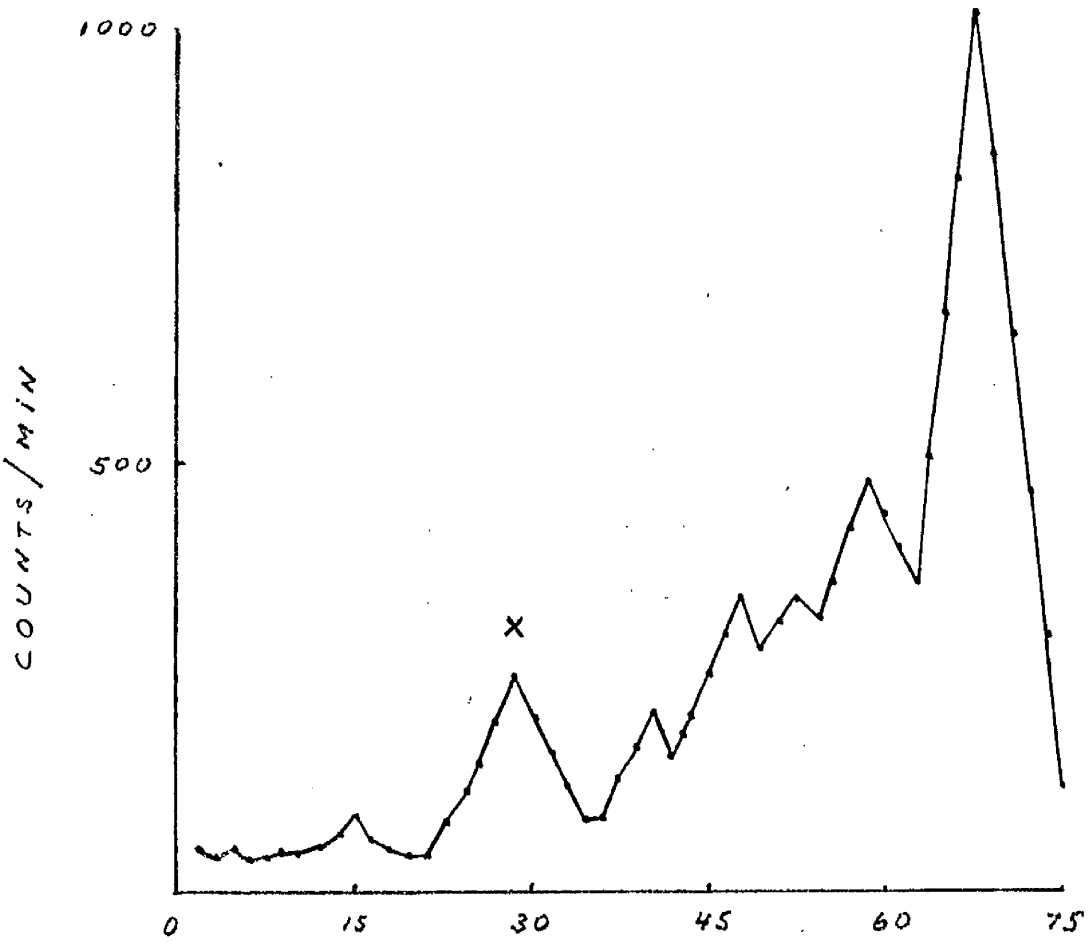
Irradiation: 850 R.

Transplant: 40 x $10^6$ adult rat bone marrow cells.

Spleen collection: 12th day post-transplantation.

The radioactivity peak 'X' did not correspond to any haemoglobin component.

FIGURE 6



necessary to obtain synthesis of rat type haemoglobin in the recolonized spleens, and the optimum period for spleen collection around the 14th to 16th day, post-transplantation.

(b) In vitro labelling experiments. Experimental groups were injected with different doses of massive transplant and the spleen-recolonizing cells labelled in vitro, following the procedures previously described. Table 10 gives a summary of the conditions and results of these experiments.

A massive dose of 20 and 40 x $10^6$ cells of 15½ day old F.M.L. yielded a radioactivity profile of a rat type of haemoglobin, obscured by the presence of a radioactivity peak which did not correspond to any of the fractions of haemoglobin. Other experiments gave similar results. Figure 7 shows a typical example of these results. Here also both experimental spleen haemoglobins and normal peripheral blood haemoglobins showed different patterns from those of rat haemoglobins described by Hunter and Paul (1969). The best time for spleen collection seemed to be around the 13th and 15th day post-transplantation.

Problems at issue. As the result of the preceding experiments two different problems emerge. 1. The patterns obtained from recolonized spleen haemoglobins

Table 10.

Conditions and results of experiments designed to determine <u>in vitro</u> labelled Hb type in spleens of irradiated mice transplanted with rat haemopoietic cells.

| Exptl. Group | Sex | Age (mths) | Weight (g) | X-ray dose (R) | Transplant | | Spleen Collection (days) | Hb Type | Presence of Peak 'X' |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | Type | No.of cells | | | |
| 1 | ♂ | 4 | 30 - 40 | 850 | 15½d.FRL | $20 \times 10^6$ | 13 | R | + |
| 2 | ♂ | 5 | 35 - 40 | 900 | 15½d.FRL | $40 \times 10^6$ | 14 | R | + |
| 3 | ♂ | 4 | 35 - 40 | 850 | ARBM | $35 \times 10^6$ | 17 | R | + |
| 4 | ♂ | 3½ | 35 - 40 | 850 | 15½d.FRL | $17 \times 10^6$ | 11 | R | + |
| 5 | ♂ | 3 | 35 - 40 | 850 | ARBM | $45 \times 10^6$ | 15 | R | + |
| 6 | ♂ | 3 | 35 - 40 | 850 | 15½d.FRL | $25 \times 10^6$ | 12 | R | + |

ARBM = adult rat bone marrow.
FRL = foetal rat liver.
R = rat.

Figure 7.

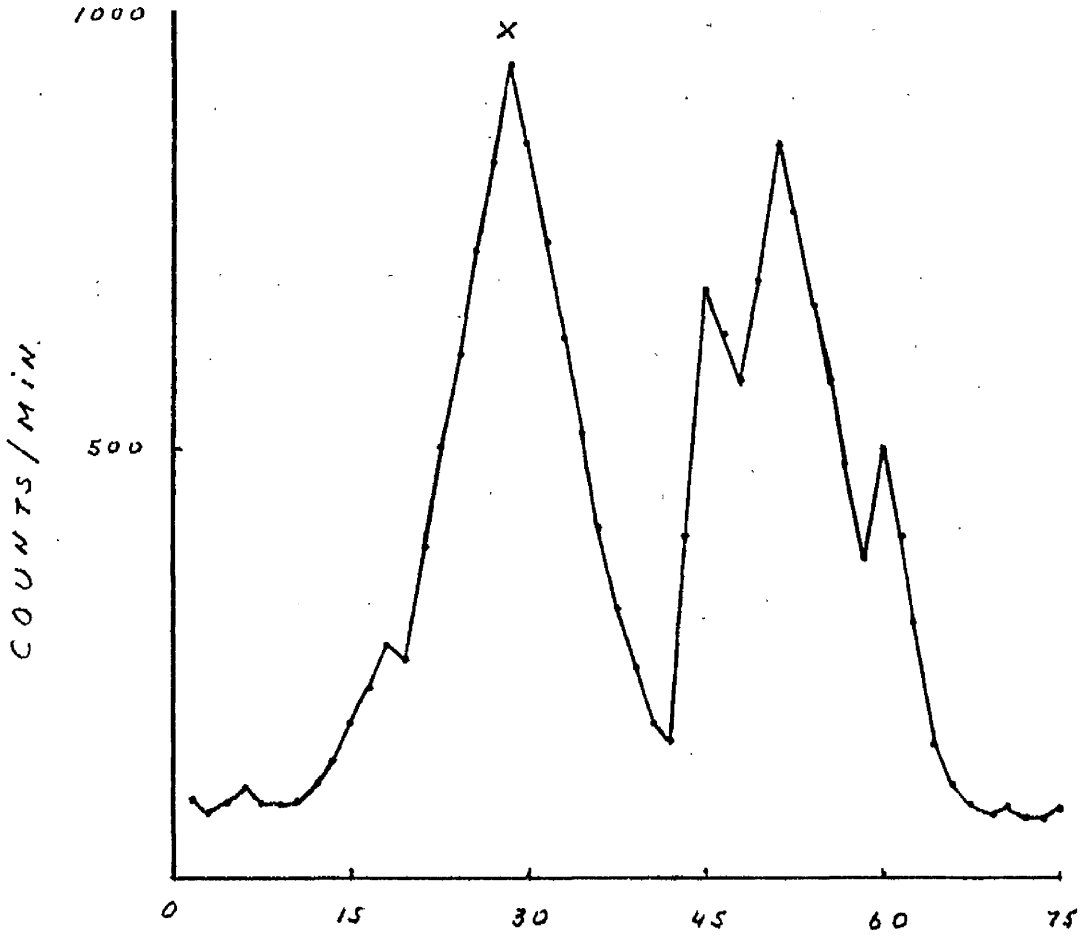
Incorporation of $^{59}Fe$ into experimentally recolonized spleen haemoglobin.

Mice were irradiated with 850 R, and transplanted with 20 x $10^6$ cells of 15½-day foetal rat liver. Experimental conditions in Table 10.

On the 13th day post-transplantation, confluent spleen colonies were isolated, disaggregated and cultured. The cells were incubated with $^{59}Fe$ for 8 hrs. Haemoglobin was prepared by chloroform extraction and fractionated by starch gel electrophoresis (Smithies, 1955) and radioactivity incorporation determined as described in the text.

The radioactivity peak 'X' did not correspond to any haemoglobin component.

FIGURE 7



and normal peripheral blood haemoglobins differed from the adult and foetal rat haemoglobins described by Hunter and Paul (1969), and 2. The presence of a radio-activity peak not corresponding to a haemoglobin fraction, interfered with the analysis of the differential characteristics of experimental haemoglobins.

B. Electrophoretic studies on normal haemoglobins.

Materials and Techniques : Development of Assays.

An important issue had, then, arisen, and had to be resolved to clarify the preliminary results, and permit the undertaking of the primary purpose of the present work. This was the reproducibility of the findings of Hunter and Paul (1969), on the -

Differentiation of foetal and adult rat haemoglobins.

Basic information. Hunter and Paul (1969) found 5 components in both the foetus and adult Wistar albino rat from the 15th day of foetal life onwards. However, the rates of synthesis of the components were different at different stages. The basic difference between adult and foetal rat haemoglobins was the fact that fraction 'c' accounted for nearly 70% of the total Hb in the 15-day-old foetal liver, and scarcely 3% in the adult bone marrow (6 months old).

Methods. Technical procedures were based on those

described by Hunter (1968). Adult albino rats, Wistar strain, were bled from the jugular vein. The blood was collected in a great excess of ice-cold normal saline or Hank's BSS and mixed immediately to avoid clotting. Rat foetuses were washed free of maternal blood with Hank's BSS and allowed to bleed freely from the cord into ice-cold BSS. A pellet of cells was obtained by centrifuging. The cells were washed 3 times with cold normal saline or Hank's BSS and lysed by adding 1 and 4 volumes of deionized distilled water to the foetal and adult cells respectively, with thorough mixing alternating with freezing and thawing 3 times. An equal or greater volume of chloroform was then mixed with the haemolysate and removed by centrifuging at 1,500 g for 10 mins, following the procedures already described in the preparation of haemoglobin from recolonized spleens.

Electrophoretic fractionation of haemoglobins was carried out immediately, in order to prevent any spontaneous precipitation, under the conditions previously described. On completion of the electrophoretic run, the gels were horizontally split into two halves, the different haemoglobin fractions identified by staining with o-dianisidine (Lehmann and Huntsman, 1966) and the proteins in general with naphthalene black (Smith, 1968).

Results: These revealed -

Electrophoretic heterogeneity of rat haemoglobins.

Individual samples of peripheral blood haemoglobin from different rats were prepared and electrophoresed. Their electrophoretic patterns were different from those described by Hunter and Paul (1969). Figures 8 and 9 are typical examples of the optical density profiles obtained.

The reasons for the discrepancy had to be clarified. Addition of dithiothreitol, a -SH group reducing agent (Cleland, 1964), did not have any effect on the electrophoretic patterns. Variations in the pH of the electrophoretic buffers had little effect between 8.3 and 9.5, and gel buffers of low ionic strength produced cathodic migration of the slower fractions, (Fig.10), but the patterns were maintained. Freezing (used during haemolysis) did not show any effect either. However, on storing the haemoglobin solution, i.e. by extending the exposure of haemoglobin to oxygen, a crystalline precipitate formed. Fraction 4 proved to be an extremely labile component of adult rat haemoglobins; it precipitated spontaneously, precipitation beginning during the first hour after haemoglobin preparation, especially in concentrated solutions, even at 4°C. The optical density profiles in Figure 11 illustrate

Figure 8.

Optical density profiles of adult rat haemoglobin from peripheral blood.

Haemoglobin was prepared and fractionated by starch gel electrophoresis, as described in the text.

(a) With the continuous system of buffers of Smithies (1955).

(b) With the discontinuous system of buffers of Poulik (1957).

FIGURE 8 (a)

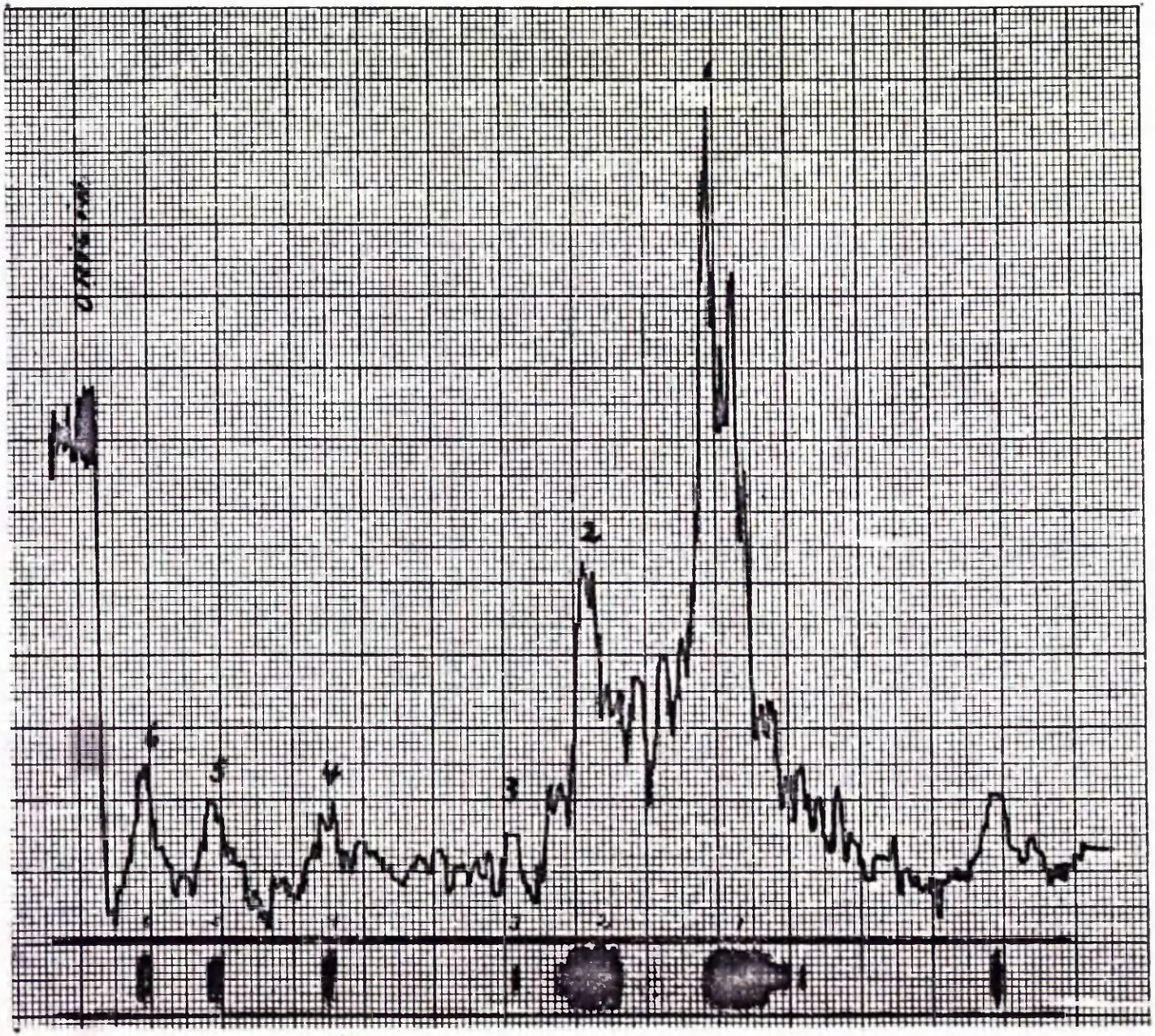


FIGURE 8 (b)

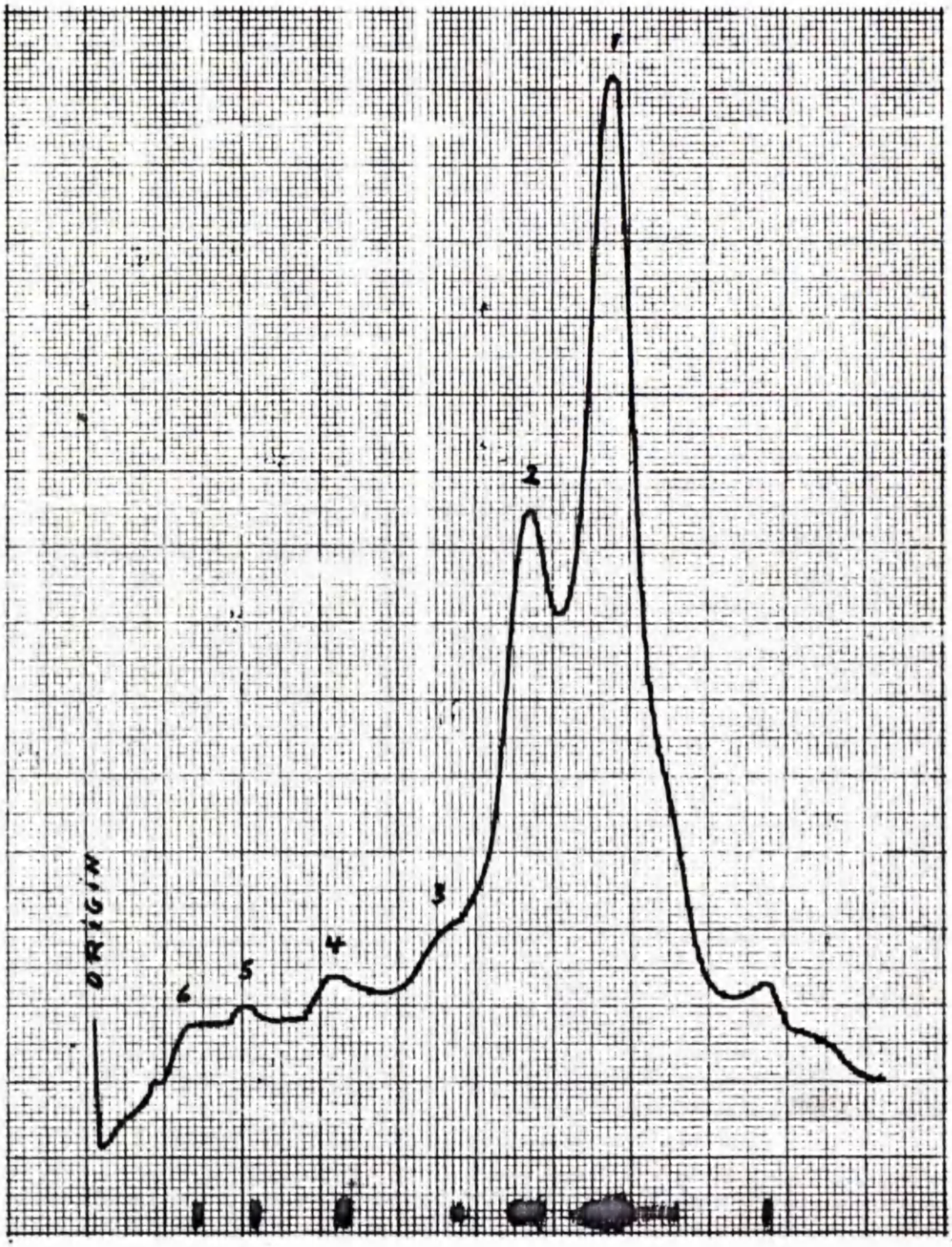


Figure 9.

Optical density profile of 15-day foetal rat haemoglobin from the cord.

The haemoglobin was prepared and fractionated by starch gel electrophoresis (Poulik, 1957) as described in the text.

FIGURE 9

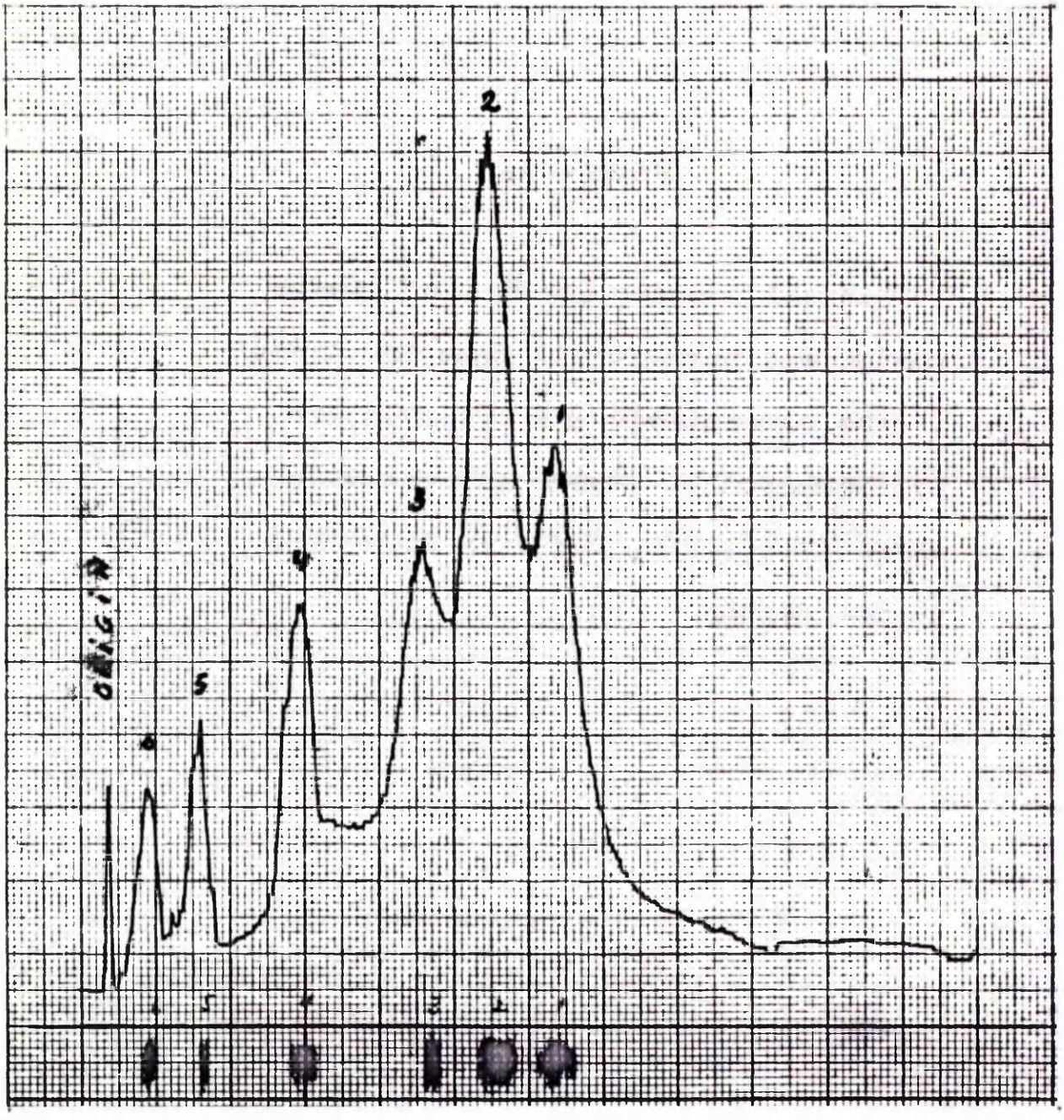


Figure 10.

Optical density profiles of peripheral blood haemoglobins.

The haemoglobins were prepared and fractionated by starch gel electrophoresis (Smithies, 1955) at low ionic strength, as described in the text.

Note the cathodic migration of fraction 6.

(a) Adult rat Hb (Wistar).

(b) 15-day foetal rat Hb (Wistar).

(c) Adult mouse Hb (Porton).

FIGURE 10 (a)

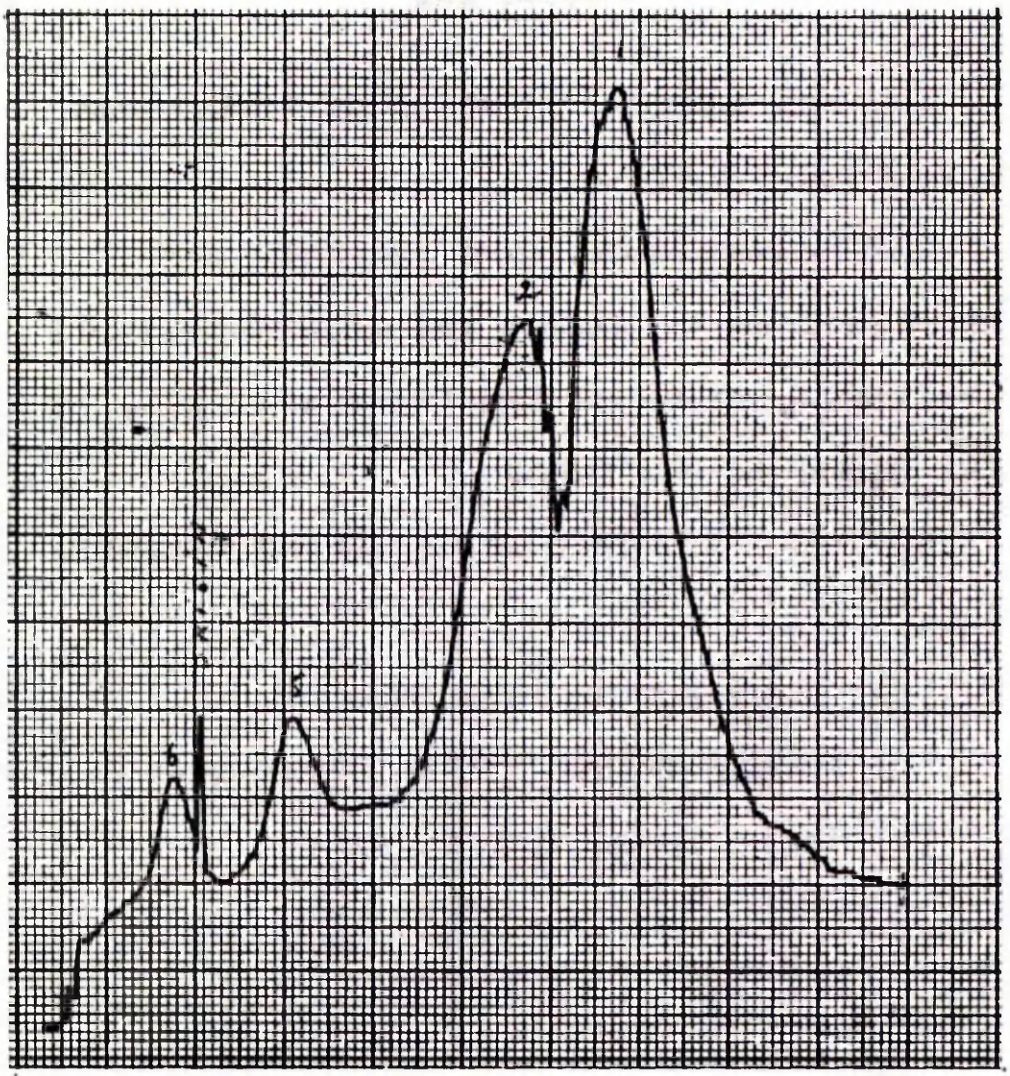


FIGURE 10 (b)

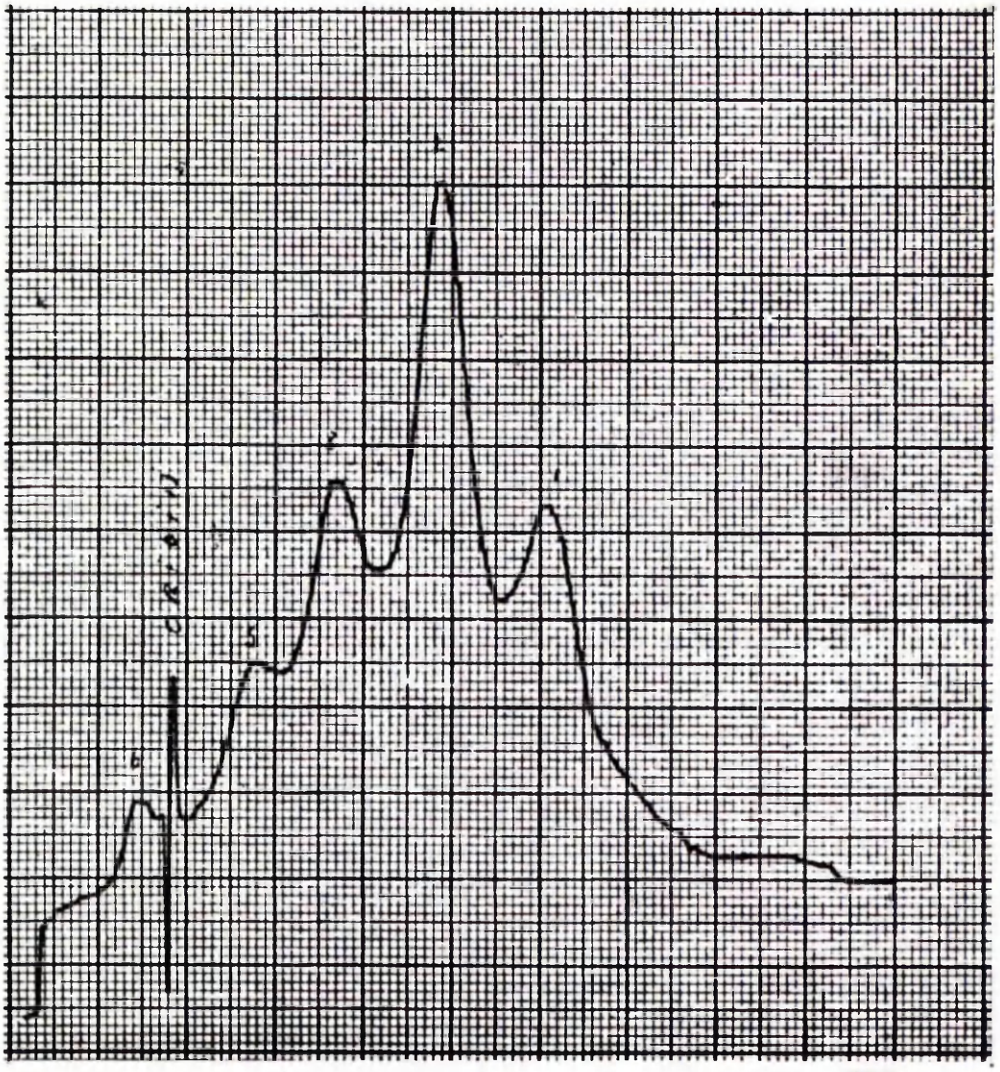


FIGURE 10 (c)

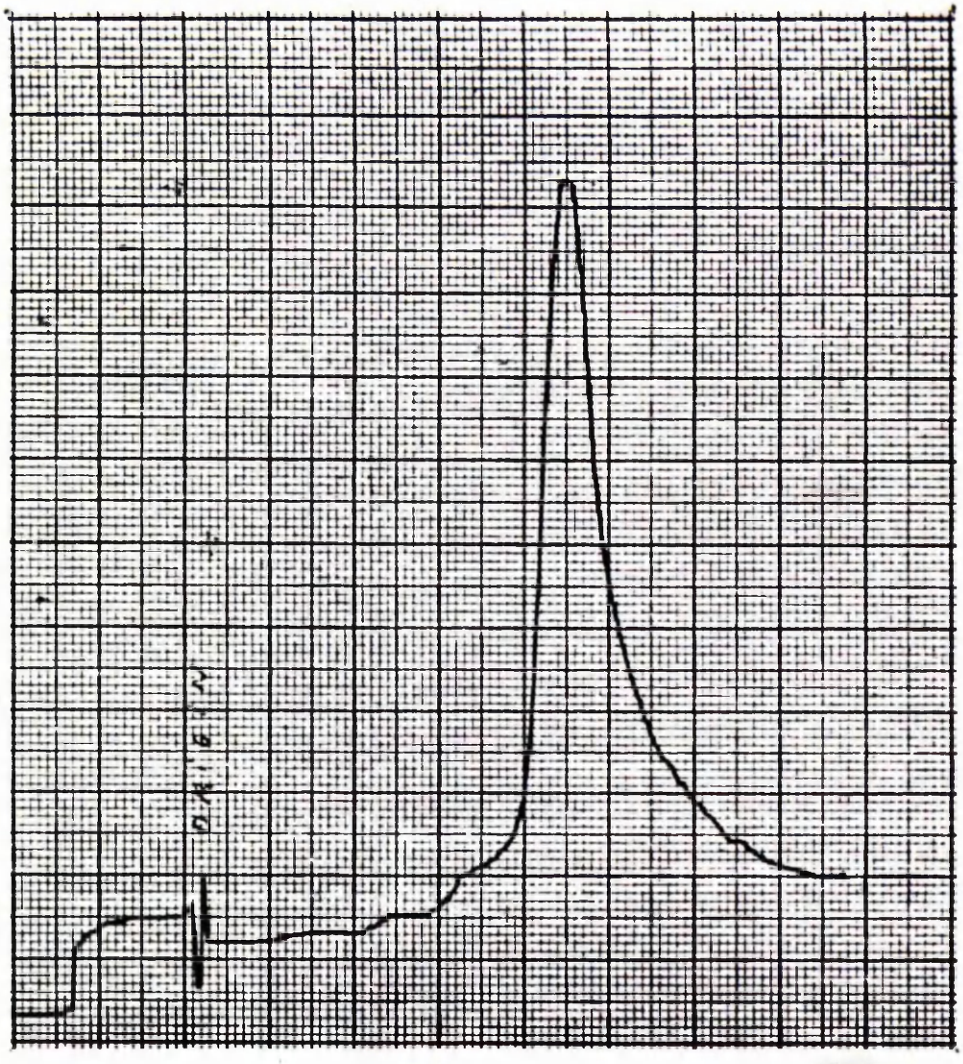


Figure 11.

Optical density profiles of adult rat haemoglobin.

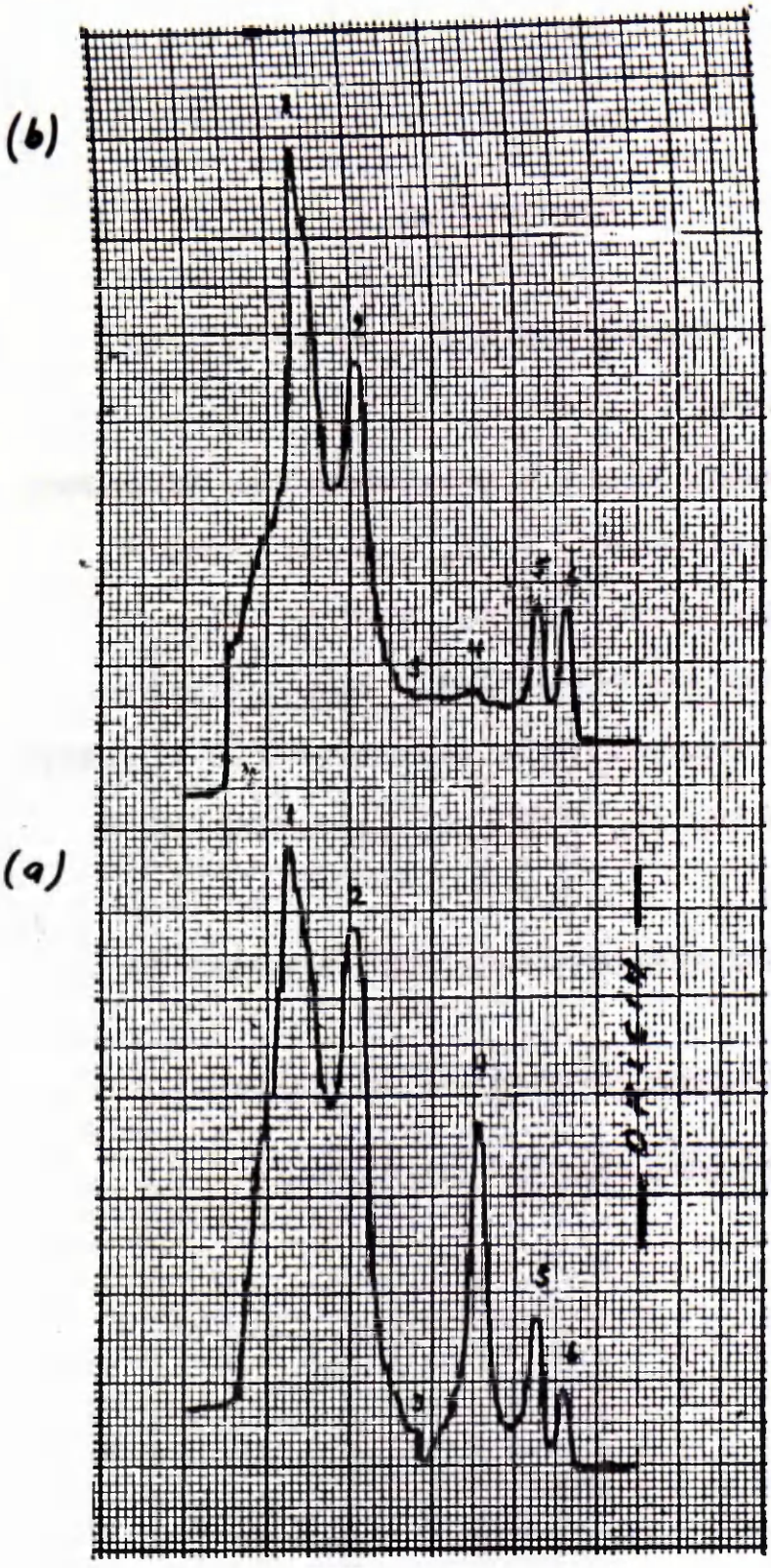
Peripheral blood haemoglobin was prepared as described in the text, and an aliquot kept in the vacuum while another aliquot was kept in air overnight. Both aliquots were then fractionated by starch gel electrophoresis (Poulik, 1957) as described in the text.

Note the disappearance of fraction 4.

(a) Aliquot maintained in vacuum.

(b) Aliquot exposed to oxygen.

FIGURE 11



this problem.

Other workers (Brada and Tobiška, 1964; Wacks et al., 1968) have also reported the tendency of adult rat Hb to crystallise spontaneously, and that the ease of crystallization increased with Hb concentration. The crystals can be redissolved by gassing the sample with $CO_2$, adding urea, raising the ionic strength of the Hb solution (Brada and Tobiška, 1964) or raising its pH with N.KOH solution (Wacks et al., 1968).

Dilution of the Hb sample delayed the appearance of this crystallization, but decreased the amount of the minor components so that they were no longer demonstrable after electrophoresis, by staining with o-dianisidine, or even with naphthalene black (which was found to be more sensitive, although not specific for haem). This led to a study of the minimal amount of Hb load under starch gel electrophoretic conditions. At least 10 $mm^3$ of a 2% Hb solution was necessary to get a complete spectrum visible by staining. Lower concentrations yielded a more discrete separation of the fractions but at the sacrifice of visibility of the minor components.

In view of these preliminary results, it was felt that different samples of haemoglobins, both foetal and adult, should be electrophoresed.

In haemoglobin from 13-day-old rat foetuses 4 fractions were clearly discernible. Figure 12 shows the optical absorption of this haemoglobin pattern. As the foetus grew older, other fractions progressively appeared, and on the 15th day a complete spectrum of components was present. In this way the fractions which persist into adult life were established. The only difference from this stage on, was in the relative amount of each fraction. Figure 13 shows the optical density profile, and Table 11 the relative amount of haemoglobin in each component. Fraction 2 was the most prominent in the foetal rat haemoglobin. In the older foetus the most noticeable difference was that while fraction 3 diminished, fraction 1 increased. Hence the preponderance of fraction 1 was established both in the newborn and in the adult. Figure 13 also shows the optical density profile of a sample of adult rat haemoglobin, and Table 11 the relative amount of each component. Fraction 1' was quite often not discretely separated from component 1. Many samples showed it only as a shoulder of fraction 1, and at other times it was absent, appearing to have been completely integrated into this fraction.

The fast migrating band 'v' ahead of the haemoglobin components, was also present, as a single band or as a

Figure 12.

Optical density profiles of peripheral blood haemoglobins.

Haemoglobins were prepared and fractionated by starch gel electrophoresis (Poulik, 1957) as described in the text.

(a) 13-day foetal rat Hb (Wistar).

(b) Adult mouse Hb (Porton).

FIGURE 12 (a)

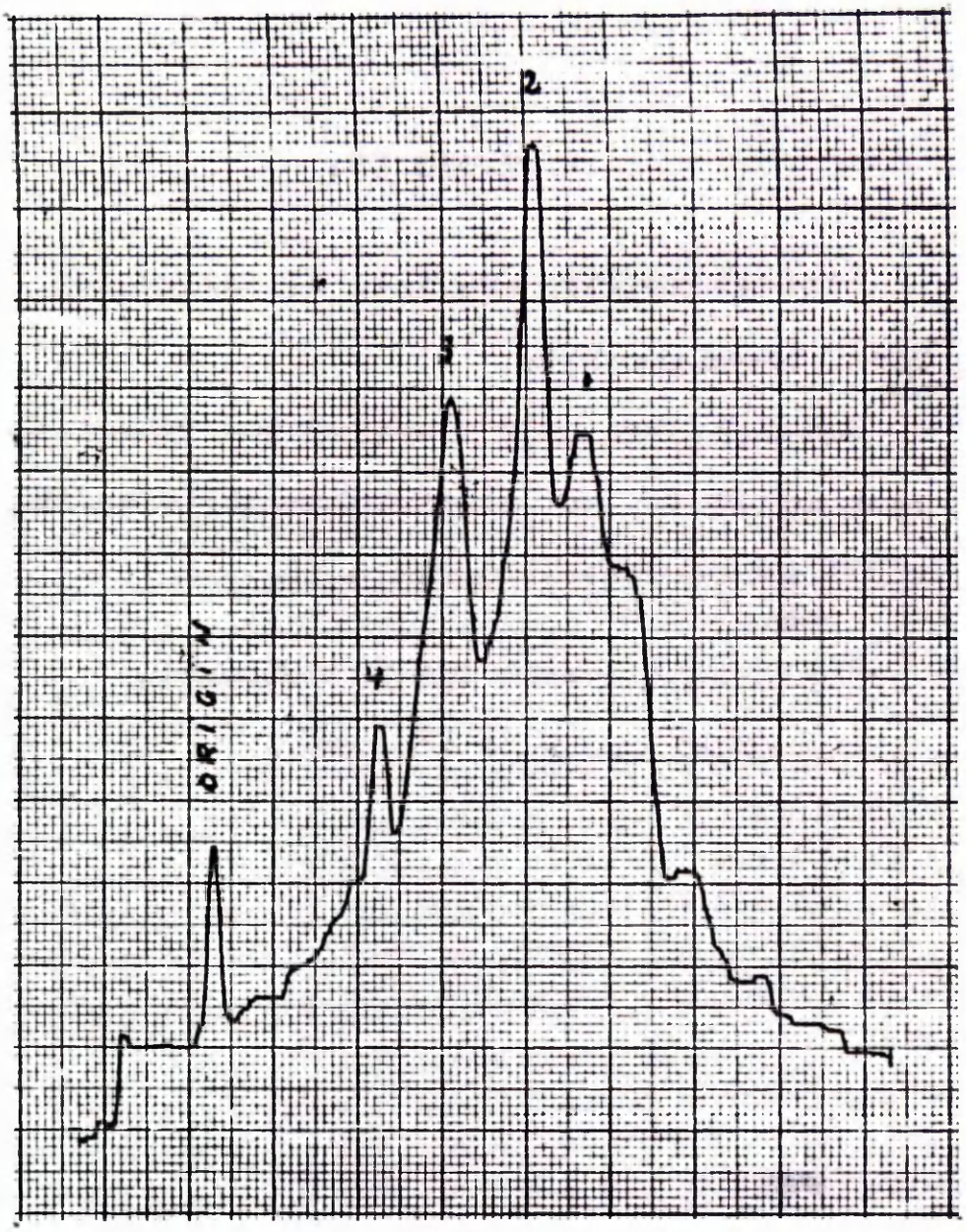


FIGURE 12 (b)

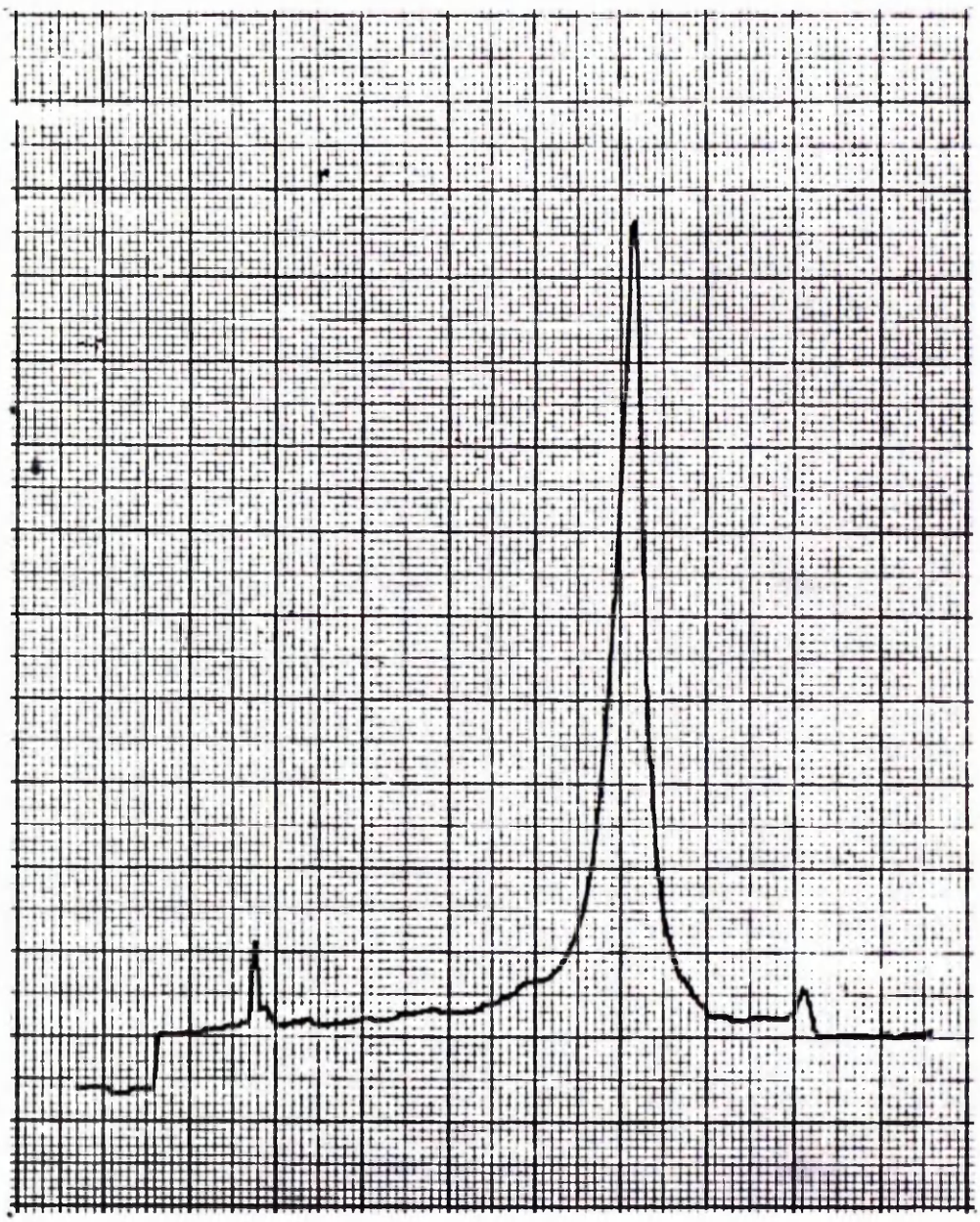


Figure 13.

Optical density profiles of peripheral blood haemoglobin.

Technical procedures as in Figure 12.

(a) 15-day foetal rat Hb (Wistar).

(b) Adult rat Hb (Wistar).

(c) Adult mouse Hb (Porton).

FIGURE 13 (a)

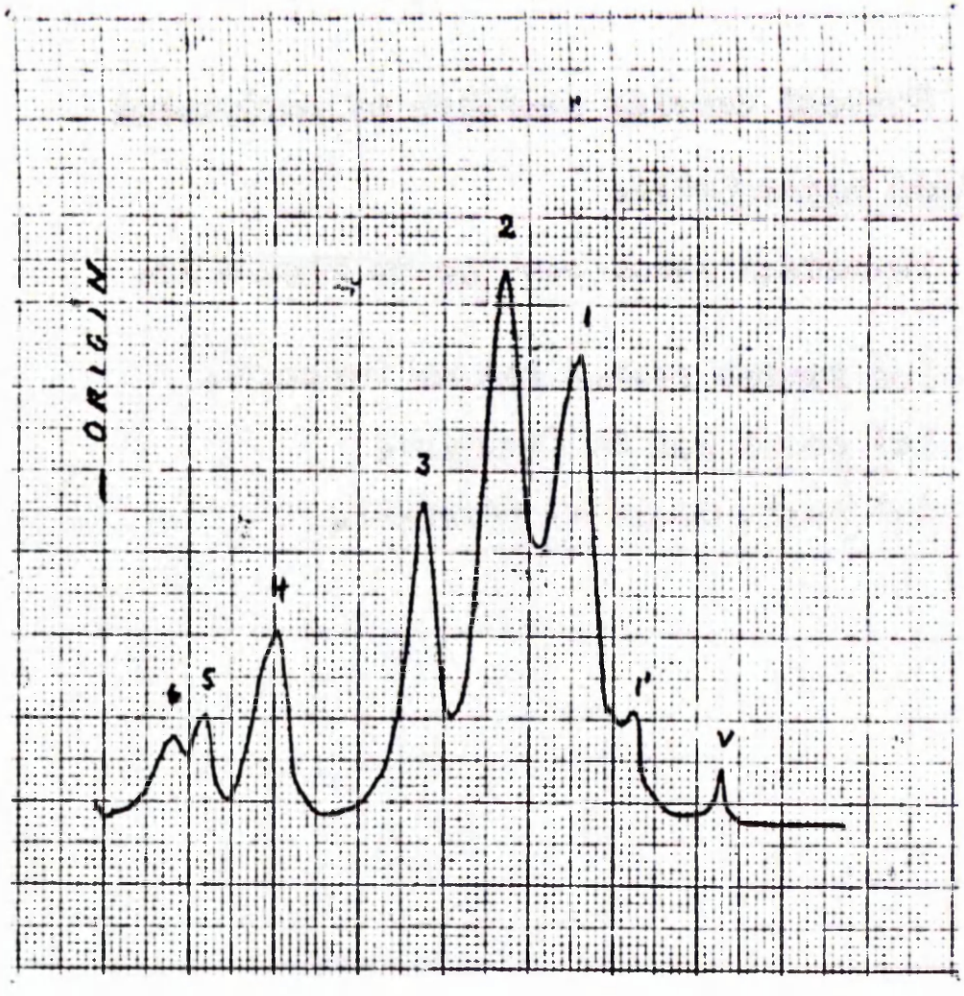


FIGURE 13 (6)

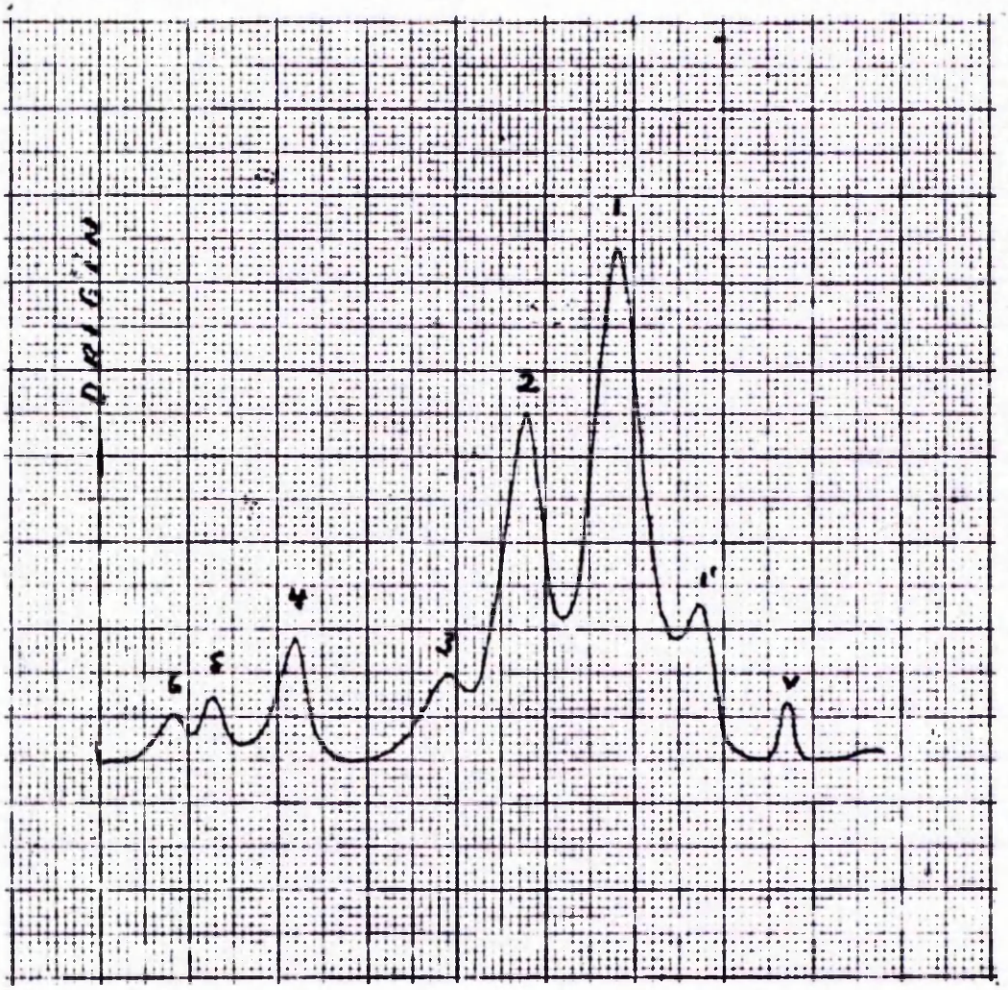


FIGURE 13 (c)

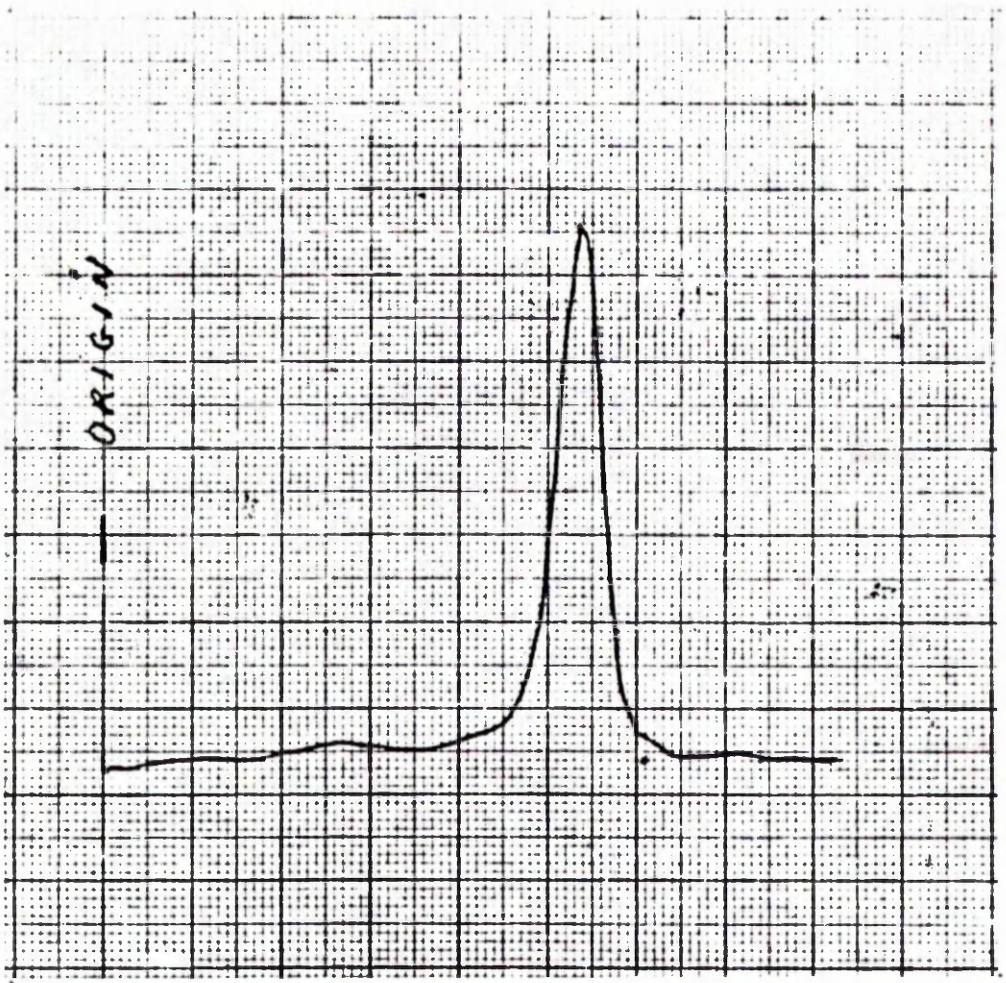


Table 11

Relative distribution of adult and 15-day foetal rat haemoglobin components.

| Hb Type | Hb components as % of total Hb | | | | | | |
|---|---|---|---|---|---|---|---|
| | 1' | 1 | 2 | 3 | 4 | 5 | 6 |
| 15 day foetal rat liver | 6.60 | 24.30 | 29.42 | 17.85 | 13.13 | 4.59 | 4.11 |
| Adult rat marrow (8 mths) | 7.92 | 39.89 | 23.33 | 9.83 | 10.02 | 4.19 | 3.82 |

doublet at times. This has been interpreted as a haemoglobin-haptoglobin complex by some workers (Brada and Tobiška, 1964; Lehmann and Huntsman, 1966). Although these were the most common patterns obtained, however, other different patterns of adult and foetal rat haemoglobin were also found. Figure 14 and attached photographs illustrate these findings.

The electrophoretic heterogeneity of rat haemoglobins, whether genetically controlled or as a result of interactions and aggregations among the haemoglobin components, added a new problem to the present research work.

Radioactivity profiles of foetal and adult rat haemoglobin.

Basic information. Regardless of the visible electrophoretic spectrum, the radioactivity profiles of foetal and adult rat haemoglobins were reported to be very different by Hunter and Paul (1969) and were readily distinguishable by their *in vitro* incorporation of $^{59}Fe$ (Figure 15). Hence experiments had to be carried out to reproduce these findings.

Methods. The technical procedures used were those described by Hunter (1968). Foetal rat liver cells from 15½-day-old foetuses, and bone marrow cells from femurs and tibias of 8 to 12 month old Wistar rats were obtained in ice-cold culture medium, using the procedure already

Figures 14.
(1-17)

Different patterns and optical density profiles of peripheral blood haemoglobin.

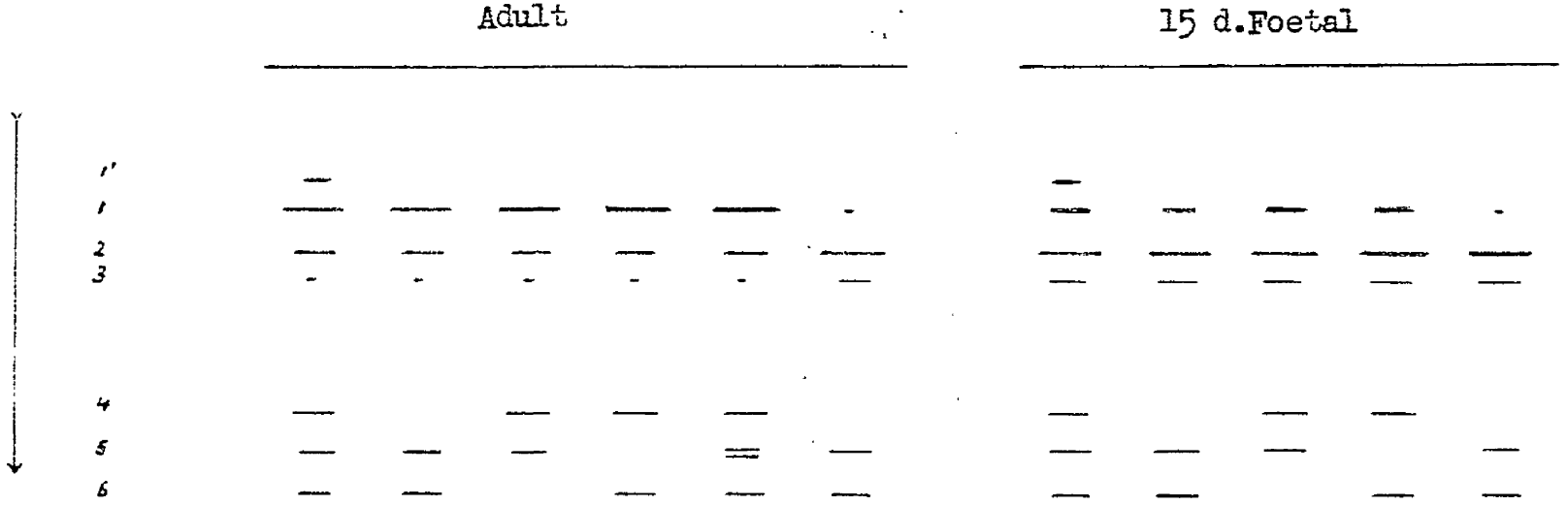
Technical procedures as in Figure 12.

These figures demonstrate the heterogeneity of rat haemoglobins.

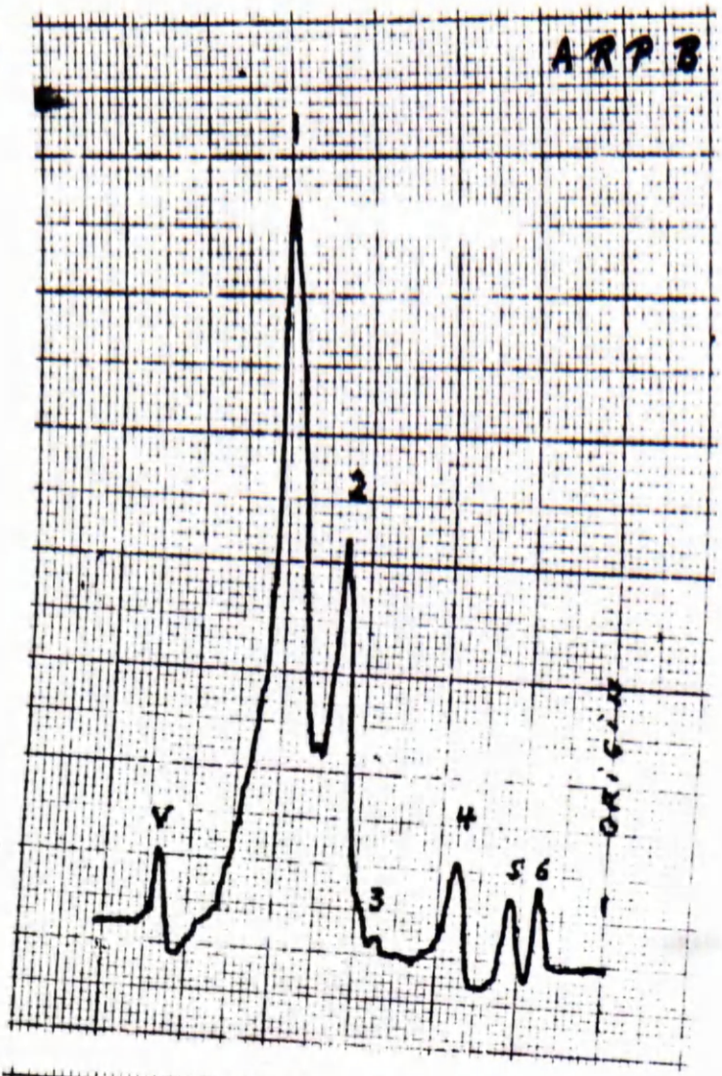
Figure 14 (i)

Patterns of Rat Haemaglobin in Starch Gel Electroforesis.

(Peripheral Blood).



(2)




(3)

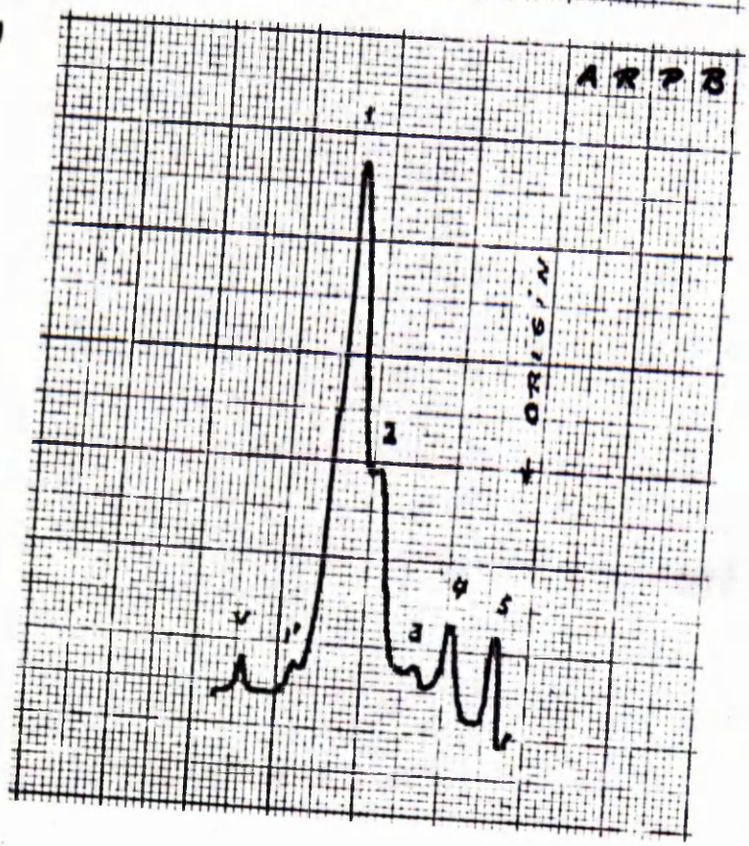


FIGURE 14

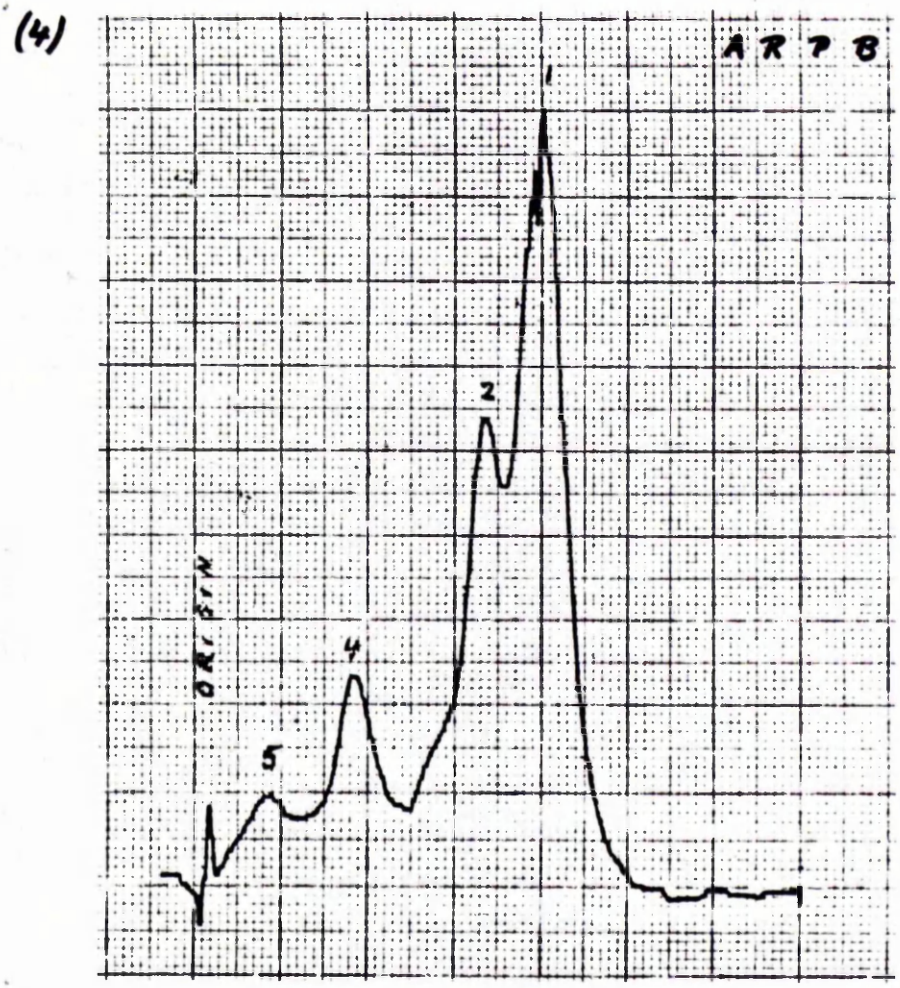


FIGURE 14

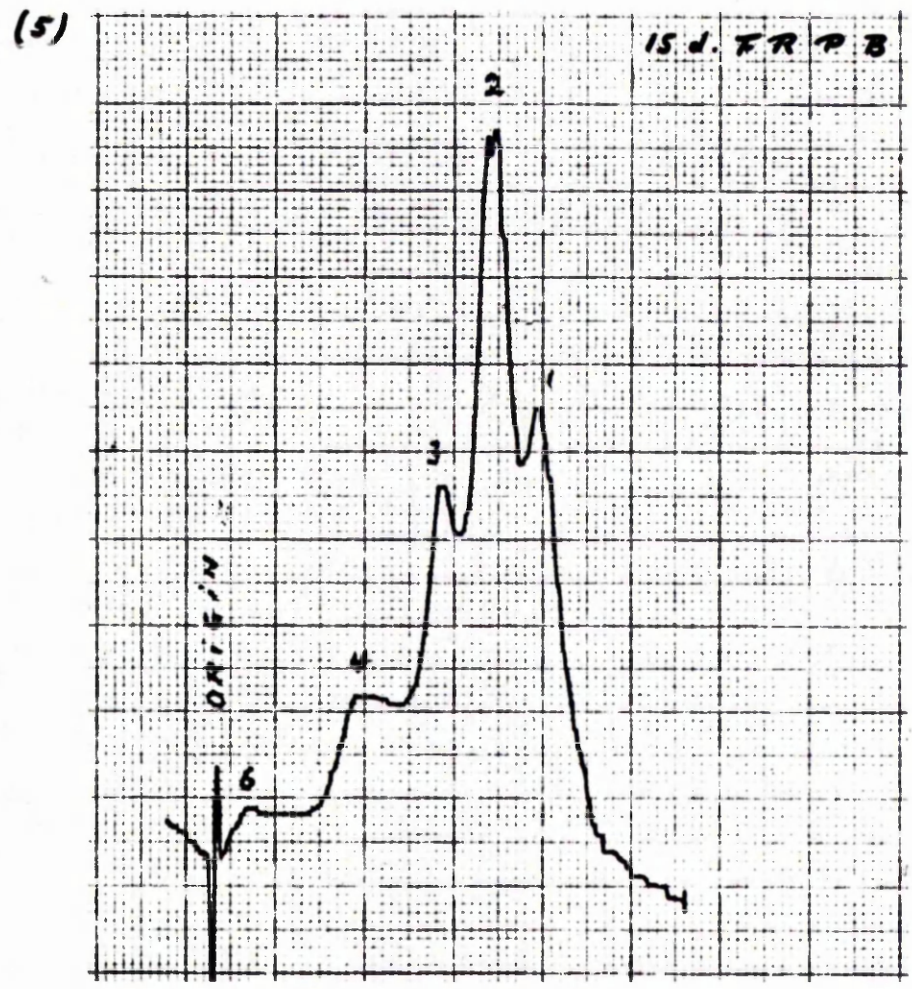


FIGURE 14

(c)

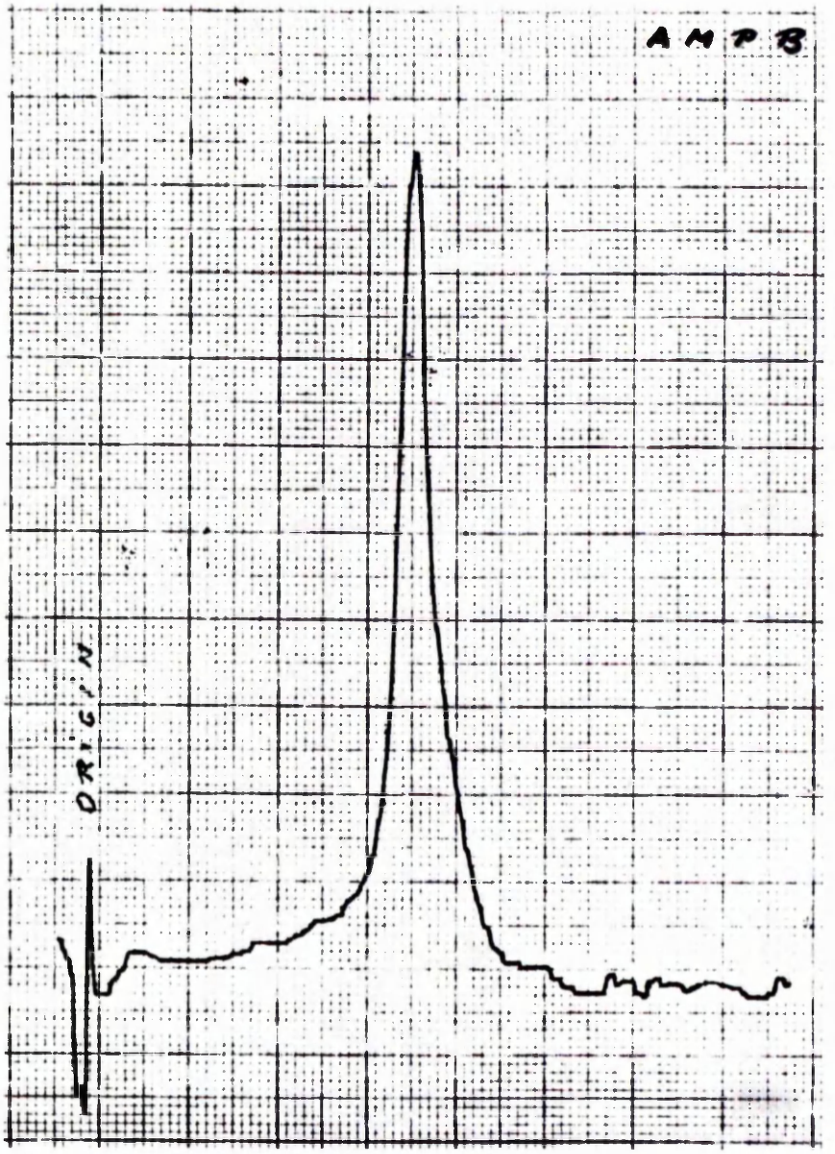


FIGURE 14

(7)

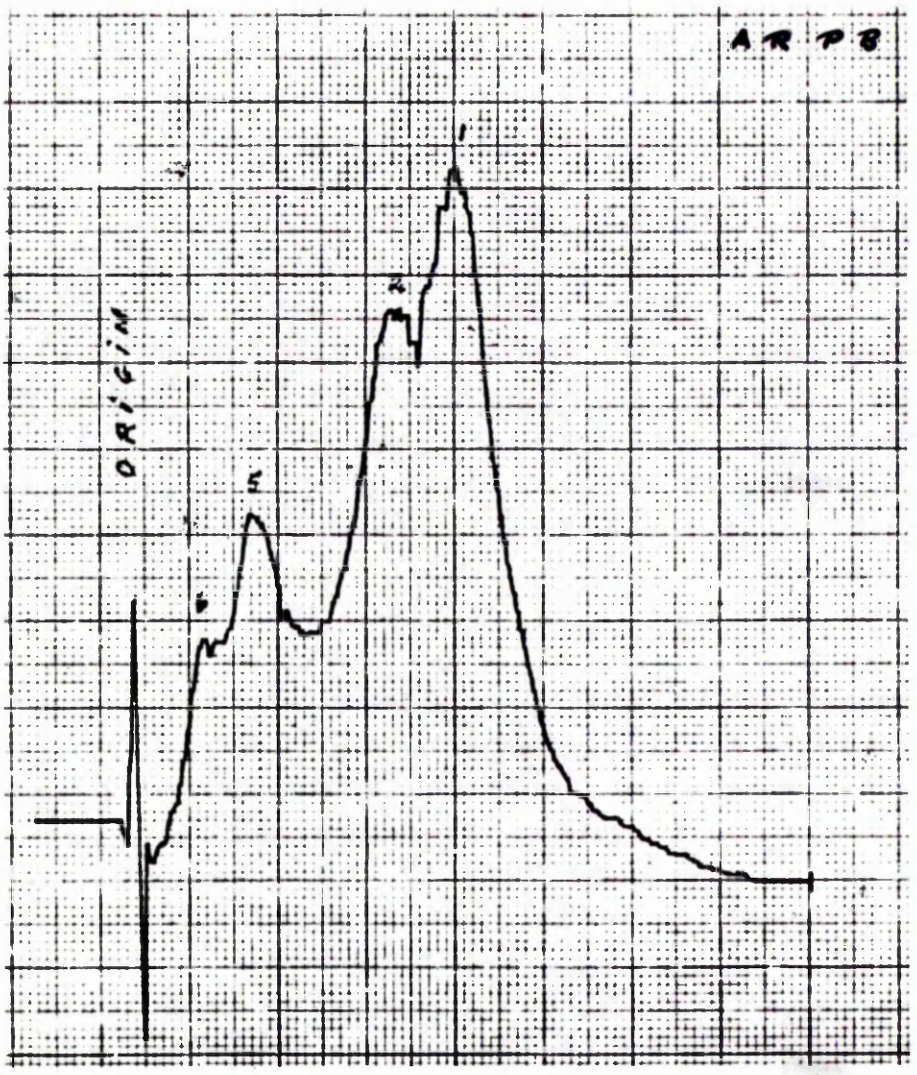


FIGURE 14

(8)

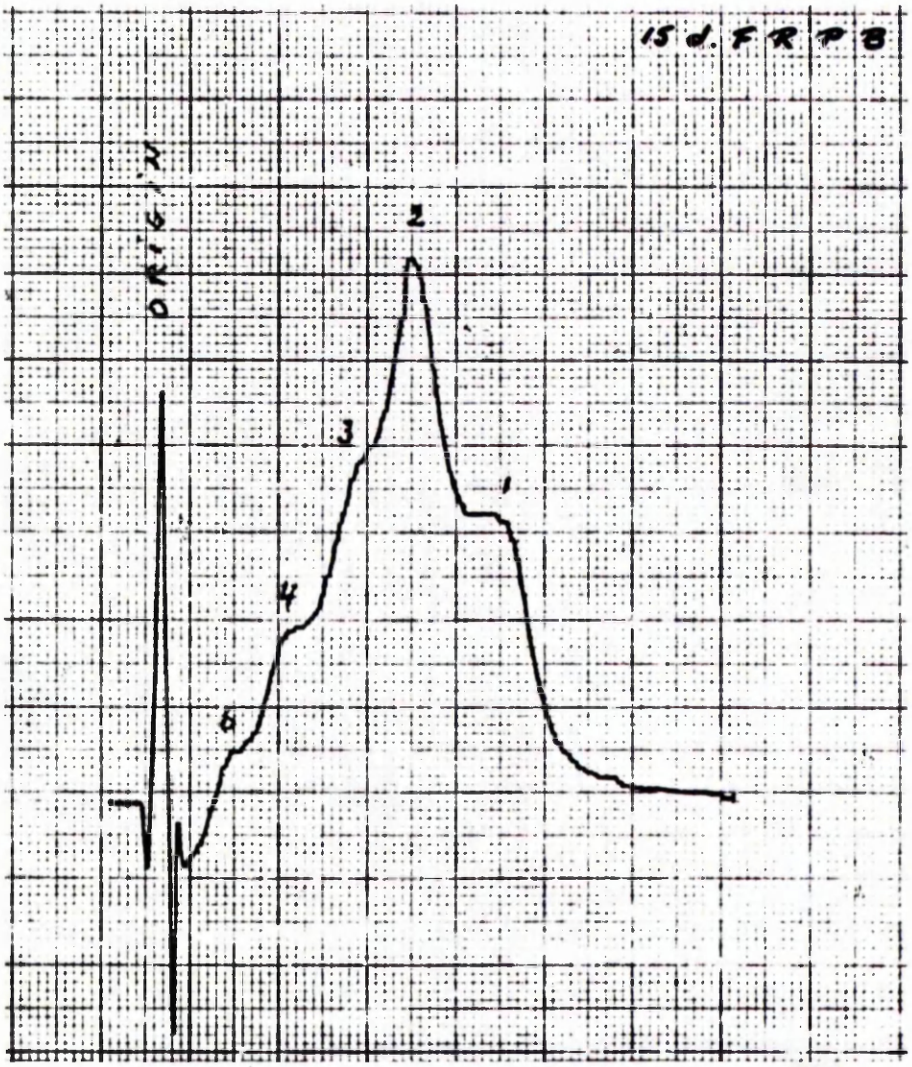


FIGURE 14

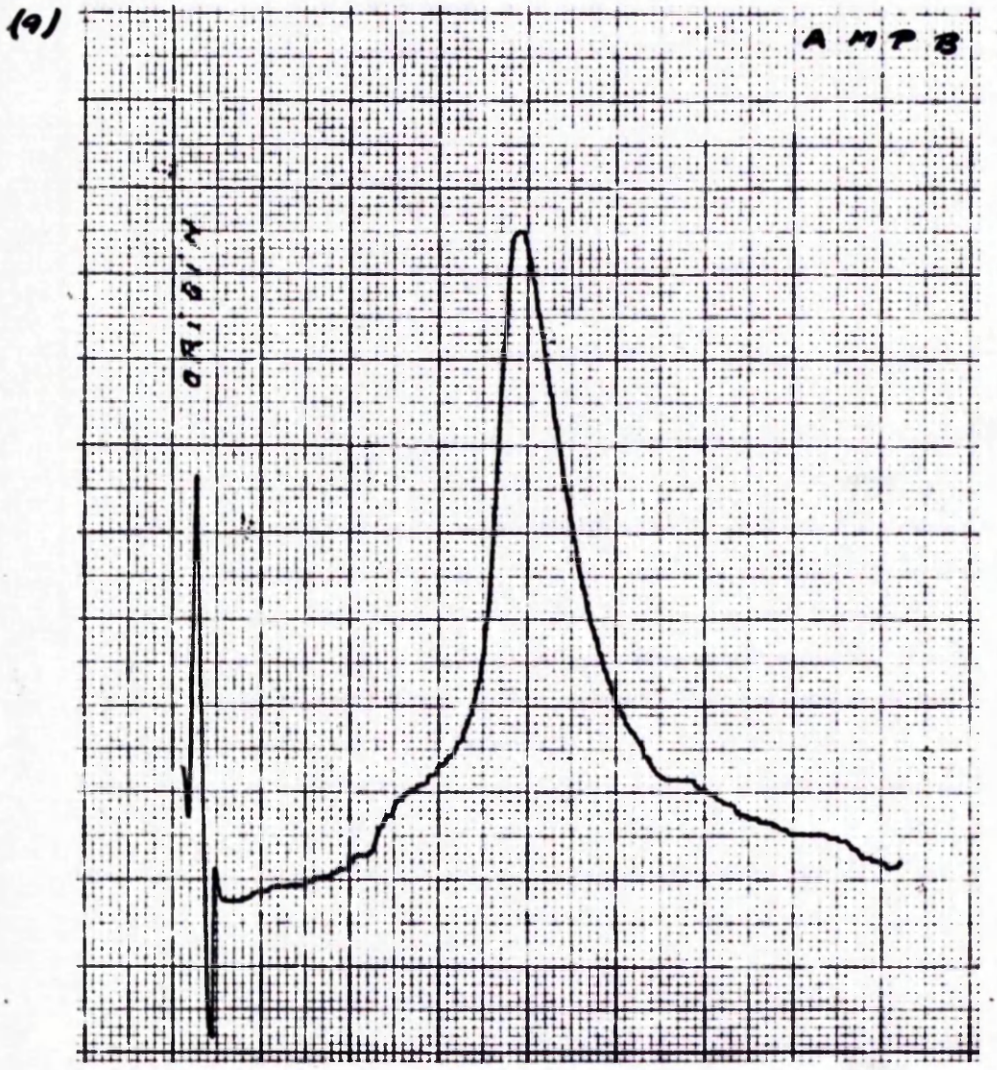


FIGURE 14

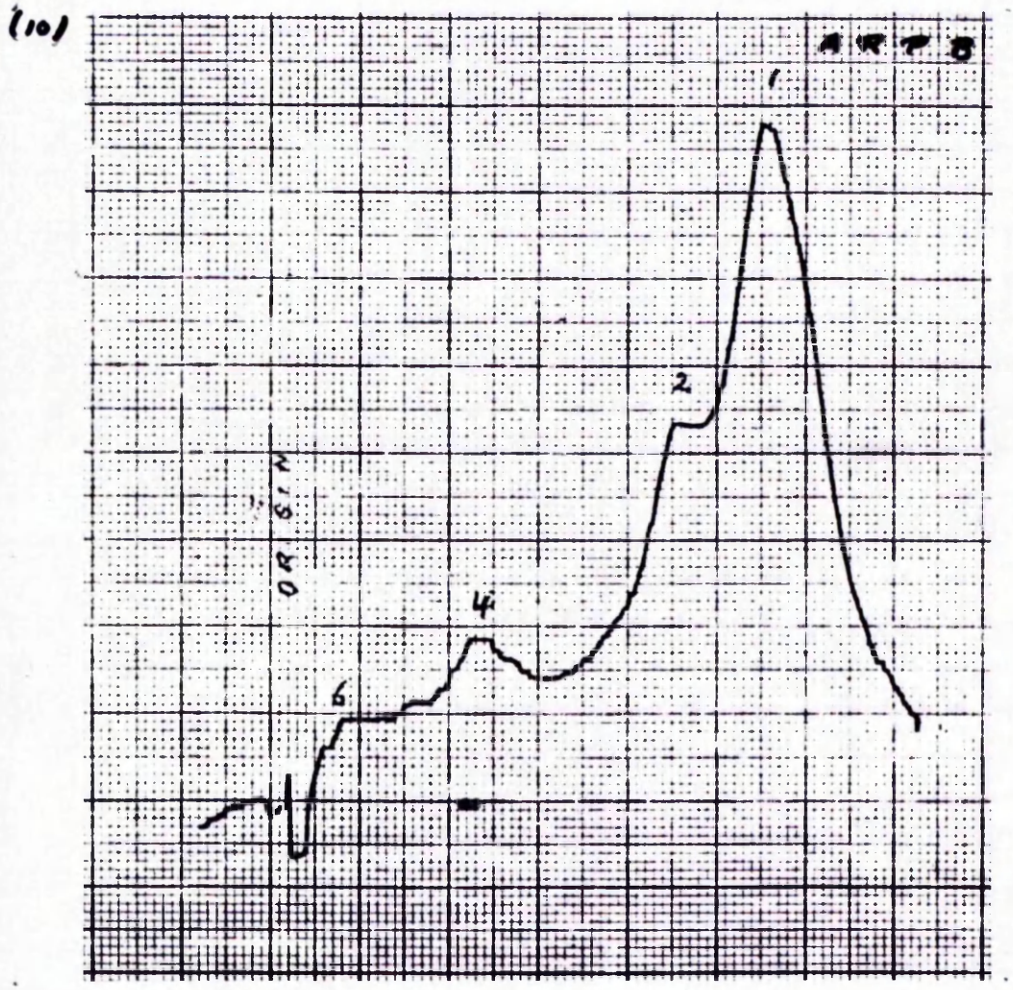


FIGURE 14

(11)

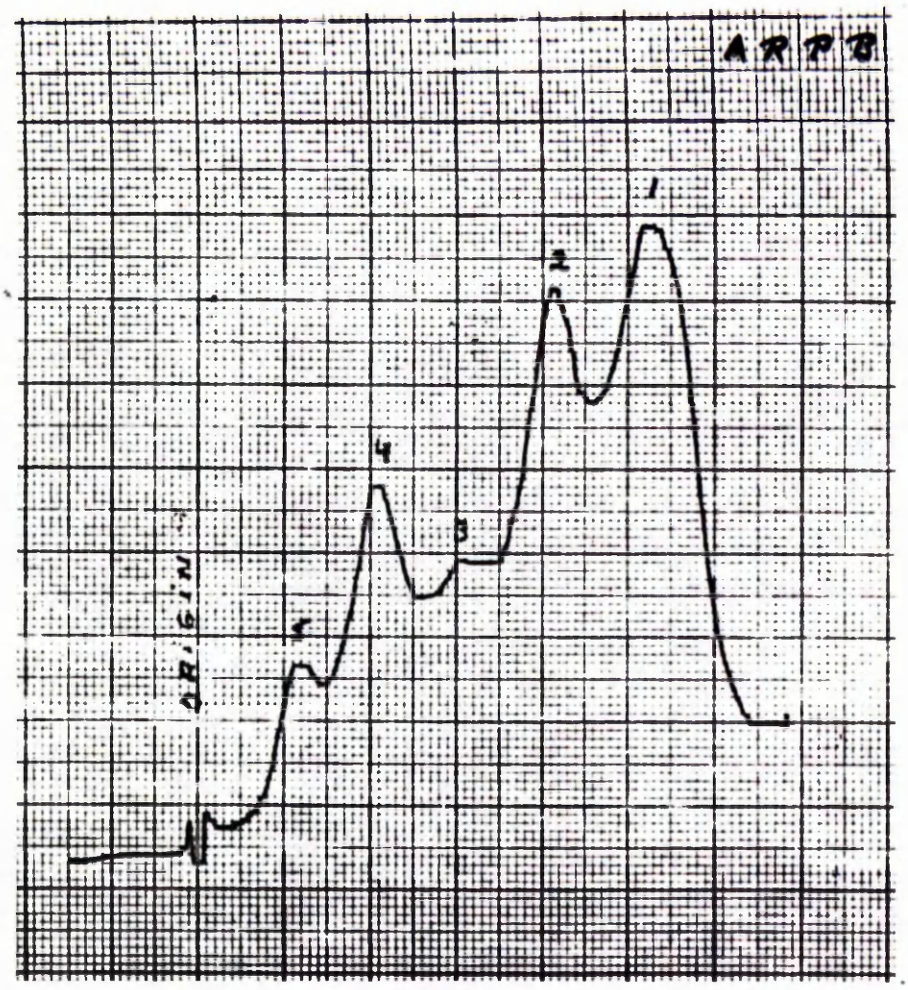


FIGURE 14

(12)

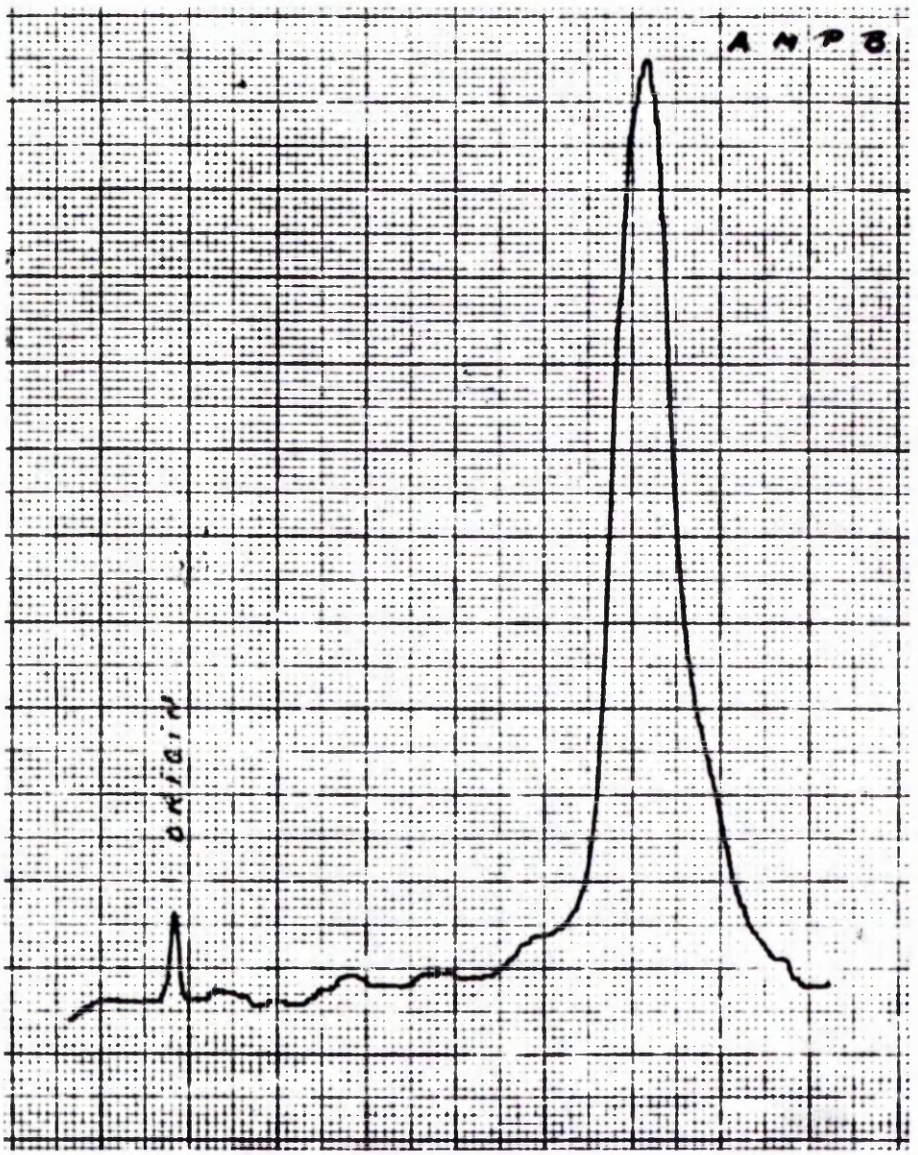


FIGURE 14

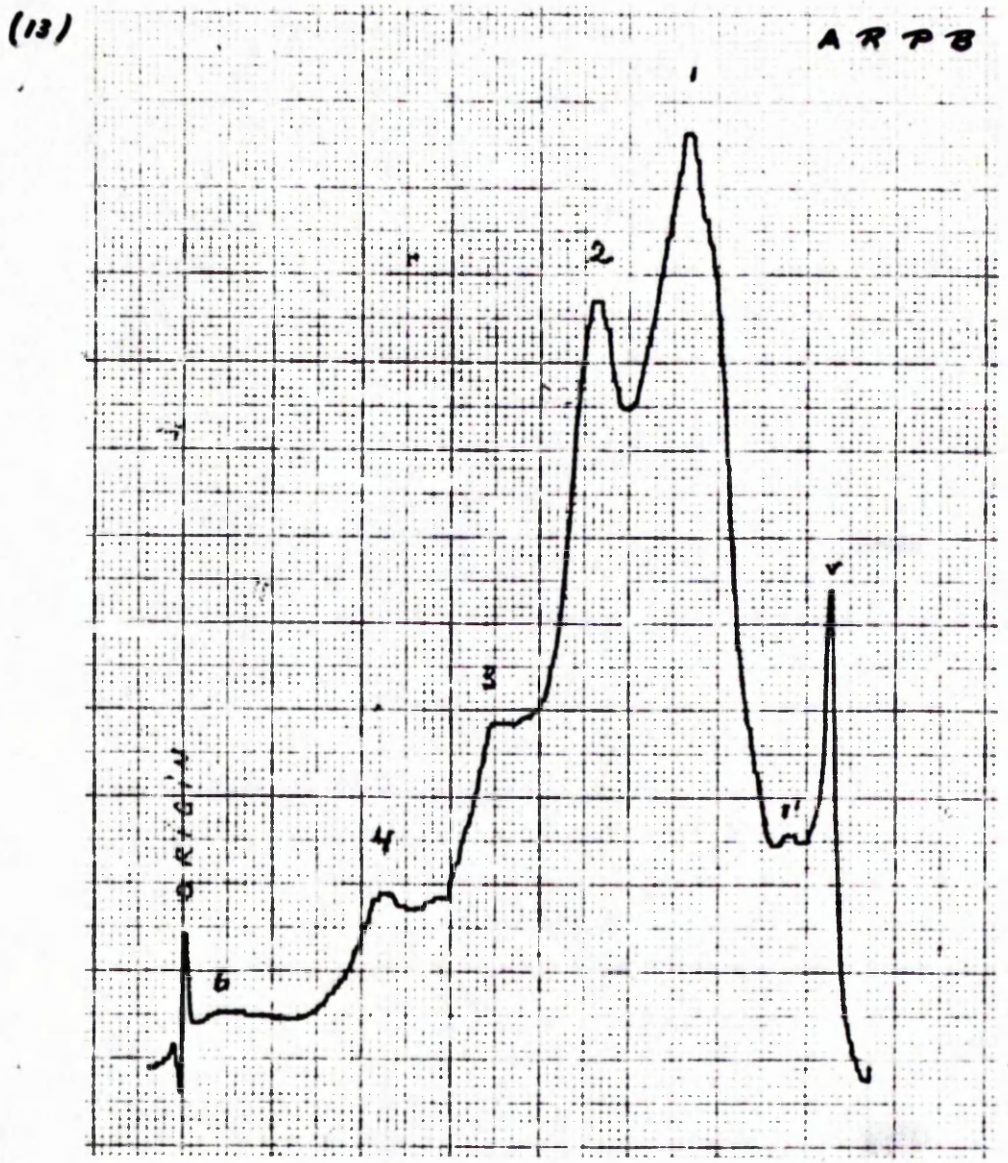


FIGURE 14

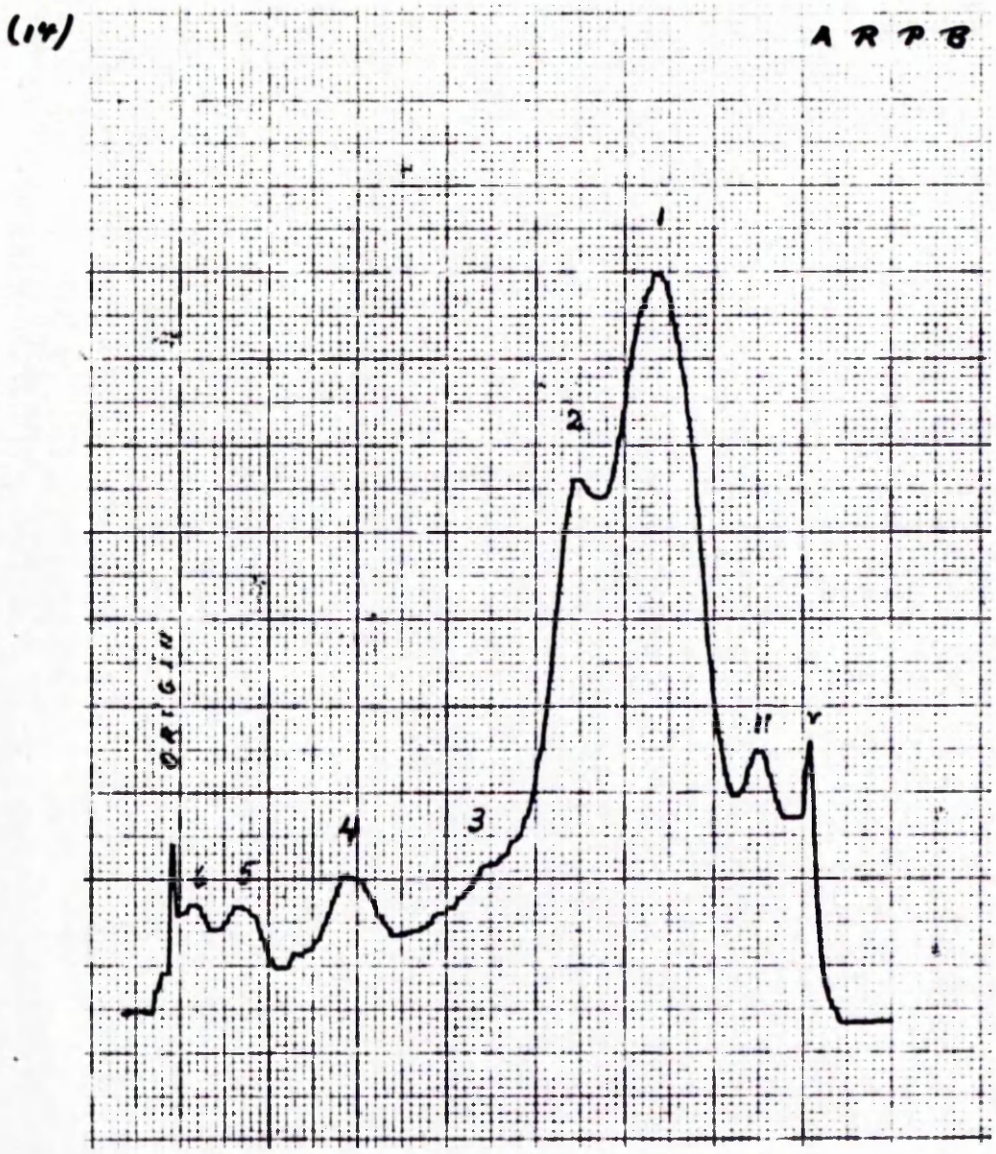
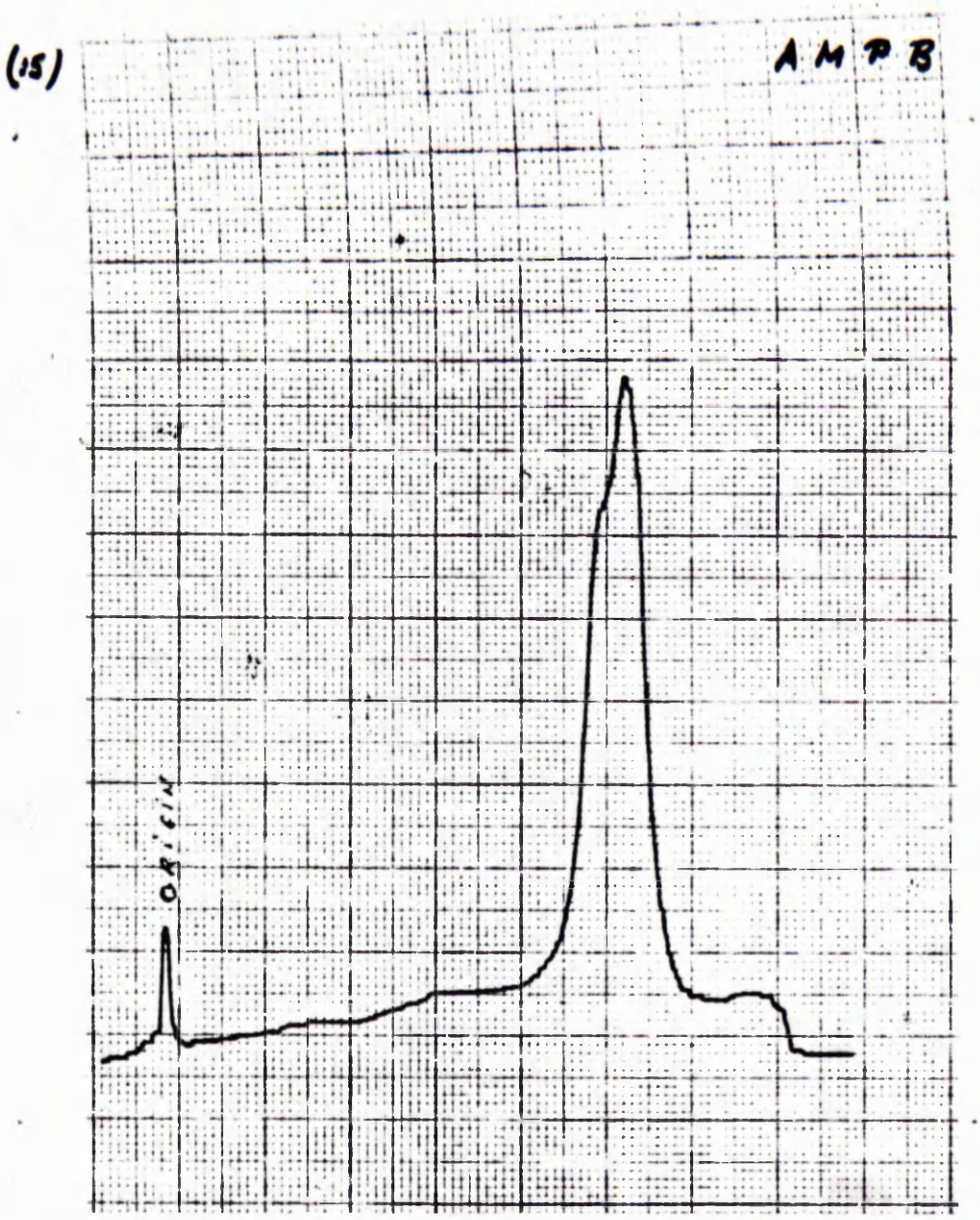
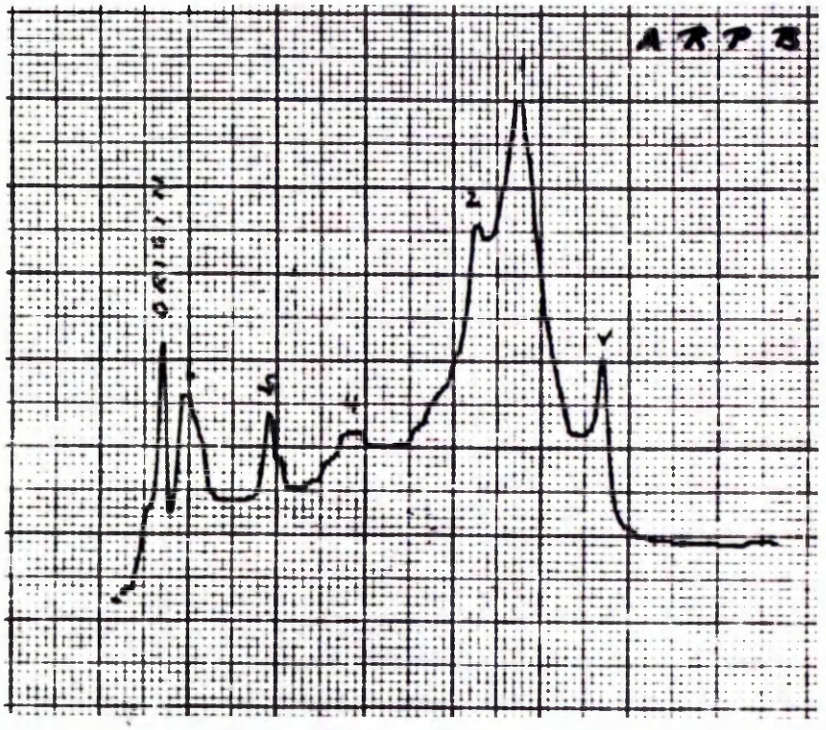


FIGURE 14



# FIGURE 14

(16)




(17)

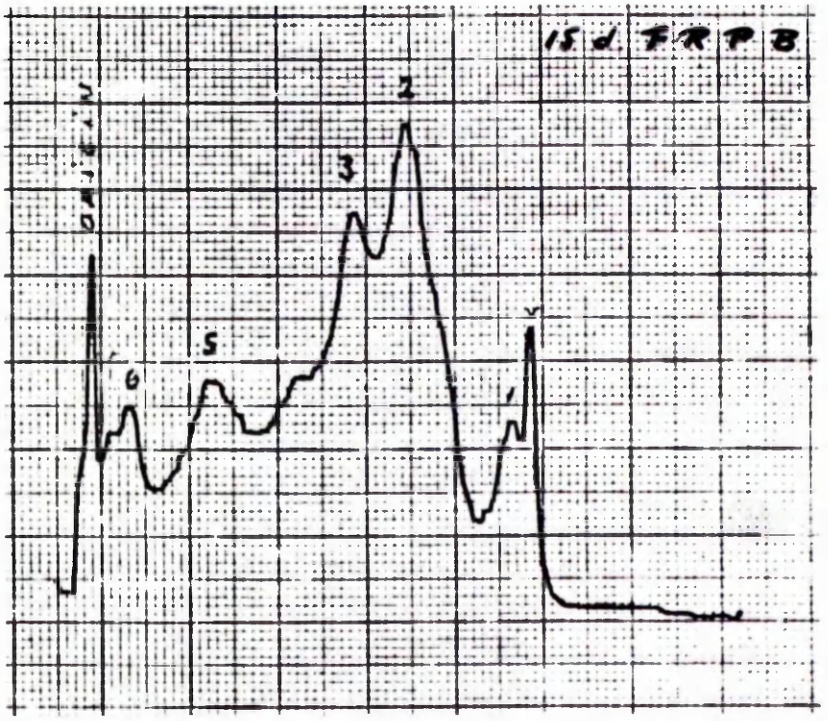


Plate 2.

Some patterns of haemoglobin fractionated by starch gel electrophoresis (Poulik, 1957), and stained with naphthalene black:

Rat - Wistar strain. Mouse - Porton strain.

(a) Left: adult rat Hb.
Middle: adult mouse Hb.
Right: 15-day foetal rat Hb.

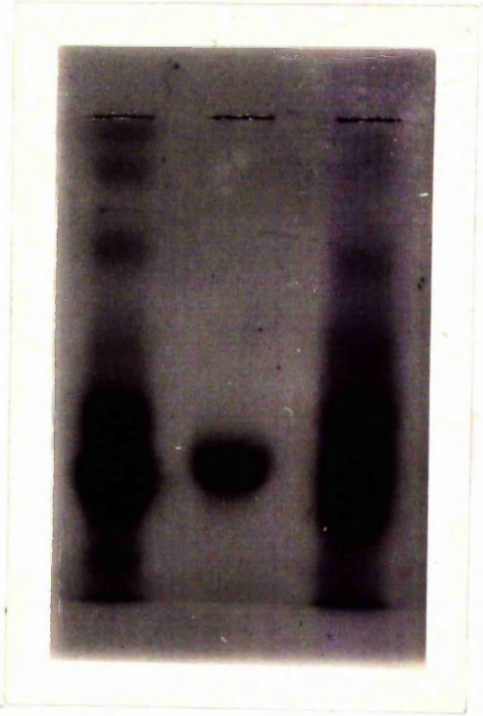
(b) Left: 13-day foetal rat Hb.
Middle: adult mouse Hb.
Right: adult rat Hb.

(c) Left: adult rat Hb.
Middle: adult mouse Hb.
Right: 15-day foetal rat Hb.

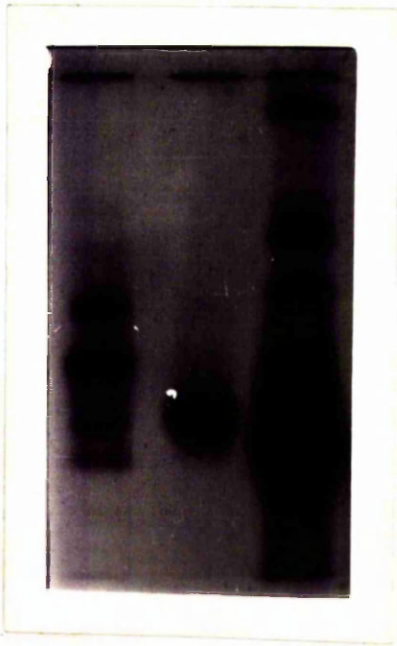
(d) Left: 15-day foetal rat Hb.
Middle: adult mouse Hb.
Right: adult rat Hb.

PLATE 2

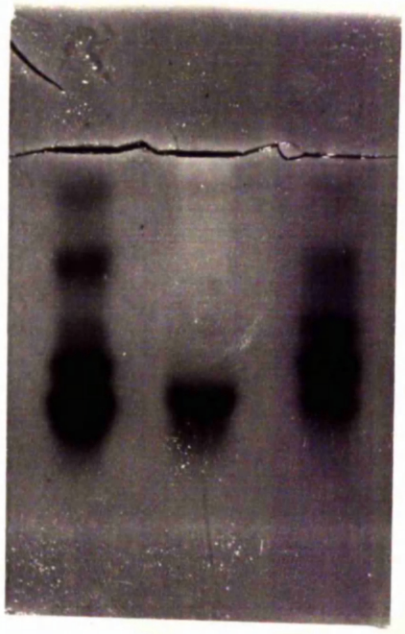
a)



b)



c)



d)

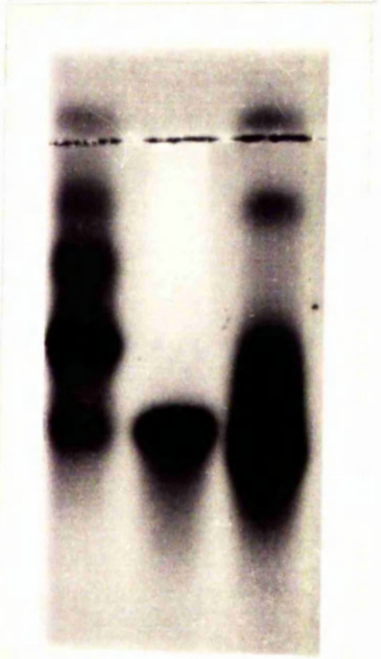


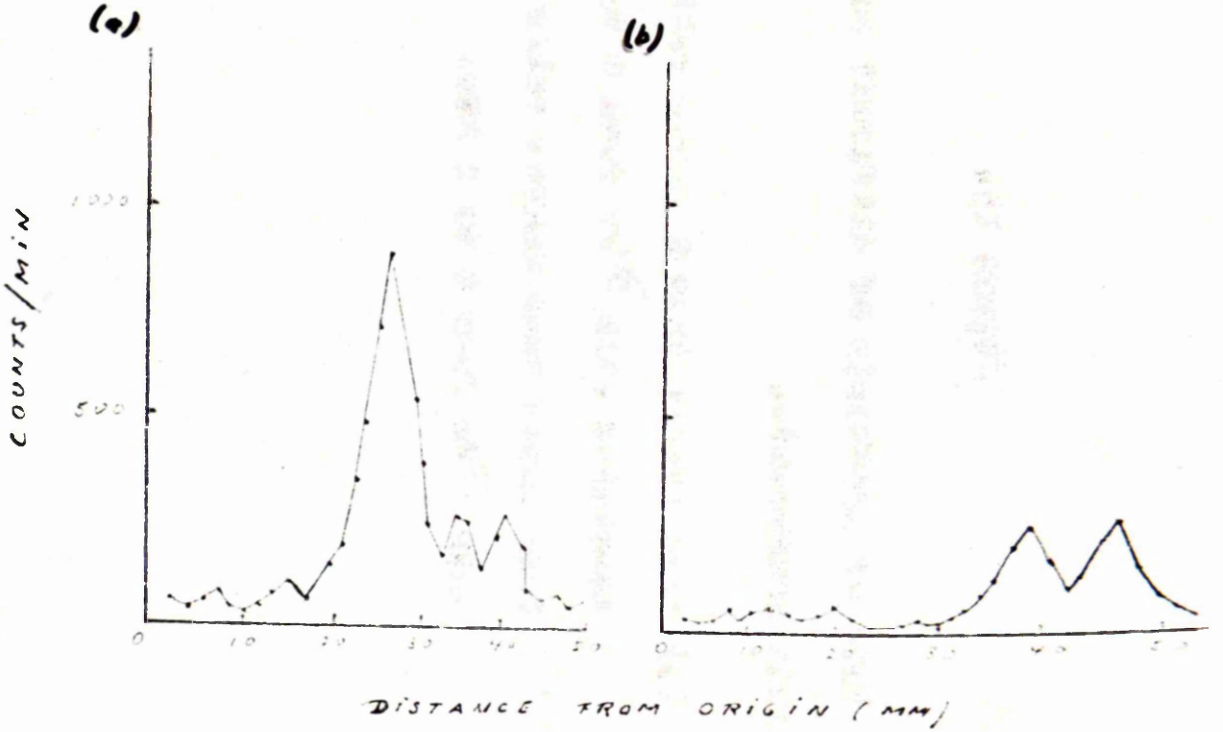
Figure 15.

Rate of synthesis of different rat haemoglobin components.

(a) From 15-day foetal liver cells incubated with $^{59}Fe$ from 0 to 6 hrs.

(b) From adult bone marrow cells incubated with $^{59}Fe$ from 0 to 4 hrs.

FIGURE 15




FROM: HUNTER & PAUL (1969)
J. EMBRYOL EXP. MORPHOL. 21: 361

described for the preparation of cell suspensions for transplantation.

Cultures containing 1 to 3 x $10^6$ cells in 1 ml culture medium were set up in tubes (Flow Laboratories Ltd.) and $^{59}FeCl_3$ transferrin, equilibrated under the conditions previously described, added at a level of 0.5 to 1 μc/ml. The medium was removed after incubation and the cells washed 3 times with cold normal saline or Hank's BSS. The pellet of cells obtained was lysed in 0.5 ml of deionized distilled water, and frozen and thawed 3 times. 1 ml chloroform was then mixed with the haemolysate and removed by centrifuging at 1,500 g for 5 mins. A volume of 0.4 ml supernatant haemoglobin solution was collected, 0.2 ml of marker (carrier) non-labelled peripheral blood haemoglobin added and 0.05 ml of this mixture electrophoresed and counted under the same conditions as before.

Results: These revealed -

(1) Interactions and aggregations among haemoglobin components, and

(2) The presence of a contaminating labelled protein in the haemoglobin solution.

Following the methods just described, labelled haemoglobins of 15½ day foetal rat livers (F.R.L.) with added foetal rat peripheral blood (F.R.P.B.) as

marker, and labelled haemoglobins of adult rat bone marrow (A.R.B.M.), with adult rat peripheral blood (A.R.P.B.) as marker, were processed. The radioactivity profiles obtained are shown in Figures 16 and 17.

A disturbing observation was made in these results. The radioactivity peak 'x' did not correspond exactly with any of the fractions of haemoglobin, although it was obliterating the nearest component. At first it was thought that this was due to a technical error; but different electrophoretic runs showed the same results. Other samples of F.R.L. and A.R.B.M. haemoglobins, with added markers, were prepared and processed. Their radioactivity profiles were somewhat similar. Figures 18 and 19 are typical examples of these results.

The possibility that aggregations were taking place among the different haemoglobin components, was considered. Hence methods of eliminating contributory factors influencing and favouring these aggregations had to be investigated. Two possible factors were -

(a) the non-labelled haemoglobin added as carrier and marker; and

(b) the chloroform used as clearing agent in haemoglobin extraction.

Elimination of carrier (marker) haemoglobin.

Experiments were set up to find out whether the

Figure 16.

Incorporation of $^{59}Fe$ into 15½-day foetal rat liver haemoglobin.

The cells were incubated with $^{59}Fe$ for 8 hrs. Haemoglobin was extracted with chloroform, mixed with Hb marker of the same type, and the mixture fractionated by starch gel electrophoresis (Poulik, 1957). Radioactivity incorporation was determined as described in the text.

The radioactivity peak 'X' did not correspond to any haemoglobin component.

FIGURE 16

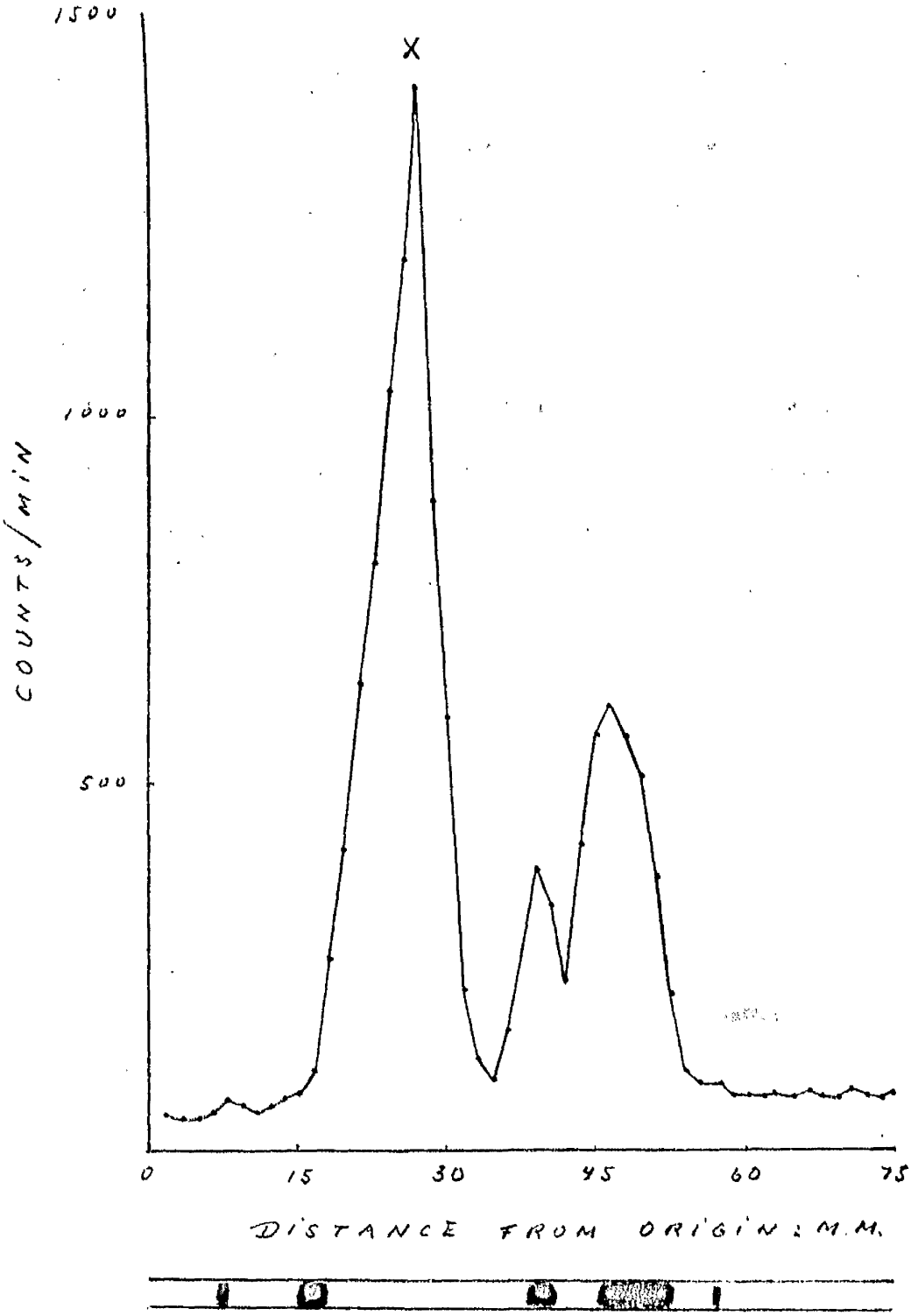

1500
1000
500
X
COUNTS/MIN
0
15
30
45
60
75
DISTANCE FROM ORIGIN: M.M.

Figure 17.

Incorporation of $^{59}Fe$ into adult rat bone marrow haemoglobin.

Technical procedures as in Figure 16.

Age of animal - 8 months.

The radioactivity peak 'X' did not correspond to any haemoglobin component.

FIGURE 17

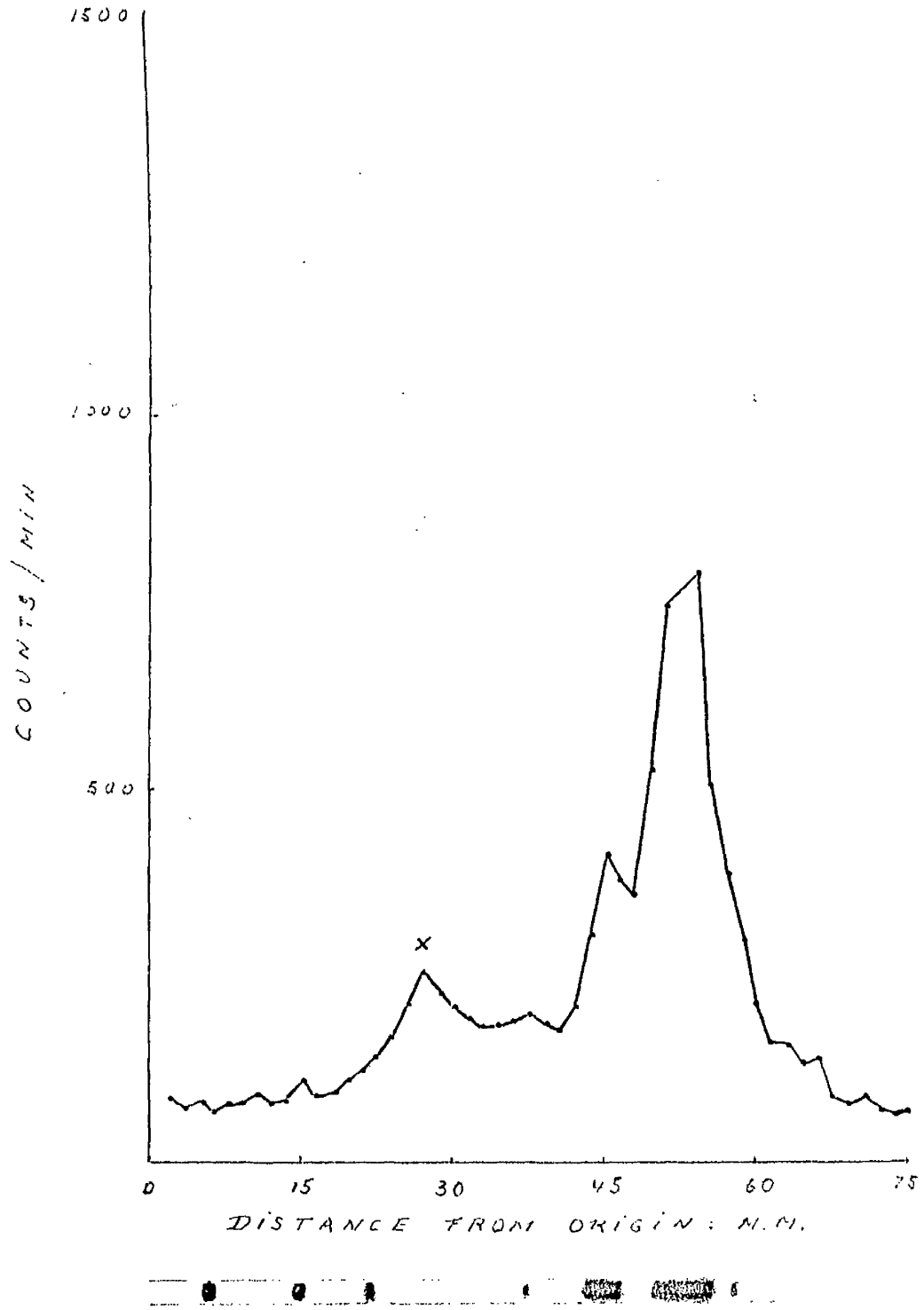

1500
1000
500
COUNTS / MIN
X
0
15
30
45
60
75
DISTANCE FROM ORIGIN : N.M.

Figure 18.

Incorporation of $^{59}Fe$ into 15½-day foetal rat liver haemoglobin.

Technical procedures as in Figure 16.

The radioactivity peak 'X' did not correspond to any haemoglobin component.

FIGURE 18

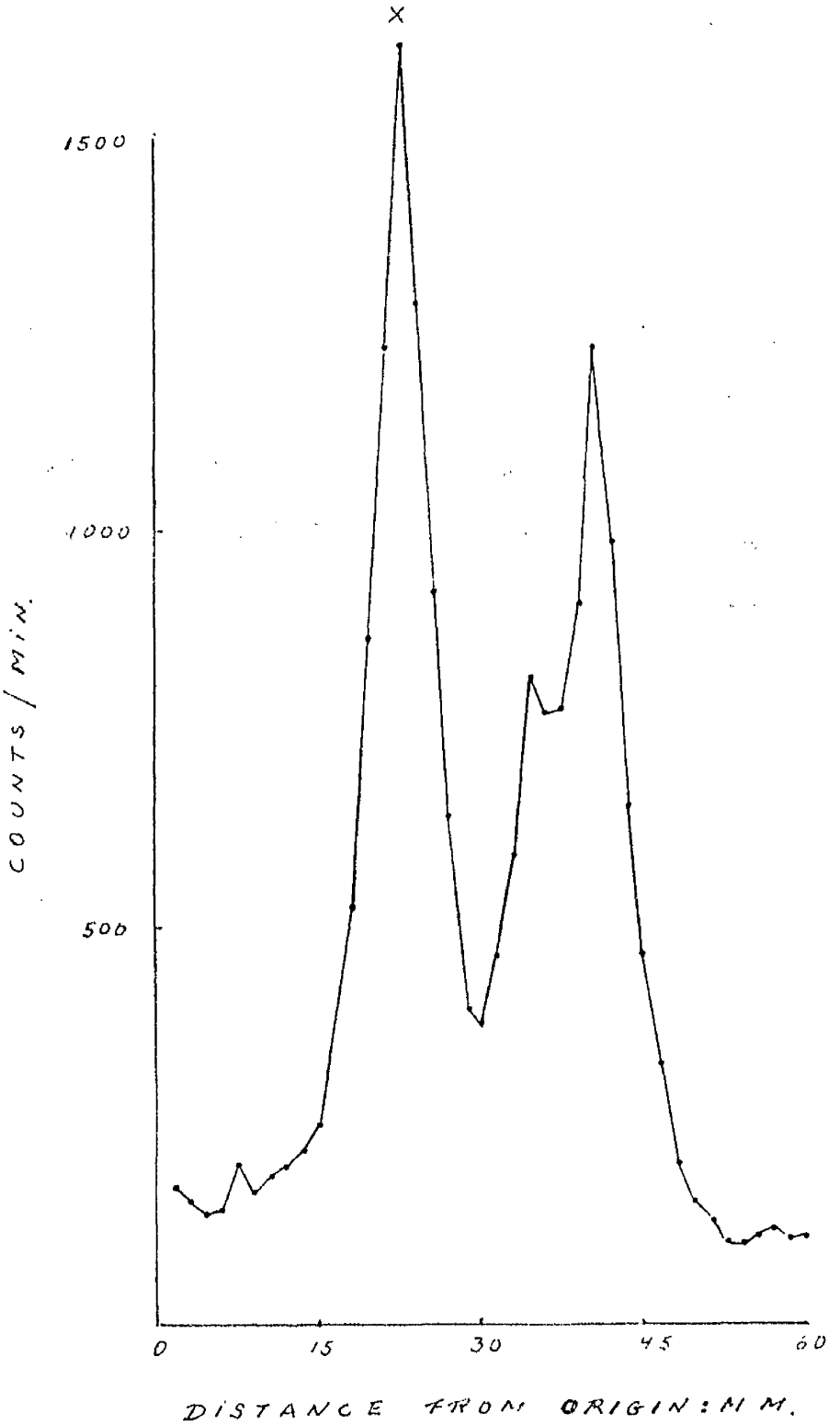

X
1500
1000
500
COUNTS / MIN.
0
15
30
45
60
DISTANCE FROM ORIGIN: MM.

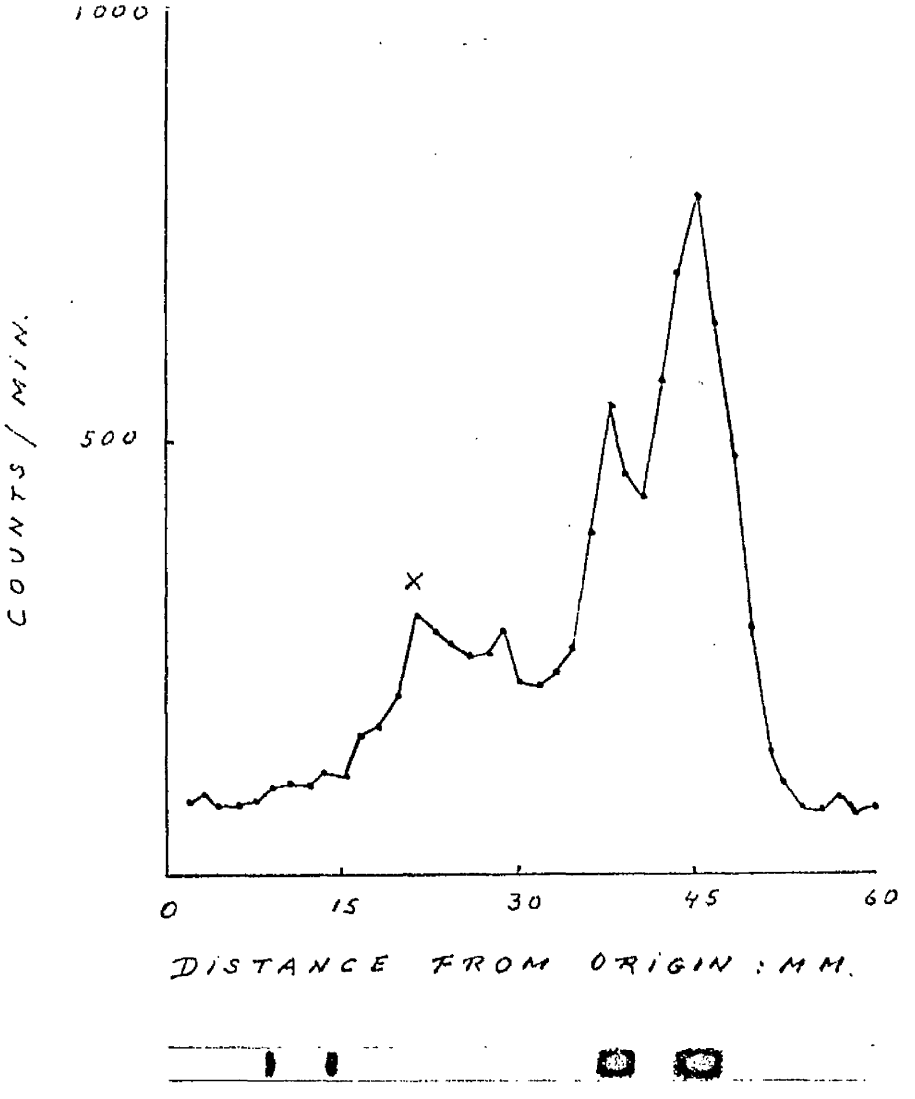
Figure 19.

Incorporation of $^{59}Fe$ into adult rat bone marrow haemoglobin.

Technical procedures as in Figure 16.

The radioactivity peak 'X' did not correspond to any haemoglobin component.

FIGURE 19



addition of peripheral blood haemoglobin marker had any effect or not on the electrophoretic pattern of the experimentally labelled haemoglobins. Labelled samples of F.R.L. and A.R.B.M. haemoglobins were prepared and mixed with peripheral blood markers of the same type (control samples: F.R.L. + F.R.P.B., A.R.B.M. + A.R.P.B.) and different type (experimental samples: F.R.L. + A.R.P.B., A.R.B.M. + F.R.P.B., F.R.L. + M.P.B., A.R.B.M. + M.P.B.). The samples were electrophoresed and their radioactivity counted. Figures 20, 21 and 22 show the results obtained. Other haemoglobin samples, with and without markers, were also processed. Some of these did not show any real difference in pattern, but others presented a marked difference between the pattern without marker and the one with added marker. The larger haemoglobin components of the marker (carrier) trapped the smaller fractions of the labelled haemoglobin and "dragged" them during electrophoresis, causing variations in their distribution patterns. Figures 23, 24 and 25 show typical examples of this effect.

The main reason for the addition of marker haemoglobin was to obtain visible bands in the electrophoretic gel so that the migratory distance of each fraction could be measured before slicing the gel in order later to compare the radioactivity profile with the visible haemoglobin spectrum. It was not possible to see all

Figure 20.

Incorporation of $^{59}Fe$ into 15½-day foetal rat liver haemoglobin, and optical density profiles.

The cells were incubated with $^{59}Fe$ for 8 hrs. Haemoglobin was extracted with chloroform and aliquots of the Hb sample mixed with foetal rat Hb marker and adult rat Hb marker. The mixtures were then fractionated by starch gel electrophoresis (Poulik, 1957) and radioactivity incorporation determined as described in the text.

Incorporation of $^{59}Fe$:

(a) 15-day foetal rat liver Hb + foetal rat Hb marker.

(b) 15-day foetal rat liver Hb + adult rat Hb marker.

Optical density profiles:

(c) 15-day foetal rat liver Hb + foetal rat Hb marker.

(d) 15-day foetal rat liver Hb + adult rat Hb marker.

FIGURE 20

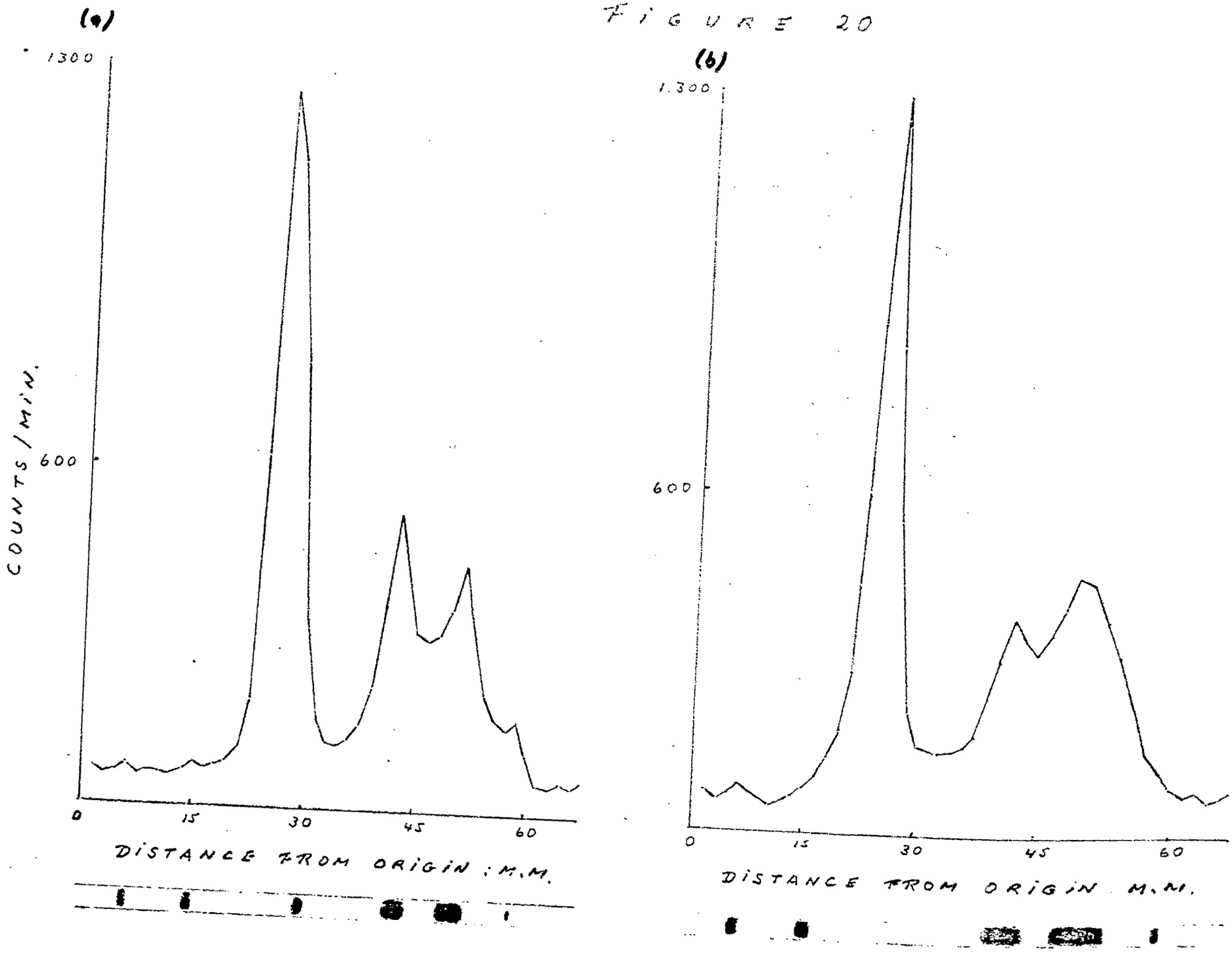

(a)
1300
600
COUNTS / MIN.
0
15
30
45
60
DISTANCE FROM ORIGIN : M.M.
(b)
1.300
600
0
15
30
45
60
DISTANCE FROM ORIGIN M.M.

FIGURE 20

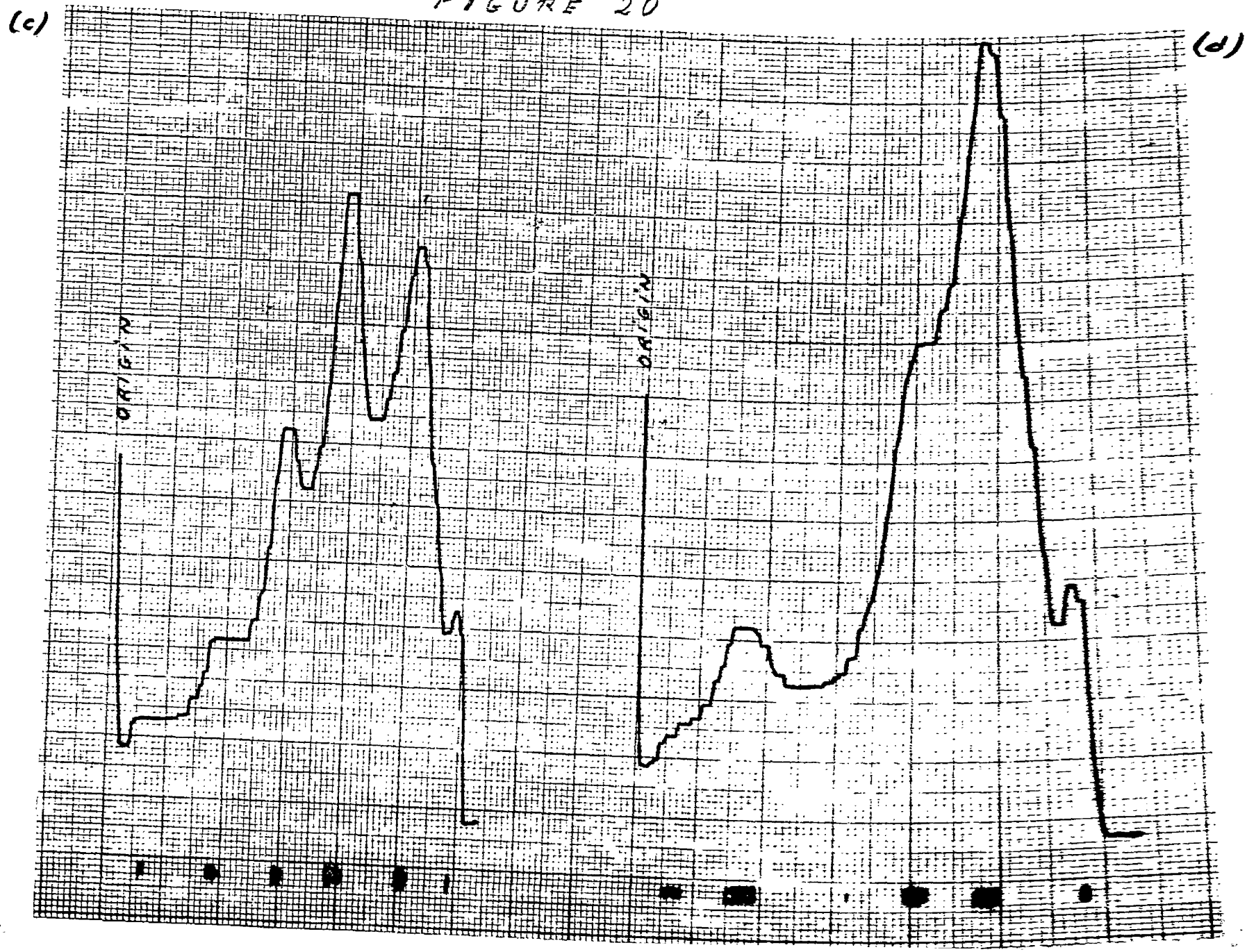


Figure 21.

Incorporation of $^{59}Fe$ into adult rat bone marrow haemoglobins, and optical density profiles.

Technical procedures as in Figure 20.

Incorporating $^{59}Fe$:

(a) adult rat bone marrow Hb + adult rat Hb marker.

(b) adult rat bone marrow Hb + foetal rat Hb marker.

Optical density profiles:

(c) adult rat bone marrow Hb + adult rat Hb marker.

(d) adult rat bone marrow Hb + foetal rat Hb marker.

FIGURE 21

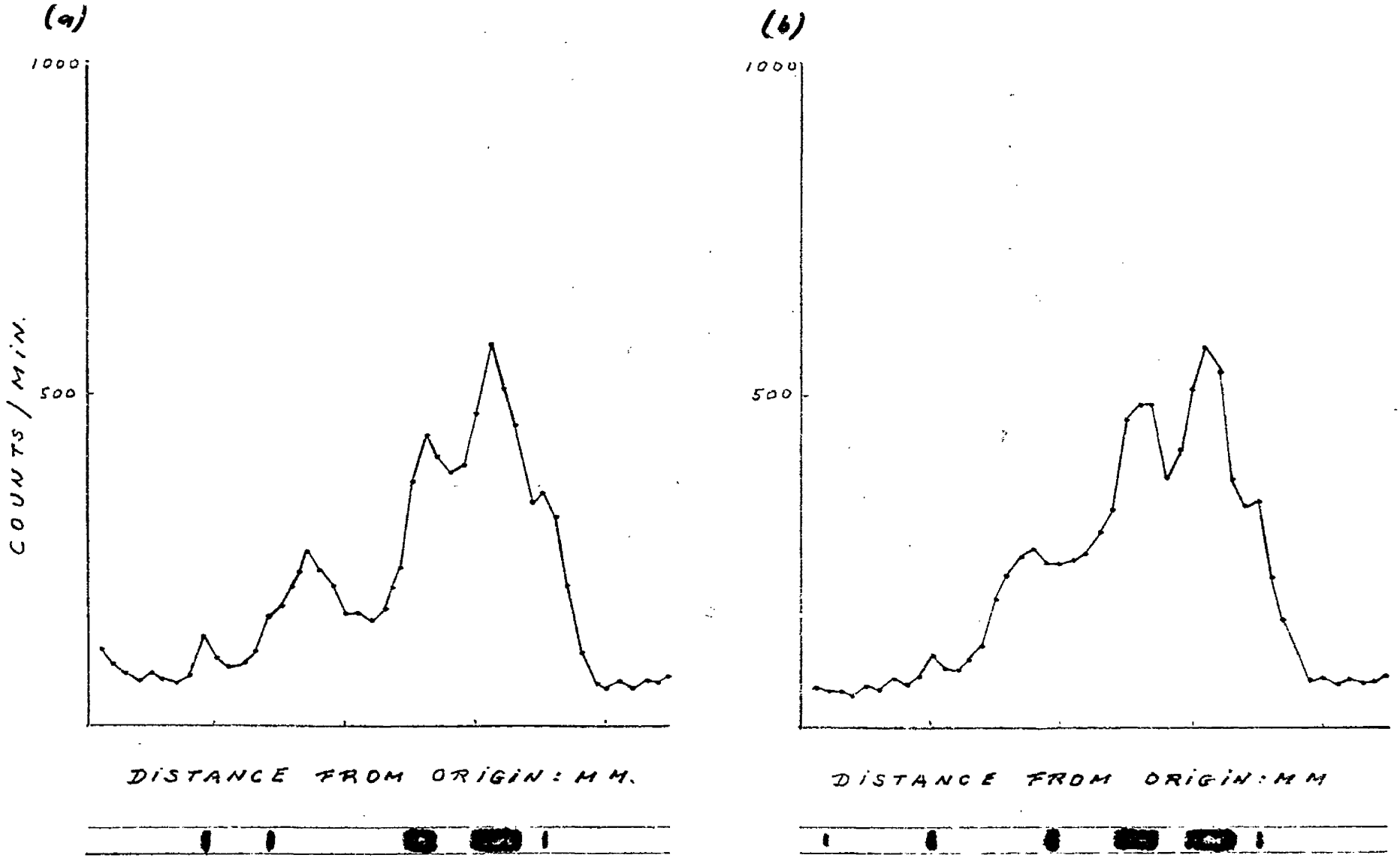

(a)
1000
500
COUNTS / MIN.
DISTANCE FROM ORIGIN: MM.
(b)
1000
500
DISTANCE FROM ORIGIN: MM

FIGURE 21

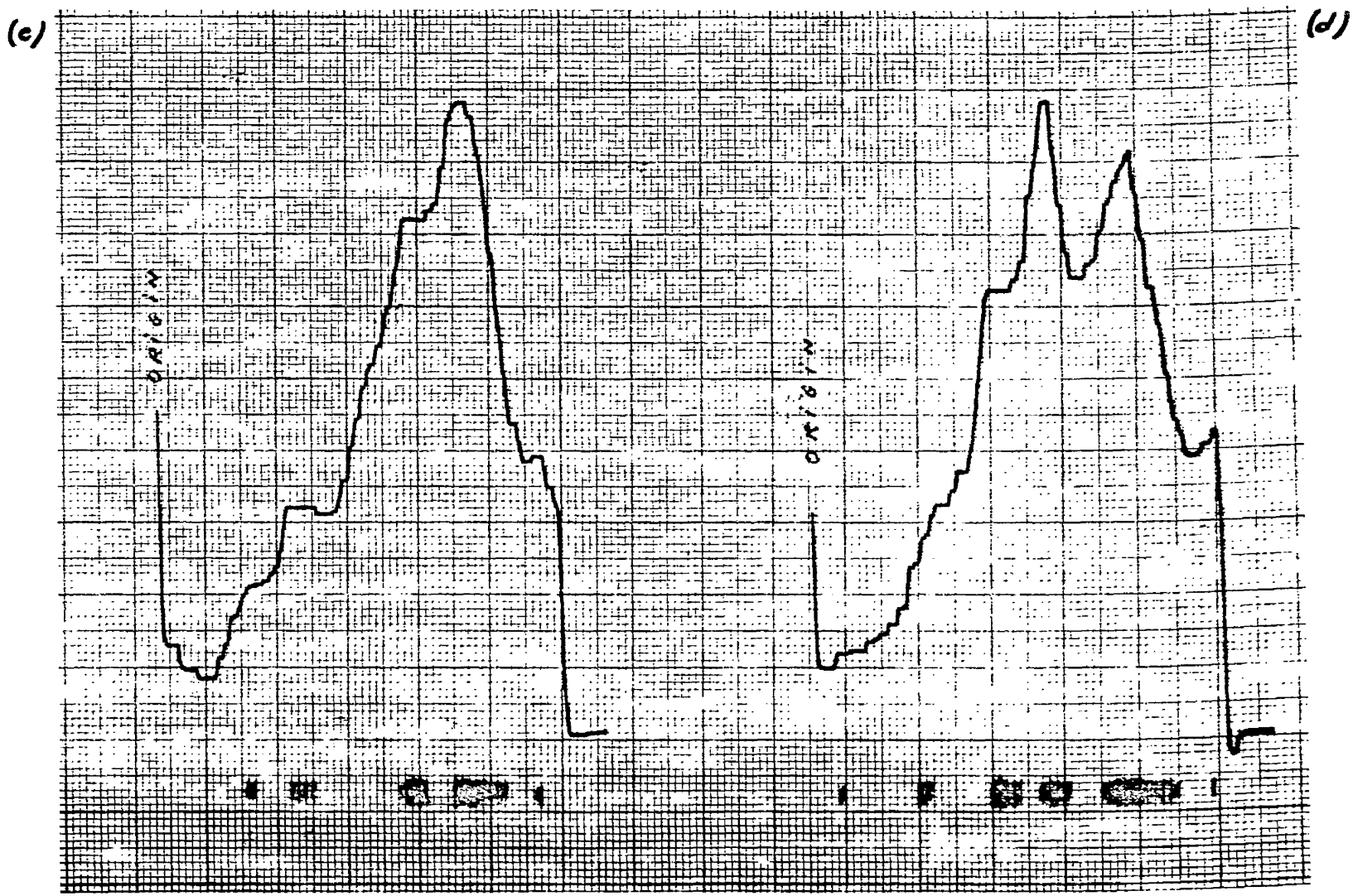


Figure 22.

Incorporation of $^{59}Fe$ into adult rat bone marrow haemoglobin, and optical density profiles.

Technical procedures as in Figure 20.

Incorporation of $^{59}Fe$:

(a) adult rat bone marrow Hb + adult rat Hb marker.

(b) adult rat bone marrow Hb + adult mouse Hb marker.

Optical density profiles:

(c) adult rat bone marrow Hb + adult rat Hb marker.

(d) adult rat bone marrow Hb + adult mouse Hb marker.

FIGURE 22

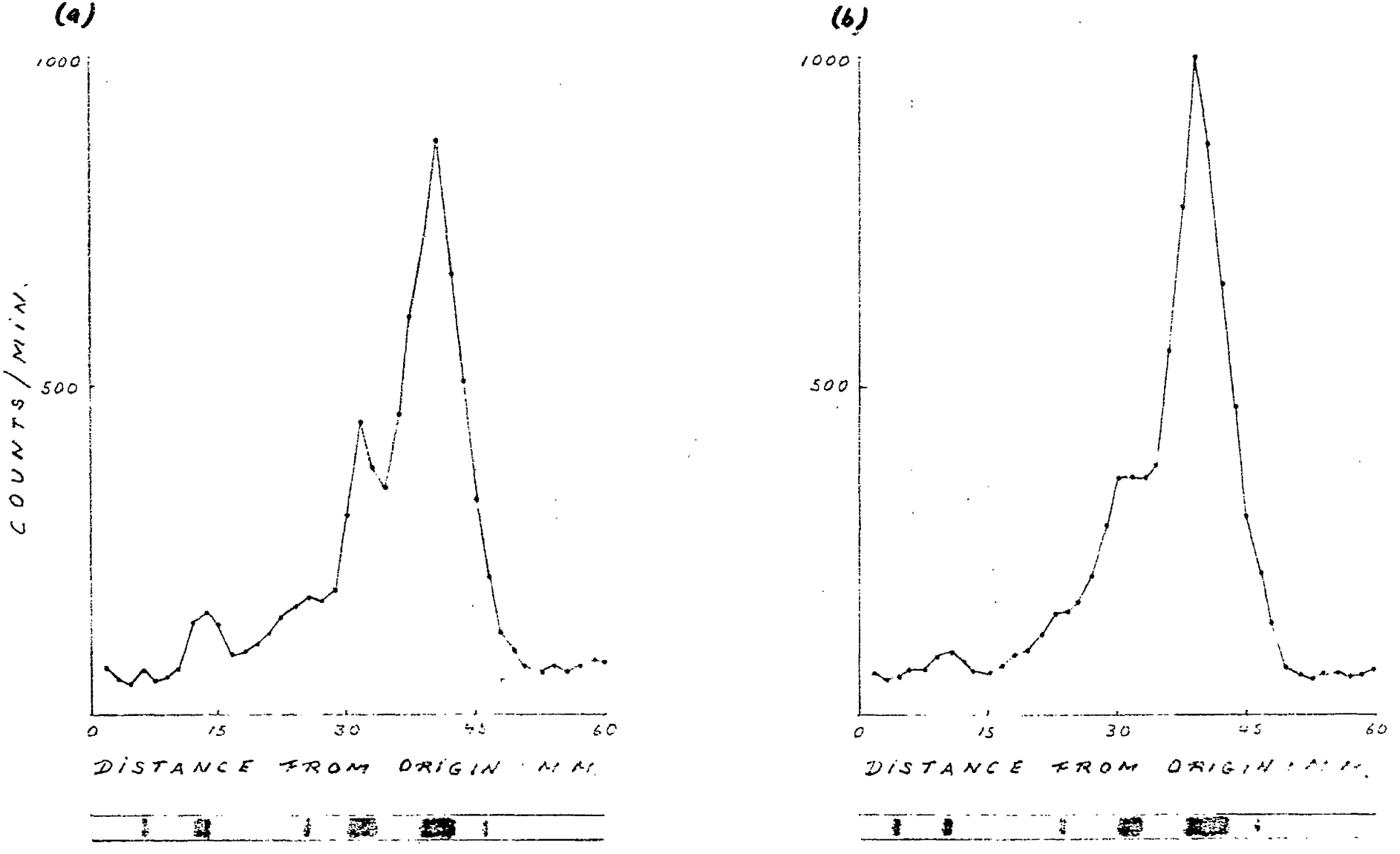

(a)
1000
500
COUNTS / MIN.
0
15
30
45
60
DISTANCE FROM ORIGIN · M M.
(b)
1000
500
0
15
30
45
60
DISTANCE FROM ORIGIN · M M.

FIGURE 22

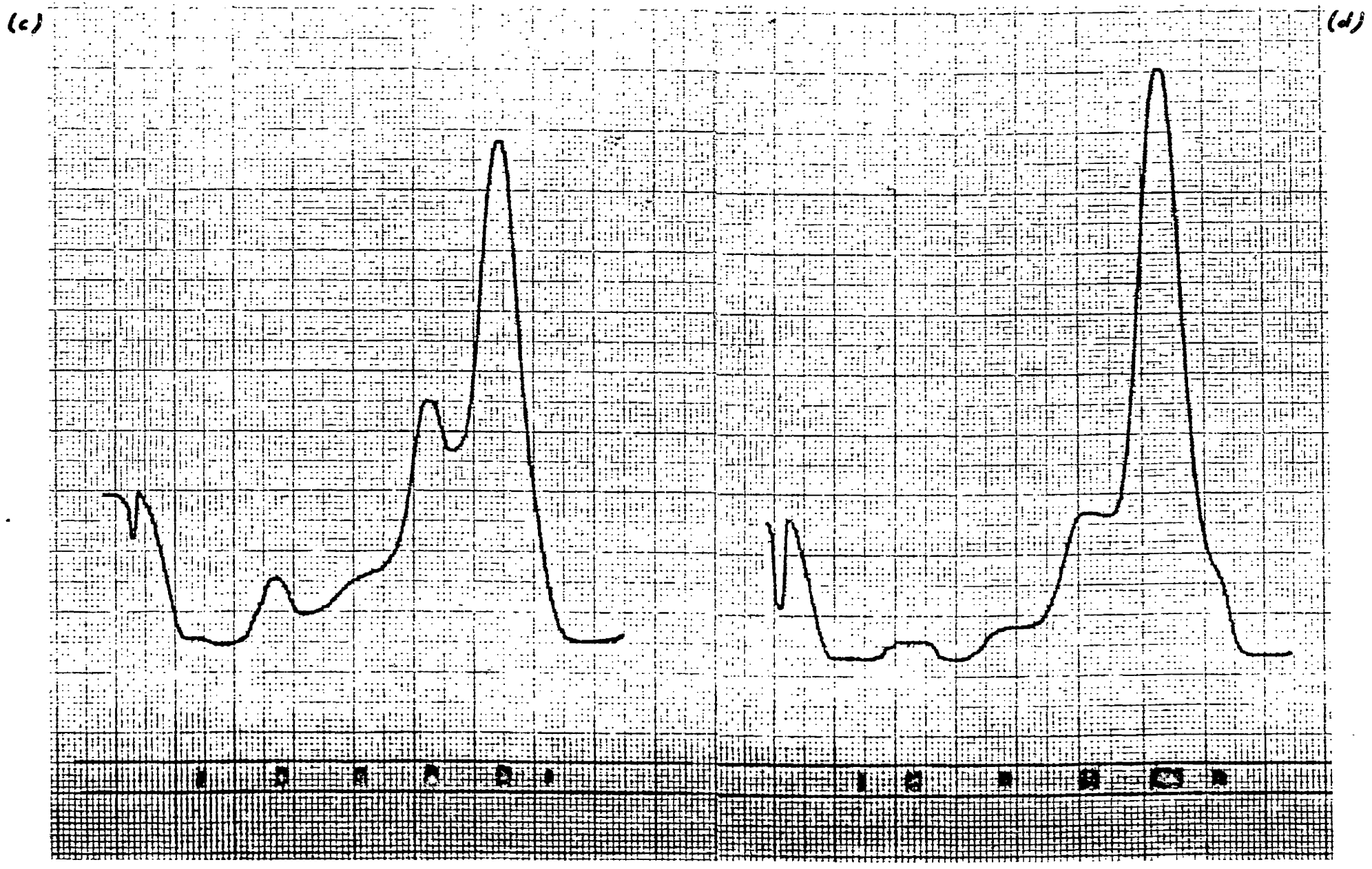


Figure 23.

Incorporation of $^{59}Fe$ into adult rat bone marrow haemoglobin.

The cells were incubated with $^{59}Fe$ for 8 hrs. Haemoglobin was extracted with chloroform, and an aliquot was mixed with Hb marker of the same type. Both haemoglobin samples (with and without Hb marker) were fractionated by starch gel electrophoresis (Poulik, 1957), and radioactivity incorporation determined as described in the text.

(a) adult rat bone marrow Hb (no marker).

(b) adult rat bone marrow Hb + adult rat Hb marker.

FIGURE 23

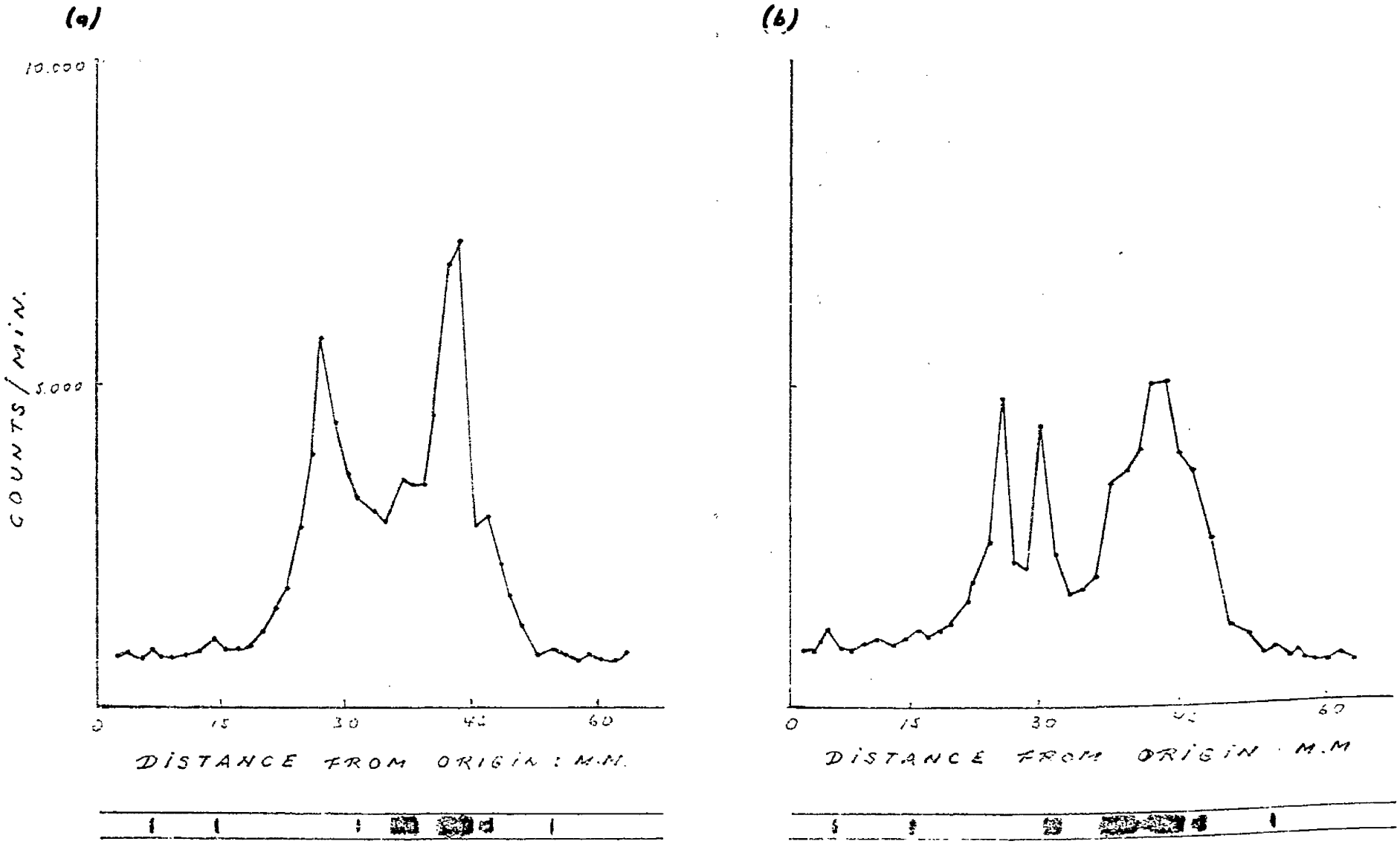

(a)
10.000
5.000
COUNTS / MIN.
0
15
30
45
60
DISTANCE FROM ORIGIN : M.M.
(b)
0
15
30
60
DISTANCE FROM ORIGIN · M.M

Figure 24.

Incorporation of $^{59}Fe$ into adult rat bone marrow haemoglobin.

Technical procedures as in Figure 23.

(a) adult rat bone marrow Hb (no marker).

(b) adult rat bone marrow Hb + adult rat Hb marker.

FIGURE 24

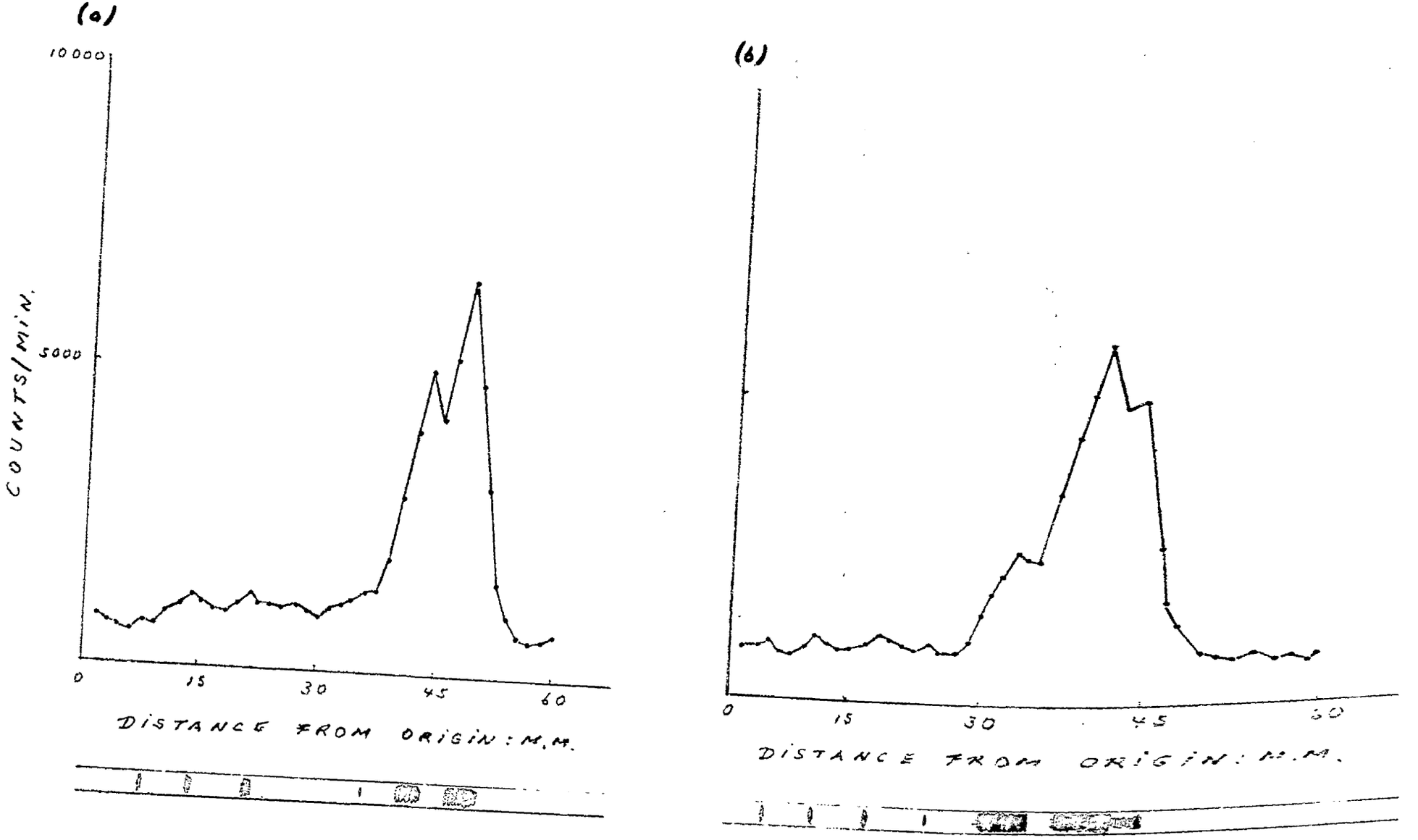

(a)
10000
5000
COUNTS/MIN.
0
15
30
45
60
DISTANCE FROM ORIGIN: M.M.
(b)
0
15
30
45
60
DISTANCE FROM ORIGIN: M.M.

Figure 25.

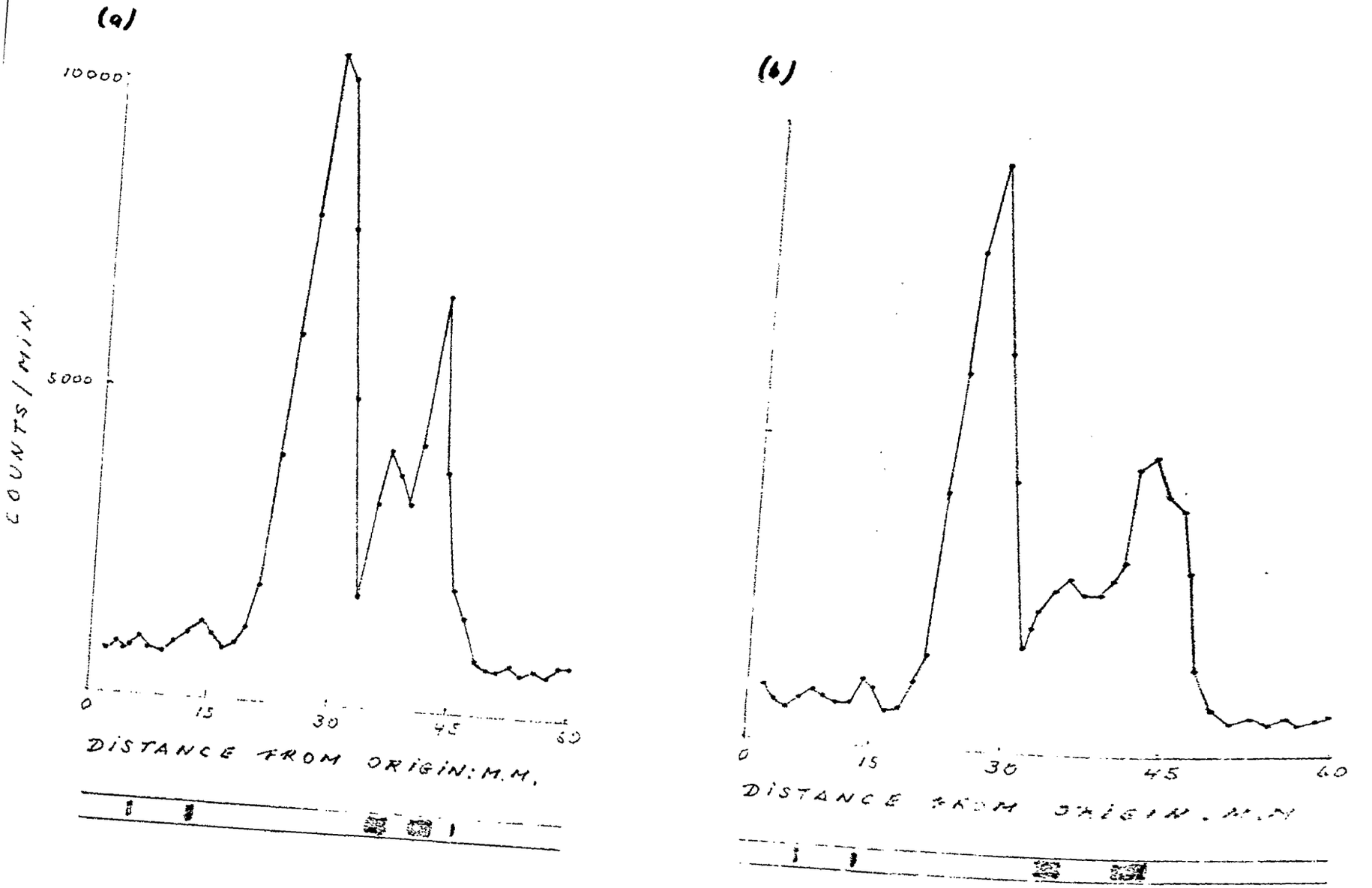
Incorporation of $^{59}Fe$ into 16½-day foetal rat liver haemoglobin.

Technical procedures as in Figure 23.

(a) 16½-day foetal rat liver Hb (no marker).

(b) 16½-day foetal rat liver Hb + foetal rat Hb marker.

FIGURE 25


(a)
10000
5000
COUNTS / MIN.
0
15
30
45
60
DISTANCE FROM ORIGIN: M.M.
(b)
0
15
30
45
60
DISTANCE FROM ORIGIN. M.M.

fractions with the low concentration of haemoglobin solution experimentally labelled. An added advantage of the marker may be that it gives a visible assessment of the electrophoretic fractionation without the tedious procedure for radioactivity counting, if the visible pattern looked unpromising. The alternative, of co-electrophoresing a marker haemoglobin along with the experimental sample, however, was not absolutely reliable, because a perfect fractionation of the marker was not necessarily accompanied by a similar fractionation of the latter. On the other hand, by using labelled haemoglobin alone, without the addition of marker, greater amounts of more concentrated experimental haemoglobin solutions could be electrophoresed. Concentration of experimental haemoglobin solutions were sufficient to make the main components visible, and their migratory distance could be measured. Migration of the minor haemoglobin components was not relevant for practical purposes. Moreover, since the radioactivity profile (not the visible spectrum) would give the differential characteristic between foetal and adult rat haemoglobins, it was decided to abandon the addition of markers (carriers) because of the effect they might have on the experimental haemoglobins.

Elimination of chloroform as clearing agent in haemoglobin extraction.

Some workers (Boyer, Lardy and Myrback, 1963; Huehns, 1968) have reported the adverse effect of chloroform and other organic solvents on haemoglobins. Experiments were performed to see if chloroform had any effect on the distribution of the radioactivity profiles of experimentally labelled F.R.L. and A.R.B.M. haemoglobins. The results obtained were variable. In some cases there was no harmful effect; on the contrary it decreased the radioactivity peak x, and some samples of A.R.B.M. haemoglobin were devoid of it. However, in other instances a dramatic enhancement of haemoglobin interactions and aggregations took place. Figures 26, 27 and 28 show typical examples of these aggregations. It seemed that factors related to the amount of chloroform, the time in contact with haemoglobins, the degree of agitation of the mixture, and the temperature, along with the instability of the haemoglobin sample itself, all played some critical role in this respect. In any case, complete elimination of the radioactivity peak 'x' was not achieved in samples of F.R.L. haemoglobins, and often a haemoglobin-protein complex containing a great amount of radioactivity appeared to be formed.

Figure 26.

Incorporation of $^{59}Fe$ into adult rat bone marrow haemoglobin.

The cells were incubated with $^{59}Fe$ for 8 hrs, and lysed. An aliquot of the haemolysate was centrifuged at 48,000 rpm x 1 hr. and another aliquot extracted with chloroform. Both haemoglobin samples (with and without chloroform extraction) were fractionated by starch gel electrophoresis (Poulik, 1957) and radioactivity incorporation determined as described in the text.

The radioactivity peak 'X' did not correspond to any haemoglobin component.

(a) haemolysate centrifuged at high speed (no chloroform).

(b) chloroform-extracted Hb sample.

FIGURE 26

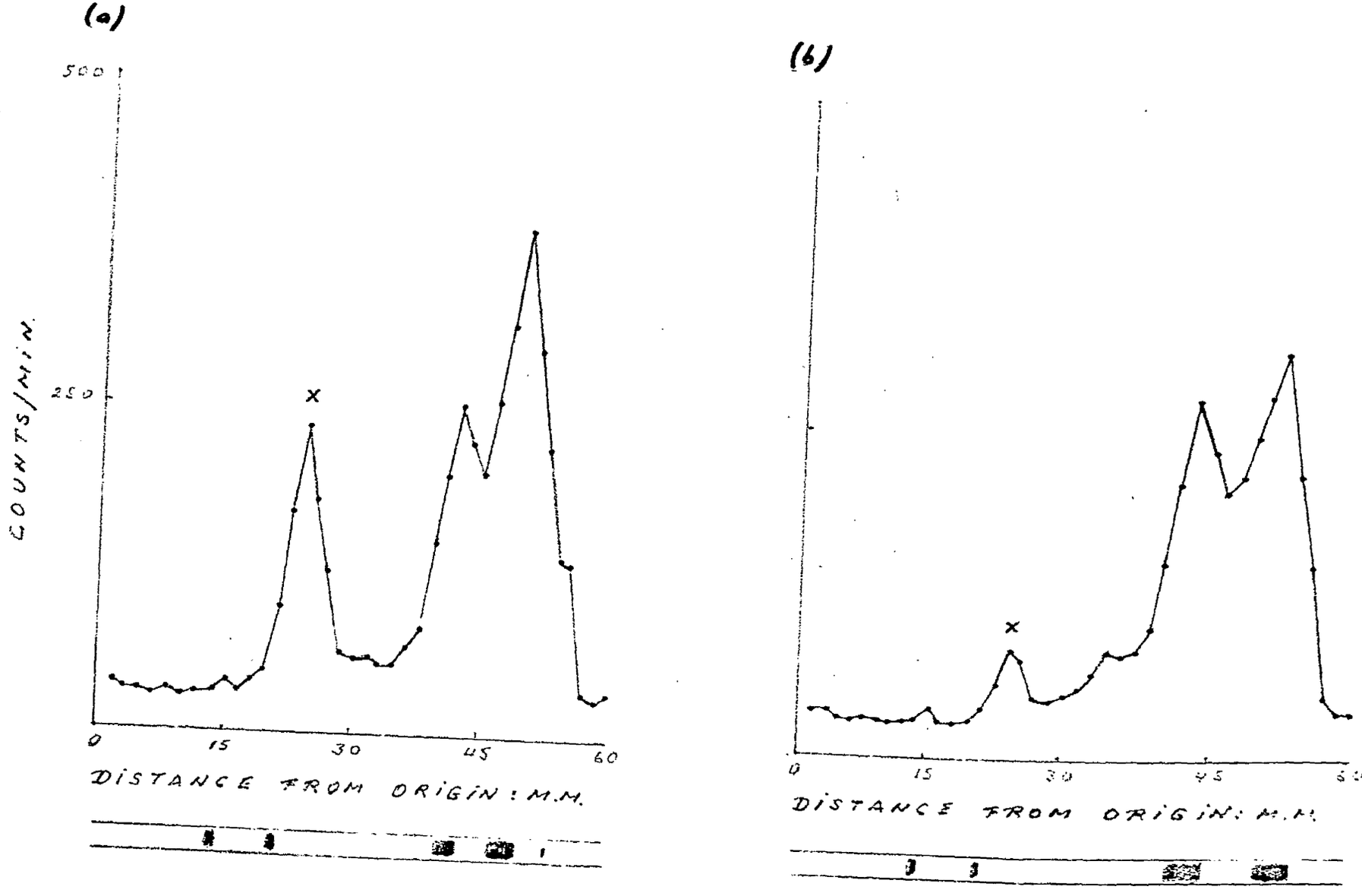

(a)
500
250
COUNTS/MIN.
X
0
15
30
45
60
DISTANCE FROM ORIGIN: M.M.
(b)
X
0
15
30
45
60
DISTANCE FROM ORIGIN: M.M.

Figure 27.

Incorporation of $^{59}Fe$ into 14½-day foetal rat haemoglobin.

Technical procedures as in Figure 26.

The radioactivity peak 'X' did not correspond to any haemoglobin component.

(a) Haemolysate with no chloroform extraction.

(b) Chloroform-extracted Hb sample.

FIGURE 27

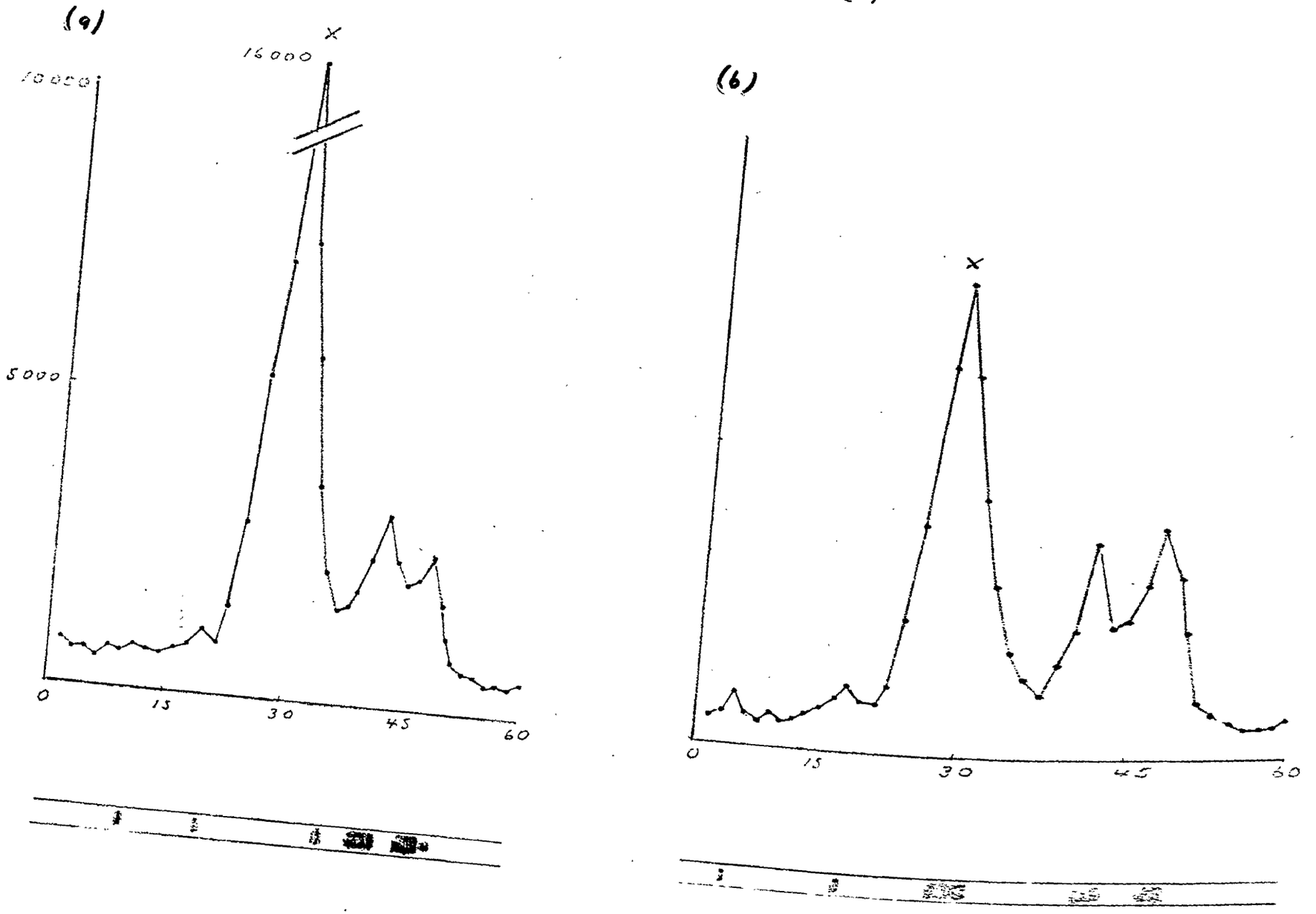


Figure 28.

Incorporation of $^{59}Fe$ into 15½-day foetal rat haemoglobin.

Technical procedures as in Figure 26.

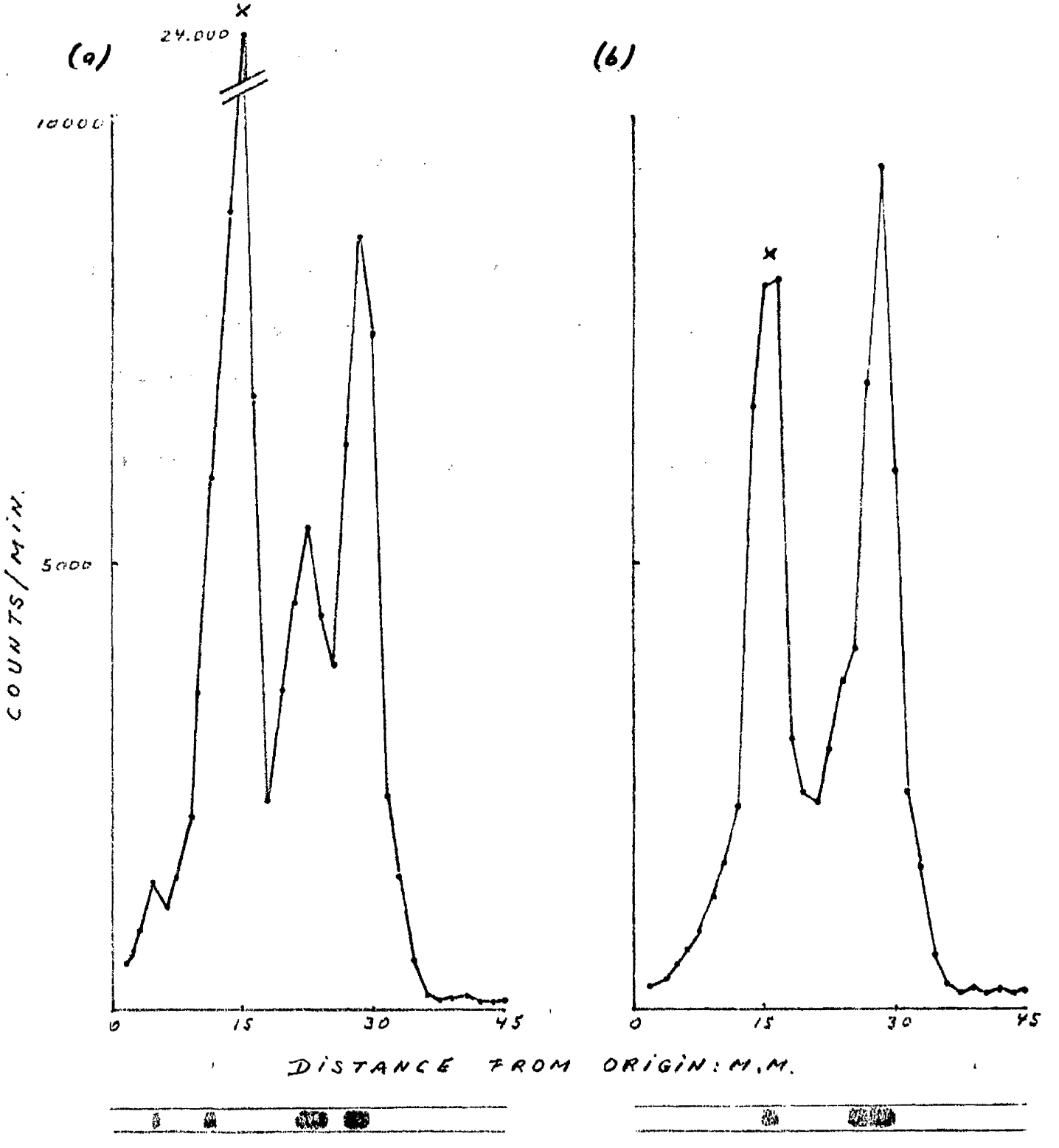
Labelling : 12 hrs.

The radioactivity peak 'X' in figure (a) did not correspond to any haemoglobin component, while peaks in figure (b) appeared to form aggregates.

(a) Haemolysate with no chloroform extraction.

(b) Chloroform-extracted Hb sample (5 vols., at -14°C).

FIGURE 28


(a)
(b)
X
24,000
10000
5000
COUNTS/MIN.
0
15
30
45
DISTANCE FROM ORIGIN: M.M.

Other organic solvents, such as toluene and carbon tetrachloride, were also tried, but with similar results. In spite of the partial purification of the haemoglobin obtained with these organic solvents, it was decided to abandon their use, because of their tendency to cause haemoglobin aggregations.

Elimination of the radioactive contaminant from haemoglobin solutions.

As seen in the previous results, a radioactive contaminant was present in many samples of A.R.B.M. haemoglobins, and in larger amounts in samples of F.R.L. haemoglobins. It sometimes appeared as multiple, at other times as a single peak, and occasionally it formed a complex with one of the haemoglobin fractions. In order to be certain of its position, haemoglobin samples were also electrophoresed in a continuous system of buffers (Smithies, 1955) to get a wider separation between fractions. The gels were stained for a more accurate location of the haemoglobin components and then sliced for radioactivity counting. Figures 29 and 30 show typical examples of these results. The location of peak 'x' between haemoglobin bands is readily seen.

The first and obvious thought was that such contamination was caused by incomplete removal of the labelled transferrin added to the culture medium. An

Figure 29.

Incorporation of $^{59}Fe$ into adult rat bone marrow haemoglobin.

Technical procedures as in Figure 16, using Smithies' (1955) continuous system of buffers, followed by O-dianisidine-staining before gel slicing and radioactivity counting.

The radioactivity peak 'X' did not correspond to any haemoglobin component.

FIGURE 29

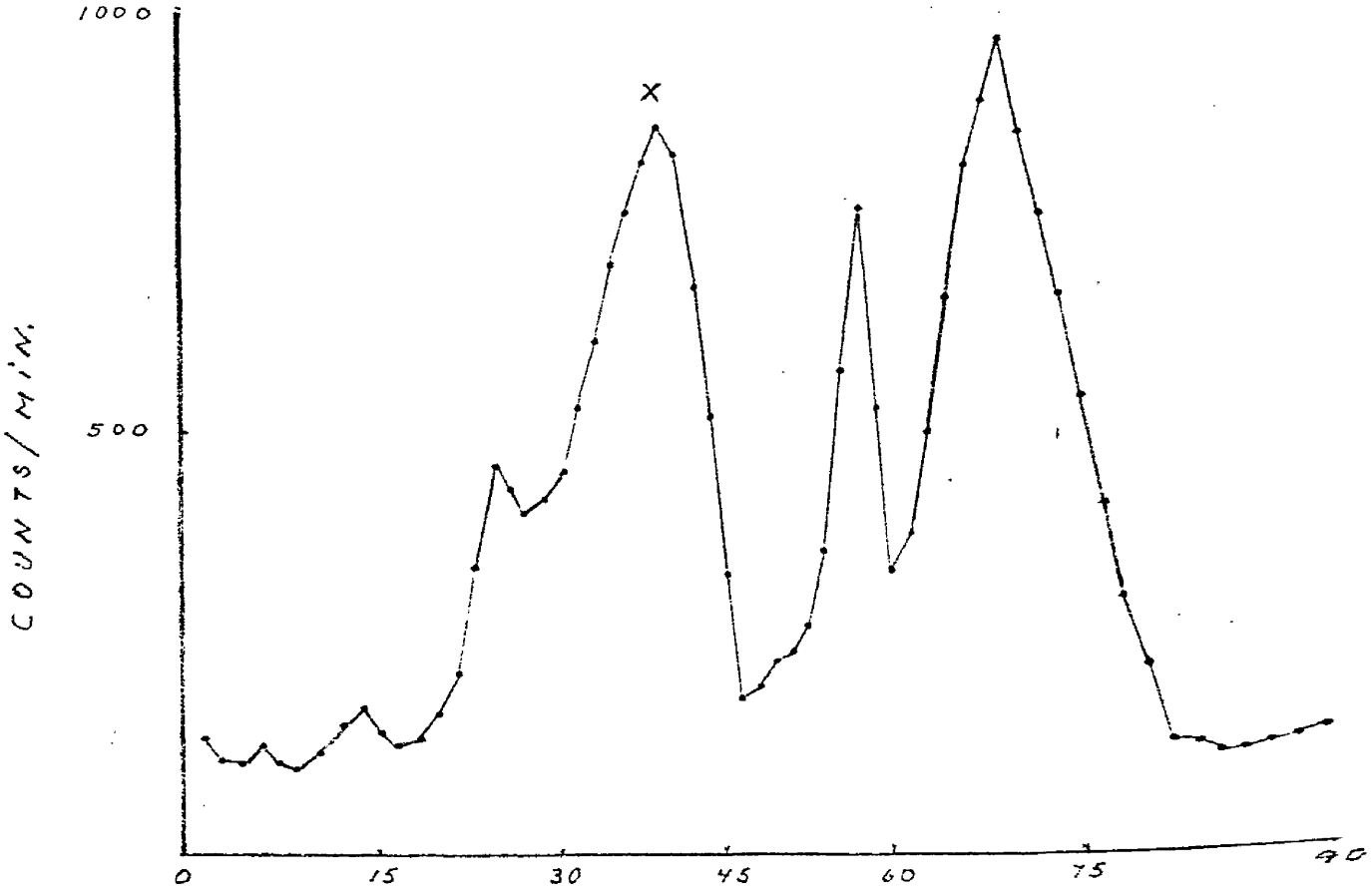

1000
500
COUNTS / MIN.
X
0
15
30
45
60
75
90
DISTANCE FROM ORIGIN: M M.

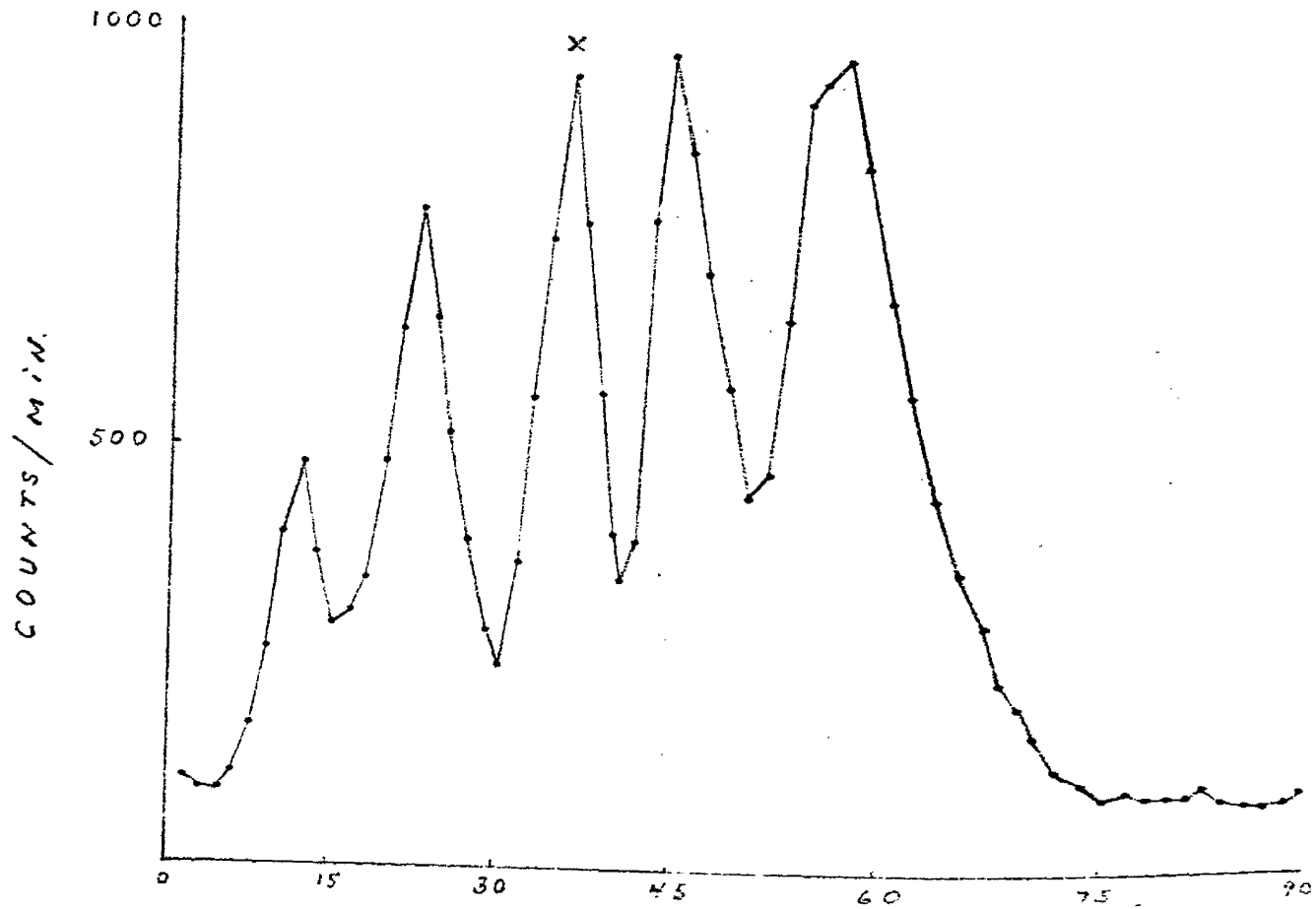
Figure 30.

Incorporation of $^{59}Fe$ into 17-day foetal rat liver haemoglobin.

Technical procedures as in Figure 29.

The radioactivity peak 'X' did not correspond to any haemoglobin component.

FIGURE 30


1000
500
COUNTS / MIN.
X
0
15
30
45
60
75
90
DISTANCE FROM ORIGIN : M.M.

experiment with exhaustive washing of the cells after culture was performed, and the results are shown in Figure 31. The radioactivity peak 'x' was present in increased amount, and it appeared to have formed a complex with one of the haemoglobin components. This result suggested that the exhaustive washing caused an increased release of contaminant. Since iron-transferrin complex becomes attached to the cell membrane of the erythroblast (Nissim, 1966), and transferrin has been identified in lysates of human erythrocytes (Cawley et al., 1965), labelled rat transferrin was co-electrophoresed with a labelled experimental haemoglobin. This showed that peak 'x' had a fairly similar migration to transferrin and suggested that transferrin might be responsible for the contamination, although it has been reported that transferrin is precipitated by chloroform (Gitlin and Janeway, 1960). It has also been reported that iron in the cell membrane and the stroma in general is in the form of ferritin (Bishop and Surgenor, 1964). It was thought that chelating agents could remove this iron, whether the contaminant was ferritin or transferrin. An experiment was performed in which, after 8 hour labelling, the culture medium was replaced by a new one containing non-radioactive iron-transferrin, and incubation continued for one more hour, to allow for movement of $^{59}Fe$ from

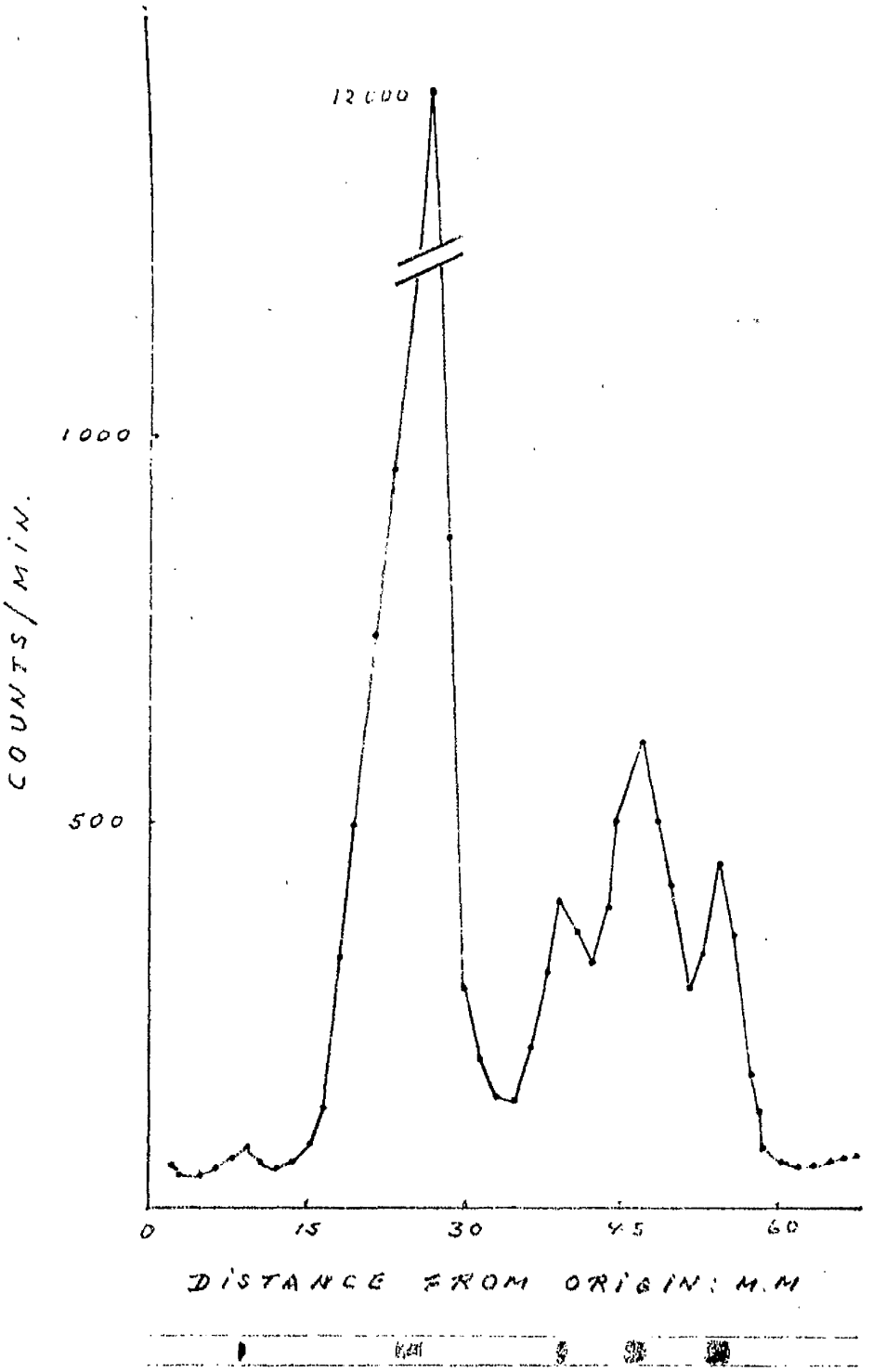
Figure 31.

Incorporation of $^{59}Fe$ into 15½-day foetal rat haemoglobin.

Technical procedures as in Figure 16, after exhaustive washing of the cells to eliminate non-incorporated label.

The radioactivity peak 'X' appeared to have aggregated with one of the haemoglobin components.

FIGURE 31


12000
1000
500
COUNTS/MIN.
0
15
30
45
60
DISTANCE FROM ORIGIN: M.M

the cell membrane. Washing of the cells was performed with BSS containing non-radioactive iron, then with normal saline, followed by normal saline containing EDTA at a final concentration of 0.001 N solution, and lastly with normal saline. Figure 32 shows the result obtained. The radioactivity peak x was still present.

Perhaps EDTA at such concentration had an insufficient chelating capacity (higher concentrations produced haemolysis). Therefore, a re-incubation of the labelled cells in trypsin/CMC for 3 and 6 minutes was tested. However, this procedure caused release of DNA, especially in cultures of foetal liver cells, and cell lysis. A different chelating agent, citric acid/sodium citrate, was also tried. To find the pH at which splitting of iron from rat transferrin took place, samples of $^{59}Fe$ rat transferrin were dialysed against M.citric acid/ M.sodium citrate buffer at different pHs and an aliquot of each sample counted before and after dialysis. Table 12 summarises the results obtained. Iron was readily removed from rat transferrin between pH 4 and 7.8. Experimentally labelled haemoglobins were therefore prepared and dialysed in M.citric acid/ M. sodium citrate buffer at pH 5.4 for 20 hours in the cold, after which the pH of the haemoglobin samples was re-adjusted to 7 with 0.1 M sodium hydroxide, electrophoresed

Figure 32.

Incorporation of $^{59}Fe$ into adult rat bone marrow haemoglobin.

Technical procedures as in Figure 16, after washing of the cells with 0.001 N solution of EDTA.

The radioactivity peak 'X' did not correspond to any haemoglobin component.

FIGURE 32

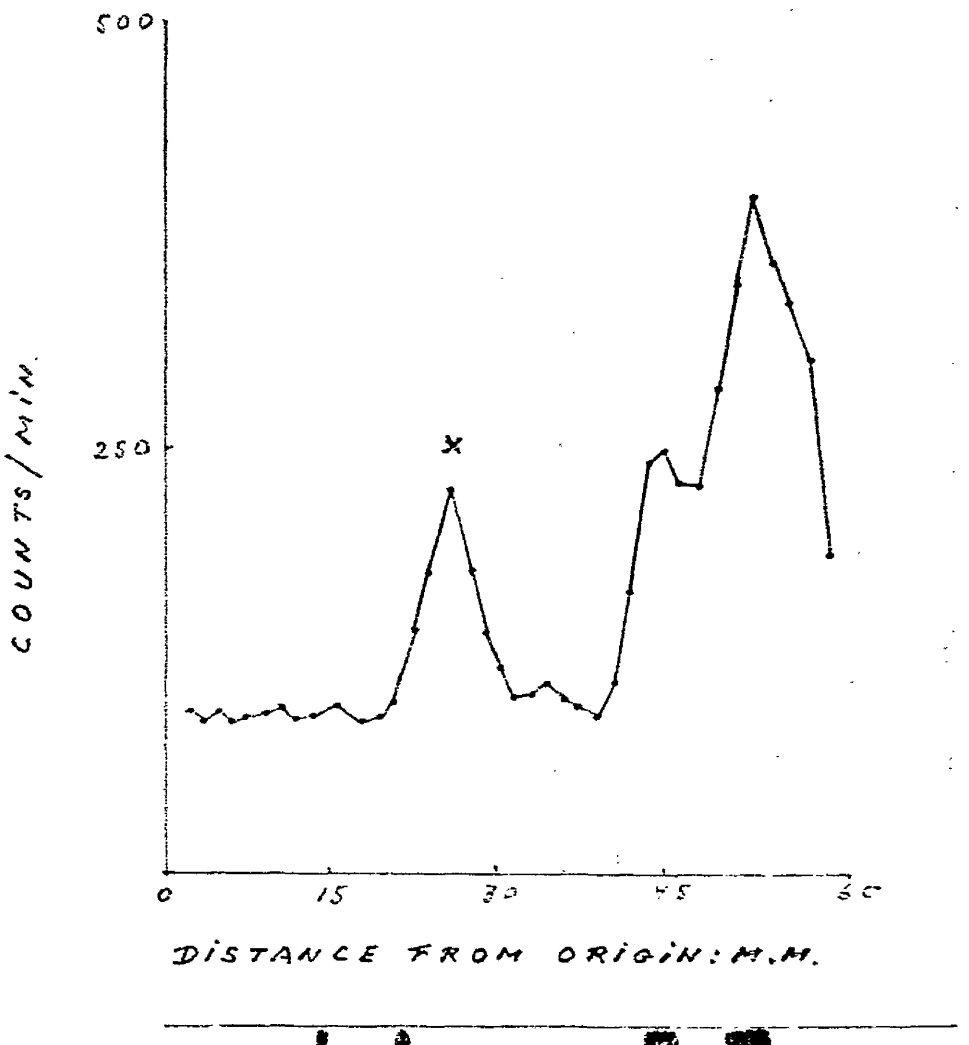


Table 12.

Effect of 24 hour dialysis against 1 M citric acid - 1 M sodium citrate on the radioactivity of $^{59}Fe$ rat transferrin.

| Sample | Buffer pH | C.P.M. | |
|---|---|---|---|
| | | Before dialysis | After dialysis |
| Transferrin | 7.8 | 966 | 73 |
| " | 5 | 964 | 62 |
| " | 4.5 | 895 | 63 |
| " | 4.2 | 623 | 62 |
| " | 4 | 633 | 60 |
| Transferrin + EDTA | 7.75 | 956 | 60 |
| " " | 5 | 878 | 70 |
| Transferrin + $FeCl_3$ | 5 | 845 | 70 |

Background radioactivity = 40 - 60 C.P.M.

and counted. The results obtained still showed the presence of the contaminant peak 'x'.

An experimental culture was set up using exclusively foetal bovine serum, both in the culture medium and in the labelled transferrin. Experimental controls comprised a culture using exclusively rat serum for medium and transferrin, and another culture using a mixture of foetal bovine serum and rat serum. Figures 33 and 34 show the results obtained. The radioactive contaminant was present in all haemoglobins. Spontaneous precipitation of the labile haemoglobin components, which was allowed to occur by storing an aliquot of the sample, did not affect the presence of the radioactivity peak 'x' either.

Purification of haemoglobin by carboxymethyl cellulose column chromatography.

In view of the foregoing, unsatisfactory results, a different approach to the methodology of haemoglobin purification had to be applied. The cation exchanger carboxymethyl cellulose method has been used by some workers (Huntsman et al., 1958; Heywood et al., 1966; Fantoni et al., 1968; Tavill et al., 1968).

Experiments were set up to purify the experimentally labelled haemoglobins following, in general, the method described by Fantoni et al. (1968). After labelling,

Figure 33.

Incorporation of $^{59}Fe$ into adult rat bone marrow haemoglobin.

The cells were incubated in Waymouth's medium supplemented with 10% rat serum or foetal bovine serum. $^{59}Fe$ transferrin, prepared in rat serum or foetal bovine serum, was added from 0 to 6 hrs. The haemolysate was extracted with carbon tetrachloride and fractionated by starch gel electrophoresis (Poulik, 1957), and radioactivity incorporation determined as described in the text.

The radioactivity peak 'X' did not correspond to any haemoglobin component.

(a) Cells incubated in Waymouth's medium, supplemented with 10% rat serum, and rat serum $^{59}Fe$ transferrin added.

(b) Cells incubated in Waymouth's medium, supplemented with 10% foetal bovine serum. Foetal bovine serum $^{59}Fe$ transferrin added.

FIGURE 33

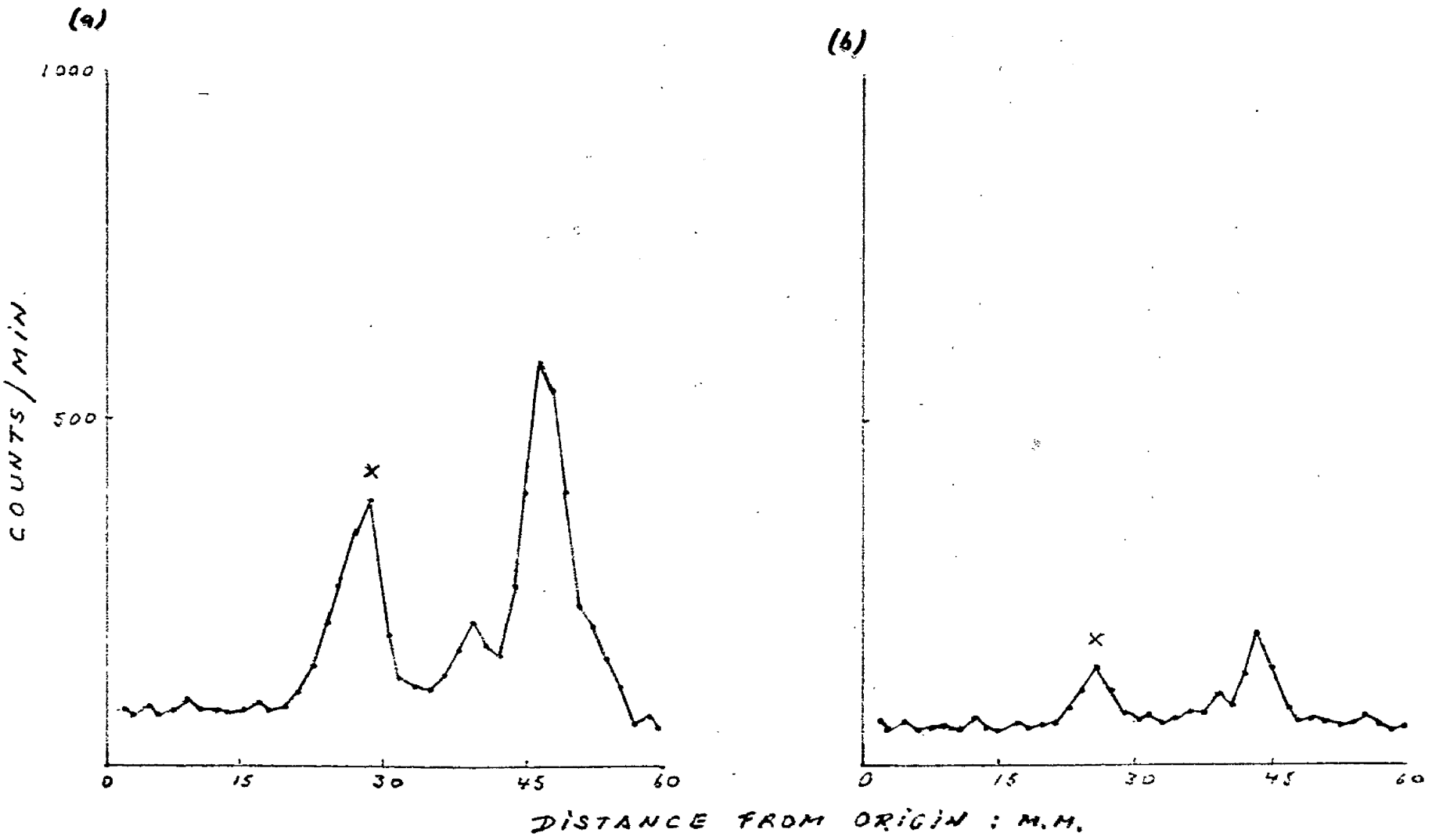


Figure 34.

Incorporation of $^{59}Fe$ into adult rat bone marrow haemoglobin.

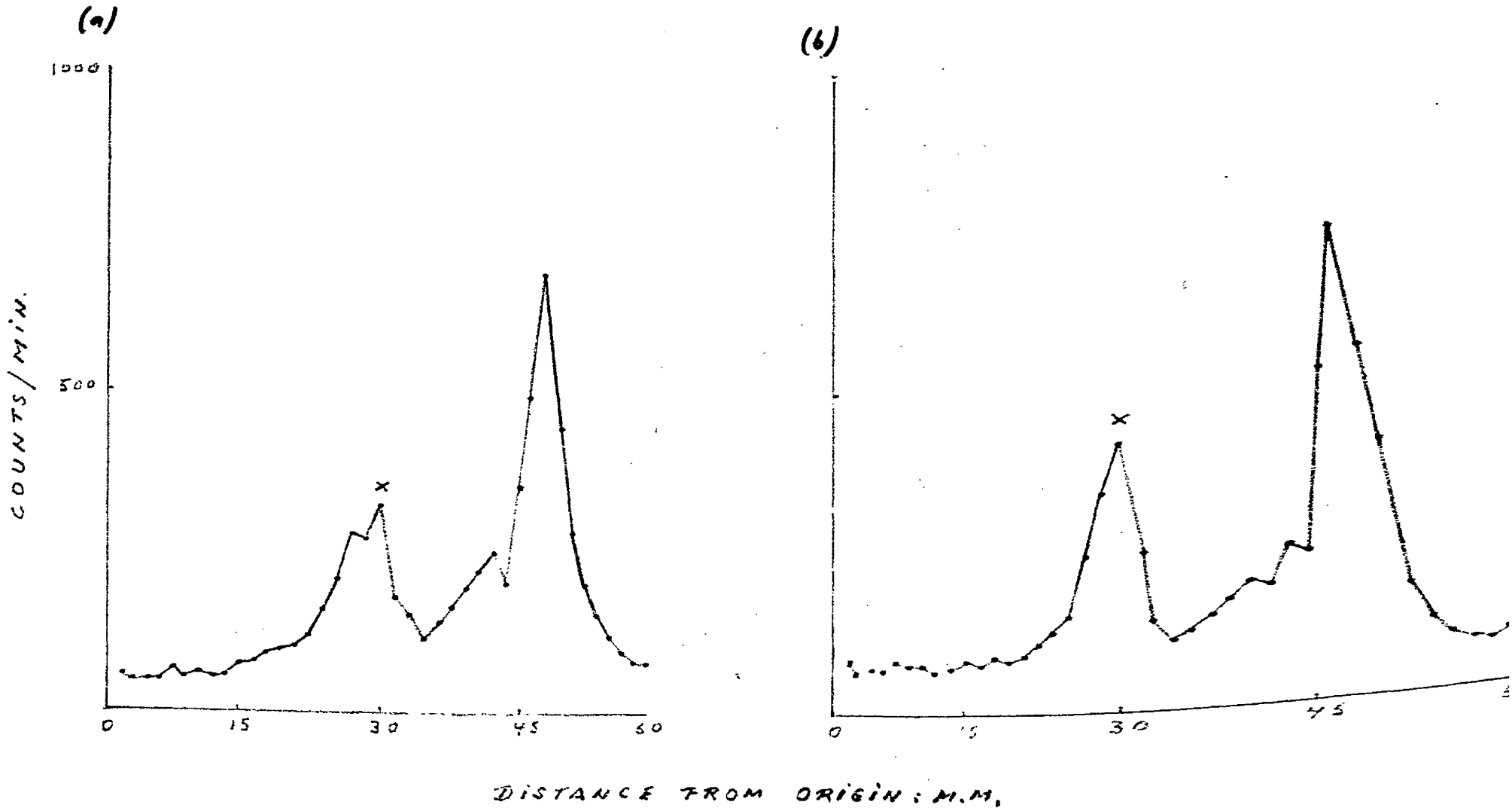
Technical procedures as in Figure 33, except that the Waymouth's medium was supplemented with 5% foetal bovine serum and 5% rat serum. $^{59}Fe$ transferrin, prepared in a mixture of foetal bovine serum and rat serum in equal parts, was added.

The radioactivity peak 'X' did not correspond to any haemoglobin component.

(a) Fresh Hb sample.

(b) Hb sample exposed to oxygen and fraction 4 precipitated.

FIGURE 34


(a)
(b)
1000
500
COUNTS / MIN.
X
X
0
15
30
45
60
0
15
30
45
DISTANCE FROM ORIGIN : M.M.

the cells were washed 4 times with cold normal saline and a cell pellet obtained. Haemolysis was produced by adding 0.5 ml of deionized distilled water and vigorous Vortex mixing, alternating with freezing and thawing 5 times, in order to ensure lysis of most of the cells. The stroma was removed by centrifuging at 30,000 g for 30 mins, and the supernatant haemolysate dialysed against 0.01 M phosphate buffer at pH 6.3 overnight at 4°C. It was then placed on a column (0.5 x 5 cm) of Whatman CM 11 carboxymethyl cellulose, previously equilibrated with 0.01 M phosphate buffer at pH 6.3 overnight at 4°C.

Non-haemoglobin proteins were eluted by passing 0.01 M phosphate buffer, pH 6.3, through the column until their extinction, evaluated by absorption spectrum. Haemoglobins were eluted with 0.01 M phosphate buffer pH 9.1. The flow rate was 4 ml/hour. Figures 35 and 36 show typical examples of the results obtained. The haemoglobin solutions were free from other radioactive contaminants.

Determining some characteristics of the CMC-eluted fractions.

To study some of the characteristics of the haemoglobin contaminants, optical density absorptions of the eluted fractions were obtained in a spectro-

Figure 35.

Incorporation of $^{59}Fe$ into adult rat bone marrow haemoglobin.

The cells were incubated with $^{59}Fe$ for 8 hrs, and lysed. Haemoglobin was purified by carboxymethyl cellulose chromatography (CMC). The cells were incubated with $^{59}Fe$ for 8 hrs, fractionated by starch gel electrophoresis (Poulik, 1957). Radioactivity was determined as described in the text.

The radioactivity peak 'X' (which did not correspond to any haemoglobin component) present in the haemolysate (figure a), disappeared after Hb purification by CMC chromatography (figure b).

FIGURE 35

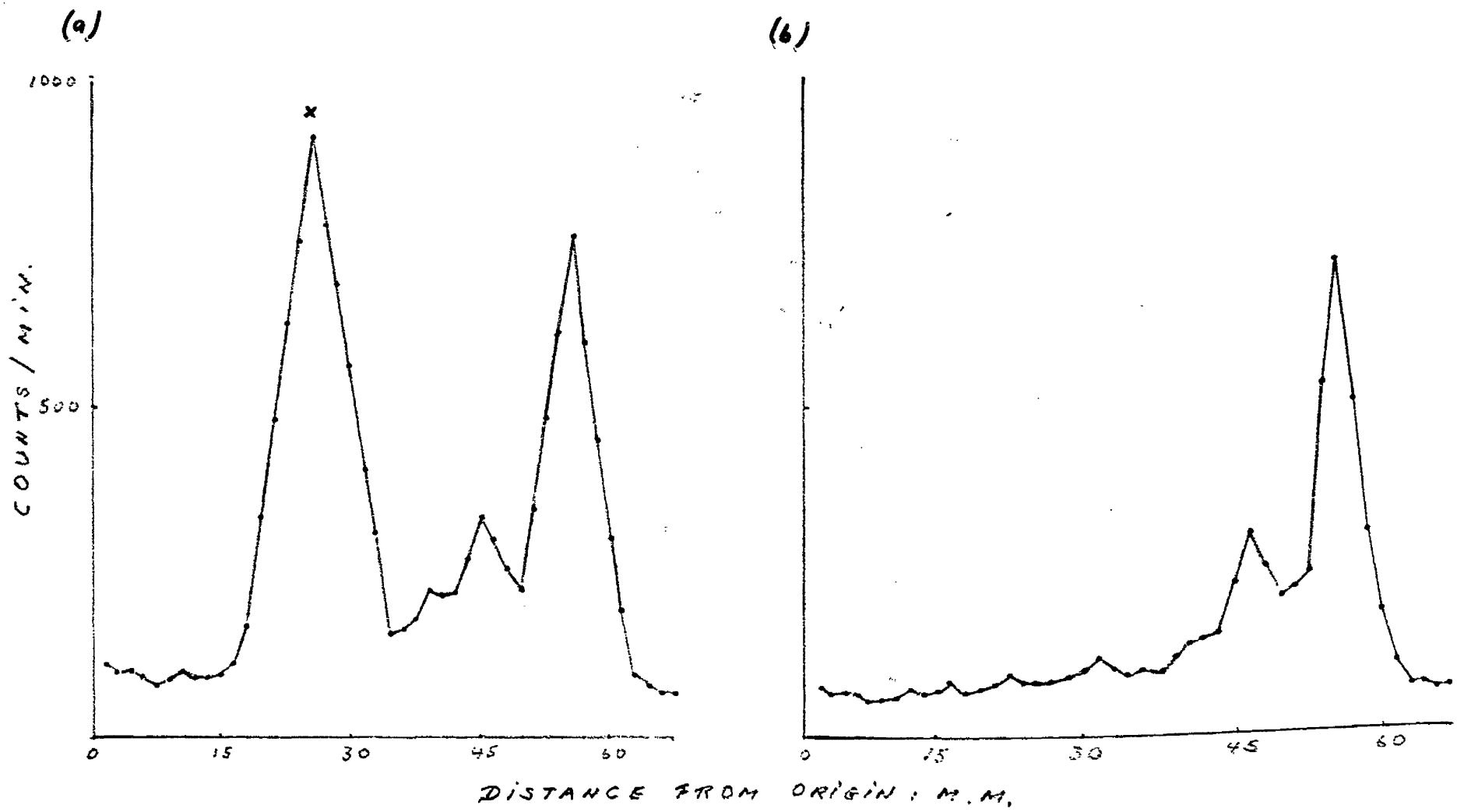


Figure 36.

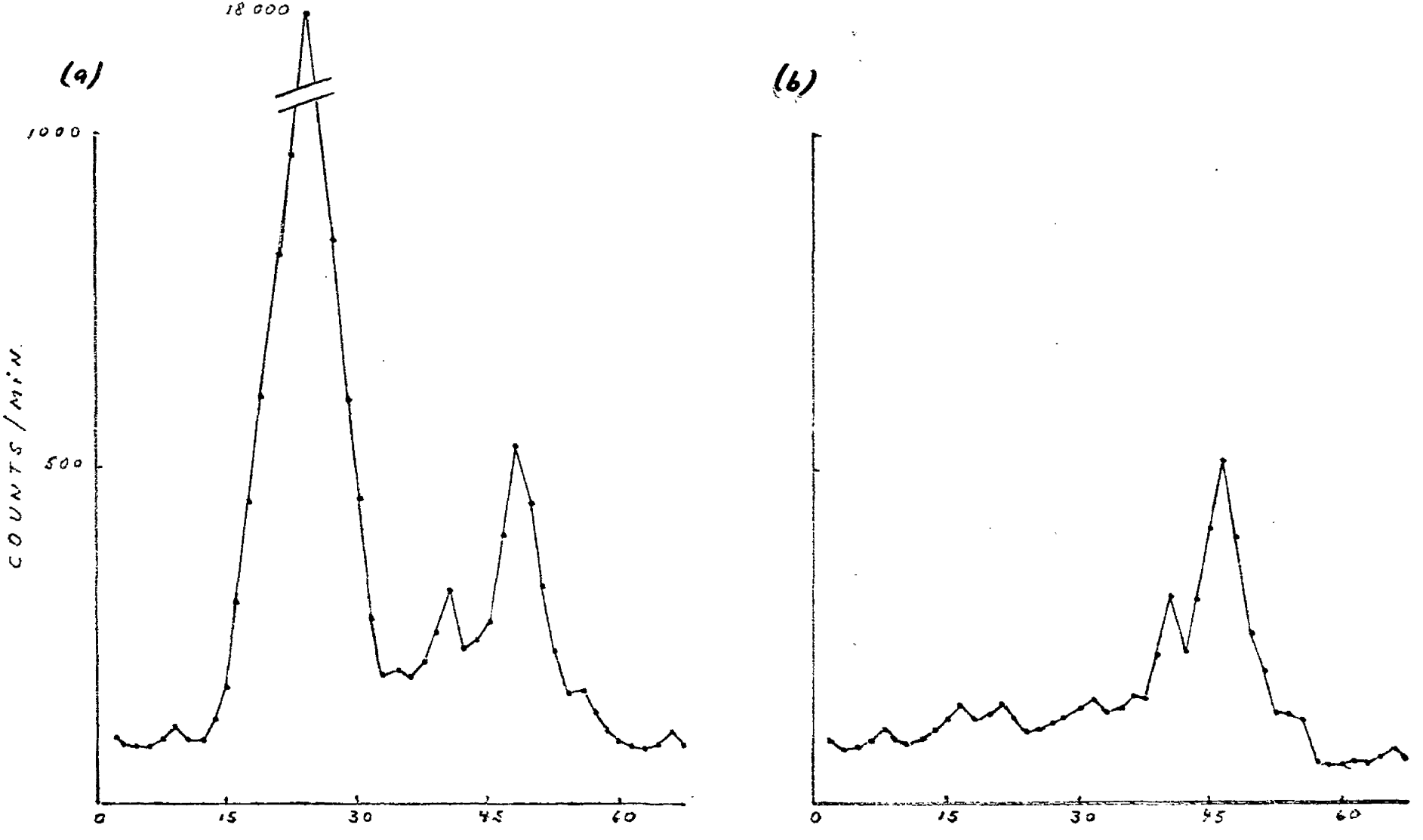
Incorporation of $^{59}Fe$ into 17-day foetal rat haemoglobin.

Technical procedures as in Figure 35.

(a) Haemolysate before CMC chromatography.

(b) Hb purified by CMC chromatography.

FIGURE 36


(a)
(b)
18 000
1000
500
COUNTS / MIN.
0
15
30
45
60
0
15
30
45
60
DISTANCE FROM ORIGIN: M.M.

photometer, Unicam SP 800. Figure 37 shows a typical example of these results. While the first eluted fractions showed slight absorption in the region of the Soret band (405 mμ) and a heavy absorption at 280 mμ, the fraction corresponding to haemoglobin showed heavy absorption in the region of the Soret band, slight absorption at 280 mμ, and the four typical haemoglobin absorption bands at 353, 405, 546 and 664 mμ.

A crude test for catalase activity was performed by adding a small amount of $H_2O_2$ to an aliquot of each eluted fraction. This revealed high activity in the first eluted fractions, while in the haemoglobin fraction the activity was very feeble and almost absent.

Degradation of a $H_2O_2$ solution by aliquots of the early eluted fraction (non-haemoglobin proteins) and the haemoglobin fraction was measured at 240 mμ. Figure 38 shows a typical result. For comparative purposes, the same Figure shows the degradation produced by 120 Sigma units of purified powdered beef liver catalase (Sigma stock No.C.40) under similar conditions. Figure 39 shows the absorption spectrum of catalase from bovine liver.

Foetal rat liver haemoglobin was prepared and half of it purified by CMC. Both purified and non-purified haemoglobins were co-electrophoresed and the starch gel

Figure 37.

Absorption spectra of fractions of haemolysate eluted by carboxymethyl cellulose column chromatography.

Non-haemoglobin components were eluted by passing 0.01 M phosphate buffer, pH 6.3, through the column, and haemoglobin was eluted with the same buffer at pH 9.1, as described in the text.

Reading at room temperature:

(a) Fractions of adult rat bone marrow haemolysate. The fraction 9 corresponding to Hb, was diluted 1:10.

| pH | 6.4 | 6.3 | 7.4 |
|---|---|---|---|
| Fraction | 1-2 | 3-8 | 9 |

$\frac{A\ 405}{A\ 280}$ of fraction 9 = $\frac{1.4}{0.5}$ = 2.8.

(b) Fractions of 16-day foetal rat liver haemolysate. The fraction 9 corresponding to Hb was diluted 1:10.

| pH | 6.4 | 6.3 | 7.5 |
|---|---|---|---|
| Fraction | 1-2 | 3-8 | 9 |

$\frac{A\ 405}{A\ 280}$ of fraction 9 = $\frac{1.5}{0.6}$ = 2.5

FIGURE 37 (a)

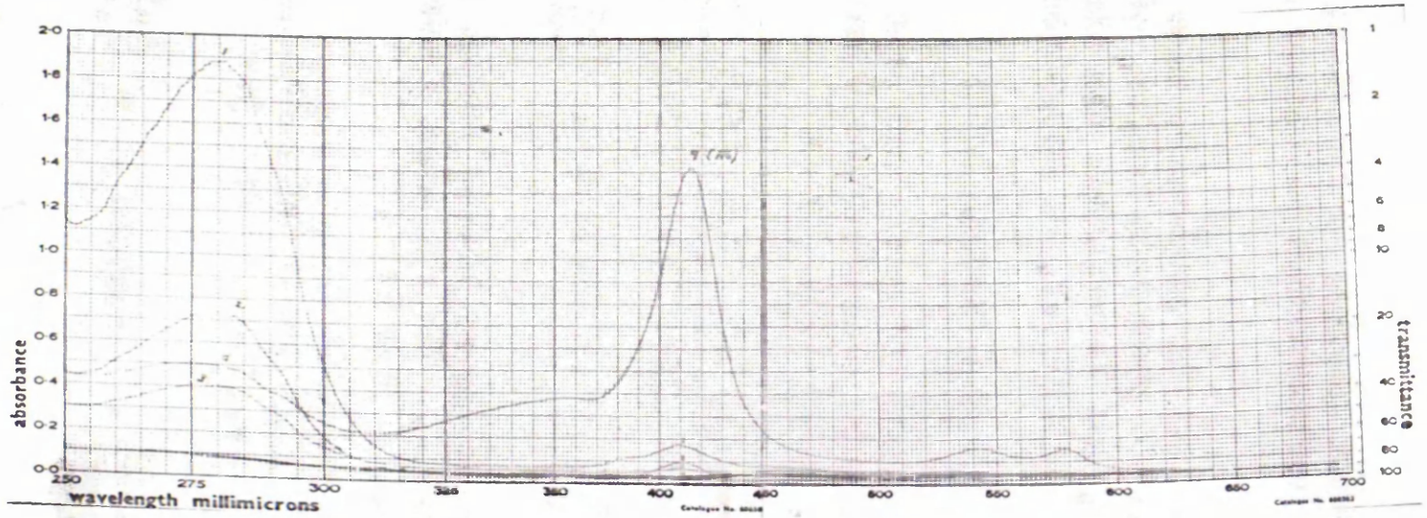

absorbance
2·0
1·8
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
0·0
250
275
300
350
400
450
500
550
600
650
700
wavelength millimicrons
transmittance
1
2
4
6
8
10
20
40
60
80
100

FIGURE 37 (b)

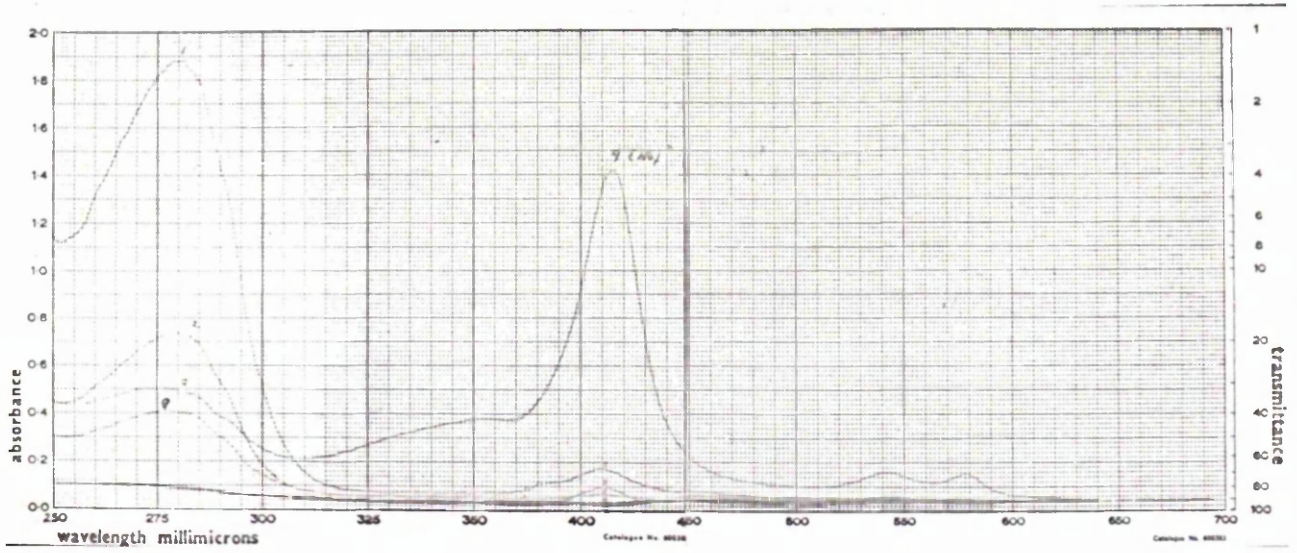

absorbance
wavelength millimicrons
transmittance
250
275
300
325
350
400
450
500
550
600
650
700

Figure 38.

Decomposition of hydrogen peroxide by fractions 1 and 9 from 15-day foetal rat liver haemolysate eluted by carboxymethyl cellulose column chromatography.

Technical procedures as described in Figure 37.

On the left, the decomposition curve of 1 ml of 0.5% $H_2O_2$ (100 Vol.) solution by 20 $mm^3$ of fractions 1 and 9. Reading at 240 mμ, at room temperature.

On the right, the decomposition caused by 120 Sigma units of catalase (purified, powdered beef liver catalase, Sigma stock No. C-40) under similar conditions.

FIGURE 38

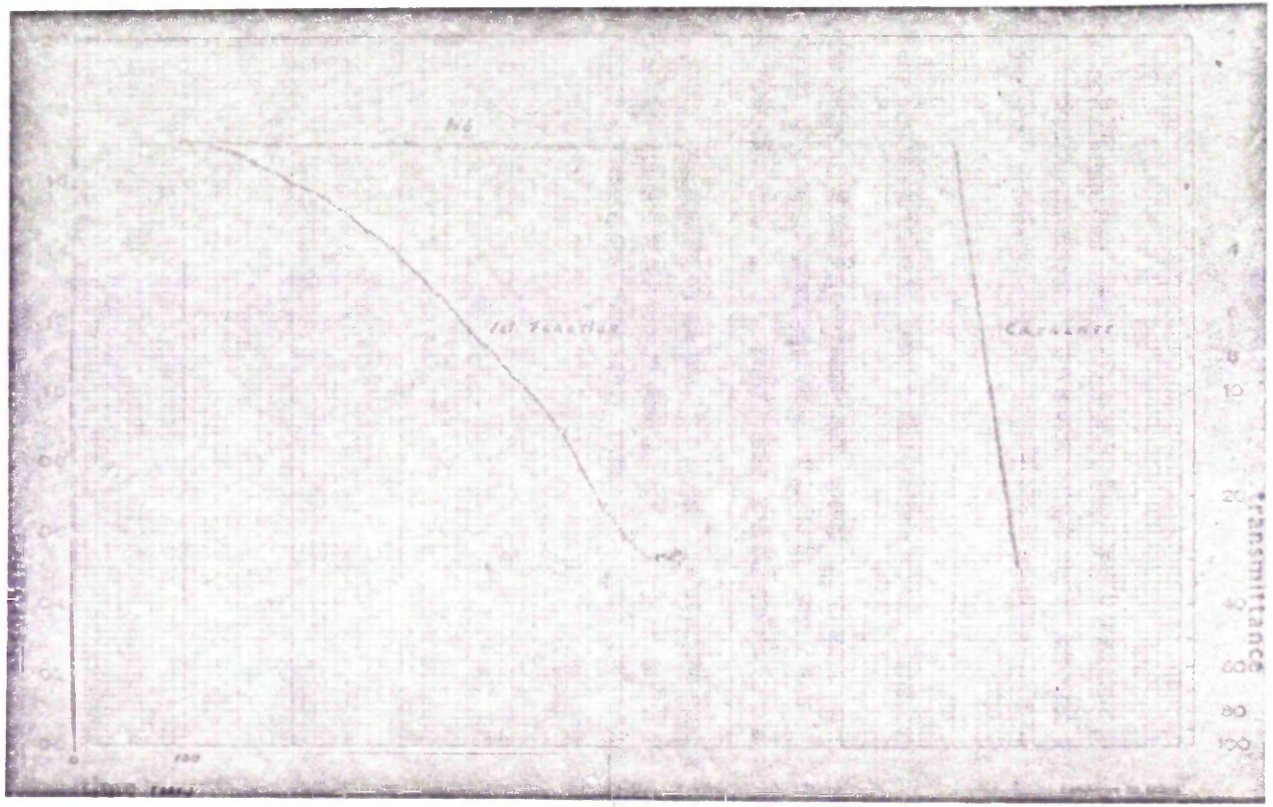
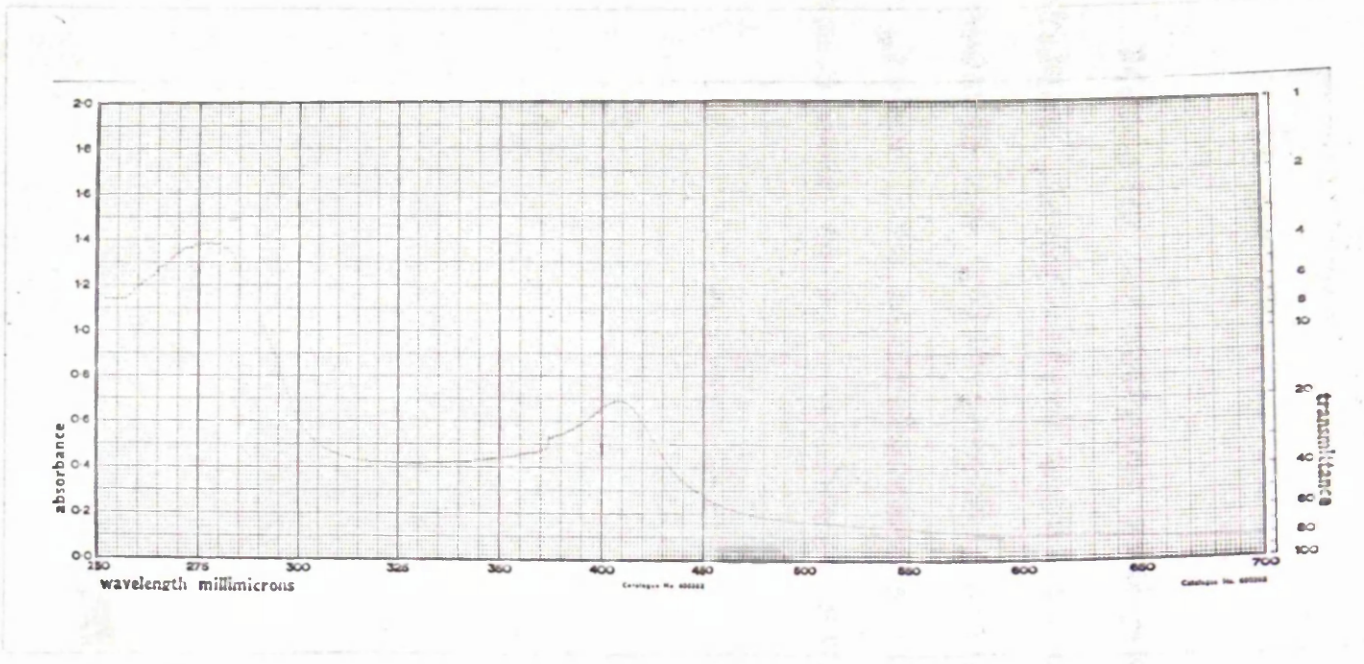


Figure 39.

Absorption spectrum of catalase:

Catalase from bovine liver, purified powder, thymol-free, Sigma, C-40, was diluted in distilled water at a concentration of 1 mg/ml, and the absorption spectrum read at room temperature.

FIGURE 39



stained using hydrogen peroxide and acidic potassium iodide (Hale and Renwick, 1961). This staining gave a positive reaction for catalase in the non-purified sample at a level corresponding to the radioactivity contaminant.

Establishing criteria for differentiation between haemoglobins of foetal rat liver and adult rat bone marrow.

Experiments were performed to establish the differential characteristics of the haemoglobins of 15-day F.R.L. and A.R.B.M. purified by carboxymethyl cellulose column chromatography.

Since the number and migration of the minor haemoglobin components were variable and did not show any reliable differential characteristics, attention was focused on the main haemoglobin components. The migration of the main fractions showed a definite relationship which could be taken as criteria for differentiation. Figures 40, 41, 42 and 43 show these results. Three migratory positions, designated I, II and III, were recognized. While the major fraction of A.R.B.M. haemoglobin migrated to position I and the following fraction to position II, in the F.R.L. haemoglobin the major fraction migrated to position II and the following fraction to position III. In other

Figure 40.

Relative migration of rat haemoglobin components in starch gel electrophoresis:

Haemoglobins were labelled with $^{59}Fe$, prepared and purified by CMC, and fractionated by starch gel electrophoresis (Poulik, 1957). Radioactivity incorporation was determined as described in the text.

——— adult rat bone marrow Hb.

·_·_· 17-day foetal rat liver Hb.

FIGURE 40

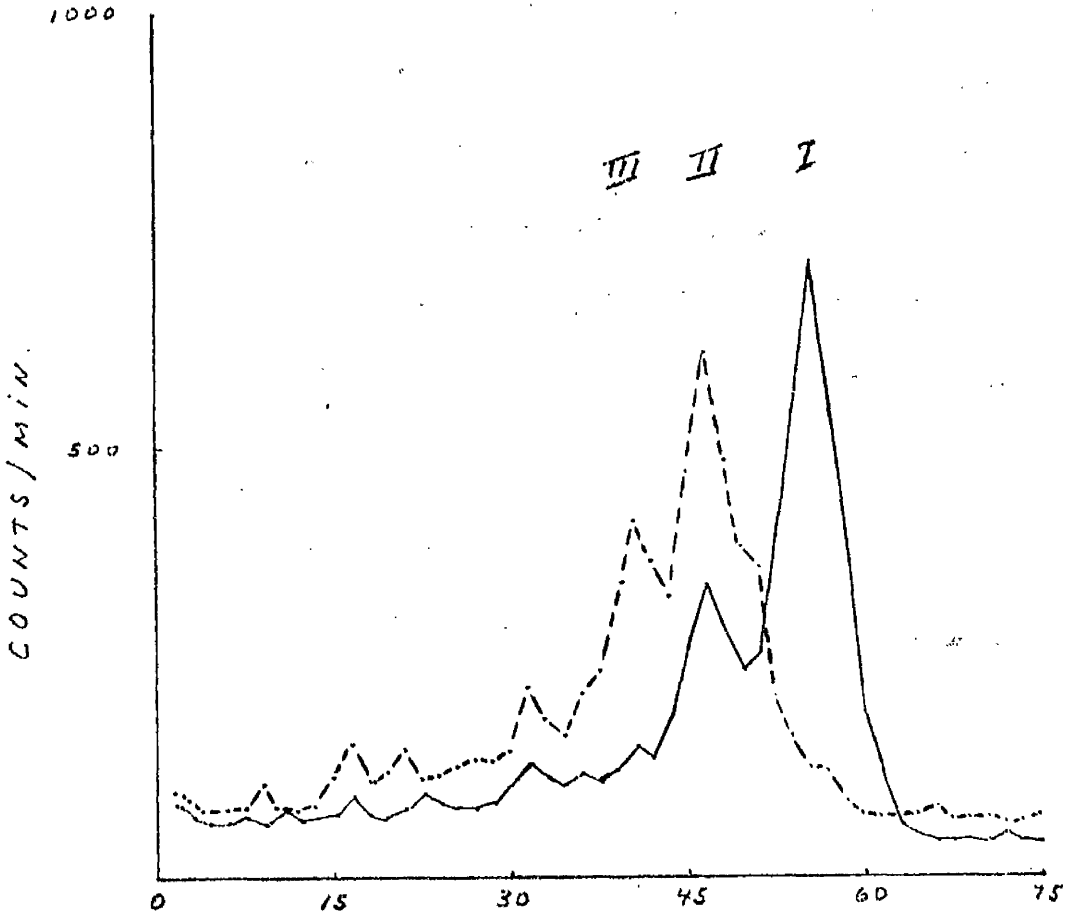

1000
500
COUNTS / MIN.
III
II
I
0
15
30
45
60
75
DISTANCE FROM ORIGIN : M.M.

Figure 41.

Relative migration of rat haemoglobin components in starch gel electrophoresis: Technical procedures as in Figure 40.

———— adult rat bone marrow Hb.

.-.-. 16-day foetal rat liver Hb.

FIGURE 41

III II I

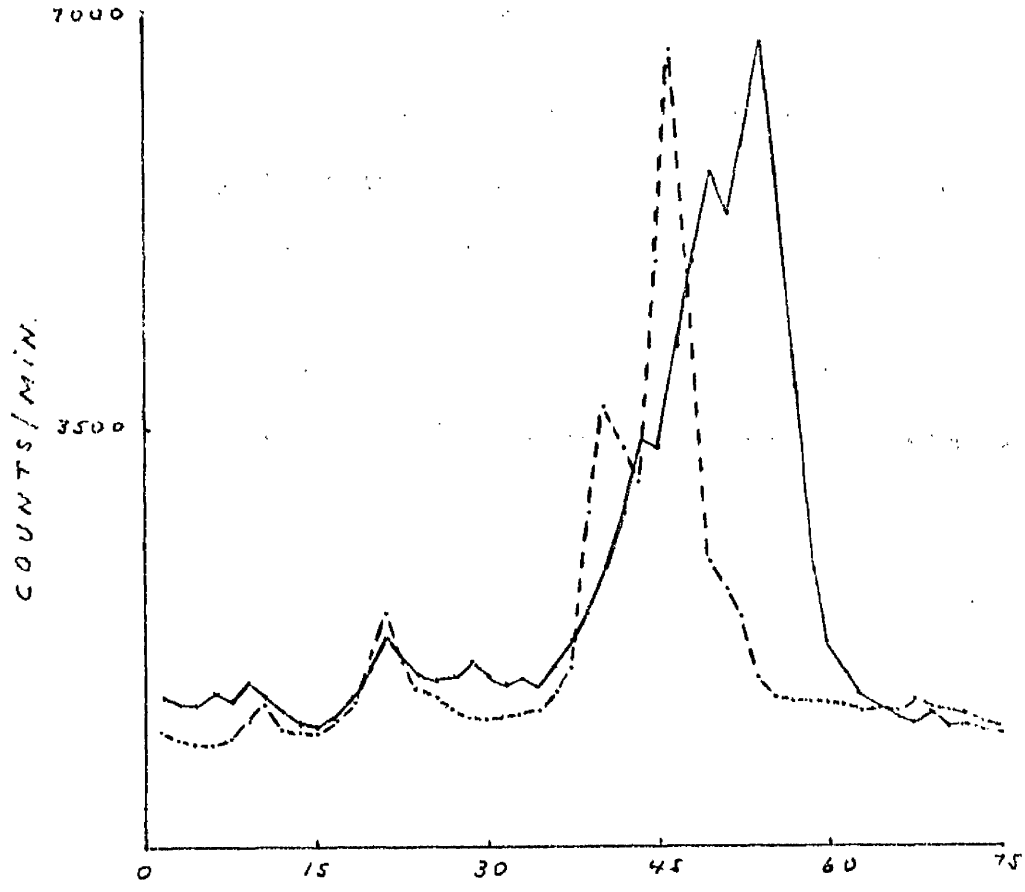


Figure 42.

Relative migration of rat haemoglobin components in starch gel electrophoresis: Technical procedures as in Figure 40.

——— adult rat bone marrow Hb.

.-.-. 15-day foetal rat liver Hb.

FIGURE 42

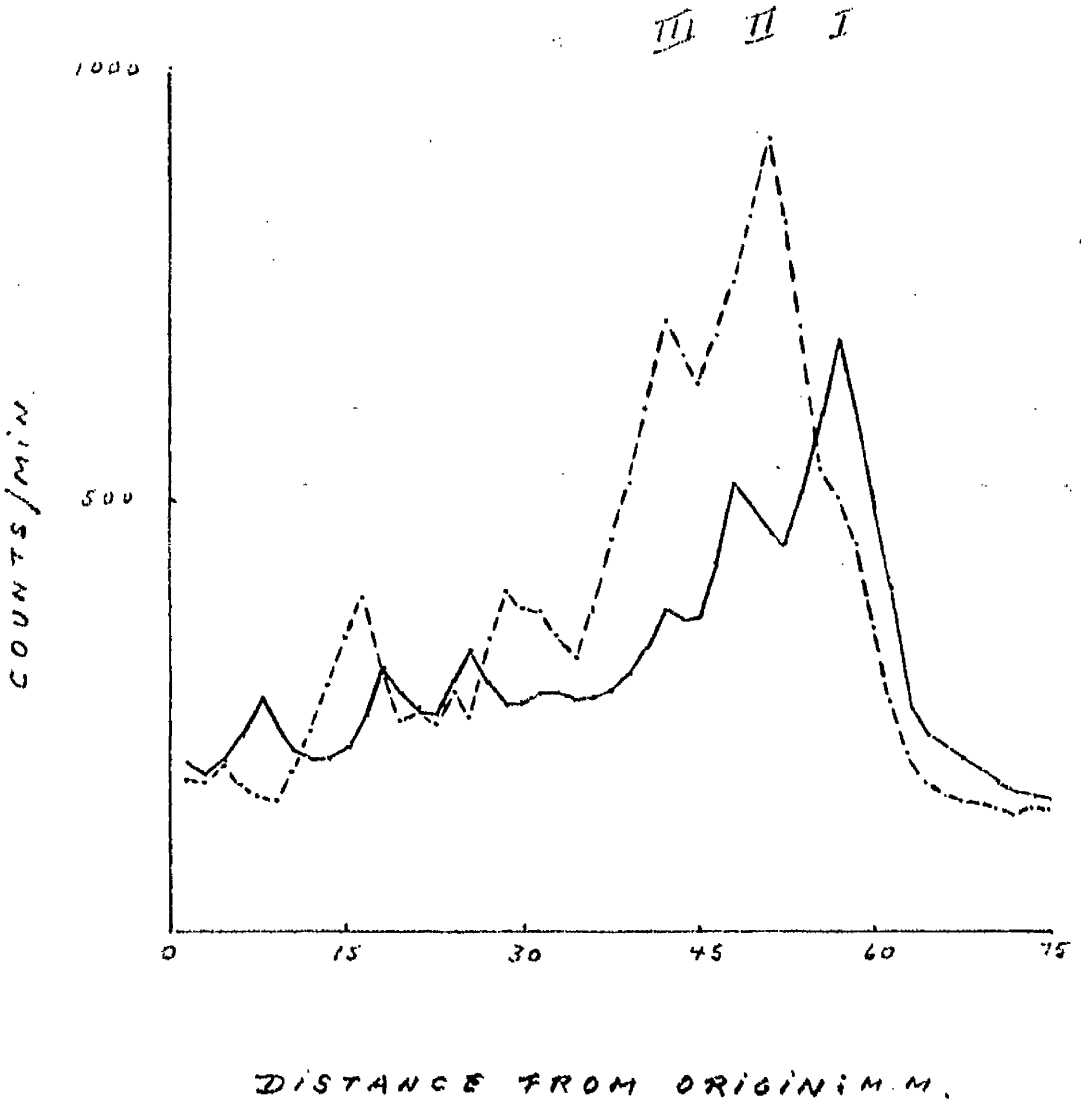

III II I
1000
500
COUNTS/MIN.
0
15
30
45
60
75
DISTANCE FROM ORIGIN IN M.M.

Figure 43.

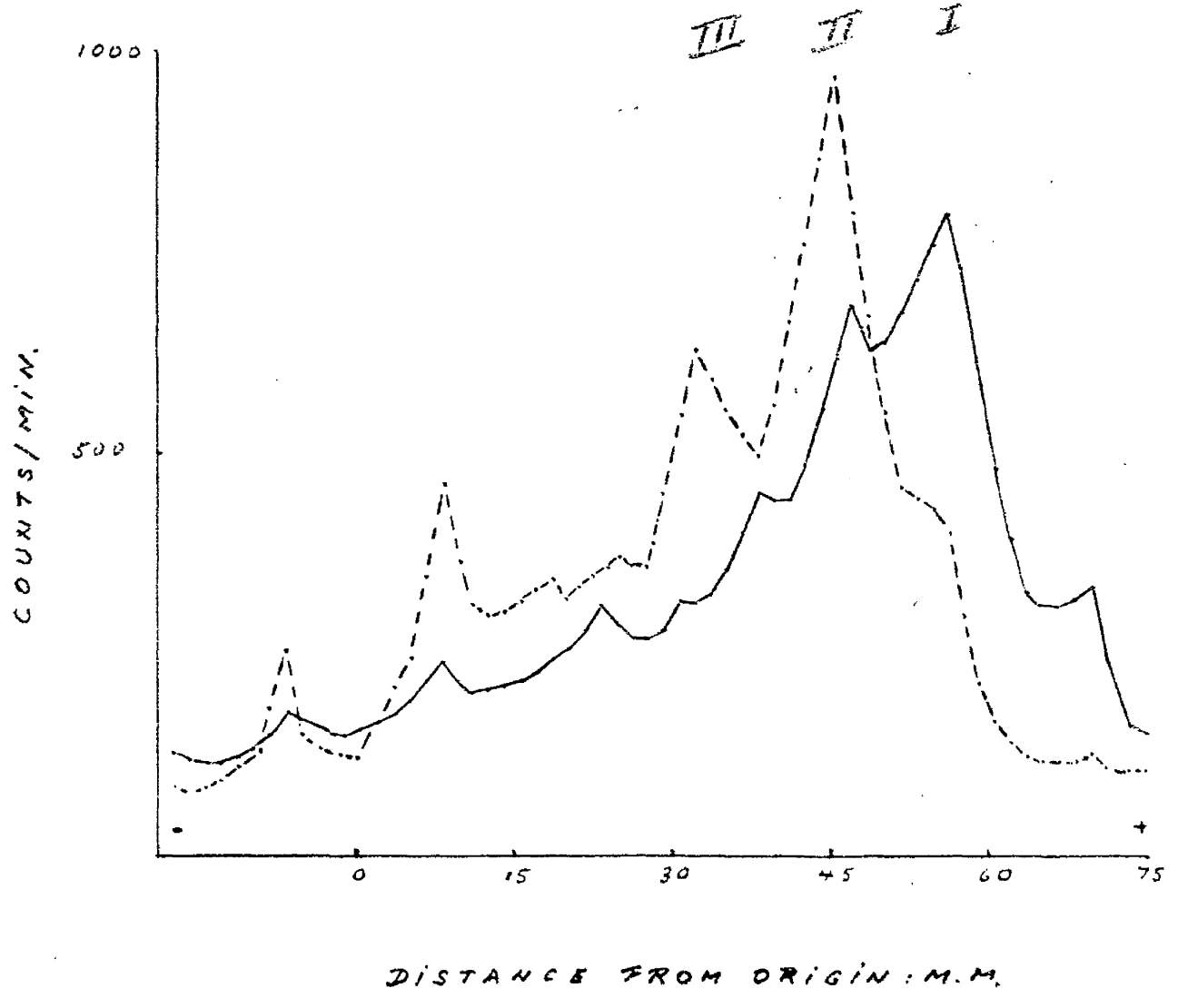
Relative migration of rat haemoglobin components in starch gel electrophoresis:

Technical procedures as in Figure 40, except that the continuous system of buffers of Smithies (1955) at low ionic concentration was used as described in the text.

_____ adult rat bone marrow Hb.

.-.-. 15-day foetal rat liver Hb.

FIGURE 43


III
II
I
1000
500
COUNTS/MIN.
-
+
0
15
30
45
60
75
DISTANCE FROM ORIGIN: M.M.

words, in A.R.B.M. haemoglobin there was a preponderance of component 1 (corresponding to position I) while in 15-day F.R.L. haemoglobin there was a preponderance of component 2 (position II). These criteria would hold good for any of the patterns of haemoglobin, regardless of variations in number and migration of the smaller components.

C. Finding the type of haemoglobin synthesized by heterologous (rat) spleen-recolonizing cells.

Having established the experimental conditions and techniques for obtaining purified labelled haemoglobins from rat cell recolonized spleens, and new criteria for differentiation between A.R.B.M. and F.R.L. haemoglobins, it was now possible to continue with the next stage of the experiment.

Irradiated experimental mice were transplanted with A.R.B.M. and F.R.L. cells, the recolonized spleens collected around the 13th day, the haemoglobins were labelled, purified by CMC, electrophoresed and counted as previously described. Table 13 shows the conditions under which the first experiment was carried out. Experimental groups 1 and 2 were transplanted with $30 \times 10^6$ cells of 15-day F.R.L. and A.R.B.M. respectively, the spleens collected on the 13th and 14th day, and

Table 13.

Conditions of experiments designed to determine the type of rat Hb synthesized in recolonized spleens of irradiated mice transplanted with rat haemopoietic cells.

| Exptl. Group | Sex | Age (mths) | Weight (g) | X-ray dose (R) | Transplant | | Spleen Collection (day) |
|---|---|---|---|---|---|---|---|
| | | | | | Type | No.of cells | |
| 1 | ♂ | 3 | 35 - 40 | 950 | 15d.FRL | $30 \times 10^6$ | 13 |
| 2 | ♂ | 3 | 35 - 40 | 950 | ARBM | $30 \times 10^6$ | 14 |

ARBM = adult rat bone marrow.
FRL = foetal rat liver.

the haemoglobins processed. Figure 44 shows the results obtained. The radioactivity peaks in position II were the most prominent in both cases, that is they both showed a characteristic foetal type of haemoglobin. However, a question arose: Were peaks in position II due to exogenous (rat) haemoglobin solely, or was there any endogenous (mouse) haemoglobin added?

To clarify this situation, the experimental haemoglobins were co-electrophoresed with a sample of adult mouse haemoglobin labelled *in vivo* with 10 $\mu$ $^{59}FeCl_3$ for 6 hours. Figure 45 shows these results. The summit of the peak corresponding to adult mouse haemoglobin migrated to an intermediate location between the summits of peaks in positions I and II; but its base overlapped both the area of peak in position I and in position II. This result suggested the possibility of aggregation of mouse endogenous haemoglobin with the exogenous (rat) haemoglobin in fractions in position II.

Other edperimental groups were set up and electrophoresed with different control haemoglobin samples. Table 14 shows the experimental conditions. Experimental groups 1 and 3 were transplanted with F.R.L. cells and group 2 with A.R.D.M. cells. Group 4 was irradiated with 600 R only and received no transplant to allow the formation of endogenous spleen colonies, so that endogenous

Figure 44.

Incorporation of $^{59}Fe$ into experimentally recolonized spleen haemoglobins.

Mice were irradiated with 950 R, and transplanted with 30 x $10^6$ cells of 15-day foetal rat liver or adult rat bone marrow. Experimental conditions in Table 13.

On the 13th day post-transplantation, confluent spleen colonies were isolated, disaggregated and cultured. The cells were incubated with $^{59}Fe$ for 8 hrs. Haemoglobin was prepared, purified by CMC chromatography and fractionated by starch gel electrophoresis (Poulik, 1957). Radioactivity incorporation was determined as described in the text.

——— experimental Hb from spleen recolonized by adult rat bone marrow cells.

.-.-. experimental Hb from spleen recolonized by 15-day foetal rat liver cells.

FIGURE 44

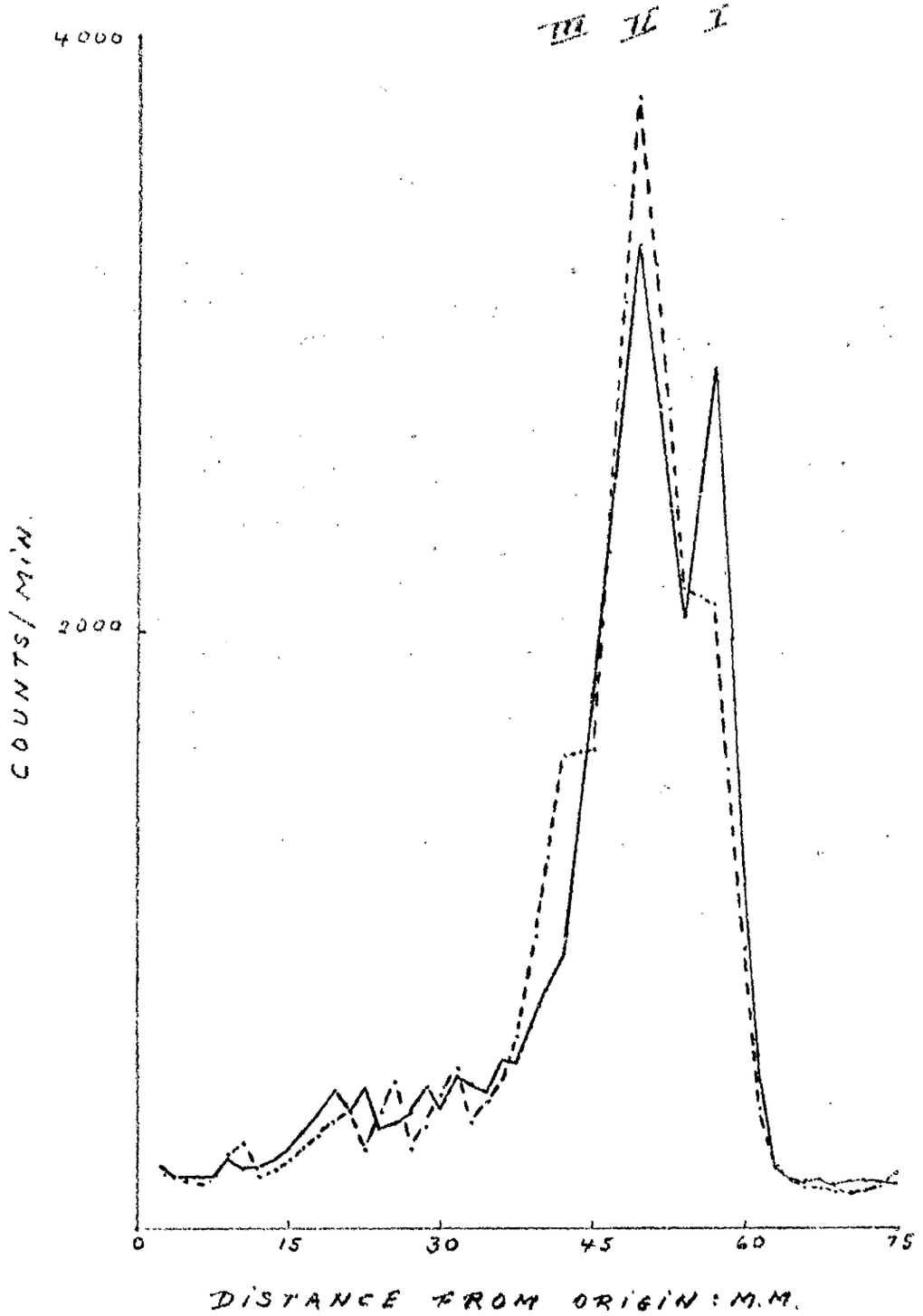

III IL I
4000
2000
COUNTS/MIN.
0
15
30
45
60
75
DISTANCE FROM ORIGIN: M.M.

Figure 45.

Relative migration of haemoglobin components in starch gel electrophoresis.

The experimentally recolonized spleen haemoglobins of Figure 44 were co-electrophoresed with adult mouse Hb, *in vivo* labelled with 10 µc $^{59}FeCl_3$ for 6 hours.

Technical procedures as in Figure 44.

——— experimental Hb from spleen recolonized by adult rat bone marrow cells.

·-·-· experimental Hb from spleen recolonized by 15-day foetal rat liver cells.

x——x adult mouse Hb.

Note: 1000 cpm corresponds to 200 cpm for the adult mouse Hb.

FIGURE 45

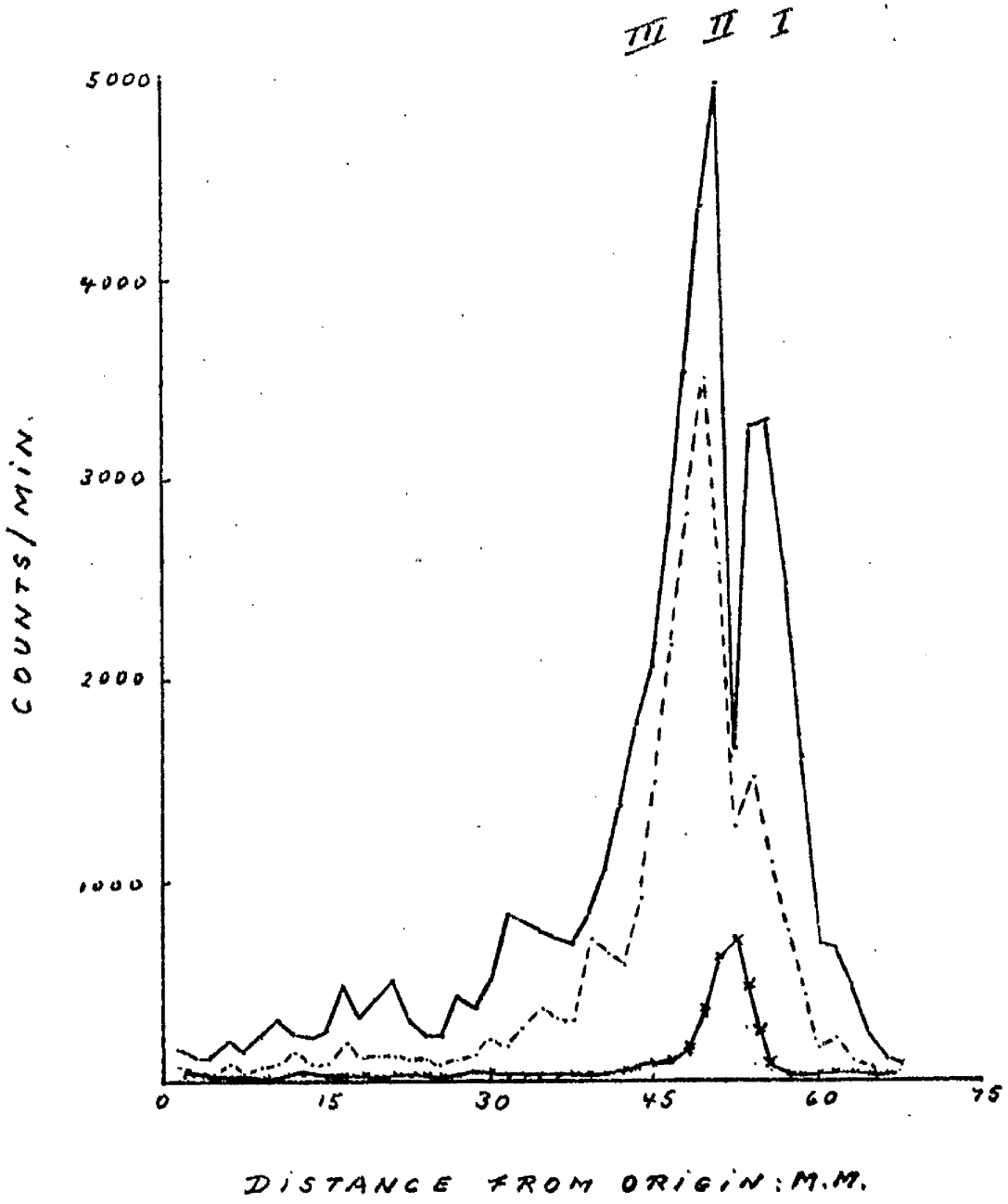

III II I
5000
4000
3000
2000
1000
COUNTS/MIN.
0
15
30
45
60
75
DISTANCE FROM ORIGIN: M.M.

Table 14.

Conditions of experiments designed to determine the type of rat Hb synthesized in recolonized spleens of irradiated mice transplanted with rat haemopoietic cells.

| Exptl. Group | Sex | Age (mths) | Weight (g) | X-ray dose (R) | Transplant | | Spleen Collection (day) |
|---|---|---|---|---|---|---|---|
| | | | | | Type | No.of cells | |
| 1 | ♂ | 3 | 35 - 40 | 950 | 15d.FRL | $20 \times 10^6$ | 14 |
| 2 | ♂ | 3 | 35 - 40 | 950 | ARBM | $15 \times 10^6$ | 14 |
| 3 | ♂ | 3 | 35 - 40 | 950 | 17d.FRL | $30 \times 10^6$ | 14 |
| 4 | ♂ | 3 | 35 - 40 | 600 | - | - | 11 |

ARBM = adult rat bone marrow.
FRL = foetal rat liver.

mouse haemoglobin could be labelled to serve as control by electrophoresing adjacent to the experimental haemoglobins, and mixed with them. Endogenous repopulation was achieved with a lower irradiation dose because experimental control animals very seldom survived the length of time necessary for spleen collection. Normal A.R.B.M. and F.R.L. labelled haemoglobins were also prepared for electrophoretic control purposes. Figure 46 shows these results. A.R.B.M. recolonized spleen haemoglobin had its peak at position I, slightly higher than the one in position II, while F.R.L. recolonized spleen haemoglobins gave their maximum peak in position II. However, the electrophoretic controls, shown in Figures 47 and 48, suggested the possibility of aggregation of mouse endogenous haemoglobin with peaks in position II. On the other hand, a new experimental group (conditions shown in Table 15) suggested the possibility of aggregation of mouse endogenous haemoglobin with peaks in position I, as shown in Figure 49.

Finding the relative electrophoretic migration of adult mouse haemoglobin.

Electrophoretic runs using different buffers were performed in order to establish the relative migration of adult mouse haemoglobin (A.M.Hb) compared with the main components of A and F rat haemoglobins. A.M.Hb

Figure 46.

Incorporation of $^{59}Fe$ into experimentally recolonized spleen haemoglobins.

Technical procedures as in Figure 44.
Experimental conditions in Table 14.

(a)

——— experimental Hb from spleen recolonized by adult rat bone marrow cells.

·-·-· experimental Hb from spleen recolonized by 15-day foetal rat liver cells.

(b)

——— experimental Hb from spleen recolonized by adult rat bone marrow cells.

·-·-· experimental Hb from spleen recolonized by 17-day foetal rat liver cells.

FIGURE 46 (a)

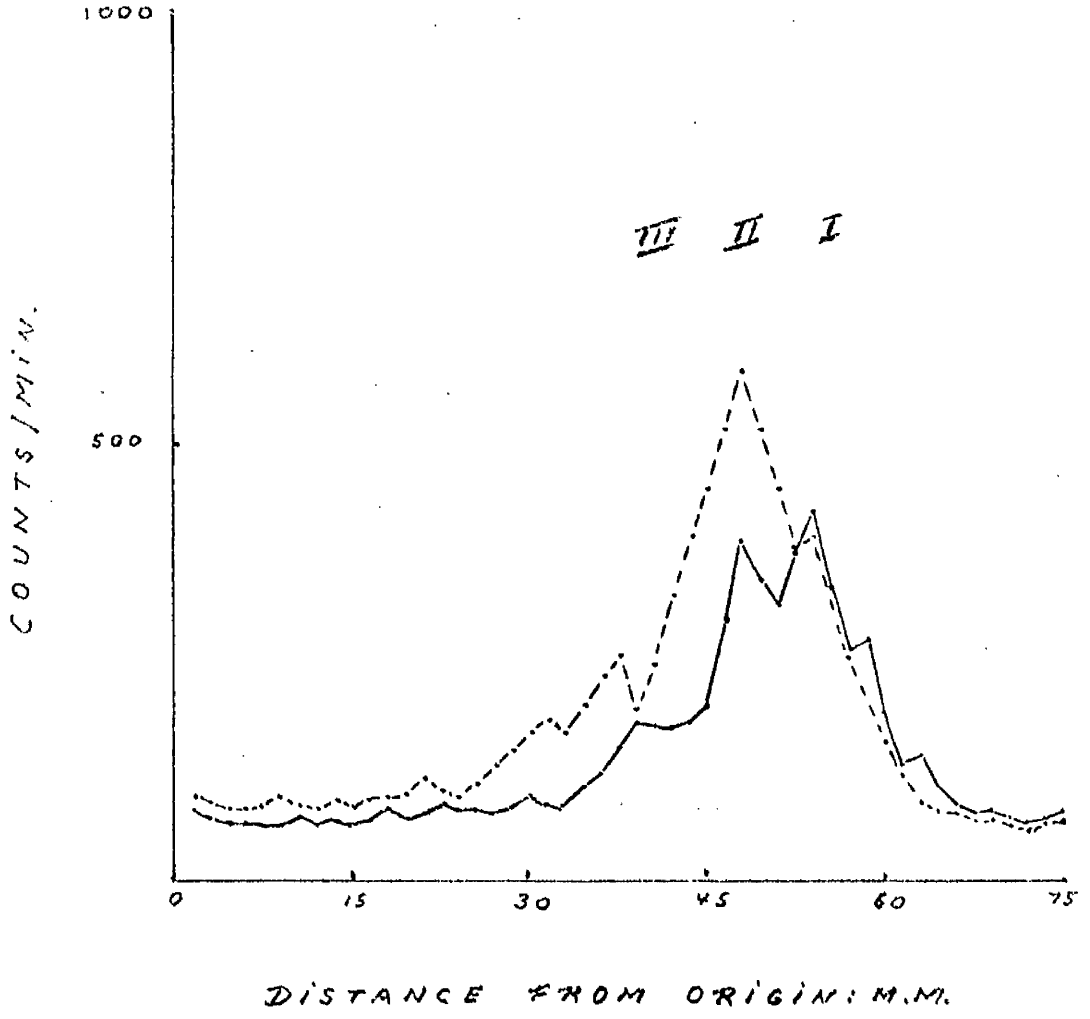

1000
500
III II I
COUNTS / MIN.
0
15
30
45
60
75
DISTANCE FROM ORIGIN: M.M.

FIGURE 46 (b)

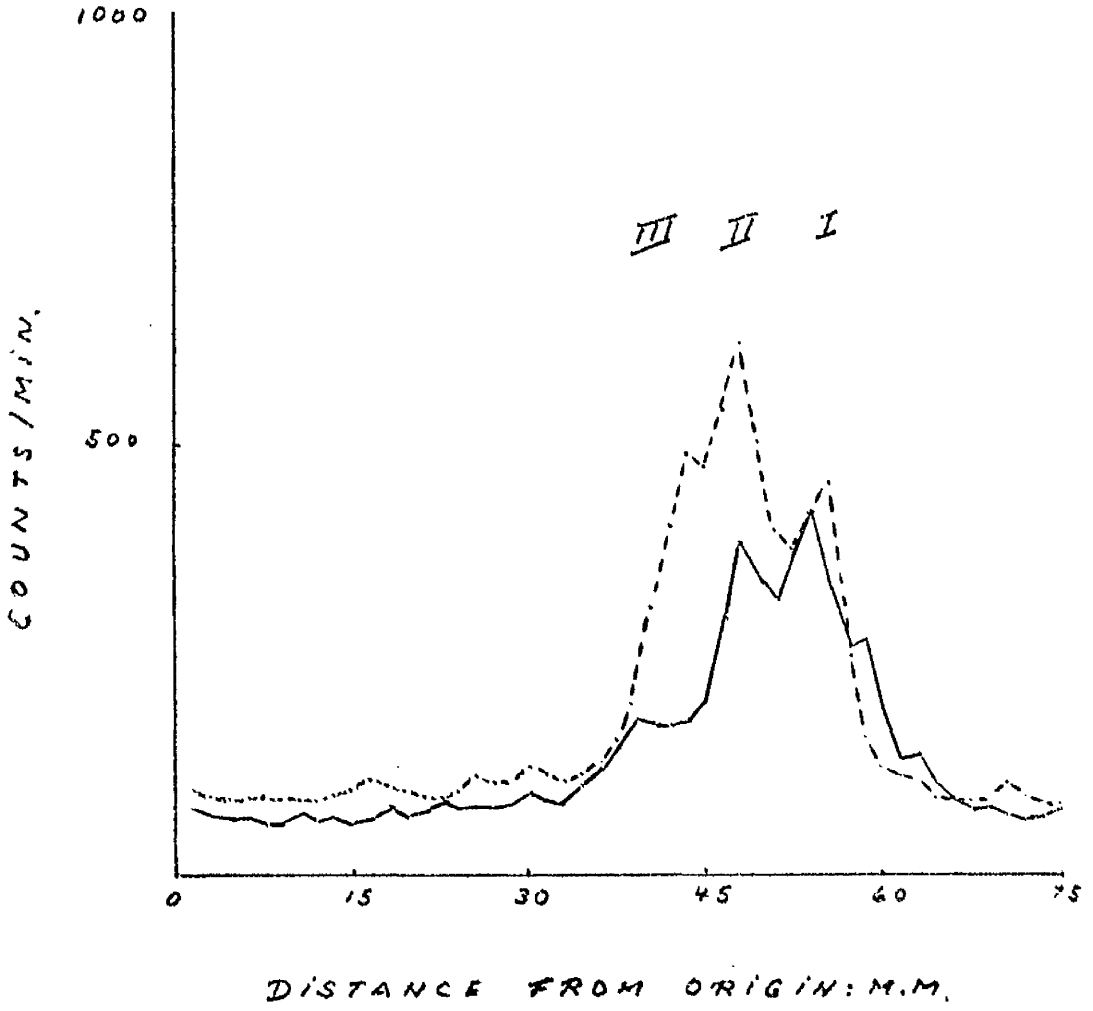

1000
500
COUNTS/MIN.
III
II
I
0
15
30
45
60
75
DISTANCE FROM ORIGIN: M.M.

Figure 47.

Relative migration of experimental haemoglobin components in starch gel electrophoresis.

The experimentally recolonized spleen haemoglobins of Figure 46 were mixed with endogenously recolonized spleen haemoglobin.

Technical procedures as in Figure 44.
Experimental conditions in Table 14.

(a)

——— experimental Hb from spleen recolonized by adult rat bone marrow cells + mouse Hb from spleen endogenously recolonized.

.-.-. experimental Hb from spleen recolonized by 15-day foetal rat liver cells + mouse Hb from spleen endogenously recolonized.

(b)

——— experimental Hb from spleen recolonized by adult rat bone marrow cells + mouse Hb from spleen endogenously recolonized.

.-.-. experimental Hb from spleen recolonized by 17-day foetal rat liver cells + mouse Hb from spleen endogenously recolonized.

FIGURE 47 (a)

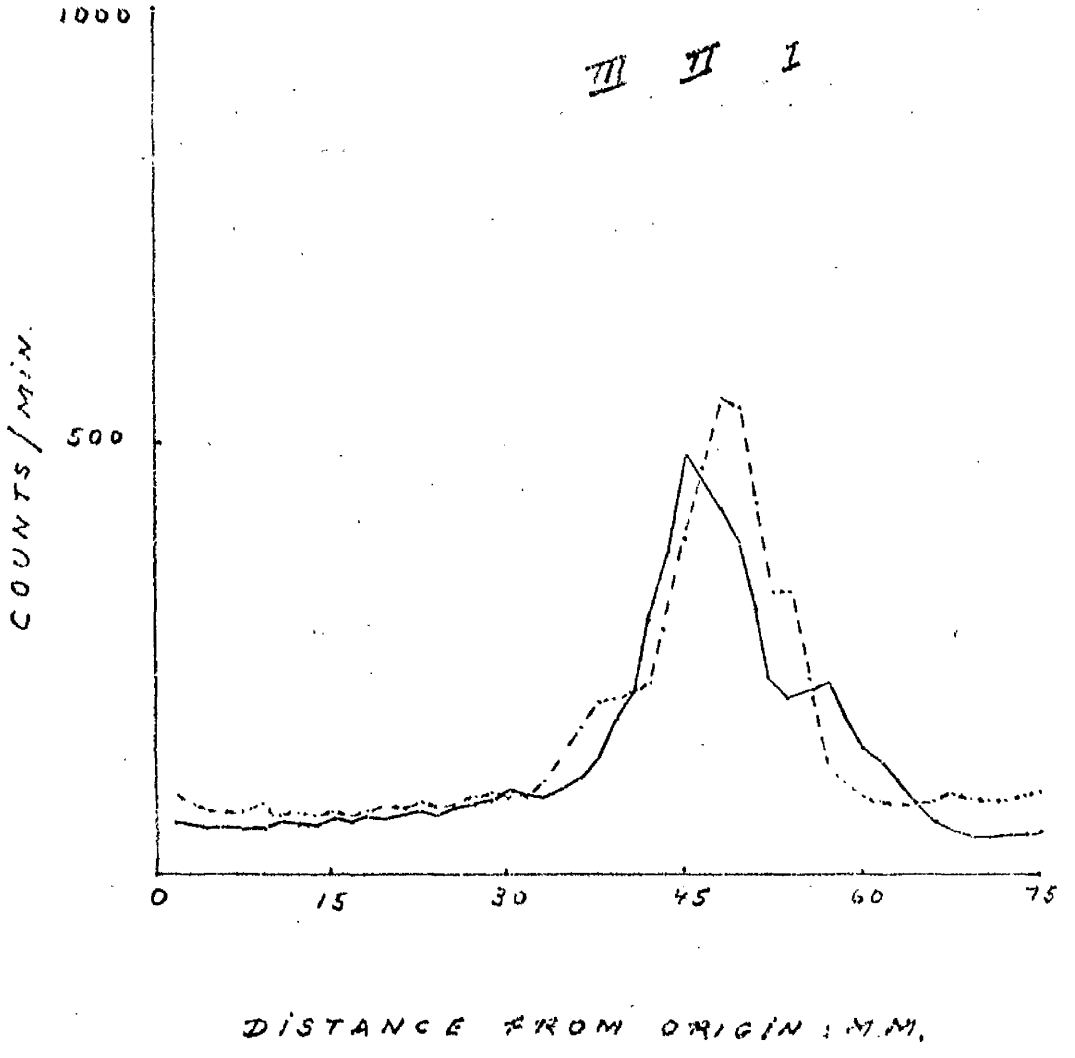

1000
500
COUNTS / MIN.
III
II
I
0
15
30
45
60
75
DISTANCE FROM ORIGIN : MM.

FIGURE 47 (b)

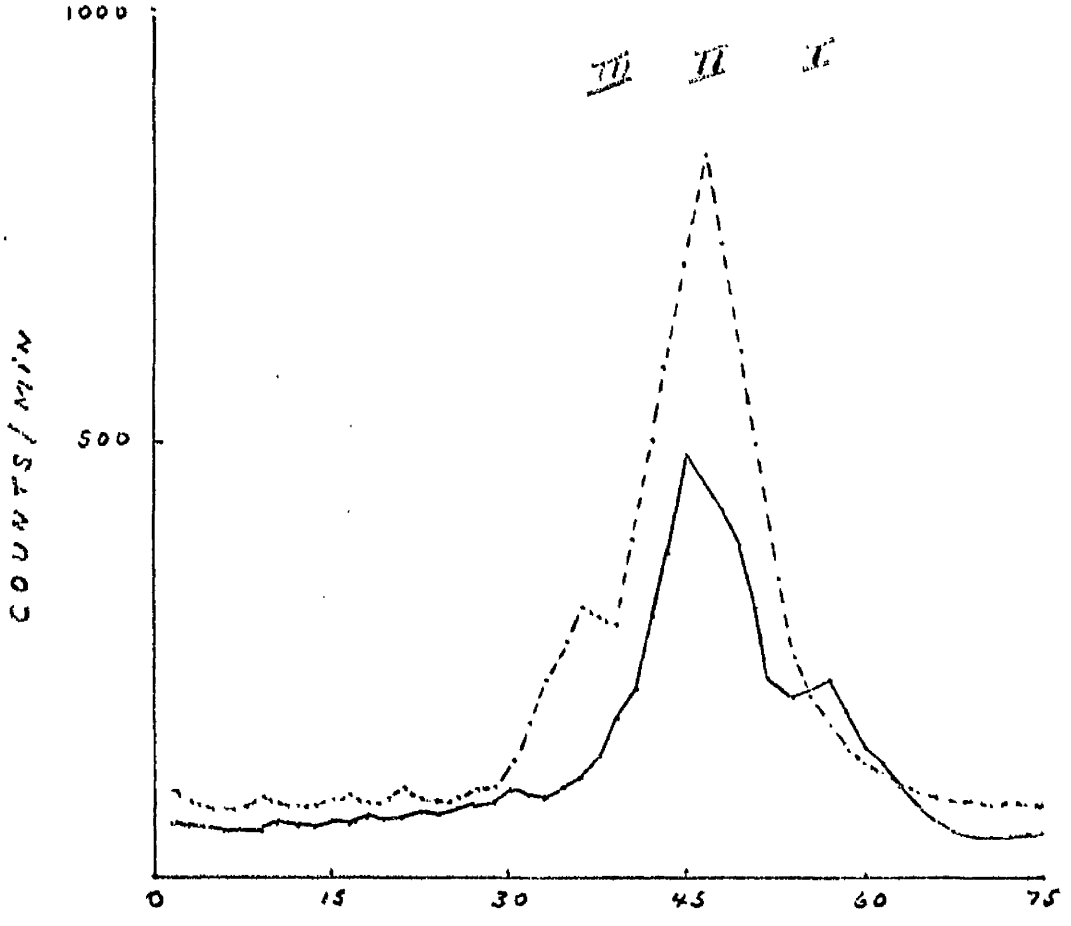

1000
500
COUNTS/MIN
III
II
I
0
15
30
45
60
75
DISTANCE FROM ORIGIN - MM

Figure 48.

Relative migration of experimental haemoglobin components in starch gel electrophoresis.

The experimentally recolonized spleen haemoglobins of Figure 46 were co-electrophoresed with endogenously recolonized spleen haemoglobin.

Technical procedures as in Figure 44.
Experimental conditions in Table 14.

——— experimental Hb from spleen recolonized by adult rat bone marrow cells.

·-·-· experimental Hb from spleen recolonized by 15-day foetal rat liver cells.

x——x mouse Hb from spleen endogenously recolonized.

FIGURE 48

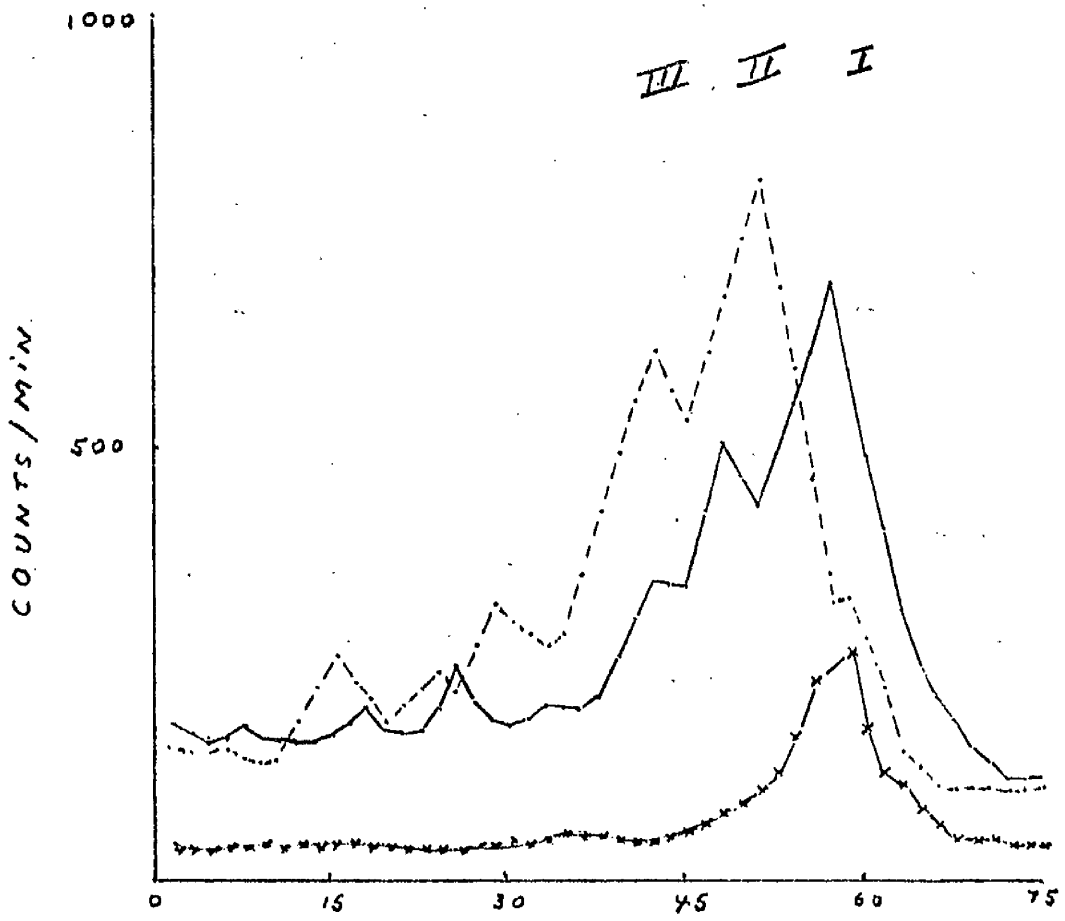

1000
500
III II I
COUNTS/MIN
0
15
30
45
60
75
DISTANCE FROM ORIGIN: M.M.

Table 15.

Conditions of experiments designed to determine the type of rat Hb synthesized in recolonized spleens of irradiated mice transplanted with rat haemopoietic cells.

| Exptl. Group | Sex | Age (mths) | Weight (g) | X-ray dose (R) | Transplant | | Spleen Collection (day) |
|---|---|---|---|---|---|---|---|
| | | | | | Type | No.of cells | |
| 1 | ♂ | 3 | 35 - 40 | 950 | 15d.FRL | $30 \times 10^6$ | 14 |
| 2 | ♂ | 3 | 35 - 40 | 950 | ARBM | $30 \times 10^6$ | 14 |
| 3 | ♂ | 3 | 35 - 40 | 620 | - | - | 14 |

ARBM = adult rat bone marrow.
FRL = foetal rat liver.

Figure 49.

Relative migration of experimental haemoglobins in starch gel electrophoresis.

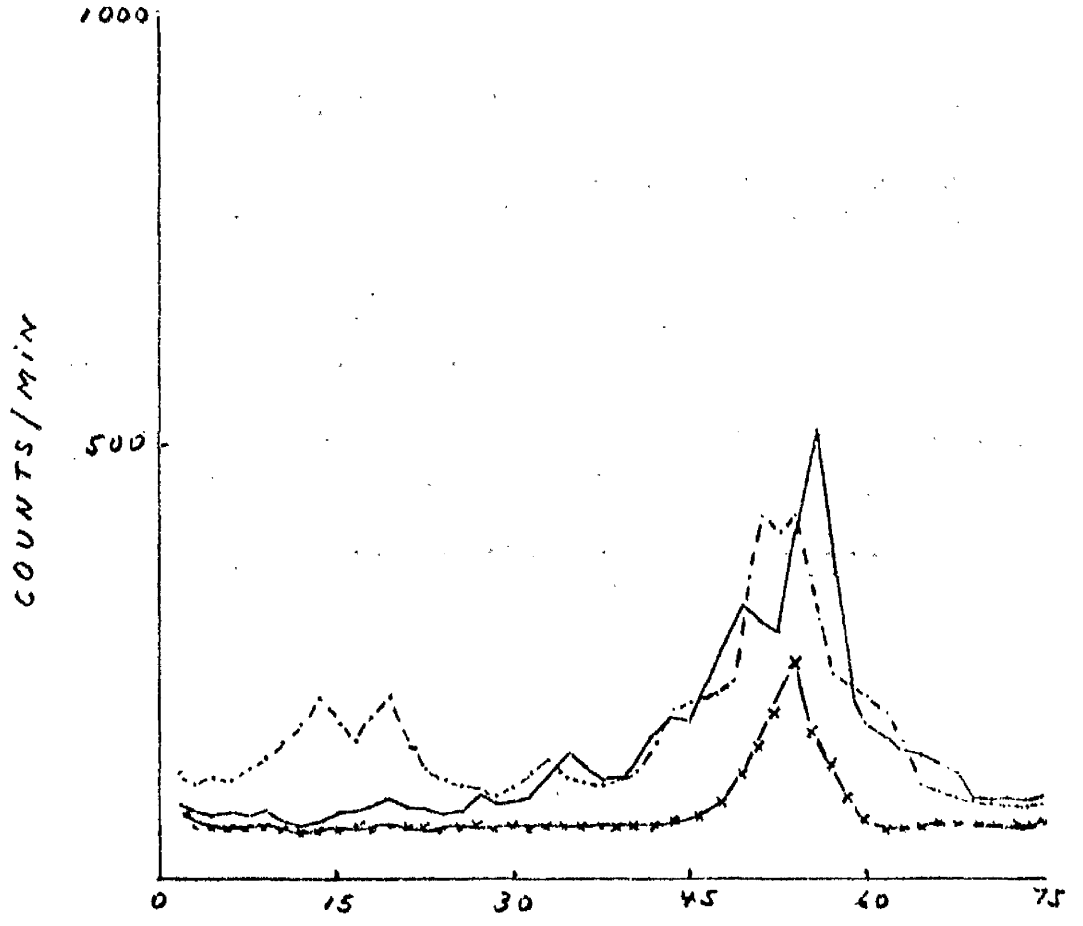
Technical procedures as in Figure 48.
Experimental conditions in Table 15.

——— experimental Hb from spleen recolonized by adult rat bone marrow cells.

·-·-· experimental Hb from spleen recolonized by 15-day foetal rat liver cells.

x——x mouse Hb from spleen endogenously recolonized.

FIGURE 49



usually migrated slightly behind, but overlapping the major component of adult rat haemoglobin. Photographs in the Heterogeneity of Rat Haemoglobin section illustrate these results.

On the other hand, the radioactivity profile of A.M.Hb overlapped the areas corresponding to both positions I and II, although its summit was located slightly behind that of the peak in position I of A.R.B.M. haemoglobin and ahead of the peak in position II of F.R.L. haemoglobin. Figures 45 and 48 illustrate these results.

Double labelling haemoglobin electrophoresis and counting.

In order to recognise the positions of the summits of radioactivity peaks of the major components of the experimental (A.R.B.M. and F.R.L.) haemoglobins in relation to the summit of the mouse haemoglobin peak, in spite of any overlapping of their radioactivity areas, a double labelling device was tested. An aliquot of the experimentally recolonized spleen haemoglobin labelled with $^{59}Fe$ was mixed with adult mouse haemoglobin labelled with $^{3}H$, electrophoresed, and counted for both isotopes, and their radiactivity profiles obtained. Due to the overlapping of the energy spectra of $^{59}Fe$ and $^{3}H$ and to the effect of different background radioactivity

on the different parts of the spectra, and because of the quenching effect of the haemoglobin-containing starch, a counting correction was established.

To obtain $^{3}H$ labelled haemoglobin, adult mice were made anaemic by intraperitoneal injection of 0.07 ml of a 25 mg/ml solution of phenylhydrazine chloride daily for 5 days. On the 7th day blood was collected by cardiac puncture. The haemoglobin synthesized in reticulocytes was labelled *in vitro* with $^{3}H$-phenylalanine (The Radiochemical Centre, Amersham, England) following the procedure of Borsook *et al.* (1957). The $^{3}H$ labelled haemoglobin was obtained and purified on carboxymethyl cellulose, as previously described.

Irradiated mice were transplanted with A.R.B.M. and F.R.L. cells, and the recolonized spleen haemoglobins labelled with $^{59}Fe$ and purified. Table 16 shows the experimental conditions. The experimental haemoglobins were co-electrophoresed with aliquot mixtures of experimental haemoglobin plus tritium-labelled mouse haemoglobin and counted for single and double isotope accordingly. Figures 50 and 51 are typical examples of the results obtained. The $^{3}H$ labelled mouse haemoglobin migrated with the components in position I of the experimental haemoglobins.

This finding and the comparative study of the

Table 16.

Conditions of experiments designed to determine the type of rat Hb synthesized in recolonized spleens of irradiated mice transplanted with rat haemopoietic cells.

| Exptl. Group | Sex | Age (mths) | Weight (g) | X-ray dose (R) | Transplant | | Spleen Collection (day) |
|---|---|---|---|---|---|---|---|
| | | | | | Type | No.of cells | |
| 1 | ♂ | 3 | 35 - 40 | 950 | 15d.FRL | $30 \times 10^6$ | 14 |
| 2 | ♂ | 3 | 35 - 40 | 950 | ARBM | $30 \times 10^6$ | 13 |
| 3 | ♂ | 3 | 35 - 40 | 950 | 15d.FRL | $30 \times 10^6$ | 15 |
| 4 | ♂ | 3 | 35 - 40 | 950 | ARBM | $20 \times 10^6$ | 11 |
| 5 | ♂ | 3 | 35 - 40 | 950 | 15d.FRL | $25 \times 10^6$ | 11 |

ARBM = adult rat bone marrow.
FRL = foetal rat liver.

Figure 50.

Incorporation of $^{59}Fe$ into experimentally recolonized spleen haemoglobins.

Technical procedures as in Figure 44.
Experimental conditions in Table 16.

——— experimental Hb from spleen recolonized by adult rat bone marrow cells.

·–·–· experimental Hb from spleen recolonized by 15-day foetal rat liver cells.

● adult mouse Hb marker.

FIGURE 50

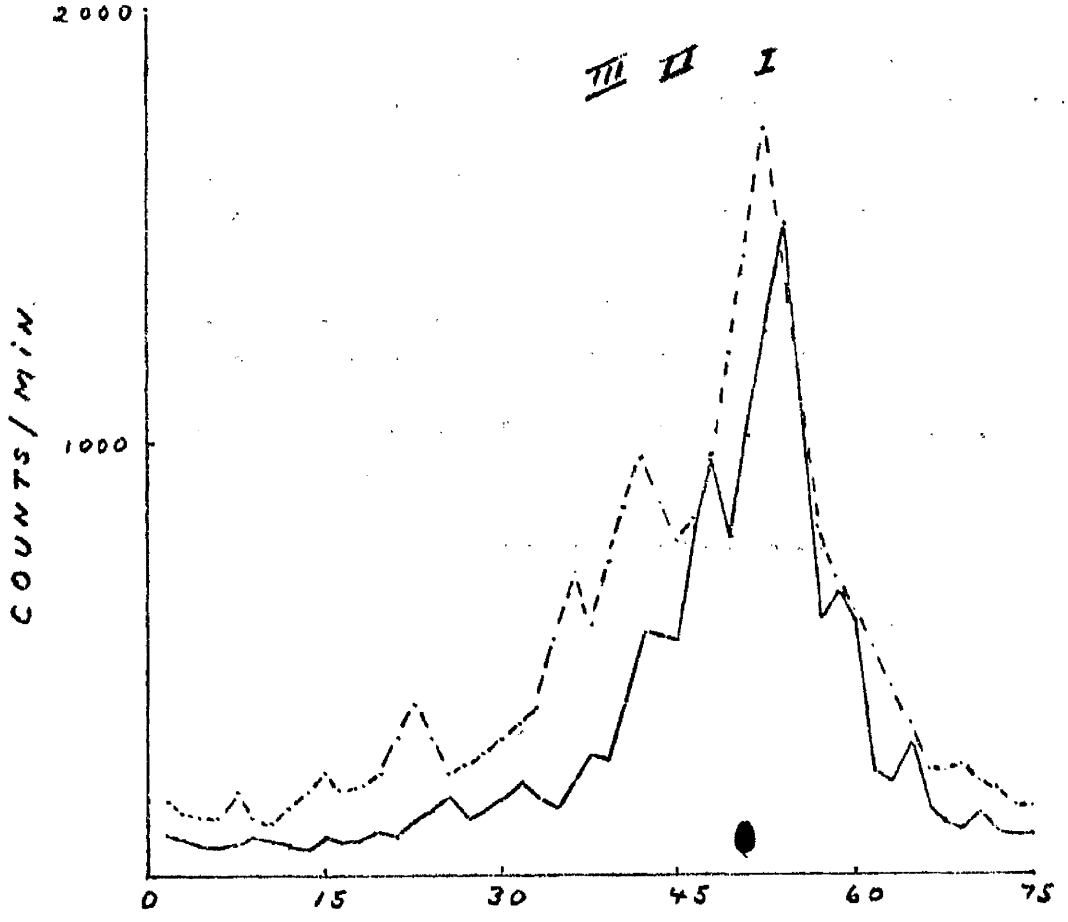

2000
1000
COUNTS / MIN.
III II I
0
15
30
45
60
75
DISTANCE FROM ORIGIN : M.M.

Figure 51.

Relative migration of experimental haemoglobins in starch gel electrophoresis.

The experimental spleen haemoglobins of Figure 50 were mixed with $^{3}H$ labelled adult mouse haemoglobin and fractionated by starch gel electrophoresis. Incorporation of $^{59}Fe$ and $^{3}H$ were determined as described in the text.

Technical procedures as in Figure 44.
Experimental conditions in Table 16.

(a)

——— $^{59}Fe$ labelled experimental Hb from spleen recolonized by adult rat bone marrow cells.

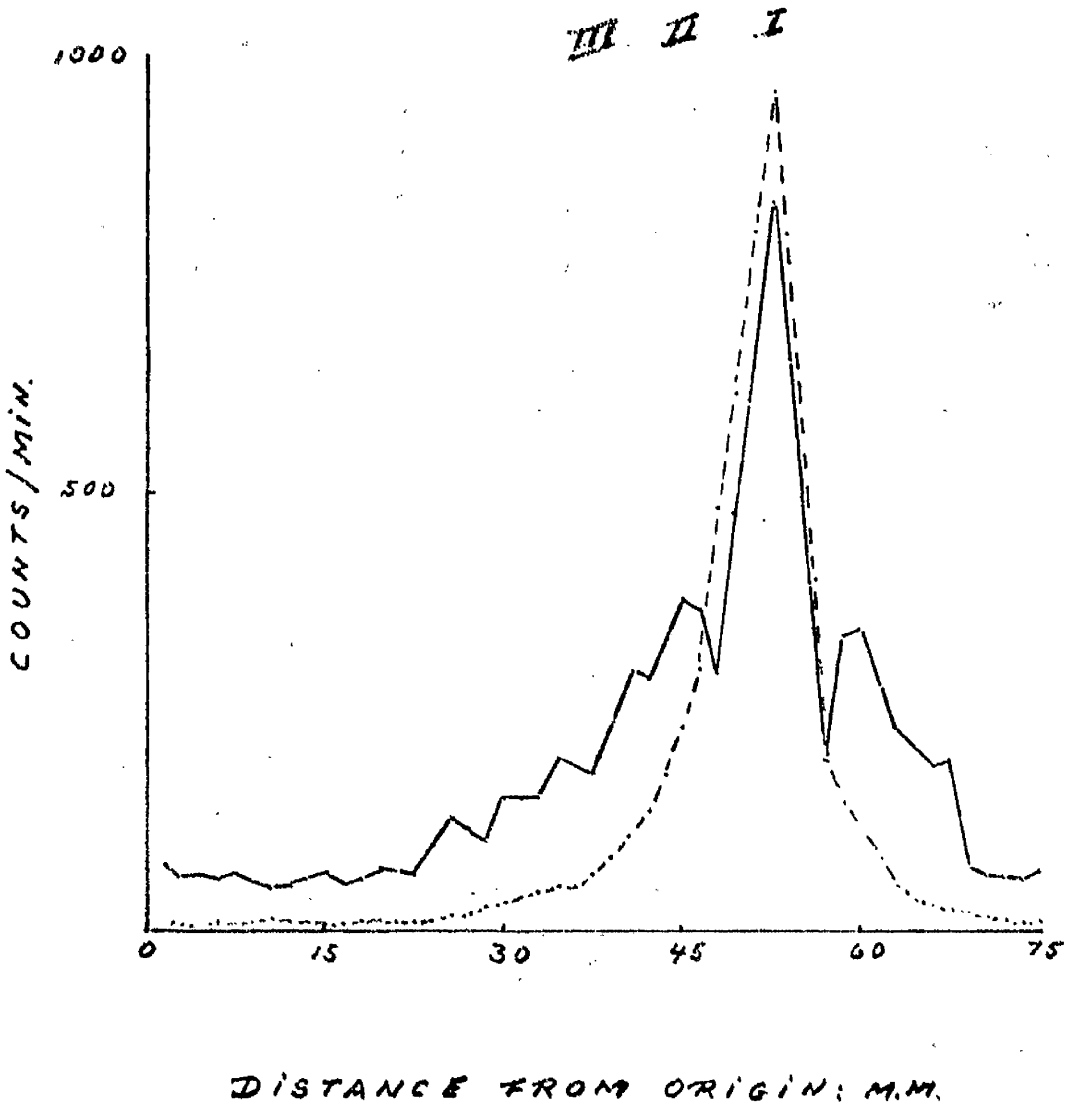
.-.-. $^{3}H$ labelled adult mouse Hb.

(b)

——— $^{59}Fe$ labelled experimental Hb from spleen recolonized by 15-day foetal rat liver cells.

.-.-. $^{3}H$ labelled adult mouse Hb.

FIGURE 51 (a)


III II I
1000
500
COUNTS/MIN.
0
15
30
45
60
75
DISTANCE FROM ORIGIN: M.M.

experimental haemoglobin profiles suggested that endogenous haemoglobin was synthesized in the experimental spleens and usually aggregated with fractions in position I. The possibility of differentiating between adult and foetal type experimental haemoglobins was therefore lost.

## D. Discussion and Conclusions.

1. Electrophoretic heterogeneity of rat haemoglobin, and Haemoglobin interactions and aggregations.

2. Synthesis of haem-proteins, and Inadequacy of haem synthesis as haemoglobin synthesis assay.

3. Origin of the iron contaminating the haemoglobin solution, and Purity of haemoglobin isolated by carboxymethyl cellulose.

4. Recolonization of irradiated mouse spleens by rat haemopoietic tissues, and Haemoglobin types synthesized by the recolonized spleens.

5. Influence of the splenic microenvironment on the production of exogenous colonies.

### 1. Electrophoretic heterogeneity of rat haemoglobin, and Haemoglobin interactions and aggregations.

The different patterns obtained upon electrophoresis of adult and foetal rat haemoglobins, both in regard to number of bands and to their relative amount, created a problem in connection with the differential characterization of experimental haemoglobins. The most

commonly observed pattern of adult rat haemoglobin was very similar to the pattern reported by Brada and Tobiška (1964) fractionated by carboxymethyl cellulose column chromatography. The other patterns found were also in accord with the different patterns reported by other workers (French and Roberts, 1965; Brdička and Šulc, 1965; Brdička, 1966) who favoured the idea that this is a genetically controlled heterogeneity. However, it has been reported that extra bands may be found due to the formation of disulphide bonds between haemoglobin molecules (Riggs, 1965) or between haemoglobin and glutathione (Baglioni, 1963). Addition of dithiothreitol, a -SH group reducing agent (Cleland, 1964), did not show any effect on the number of haemoglobin fractions. Addition of mercaptoethanol to reduce the disulphide bridges was also found to have no effect (Hunter, 1968).

The presence of interactions and aggregations among the different haemoglobin fractions was demonstrated in some of the experiments, and these may have accounted for some of the variations noted in relative amounts of each component. They may also have accounted even more for the absence of small components in some of the samples. Exposure to oxygen seemed to be the major factor responsible for the appearance of these

aggregations. Other workers have also suggested that the interaction effects on separation patterns are due to exposure to oxygen, and are dependent on glutathione concentration and thiol interaction (Shaw, 1969). By the same token, and because of oxidation, the possibility that the minor leading peaks of some haemoglobin samples (such as peak 1') could have been derived from the main adjacent fraction, similar to the "shadow" bands of globin chains reported by Garrick, Balzer and Charlton (1970), should not be disregarded.

Haemoglobin per se is such an unstable component that interaction effects in haemoglobins actually represent equilibria (Wyman, 1964). Even in physiological conditions haem groups do exchange among intact haemoglobin molecules (Bunn and Jandl, 1968) which complicates the interpretation of experimental studies.

The spontaneous precipitation of rat haemoglobin fractions was another factor that led to the appearance of false electrophoretic patterns, and in this respect the results obtained are in disagreement with the findings of Hunter (1968) who reported no effect of haemoglobin storage on these patterns.

2.- Synthesis of haem-proteins and Inadequacy of haem synthesis as haemoglobin synthesis assay.

One of the most important findings obtained during

the experimental development of the present work was the demonstrable synthesis of non-haemoglobin haem-proteins along with the synthesis of haemoglobin in cultures of foetal rodent liver cells and rodent marrow cells, and also *in vivo*. The amount of these haem-containing proteins (mainly catalase) synthesized was variable and was possibly associated with different factors connected with the control of pH and oxygen tension.

Since the haem extracted by acid methyl ethyl ketone (Teale, 1959) has been adopted as an indication of haem-synthesis in rat marrow cell cultures, and since this haem synthesis assay was considered as equivalent to the synthesis of haemoglobin (Krantz, Gallien-Lartigue and Goldwasser, 1963; Gallien-Lartigue and Goldwasser, 1964) the haem synthesis assay has been widely used as a haemoglobin synthesis assay in cultures of rodent bone marrow and foetal liver cells (Cole and Paul, 1966; Cole, Hunter and Paul, 1968; Hunter and Paul, 1969). However, from the results obtained in the present experimental work, it was clearly shown that haem-proteins other than haemoglobin are synthesized both *in vitro* and *in vivo*. Hence the haem synthesis assay is inadequate as an assay for haemoglobin synthesis unless optimal conditions are achieved both *in vivo* and

*in vitro* that will minimise the synthesis of catalase and haem-proteins of catalase activity to a negligible amount without interfering one way or another with the synthesis of haemoglobin and/or the cleavage of Fe from compartments other than haemoglobin.

On the other hand, the presence and purity of haemoglobin were controlled by the optical absorption in the region of the Soret band in the original experimental work, establishing the so-called adequacy of haem synthesis assay/for haemoglobin synthesis as assay (Gallien-Lartigue and Goldwasser, 1964). This method has a fundamental error because of the fact that the absorption in the Soret region (400-425 mμ) is due to the presence of the porphyrin ring in the haem group (Prankerd, 1961). Hence all haem-containing proteins show bands of absorption in this region (White, Handler and Smith, 1959; Deisseroth and Dounce, 1969). It is impossible, therefore, to distinguish between haemoglobin and other haem-proteins by the absorption in the Soret band.

Since absorption in the region of 280 mμ is due to the presence of the aromatic amino acids tyrosine and tryptophane (White, Handler and Smith, 1959) rather more useful information is given by the ratio of absorptions at 405 and 280 mμ.

3.- Origin of the iron contaminating the haemoglobin solution and Purity of haemoglobin isolated by carboxy-methyl cellulose.

It has been reported that lysis of *in vitro* labelled canine marrow cells by means of distilled water (pH 5.6 to 6.0) splits off iron from several different compartments so that the haemolysate contains the following proportions of iron: 29% from haemoglobin, 33% from microsomes, 19% from stroma and 19% from ferritin, other haem-proteins, and as low molecular weight iron. Differences in these relative proportions are due to the nature of the lysing vehicle, the degree of lysis achieved, the time of incubation, and other factors (Primasigh and Thomas, 1968). Catalase and lactate dehydrogenase, together with aldolase, are liberated in the process of erythrocyte lysis (Determann, 1968). Liver and erythrocytes are especially rich in catalase (Sargent, 1967; Summer and Somers, 1947; Dixon and Webb, 1966; White, Handler and Smith, 1959; West and Todd, 1951). In the rat liver, 40% of the microbody protein is catalase (Fahimi, 1968). Rat liver synthesizes 28 µg of catalase per gram of liver weight, which implies a replacement of 2.25% of the total liver catalase per hour (Neuberger and Richards, 1964). Transferrin not only becomes attached to the cell membrane of the

erythroblasts (Nissim, 1966) but actually penetrates the reticulocyte cell membrane (Morgan and Appleton, 1969). Lysates of bone marrow and foetal liver cells are, no doubt, prone to contain these haem proteins.

From the results obtained in the present work, it was shown that haem-containing proteins are only partially precipitated by organic solvents such as chloroform. This has been acknowledged by other workers. Other haem-containing proteins may remain in the aqueous haemoglobin solution after chloroform extraction (Hunter, 1968). Catalase and cytochrome are more stable in the presence of chloroform than is haemoglobin itself (Boyer, Lardy and Myrback, 1963; Hunter, 1968); but, since cytochrome 'c' migrates towards the cathode at pH 8.6 while haemoglobin migrates towards the anode, and catalase, like haemoglobin, migrates towards the anode (Hunter, 1968), this would leave catalase as the most likely contaminant of haemoglobins extracted by chloroform.

The differential radioactivity peak of the foetal rat haemoglobin described by Hunter and Paul (1969) then seems to be due to contamination of the haemoglobin solution by catalase. Its elution from the haemoglobin solutions in the first fractions obtained by carboxymethyl cellulose column chromatography, the spectrophotometric absorption curves of hydrogen peroxide degradation, and

the positive catalase activity shown in starch gel electrophoresis stained with acidic potassium iodide-hydrogen peroxide, confirm its nature. On the other hand, it has been reported that the purification of haemoglobin by carboxymethyl cellulose is still incomplete, due to its inability to separate a minor non-haem protein component (Tavill et al., 1968). If that is so, its presence did not have any effect on the radioactivity profiles. A possible effect of carboxymethyl cellulose on the stability of dimers is still unresolved (Tavill et al., 1968).

4. Recolonization of irradiated mouse spleens by rat haemopoietic tissues and Haemoglobin types synthesized by the recolonized spleens.

Injections of rat bone marrow were found to prolong the survival of irradiated mice (Congdon and Lorenz, 1954). Repopulation of irradiated mouse marrow cavities by rat marrow cells capable of proliferation has been reported by Nowell et al. (1956). Transplants of 19- and 20-day old rat foetal liver in $W^{V}W^{V}$ anaemic mice produced a totally exogenous repopulation of the marrow cavities and 99% of the spleen, and the presence of donor type haemoglobin in peripheral blood (Seller, 1968).

In the present experiments, in spite of the antigenic disparity between donor and host, recolonization of mouse

spleen was achieved by transplanting adult rat marrow and foetal rat liver cells. The time lapse between transplantation and spleen collection for labelling and study of haemoglobin type, which was usually between 13 and 15 days, suggests that proliferation of rat haemopoietic cells indeed took place in competition with the endogenous erythrocytic precursors that survived irradiation.

The presence of haemoglobin interactions, and the apparent aggregation of endogenous mouse haemoglobin with the experimentally recolonized spleen haemoglobins did not allow the drawing of a clearcut conclusion, as far as type of exogenous haemoglobin synthesized was concerned. However, by comparative analysis of the results obtained it appeared that the transplanted haemopoietic cells continued to function according to an already determined haemoglobin synthesis pathway.

From this tentative conclusion it might be suggested that there are two types of haemopoietic stem cells, one type destined to originate erythrocytic cells synthesizing adult type haemoglobin, and the other destined to originate erythrocytic cells synthesizing foetal type haemoglobin. The problem is not so simple, however, because the mechanism of haemoglobin synthesis is still unknown and moreover the identity of the colony

forming cell and the haemopoietic stem cell is still not established.

With regard to the first issue, it might well be that the single haemopoietic stem cell, having a full DNA complement capable of a complete range of cellular functions, produces a messenger for the synthesis of one type of haemoglobin or another, according to the signals it receives. This is supported by the fact that single erythrocytes may possess haemoglobin F and haemoglobin A as well. Kleinhauer and Betke (1961) and Mitchener, Thompson and Huisman (1961) demonstrated the presence of both haemoglobin F and haemoglobin A in single red cells of patients suffering from sickle cell anaemia and thalassaemia major, by elution with citric acid phosphate buffer at pH 3.3 (Betke and Kleinhauer, 1958). Hosoi (1965), using a fluorescent antibody technique, showed that erythrocytes containing haemoglobin F consisted of haemoglobin A as well, in normal human umbilical cord blood, and adult blood. The intensity of fluorescence in these cells varied, which might indicate quantitative differences in the amount of haemoglobin F (Hosoi, 1965)

The fact that haemoglobin synthesis in the rat results in the sequential appearance of the adult haemoglobins in contrast with the situation in the mouse

in which the adult haemoglobin arises as a result of sequential replacement of the foetal haemoglobin (Cole, Hunter and Paul, 1968) also supports this hypothesis.

With regard to the second issue, the fact that the recolonizing cells seemed already determined to synthesize one type of haemoglobin solely might indicate that the C.F.U. is not the haemopoietic stem cell itself, but a later stage. However, the identity of the C.F.U. and the haemopoietic stem cell is still not clear; it has been suggested that some of the characteristics of stem cells may also be typical of their immediate descendents, and that not all haemopoietic progenitor cells are pluripotent and uncommitted (Bennett and Cudkowicz, 1968). On the other hand, the possibility that the recolonized spleens produce a different type of haemoglobin, instead of a typical adult or foetal haemoglobin, should not be disregarded. It has been reported that spleen colonies obtained by homologous transplantation of bone marrow or spleen cells do not produce a normal distribution of haemoglobin components (Lord and Schofield, 1969). Nevertheless, heterologous (rat) transplantation appeared to produce an enhancement of endogenous repopulation, and consequently of endogenous haemoglobin synthesis. This is supported by the fact that haemoglobin synthesis in the spleens of

surviving controls (the few that survived) was extremely low. Even in endogenously recolonized spleens, haemoglobin synthesis was much lower than in exogenously repopulated spleens. Stimulation of endogenous colony proliferation in irradiated mice injected with human marrow cells has also been reported (Fischbarg, Lewis and Trobaugh, 1967). The mechanism whereby these cells produce such stimulation, however, remains to be elucidated (Fischbarg, Lewis and Trobaugh, 1967).

5.-Influence of the splenic micro-environment on the production of exogenous colonies.

The influence of the haemopoietic inductive microenvironment surrounding a stem cell has been suggested to determine its choice of differentiation pathway, and of self-replication (Curry and Trentin, 1967). Although it has been pointed out that since the stem cell is a multipotent entity, the microenvironment would not be the only factor involved in this, but probably intrinsic differences between the stem cells themselves would also be involved (Worton, McCulloch and Till, 1969).

That cell-cell interactions or, in other words, microenvironmental factors influence the recolonization of mouse spleens by rat transplants of haemopoietic tissues certainly seemed to be the case in the experiments performed. Relatively small transplants failed to

produce exogenous colonies or to show haemoglobin synthesis of exogenous type, while similar doses of homologous transplant, like large doses of heterologous transplant, were successful in this respect. It appeared that the large number of rat cells injected and located in the mouse spleen surrounded and favoured the growth and differentiation of rat stem cells. This microenvironment seemed to be necessary for the rat stem cell successfully to compete against host stem cell proliferation and environment, whether immunological mechanisms were active or not. The environment of a non-syngeneic host generally inhibits the spleen colony forming capacity of bone marrow cells injected into heavily irradiated mice (Lengerova and Zelíny, 1968) but large inocula appear resistant to allogeneic inhibition (Hellström and Hellström, 1965).

The possible effect of a favourable micro-environment upon synthesis of one type of haemoglobin or the other is unresolved. Hence, the commitment of the rat C.F.U. to synthesize one type of haemoglobin could not be definitely established. On the other hand, the microenvironment could exert its control and stimulatory mechanisms on the multipotent stem cell so that it would follow a specific pathway of Hb synthesis. It might be argued that with the massive

rat transplants given, there was no exogenous repopulation due to growth and differentiation of rat stem cells, but only a replenishment or refilling of the spleen by rat cells. In other words, the injected rat cells filled the empty spaces of the irradiated mouse spleen and stayed there in a somewhat passive fashion. This would certainly be the case with many of the rat cells which formed the microenvironment for the stem cells. It is reasonable to assume, however, that they must either have continued their normal process of maturation or perished, and the products would have been released into the blood stream, or disposed of. Hence, it is most likely that by the 13th or 15th day after transplantation (when the experimental spleens were collected) the only cells left in the areas of splenic recolonization capable of $^{59}Fe$ incorporation into haemoglobin were those erythroid precursors which originated from stem cells. The possibility that the release mechanism was impaired seemed unlikely. Splenomegaly was never observed and the experimental spleens never regained their normal size.

## Experimental II.

## Haemopoietic cell culture and Time Lapse Cine-micrographic Studies:

### I. Materials and Methods.

### II. Experiments and Results.

A. On the frequency distribution of mitoses in the haemopoietic cell population of MFL cells as a whole:

(1) Effect of erythropoietin on mitoses of MFL cells in plasma-clot cultures.

Time-lapse cinemicrographic continuous observations:

Normal mouse plasma.
Chicken plasma.
Hypertransfusion-polycythaemic mouse plasma.

(2) Effect of different sera on mitoses of MFL cells:

Discontinuous observations:

In fibrin-clot cultures:

Normal mouse serum.
Hypertransfusion-polycythaemic mouse serum.
Human polycythaemia vera serum.
Foetal bovine serum.

In plasma-clot cultures:

Normal mouse serum (NMS).
Hypertransfusion-polycythaemic mouse serum (HPMS).
Mixed (NMS + HPMS) serum.

(3) Effect of erythropoietin on mitoses of MFL cells.

Discontinuous observations:

In hypertransfusion-polycythaemic mouse plasma-clot cultures.

B. On the behavioural and morphocytological characteristics of the haemopoietic cell population of MFL:

(1) Behaviour and morphocytology of MFL cells in time-lapse cinemicrographic observations.

(2) Cytomorphologic characterization in fixed and stained preparations.

C. On the maturation of haemopoietic MFL cells. Time-lapse cinemicrographic and autoradiographic observations:

Effect of erythropoietin on the cell cycle.

III. Discussion and Conclusions.

## I. Materials and Methods.

In order to investigate the behaviour and characteristics of erythroid precursors in culture, and the effect of erythropoietin, time-lapse cinemicrographic observations were made. Mouse foetal liver cells, 13 days old, were chosen as the erythropoietic cell system because livers at this stage of development possess a high proportion of erythropoietic cells (Silini, Pozzi and Pons, 1967; Fantoni, et al., 1968; Paul, Conkie and Freshney, 1969). Also, they represent a very much less complex population mixture than other haemopoietic tissues suitable for this purpose, such as bone marrow. Moreover, they respond to erythropoietin stimulation (Cole and Paul, 1966; Cole, Hunter and Paul, 1968). Finally, erythroid cytologic information on the mouse foetal liver is currently being accumulated (Silini, Pozzi and Pons, 1967; Fantoni et al., 1968; Paul, Conkie and Freshney, 1969).

Plasma-clot cultures immersed in liquid medium were adopted as the main culture system because they allowed fixation and staining at the end of incubation and filming, and relocation of the field under observation afterwards.

Cell suspensions:

Following the technique of Cole and Paul (1966) livers

of 13-day-old foetuses were obtained from Swiss albino mice (Porton strain) and disaggregated to single cell suspensions by exposing them to trypsin for 6 or 18 hours, followed by the procedures described in Experimental I, Methods section. Cells were suspended in Waymouth's medium MB 752/1 (Waymouth, 1959) supplemented by 10% or 15% serum and a 0.01 mM $FeCl_3$ solution to give a final concentration of 1 : 2 µg/ml (Cole and Paul, 1966), equilibrated overnight at 37°C and adjusted to pH about 7.4 by adding a 6.6% sodium bicarbonate solution as required.

Plasma:

For each experiment 1 ml of fresh blood was obtained by cardiac puncture from a normal or hypertransfused adult mouse. The blood was mixed with 0.1 to 0.2 units of heparin (Evans Pularin Heparin BP, micous, without preservative), centrifuged at 3500 r.p.m. for 5 minutes at 4°C, and the plasma collected. When fowl plasma was required, blood from the wing vein was obtained as described by Paul (1965) and the procedure followed as described above.

Chicken embryo extract:

Aliquots of TC Chick Embryo Extract EE 100, desiccated, Difco 5355, were dispensed in disposable universal containers (Sterilin Ltd., Richmond, Surrey)

Each time a fresh extract was prepared by adding enough serum to the rehydrated embryo extract to give a 50% solution, and incubated at 37°C for 1 to 2 hours. The extract was centrifuged at 3500 r.p.m. for 5 minutes and the supernatant collected and kept overnight at 4°C.

Fibrinogen solution:

Human fibrinogen, 50% clottable (Batch No.FF 445/1, Regional Blood Transfusion Centre, Royal Infirmary, Edinburgh) was placed in an aliquot of the following solvent: 4 g of NaCl, 250 mg of KCl, 125 g of tri-sodium citrate and 500 ml of $H_2O$ (Schindler, 1959, 1964) to give a concentration of 1% and dissolved by standing at room temperature for 2 hours, followed by magnetic stirring for 15 minutes. The solution was then centrifuged at 3500 r.p.m. for 10 minutes, filtered through a 0.22 μ Millipore filter, and kept at room temperature overnight.

Thrombin stock solution:

A solution of thrombin was prepared in Hank's BSS, pH 7.4, to a concentration of 1 U/ml, and kept at -20°C:

- No. T-9000 Sigma Thrombin from Beef Plasma, Grade II, 1000 NIH Units/ml. Activity: 50-75 NIH U/mg of protein.

Erythropoietin stock solution:

Human urinary erythropoietin was dissolved in Hank's BSS, pH.7.4, to give a concentration of 20 U/ml, filtered through a 0.22 μ Millipore filter and kept at -70°C.

Details of erythropoietin stimulation will be given with the description of each set of experiments.

- Human Urinary Erythropoietin, Pool B-1 - TaLSL, Potency 16.4 U/mg. Hematology Research Laboratories, Children's Hospital of Los Angeles, California, U.S.A.

$^{3}$H-thymidine stock solution:

A solution of $^{3}$H-thymidine was prepared in Hank's BSS to give a concentration of 50 µc/ml, and kept at -10°C. Details of isotopic labelling will be given with the description of each set of experiments.

- TRA.120, Batch 49, Thymidine (methyl-T) aqueous solution, 3000 mc/mM, Radiochemical Centre, Amersham.

Sera:

Commercial foetal bovine serum and chicken serum were obtained from Flow Laboratories Ltd. Normal and hypertransfusion-polycythaemic mouse sera were pooled from normal and transfusion-polycythaemic mice respectively and filtered through a 0.22 µ Millipore filter. Normal human serum was obtained from a healthy individual and polycythaemia vera serum from a patient. Both were prepared in sterile conditions and therefore did not require filtering.

Hypertransfusion:

Transfusion-induced polycythaemia was created in mice by intravenous injections of 0.50 to 0.75 ml of

saline washed packed isologous red blood cells on each of two consecutive days, so that on the 5th day the haematocrit was 75% to 85%. Erythropoiesis was suppressed as judged by cytologic examination of the bone marrow, i.e. almost total absence of erythroid precursors. Ages of the receptor mice varied between 2½ and 3 months and their weights between 25 and 30 gm. They were all males.

Preparation of clot-cultures:

To prepare plasma-clot cultures, equal amounts of each component, cell suspension, embryo extract and plasma were placed in a Petri dish and mixed. A tiny aliquot of the mixture was transferred to a coverslip and spread out, following in general the technique described by Paul (1965). Immediately on clotting, the coverslip preparation was inverted on to a slide tissue culture chamber (Cat.No. N8/B, Sterilin Ltd., Richmond, Surrey) containing approximately 0.18 ml of Waymouth's culture medium, and the margins of the coverslip sealed with paraffin.

For relocation of the photomicrographic field (necessary to correlate the fixed and stained cells with the film) a Carter type nickel mask was stuck to the coverslip with a 0.3% solution of FORMVAR in dichloride ethylene.

Microscope and Time-Lapse equipment:

Viable observations were performed by means of a Zeiss phase-contrast (after Heine) microscope placed inside a thermostatic (37°C) chamber. A 25 x objective, without other intervening lenses, was used through a 16 mm. Reflex Bolex camera operated by an Emdeco model UT-150 timer unit (Electro-Mechanical Development Co., Houston, Texas).

After incubation and filming, cultures were fixed by adding methanol (Analar, BDH) very gently, drop by drop on to the clot-culture which was then air-dried and stained with May Grunwald and Giemsa. Identification of stained cells was made according to the criteria established by Bessis (1956).

Autoradiography:

Cultures were prepared for autoradiographic studies after fixation by immersing them in ice-cold 0.2 N perchloric acid solution for 10 minutes followed by washing in cold running water for 45 to 60 minutes, to eliminate non-incorporated isotope. Specimens were covered with Kodak autoradiographic stripping plate AR.10, and exposed at 4°C, before developing and staining following in general the procedures described by Feinendegen (1967).

## II. Experiments and Results.

### A. Frequency of distribution of mitosis.

(1) Time-lapse cinemicrographic continuous observations: Effect of erythropoietin on M.F.L. erythroid cells in plasma clot cultures:

Normal mouse plasma clot cultures: First of all a group of experiments was performed to find out the behaviour of mouse foetal liver (M.F.L.) cells cultured in normal mouse (isologous) plasma-clot cultures. Experimental cell suspensions containing 0.6 units of erythropoietin per ml, and control cell suspensions without erythropoietin, were pre-incubated at 25°C. Coverslip clot cultures were then prepared, perfusion chambers built up, incubation at 37°C and filming performed as described in the Methods section. The culture medium perfused into the chamber contained 1.2 units of erythropoietin per ml, in the case of erythropoietin-stimulated cultures.

From this experiment it was clear that mitoses could be recognized and analysed. Table 17 and Figures 52, 53 and 54 summarize the experimental conditions and results. The cells were unevenly distributed in the clot culture, and clumping made accurate cell counting very difficult sometimes. Between 8% and 14% of mitoses were not recognized as belonging to the erythroid

Table 17.

Effect of erythropoietin on mitoses of 13-day FML erythroid cells cultured in normal mouse plasma clots:

Cell suspension in Weymouth's + 5% FBS + 5% MS.
50% chicken embryo extract in FBS.
Normal mouse plasma.
} equal parts.

| Culture phases. | Erythro-poietin. | Total No. of cells | No. of cells undergoing mitosis | Mitotic Index % |
|---|---|---|---|---|
| Pre-incubation at 25°C : 1 hr.<br>Incubation at 37°C : 36 hr. | -Ep<br>+Ep | 112<br>132 | 9<br>21 | 8.04<br>15.22 |
| Pre-incubation at 25°C : 30 min.<br>Incubation at 37°C : 18 hr. | -Ep<br>+Ep | 53<br>107 | 5<br>13 | 9.43<br>12.15 |
| Pre-incubation at 25°C : 15 min.<br>Incubation at 37°C : 18 hr. | -Ep<br>+Ep | 58<br>32 | 5<br>4 | 8.62<br>12.50 |

Erythropoietin stimulation - a) at pre-incubation of cell suspension = 0.6 U/ml CM.
b) at incubation of clot culture = 1.2 U/ml CM (perfused).

FML = foetal mouse liver. FBS = foetal bovine serum. MS = mouse serum.
CM = culture medium

Figure 52.

Frequency distribution of mitoses in 13-day foetal mouse liver erythroid cells cultured in normal mouse plasma clots.

Erythropoietin-stimulated, and control, non-stimulated cells were incubated and continuously observed by time-lapse cine-micrography as described in the text.

Experimental conditions in Table 17.

FIGURE 52

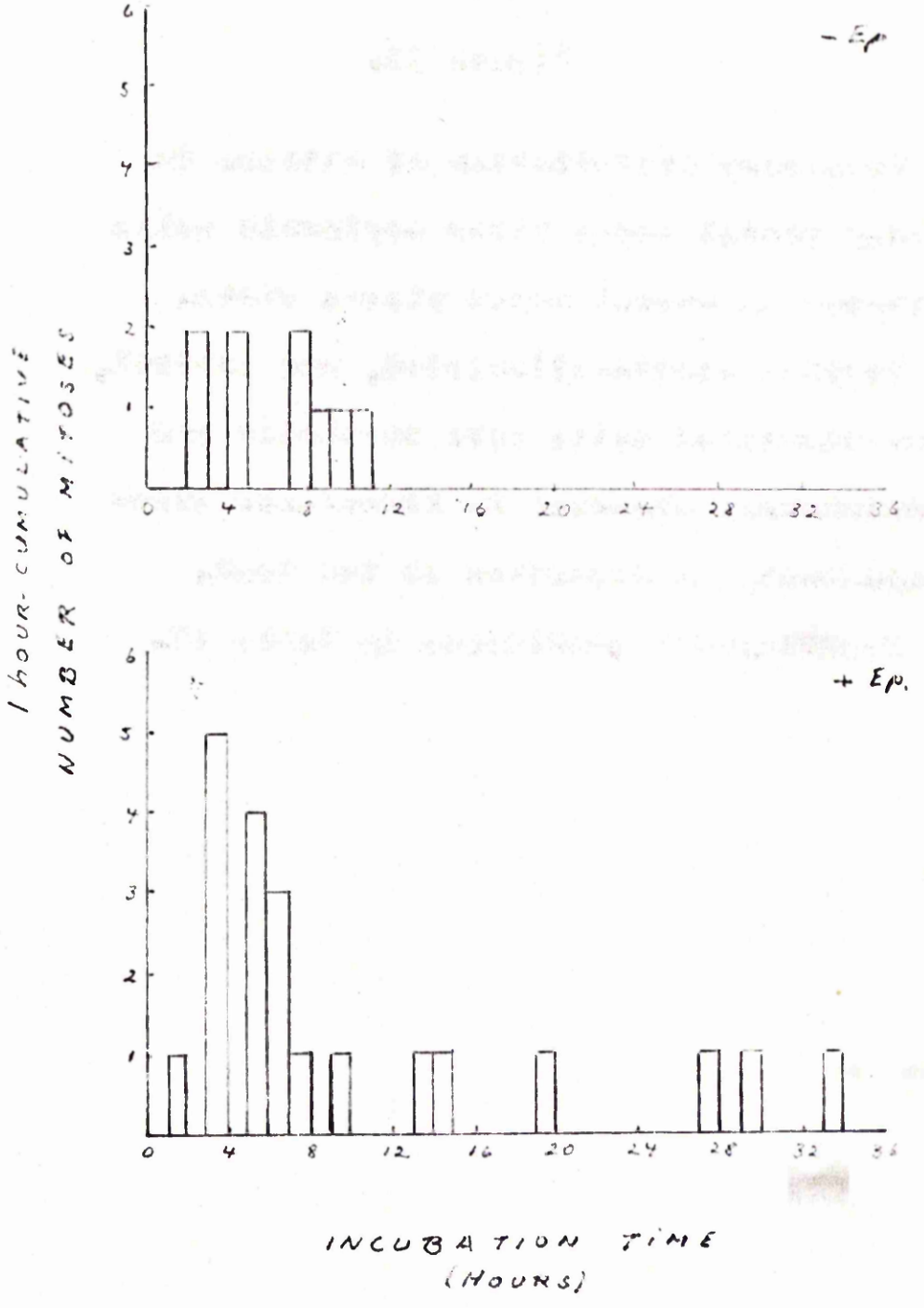

- Ep.
+ Ep.
1 HOUR CUMULATIVE NUMBER OF MITOSES
0
4
8
12
16
20
24
28
32
36
INCUBATION TIME (HOURS)

Figure 53.

Frequency distribution of mitoses in 13-day foetal mouse liver erythroid cells cultured in normal mouse plasma clots.

Technical procedures as in Figure 52.

Experimental conditions in Table 17.

FIGURE 53

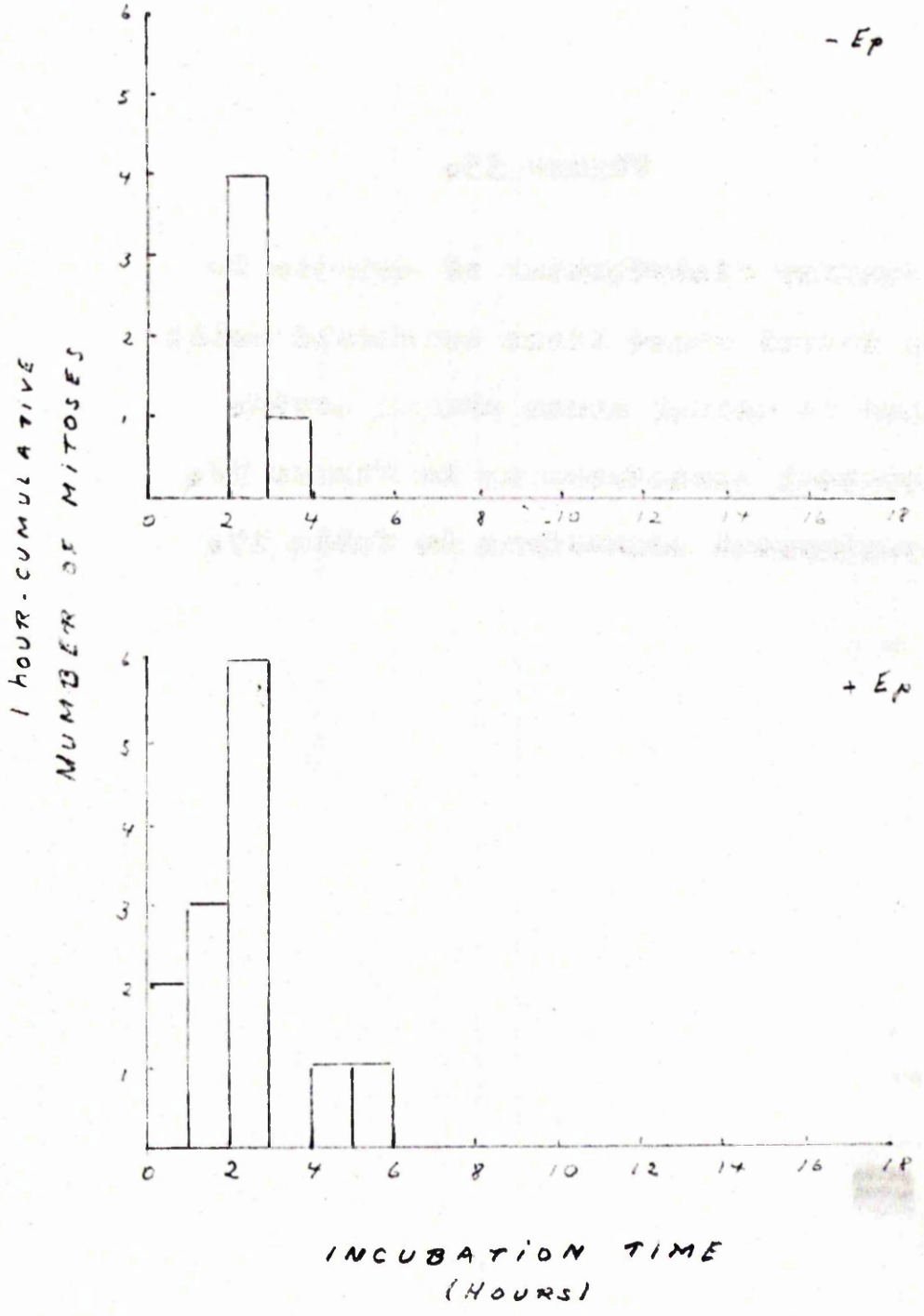

- Ep
+ Ep
1 HOUR-CUMULATIVE NUMBER OF MITOSES
0
2
4
6
8
10
12
14
16
18
INCUBATION TIME (HOURS)

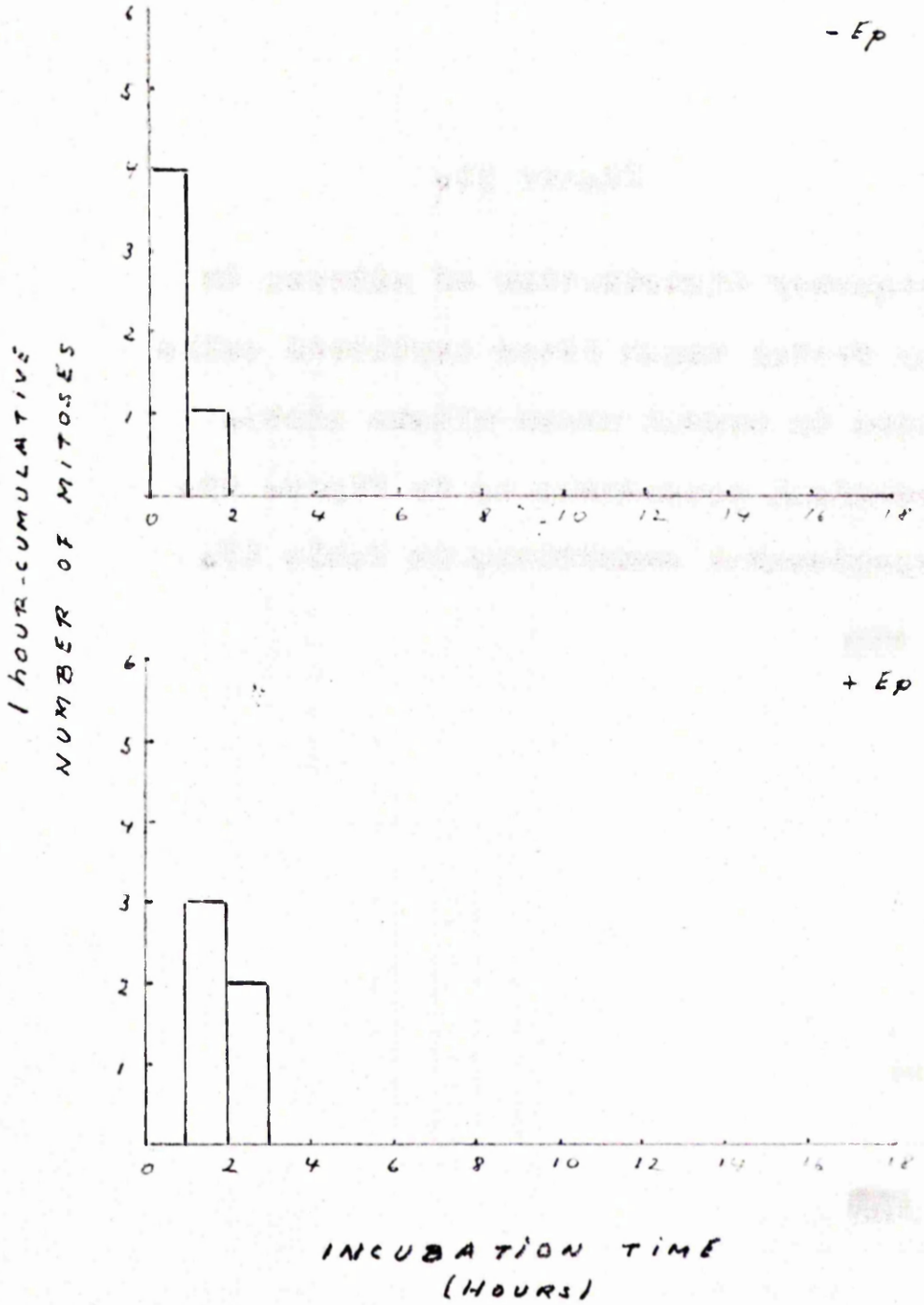
Figure 54.

Frequency distribution of mitoses in 13-day foetal mouse liver erythroid cells cultured in normal mouse plasma clots.

Technical procedures as in Figure 52.

Experimental conditions in Table 17.

FIGURE 54


- Ep
+ Ep
1 HOUR-CUMULATIVE NUMBER OF MITOSES
0
1
2
3
4
5
6
0
2
4
6
8
10
12
14
16
18
INCUBATION TIME (HOURS)

series. Culture conditions appeared unfavourable for hepatocyte survival and proliferation. The small number of lysed erythroid cells present during incubation and filming did not make a significant difference to the results.

Mitoses were recognized in the recorded films when a cell was in transition from anaphase to telophase. In other words, the transition from single cell state (o) to the obvious appearance of a constriction of the cytoplasm in the equatorial region of the cell (8), i.e. the beginning of telophase (Mazia, 1961; De Robertis, Nowinski and Saez, 1966). Incidentally, the transition took place within about 5 minutes. A wave of mitoses during the first hours of incubation occurred both in erythropoietin-stimulated cultures and in control, non-stimulated cultures. After this, the number of mitoses showed a profound reduction and became less constant and scattered. The erythropoietin-stimulated cultures showed not only a relatively higher number of mitoses, but also a longer maintenance of the mitosis phenomenon during incubation, in comparison with the control cultures. For the sake of clarity, behavioural and morphocytologic characteristics will be described later, in a separate paragraph.

Chicken plasma-clot cultures: It has been reported (Rosse, 1968) that avian erythropoietin is not active in mammalian systems, which would avoid the stimulant effect of erythropoietin normally present in plasma. With the object of improving culture conditions, cultures of M.F.L. cells in chicken plasma clots were set up. Table 18 and Figures 55 and 56 summarize the experimental conditions and results. Plasma for each experiment was obtained from the same chicken, which developed a progressive jaundice, possibly due to haemolytic anaemia. The results suggested erythroid cell stimulation by the chicken plasma.

Hypertransfusion-polycythaemia mouse plasma-clot cultures: Since it has been reported that transfusion-induced polycythaemia suppresses erythropoietin production and consequently erythropoiesis (Jacobson, Goldwasser and Gurney, 1960), cultures of M.F.L. cells in hypertransfusion-polycythaemic mouse plasma-clots were set up under the same conditions as in the preceding experiments. Table 19 and Figures 57, 58 and 59 show a summary of the experimental conditions and results.

In general, the occurrence of mitoses appeared to decrease in comparison with all previous experiments. However, the distribution of mitoses showed a similar pattern to that in the cultures with normal mouse

Table 18.

Effect of erythropoietin on mitosis of 13-day FML erythroid cells cultured in chicken plasma clots:

Cell suspension in Waymouth's + 5% FBS + 5% MS. } equal
50% chicken embryo extract in FBS. } parts.
Chicken plasma. }

| Erythropoietin stimulation | Plasma obtained on: | Total No.of cells | Cells undergoing mitosis | Mitotic Index % |
|---|---|---|---|---|
| -Ep | 1st day | 148 | 9 | 6.08 |
| +Ep | 8th day | 121 | 10 | 8.26 |
| +Ep | 10th day | 169 | 19 | 11.24 |
| -Ep | 22nd day | 125 | 19 | 15.20 |

Erythropoietin stimulation:

a) Cell suspension containing 0.6 U/ml. : 30 min. pre-incubation at 25°C.
b) Ep.perfused in the chamber's culture medium : 1.2 U/ml.

Incubation at 37°C:10 hours.

FML = foetal mouse liver.
FBS = foetal bovine serum.
MS = mouse serum.

Figure 55.

Frequency distribution of mitoses in 13-day foetal mouse liver erythroid cells cultured in chicken plasma clots.

Technical procedures as in Figure 52.

Experimental conditions in Table 18.

FIGURE 55

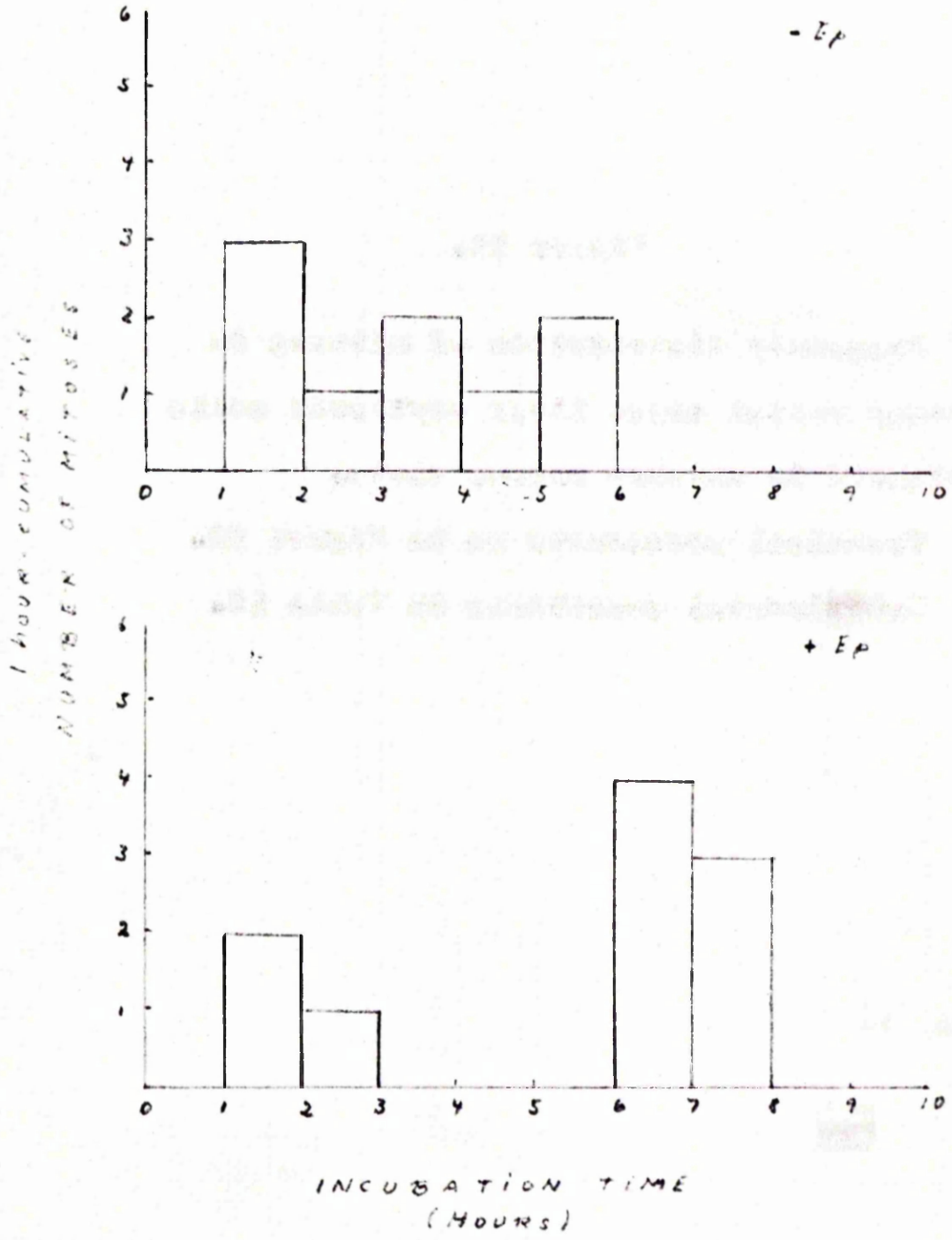

- Ep
+ Ep
1 HOUR - EQUIVALENTS
NUMBER OF MITOSES
0 1 2 3 4 5 6 7 8 9 10
INCUBATION TIME
(HOURS)

Figure 56.

Frequency distribution of mitoses in 13-day foetal mouse liver erythroid cells cultured in chicken plasma clots.

Technical procedures as in Figure 52.

Experimental conditions in Table 18.

FIGURE 56

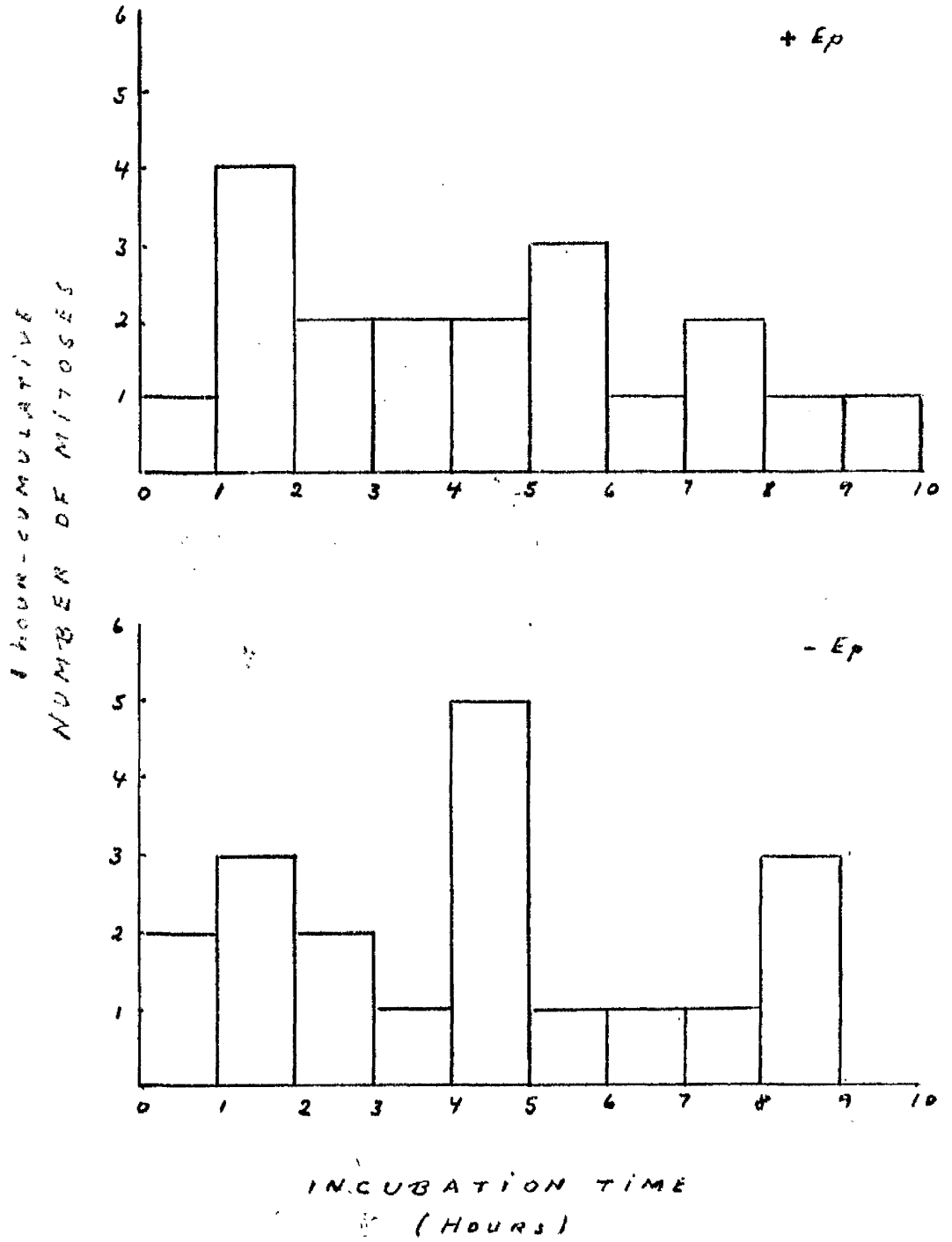

+ Ep
- Ep
1 HOUR-CUMULATIVE NUMBER OF MITOSES
INCUBATION TIME (HOURS)

Table 19.

Effect of erythropoietin on mitosis of 13-day FML erythroid cells cultured in hypertransfused-polycythaemic mouse plasma clots:

Cell suspension in Waymouth's + 5% FBS + 5% HPMS
50% chicken embryo extract in FBS
Hypertransfused-polycytgaemic mouse plasma
} equal parts.

| Erythropoietin stimulation | Total No. of cells | No. of cells undergoing mitosis | Mitotic Index % |
|---|---|---|---|
| -Ep | 38 | 1 | 3.22 |
| +Ep | 36 | 3 | 8.33 |
| -Ep | 85 | 2 | 2.35 |
| +Ep | 73 | 6 | 8.22 |
| -Ep | 57 | 3 | 5.26 |
| +Ep | 126 | 13 | 10.32 |

Erythropoietin stimulation:

a) Cell suspension containing 0.6 U of Ep/ml.: 30 min. pre-incubation at 37°C.

b) Ep. perfused in the chamber's culture medium: 1.2 U/ml.

Incubation at 37°C and filming = 10 hours.

FML = foetal mouse liver.
FBS = foetal bovine serum.
HPMS = hypertransfused-polycythaemic mouse serum.

Figure 57.

Frequency distribution of mitoses in 13-day foetal mouse liver erythroid cells cultured in hypertransfusion-polycythaemic mouse plasma clots.

Technical procedures as in Figure 52.

Experimental conditions in Table 19.

FIGURE 57

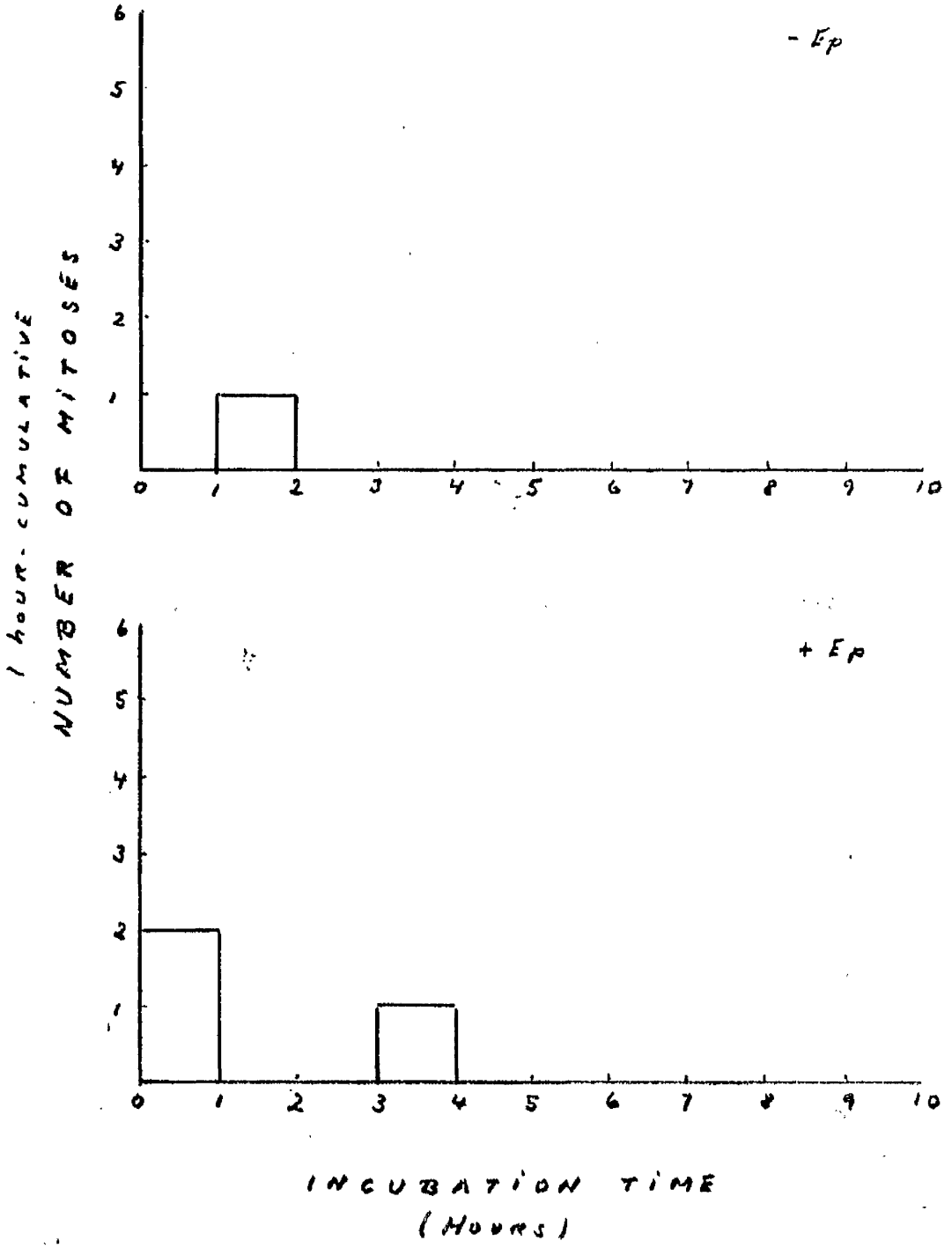

- Ep
+ Ep
1 hour cumulative
NUMBER OF MITOSES
INCUBATION TIME
(Hours)

Figure 58.

Frequency distribution of mitoses in 13-day foetal mouse liver erythroid cells cultured in hypertransfusion-polycythaemic mouse plasma clots.

Technical procedures as in Figure 52.

Experimental conditions in Table 19.

FIGURE 58

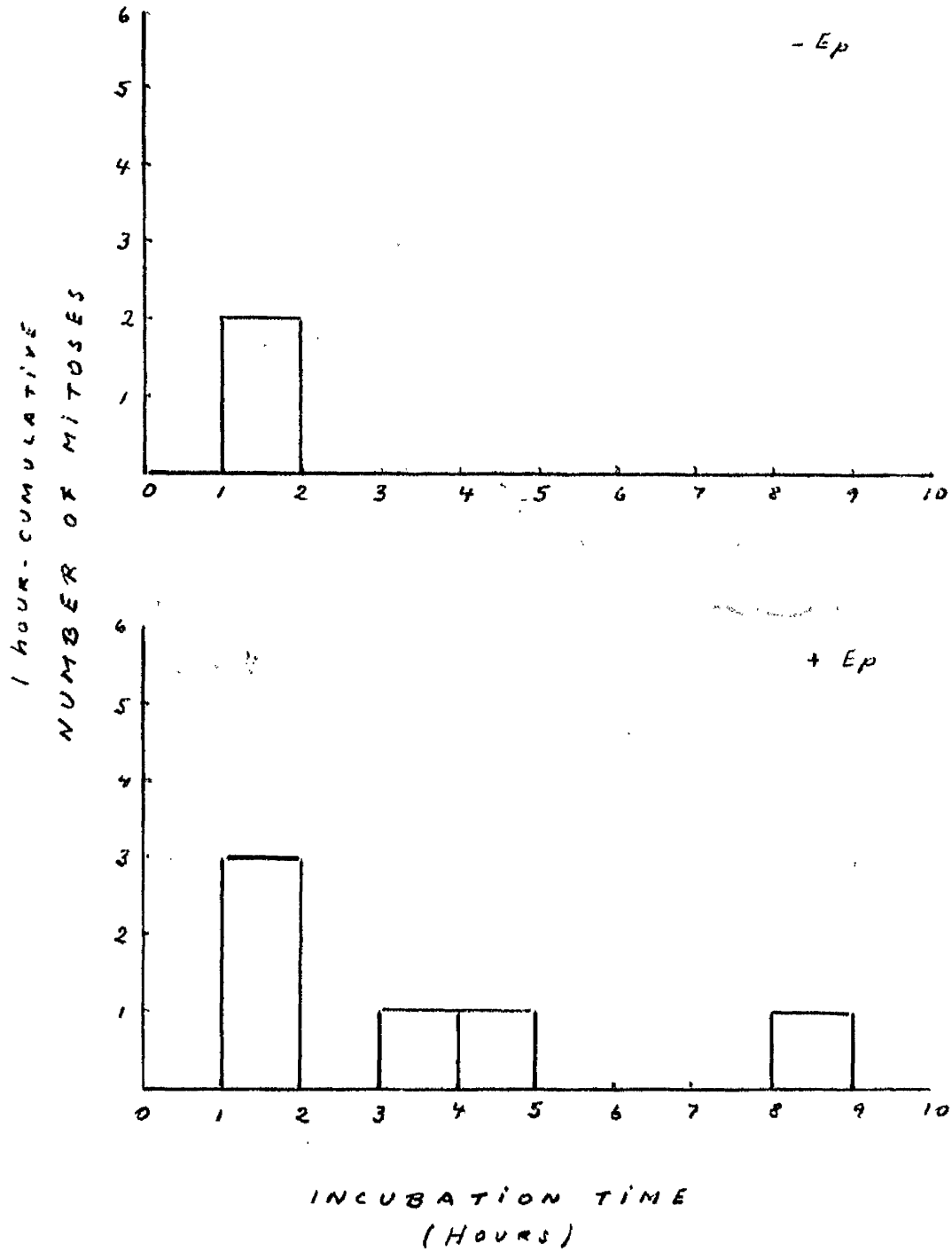

- Ep
+ Ep
1 HOUR-CUMULATIVE NUMBER OF MITOSES
0 1 2 3 4 5 6 7 8 9 10
INCUBATION TIME (HOURS)

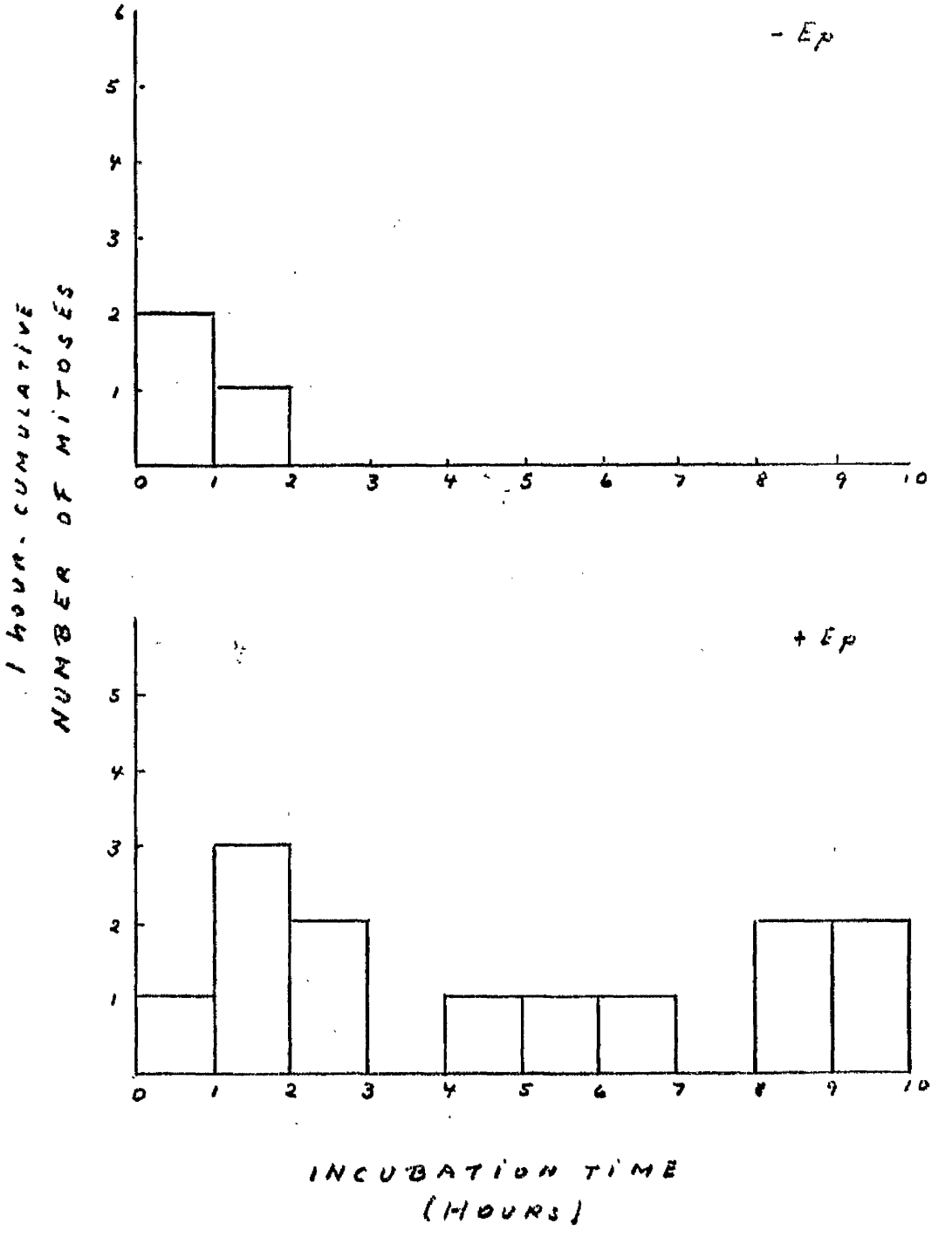
Figure 59.

Frequency distribution of mitoses in 13-day foetal mouse liver erythroid cells cultured in hypertransfusion-polycythaemic mouse plasma clots.

Technical procedures as in Figure 52.

Experimental conditions in Table 19.

FIGURE 59


- Ep
+ Ep
1 HOUR CUMULATIVE NUMBER OF MITOSES
0 1 2 3 4 5 6 7 8 9 10
INCUBATION TIME (HOURS)

plasma-clot; the increased mitotic index in erythropoietin-stimulated cultures also persisted.

(2) Discontinuous observations: Effect of different sera on mitoses of M.F.L. erythroid cells in fibrin-clot cultures:

Since the previous experiments showed a marked effect of serum or plasma on the mitotic index, a set of experiments was planned to find out the effect of different sera on this parameter. Hence plasma was replaced by fibrin as culture substrate. However, this medium presented a technical problem. During fixation the fibrin-clots broke up into flakes and minute particles and many of them became detached from the coverslip. As a result, the field of cinemicrographic observation was often lost, and correlation between film and stained field impossible to obtain.

For this reason, and since previous cinemicrographic observations showed that most of the daughter cells resulting from a mitotic parent cell remained as "doublets", cinemicrography was not used in this series of experiments, and the "doublet index" was considered to represent the mitotic index. The distribution of mitoses in relation to incubation time, of course, could not be studied by this system.

To strengthen the consistency of the clot, increas-

ing concentrations of fibrinogen were tried, but these caused the cells to become gradually swollen. The fluid intake which appeared to take place made their cytoplasm adopt the refractive characteristics of the medium.

Different sera were used in these experiments. Tables 20 and 21 summarize the experimental conditions and results. Cultures of human polycythaemia vera serum, freshly prepared from a patient, showed the highest mitotic (doublet) index, approximately twice that of the cultures containing normal mouse serum, whereas cultures containing hypertransfusion-polycythaemia mouse serum showed about half the mitotic index of the cultures with normal mouse serum. Foetal bovine serum seemed to reduce the mitotic index and a very low number of mitoses were seen in cultures with no serum. It is necessary to point out here that 8 months later the same human polycythaemia vera serum, which had been kept at -20°C, was used to try to reproduce these findings. However, the cultures presented a great proportion of naked nuclei, and "doublets" were scarce at 4-5 hour incubation.

Effect of different sera on mitoses of M.F.L. erythroid cells in plasma-clot cultures. Since previous experimental results showed a decreased mitotic

Table 20

Effect of different sera on mitosis of 13-day MFL erythroid cells cultured in fibrin clots:

Cell suspension in Waymouth's + 10% serum } equal
1% Fibrinogen (filtered through 0.22μ Millipore) } parts.
Thrombin, traces.

Incubation at 37°C : 8 hours.

| Type of serum in culture clot | Total No.of cells | No.of mitosis (Doublets) | Mitotic Index (Doublet Index) % |
|---|---|---|---|
| Normal mouse serum. | 500<br>500 | 46<br>48 | 9.2<br>9.6 |
| Hypertransfused-polycythaemic mouse serum. | 500<br>500 | 22<br>31 | 4.4<br>6.2 |
| Human polycythaemic vera serum | 500<br>500 | 91<br>105 | 18.2<br>21.0 |

MFL = foetal mouse liver.

Table 21.

Effect of different sera on mitosis of 13-day FML erythroid cells cultured in fibrin clots.

Conditions as in Table 20.

| Type of serum in culture | Total No. of cells | No. of mitosis (Doublets) | Mitotic Index (Doublet Index) % |
|---|---|---|---|
| Human polycythaemia vera serum. | 500 | 74 | 14.8 |
| | 500 | 84 | 16.8 |
| Foetal bovine serum (Normal). | 500 | 42 | 8.4 |
| | 500 | 20 | 4.0 |
| No serum. | 500 | 14 | 2.8 |
| | 500 | 21 | 4.2 |

FML = foetal mouse liver.

index in cultures containing hypertransfusion-polycythaemic mouse serum, a further investigation along these lines seemed advisable. Therefore, a set of experiments was carried out to compare the effect of normal and hypertransfusion-polycythaemic mouse plasmas and sera, and to determine whether the effect of one type was additive to the other or not. Tables 22, 23 and 24 summarize the experimental conditions and results. In all experiments, the cultures with hypertransfusion-polycythaemic mouse plasma and serum showed a decreased mitotic index compared with that shown by cultures containing normal mouse plasma and serum. Cultures with a mixture of both types of plasma and serum (with the same final concentration as in the other cultures) also showed a decreased mitotic index, proving that hyper-transfusion-polycythaemic mouse plasma and serum had an inhibitory effect on mitoses.

(3) Effect of erythropoietin on mitoses of M.F.L. erythroid cells in hypertransfusion-polycythaemic mouse plasma clot cultures:

The previous experimental results indicated the presence of a factor with a mitotic inhibiting effect in the hypertransfusion-polycythaemic mouse plasma. A new set of experiments was performed to confirm the effect of erythropoietin in a system using hypertransfusion-

Table 22.

Effect of hypertransfusion-polycythaemic mouse plasma and serum on mitosis of 13-day FML erythroid cells cultured in plasma clots.

Incubation at 37°C : 5 hours.

| | Clot components | Total No.of cells | Cells in mitosis (Doublets) | Mitotic Index (Doublet Index) % |
|---|---|---|---|---|
| N | Normal mouse plasma.<br>50% Ch.EE in FBS.<br>Waymouth's+ 15% FBS. | 500<br>507 | 64<br>60 | 12.8<br>11.8 |
| H | Hypertransfused-polycythaemic mouse plasma.<br>50% Ch EE in HPMS.<br>Waymouth's+ 15% HPMS. | 500<br>510 | 38<br>45 | 7.6<br>8.8 |
| X | Mixed.<br>N + H. | 500<br>511 | 26<br>41 | 5.2<br>8.0 |

FML = foetal mouse liver.
Waymouth's = Waymouth's medium.
Ch.EE = chicken embryo extract.
FBS = foetal bovine serum.
HPMS = hypertransfusion-polycythaemic mouse serum.

Table 23.

Effect of hypertransfusion-polycythaemia mouse plasma and serum on mitosis of 13-day FML erythroid cells cultured in plasma clots.

Conditions as in Table 22.

| Clot components | Total No.of cells | Cells in mitosis (Doublets) | Mitotic Index (Doublet Index) % |
|---|---|---|---|
| N | 523 | 42 | 8.03 |
| H | 508 | 35 | 6.88 |
| X | 516 | 30 | 5.81 |

Table 24.

Effect of hypertransfusion-polycythaemic mouse plasma and serum on mitosis of 13-day FML erythroid cells cultured in plasma clots.

Conditions as in Table 22.

| Clot components | Total No. of cells | Cells in Mitosis (Doublets) | Mitotic Index (Doublet Index) % |
|---|---|---|---|
| N | 631 | 52 | 8.24 |
| H | 581 | 30 | 5.16 |
| X | 547 | 31 | 5.67 |

polycythaemic mouse plasma and serum. Tables 25 and 26 summarize the experimental conditions and results. Erythropoietin stimulated cultures showed an increased mitotic index in spite of the inhibiting effect previously demonstrated.

B. Behavioural and Morphocytological Characteristics of M.F.L. erythroid cells in plasma and fibrin clot cultures:

Optical conditions were insufficient to attempt a detailed morphological charatterization of the cells in the recorded films. Not only the relatively low magnification used (25 x) in order to get a reasonable number of cells into the field of observation, but also the presence of "halos" aggravated by the density and sphericity of most cells, presented great optical difficulties. Increasing concentrations of albumin were tested to try to improve the optical conditions. A concentration of 20% yielded quite good optical qualities in general, but pynocytic vacuoles were induced even with about 5% concentrations; vacuolization was more intense and occurred more quickly with increasing concentrations and the rate of recovery was inversely proportional . Ficoll, dextran and polyvinyl pirrolidone were also tried but as the concentrations

Table 25.

Effect of erythropoietin on mitosis of 13-day FML erythroid cells cultured in hypertransfusion-polycythaemic mouse plasma clots:

Cell suspension in Waymouth's + 15% HPMS
50% chicken embryo extract in HPMS,
Hypertransfusion-polycythaemic mouse plasma
} equal parts.

Incubation at 37°C : 5 hours.

| Erythropoietin stimulation | Total No.of cells | Cells in mitosis (Doublets) | Mitotic Index (Doublet Index) % |
|---|---|---|---|
| -Ep | 500 | 24 | 4.8 |
| +Ep | 500 | 39 | 7.8 |
| -EP | 500 | 14 | 2.8 |
| +Ep | 500 | 31 | 6.2 |

Erythropoietin perfused at a concentration of 1.5 U/ml of Waymouth's culture medium.

FML = foetal mouse liver.
HPMS = hypertransfusion-polycythaemic mouse serum.

Table 26.

Effect of erythropoietin on mitosis of 13-day FML erythroid cells cultured in hypertransfusion-polycythaemic mouse plasma clots.

Conditions as in Table 25.

| Erythropoietin stimulation | Total No. of cells | Cells in mitosis (Doublets) | Mitotic Index (Doublet Index) % |
|---|---|---|---|
| -Ep | 500 | 27 | 5.4 |
| +Ep | 500 | 36 | 7.2 |
| -Ep | 500 | 21 | 4.2 |
| +Ep | 500 | 33 | 6.6 |

Erythropoietin mixed with clot at a concentration of 0.75 U/ml of clot culture, and perfused at a concentration of 0.75 U/ml of Waymouth's culture medium.

FML = foetal mouse liver.

needed to obtain improved optical conditions showed serious disadvantages due to cell vacuolization, they were discarded.

Cells were located at different levels within the thickness of the clot. Some of the cells were at the optimal focal plane, others were slightly over or under it. A few cells were so far from the focal plane that reasonable optical conditions were not possible. Hence a compromise focal plane had to be adopted.

General behavioural characteristics could be determined from the recorded film analysis. Cyto-morphological characteristics were analysed in the fixed and stained cells, and correlated with the films. The culture system appeared to be far from ideal, however useful for short-term experiments of the type used here. Only occasionally did cultures of more than 16 hours show no sign of deterioration. Some cells disintegrated during incubation, and some of the daughter cells resulting from mitosis broke up into small particles and lysed, especially in the erythropoietin stimulated cultures. Mitosis did not progress to completion in many instances.

Although most of the cells remained stationary in the photographic field under study, some of them showed an active locomotory movement and even migrated out of

the field at times. This movement appeared to be similar to that exhibited by the lymphocyte, a detailed description of which may be found in the review by Trowell (1965). Both a random but polarized locomotory movement of the so-called "amoeboid" type, and a "passage through a constriction ring" were often seen, mainly in cells identified as primitive type, after fixing and staining, but sometimes also in later forms, with structural and staining characteristics of pro-erythroblasts. The "passage through a constriction ring" at a certain stage may appear as a pair of daughter cells and this has to be taken into consideration in assessing mitoses.

Cells which underwent mitosis gave rise to two daughters, most of which remained as "doublets", i.e. without separation as independent cells. This happened even with many of the primitive type daughter cells that showed intense movement towards separation after mitosis. Some of them succeeded in becoming free, and actively migrated away from each other.

A different type of movement of intense activity was observed in erythroblasts which tried to extrude the nucleus. In fixed and stained cultures these cells shows a relatively small nucleus, but not as compact as that observed in mature orthochromatic erythroblasts

with pyknotic nucleus; the cytoplasm still showed some basophilia. Following the concepts of Lajtha (1952) and Berman and Powsner (1959), who defined the orthochromatic erythroblast as a cell with no traces of basophilia in the cytoplasm, with regard to its staining characteristics, these cells should be considered as late polychromatic erythroblasts. In fact, extrusion of the nucleus has been reported in polychromatic stage under conditions of anaemic stress, and occasionally even in normal conditions (Borsook et al., 1962; Brecher and Stohlman, 1959). However, these cells would be considered as early orthochromatic erythroblasts by Dacie and White (1949) and Bessis (1956) who remarked that it is rare to find completely acidophilic erythroblasts. It would appear that these two cell stages correspond to two different modes of nuclear loss; by extrusion, and by pyknosis and karyolysis, the former taking place at an earlier stage than the latter. It may well be that in cells in which the nucleus was not extruded, for one reason or another, the process of pycnosis and karyolysis occurs.

Parent cells appeared to be of variable size. Many of them were large while others were small and comparable in size to a mature orthochromatic erythroblast. After completion of mitosis, some of the daughter cells

appeared to exceed the size of the parent cell. Optical conditions did not permit of taking measurements with certainty. Besides, the measurements of rounded structures by phase contrast are not accurate, because the boundary between the bright and dark haloes does not coincide with that of the object (Leitz-Wetzlar 513 - 5c Publication).

Fibroblastic cells began to appear after some hours of incubation, and later on, macrophages which wandered around, and phagocytosed cells that, sometimes, appeared to be in a good state of vitality and mobility. On the other hand, all stages of erythroid differentiation, from the so-called haemohistioblast (Bessis, 1956) to the more mature forms, were recognized in stained cultures. Haemoglobinized macrocytic erythroblasts were observed in erythropoietin stimulated cultures.

With regard to the identification of erythroid cells in fixed and stained cultures, the criteria established by Bessis (1956) were, in general, followed. However, emphasis was given to the structure of the nucleus and nuclear chromatin rather than to other cell characteristics. Cell size was considered of less importance because variations in size were quite extensive in otherwise similar cells, especially in the megaloblastic type of differentiation in erythropoietin

stimulated cell cultures. This was aggravated by the fact that daughter cells remained as doublets in different degrees of attachment, apparently because, in some cases, mitosis did not progress, but stopped at various stages of mitotic development, and in most cases daughters did not separate from each other. In spite of the difficulty of identifying haemopoietic cells in the mouse (Dunn, 1954) an attempt was made to group the erythroid daughter cells according to stages of maturation.

Total number of mitoses, i.e. mitotic indices, seemed to be sensitive enough to demonstrate differences in the effects of different sera or plasmas on the mitoses of erythroid cells. Cumulative results of comparable experiments also seemed to be sufficiently sensitive to demonstrate the effect of erythropoietin stimulation on different erythroid cell groups. Tables 27, 28 and 29 summarize these results. Mitotic activity was more prevalent among the early erythroid precursors, the resultant daughters being mostly proerythroblasts and basophilic erythroblasts, both in the erythropoietin stimulated cell cultures and in the control, non-stimulated cells. A group of undifferentiated daughters was also identified. These cells had similar characteristics to those described by Lucarelli et al. (1966) in

Table 27.

Effect of Erythropoietin:

Differential counts of erythroid daughter cells arising from mitoses of FML cells cultured in plasma and fibrin clots.

| Cell Type | -Ep | +Ep |
|---|---|---|
| Undifferentiated cells | 14.66 | 16.23 |
| Haemocytoblasts | 8.33 | 7.69 |
| Proerythroblasts | 26.66 | 25.76 |
| Basophilic erythroblasts | 39.00 | 34.94 |
| Early polychromatic erythroblasts. | 11.35 | 15.38 |

%

FML = foetal mouse liver.

Table 28.

Effect of hypertransfusion-polycythaemic plasma and serum:

Differential counts of erythroid daughter cells arising from mitoses of FML cells in clot cultures.

| Cell Type | Normal Serum | Hypert. Poly. Serum | Mixed Serum |
|---|---|---|---|
| Undifferentiated cells | 18.00 | 17.25 | 20.00 |
| Haemocytoblasts | 7.33 | 5.24 | 7.23 |
| Proerythroblasts | 27.66 | 27.76 | 35.00 |
| Basophilic erythroblasts | 33.35 | 34.00 | 25.44 |
| Early polychromatic erythroblasts | 13.66 | 18.75 | 12.33 |

%

FML = foetal mouse liver.

Table 29.

Effect of erythropoietin:

Differential counts of erythroid daughter cells arising from mitoses of FML cells in hypertransfusion-polycythaemic mouse plasma clot cultures.

| Cell Type | -Ep | +Ep |
|---|---|---|
| Undifferentiated cells | 4.84 | 6.89 |
| Haemocytoblasts | 8.53 | 3.44 |
| Proerythroblasts | 25.07 | 30.34 |
| Basophilic erythroblasts | 49.00 | 37.96 |
| Early polychromatic erythroblasts | 12.56 | 21.37 |

%

FML = foetal mouse liver.

foetal rat liver, which have been considered to belong to the stem cell compartment ("cellule staminali") by these workers. Optical conditions did not permit characterization of the parent cell as belonging to one erythroid group or another. Hence it was not possible to discover whether the resultant daughters were in the same differential group as their parent, or in a later developmental stage. Differential counts showed certain variations from experiment to experiment, but in general no clearly significant differences appeared between the erythropoietin-stimulated cultures and the non-stimulated cultures. However, a certain shift of increased mitosis towards the more mature stages did appear in erythropoietin-stimulated cultures when hypertransfusion-polycythaemic serum and plasma were used.

C. On the maturation of M.F.L. erythroid cells: Time-lapse cinemicrographic and autoradiographic observations:

Techniques for analysis of the cell cycle with the aid of time-lapse cinemicrography have been successfully applied in human amnion and kitten lung cell cultures (Sisken, 1963). However, with the type of cells and culture system adopted for the present studies, orthodox methods of measuring the different

phases of the cell cycle were out of the question. In previous experiments neither a sufficiently long survival of the erythroid cells nor the progress of a complete cell cycle were observed. On the other hand, it was considered that some information on the effect of erythropoietin on the cell maturation time could be obtained by the combined use of time-lapse cinemicrography and autoradiography.

Since the most readily, and most sharply defined period of mitosis was the transition from anaphase to telophase, i.e. the beginning of telophase (Mazia, 1961; De Robertis, Novinski and Saez, 1966), this was adopted as the point of reference for timing. The abbreviations of Howard and Pelc (1953), $G_1$, S, $G_2$, M for the four phases of the cell cycle, were used.

To find the effect, if any, of erythropoietin on the pre-mitotic phase, M.F.L. cell suspensions were labelled with $^3$H-thymidine at a level of 5 µc/ml (Paul and Hunter, 1969) for 30 minutes at 37°C. The cells were then washed with Hank's BSS and centrifuged at 1,000 r.p.m. for 5 minutes. This procedure was repeated twice more. Plasma-clot cultures were prepared, incubated and filmed, followed by fixation, autoradiographic and staining procedures, as described in Methods. Table 30, and Figures 60, 61, 62, 63 and 64 summarize the conditions and results of these experiments.

Table 30.

Effect of erythropoietin on mitoses of 13-day FML erythroid cells in plasma clot cultures, after $^{3}H$ thymidine cell suspension labelling (5μc/ml) for 30 minutes followed by three washings in Hank's BSS.

| Culture phases | Erythro-poietin | Total No. of cells | Cells undergoing mitosis | Mitotic Index % |
|---|---|---|---|---|
| 1st - Labelling incubation<br>2nd - Washing<br>3rd - Incubation + filming : 4 hrs. | -Ep<br>+Ep | 38<br>105 | 1<br>7 | 2.63<br>6.66 |
| Ditto<br>Ditto<br>Ditto 12 hrs. | -Ep<br>+Ep | 155<br>85 | 7<br>6 | 4.57<br>7.06 |
| Ditto<br>Ditto<br>Ditto 5 hrs. | -Ep<br>+Ep | 83<br>138 | 2<br>1 | 2.41<br>0.72 |
| Ditto<br>Ditto<br>Ditto 10 hrs. | -Ep<br>+Ep | 87<br>86 | 2<br>7 | 2.30<br>8.14 |

Erythropoietin stimulation : 0.6 U/ml of Waymouth's culture medium.

Clot components: Equal parts of -

Cell suspension in Waymouth's + 10% hypertransfusion-polycythaemic mouse serum.
50% chicken embryo extract in hypertransfusion-polycythaemic mouse serum.
Hypertransfusion-polycythaemic mouse plasma.

Figure 60.

Frequency distribution of mitoses in 13-day foetal mouse liver erythroid cells cultured in hypertransfusion-polycythaemic mouse plasma clots.

Erythropoietin-stimulated, and control, non-stimulated cells were incubated with $^{3}H$-thymidine (5 μc/ml) for 30 minutes, and then washed to eliminate non-incorporated isotope. Cultures were set up in hypertransfusion-polycythaemic mouse plasma clots, incubated, and continuously observed by time-lapse cinemicrography and by autoradiography, as described in the text.

Experimental conditions in Table 30.

Crossed squares indicate incorporation of $^{3}H$-thymidine.

# FIGURE 60

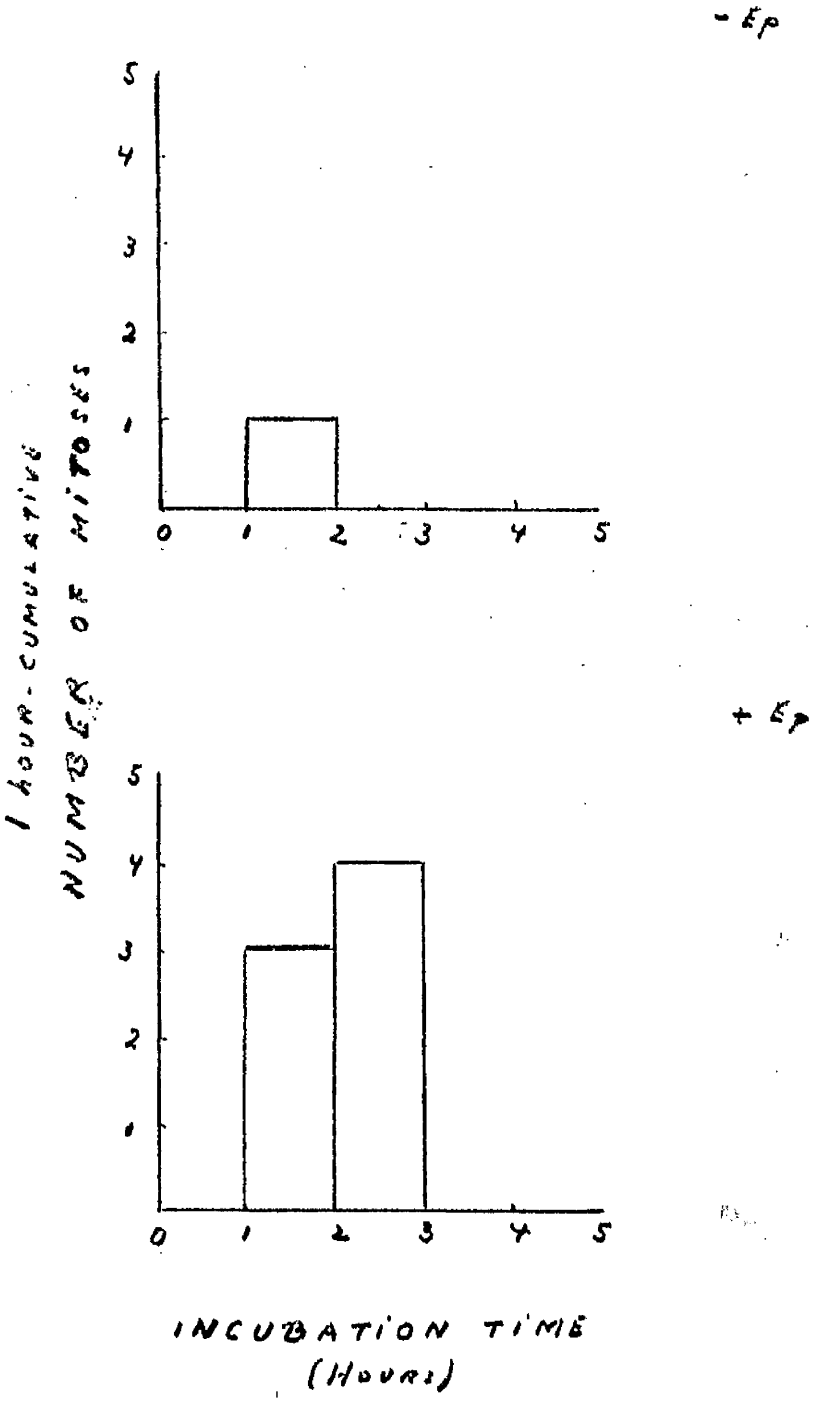

- Ep
+ Ep
1 HOUR - CUMULATIVE NUMBER OF MITOSES
0
1
2
3
4
5
INCUBATION TIME (HOURS)

Figure 61.

Frequency distribution of mitoses in 13-day foetal mouse liver erythroid cells cultured in hypertransfusion-polycythaemic mouse plasma clots.

Technical procedures as in Figure 60.

Experimental conditions in Table 30.

Crossed squares indicate incorporation of $^{3}H$-thymidine.

FIGURE 61

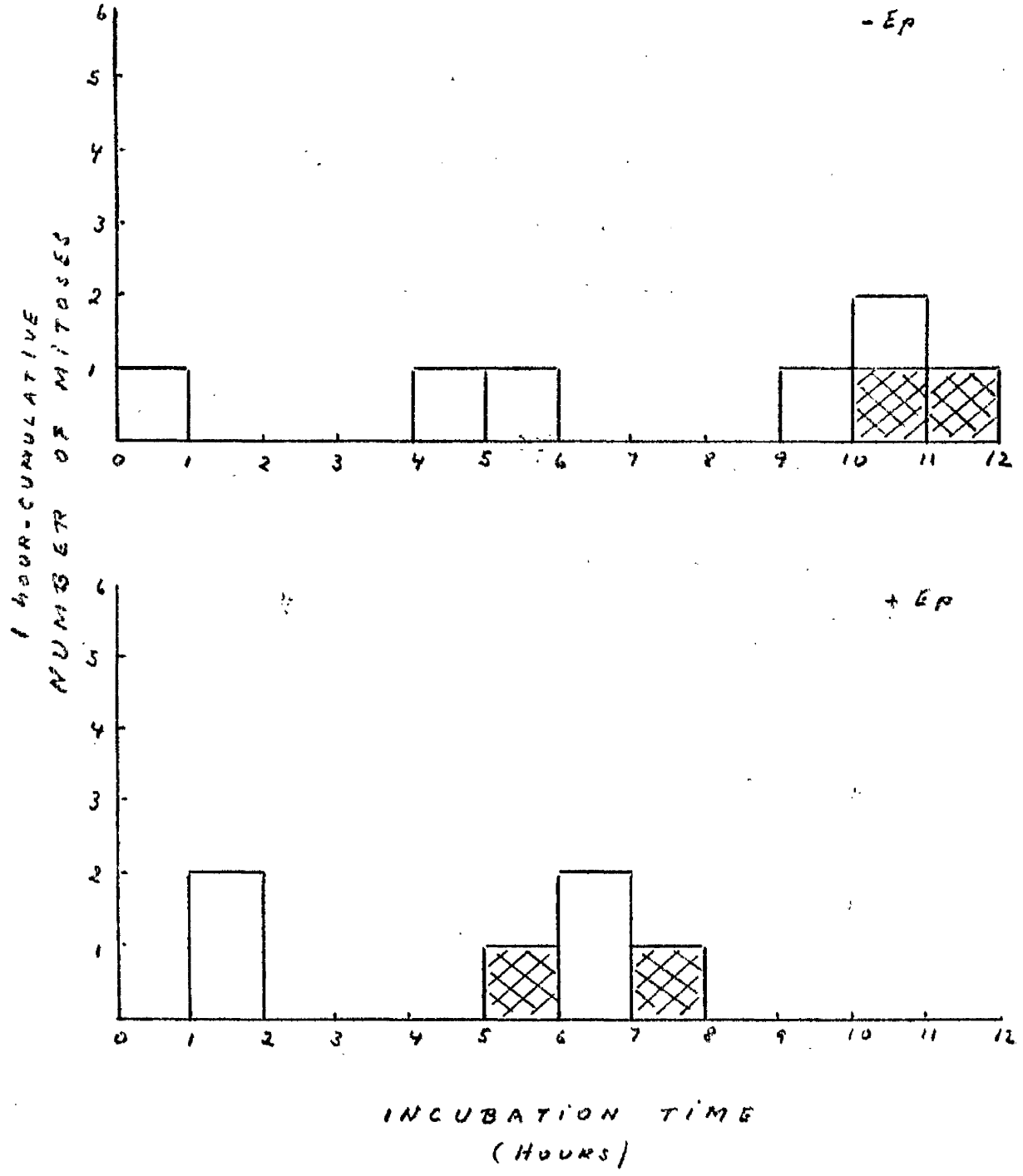

- Ep
+ Ep
1 HOUR-CUMULATIVE NUMBER OF MITOSES
0 1 2 3 4 5 6 7 8 9 10 11 12
INCUBATION TIME (HOURS)

Figure 62.

Frequency distribution of mitoses in 13-day foetal mouse liver erythroid cells cultured in hypertransfusion-polycythaemic mouse plasma clots.

Technical procedures as in Figure 60.

Experimental conditions in Table 30.

Crossed squares indicate incorporation of $^{3}H$-thymidine.

FIGURE 62

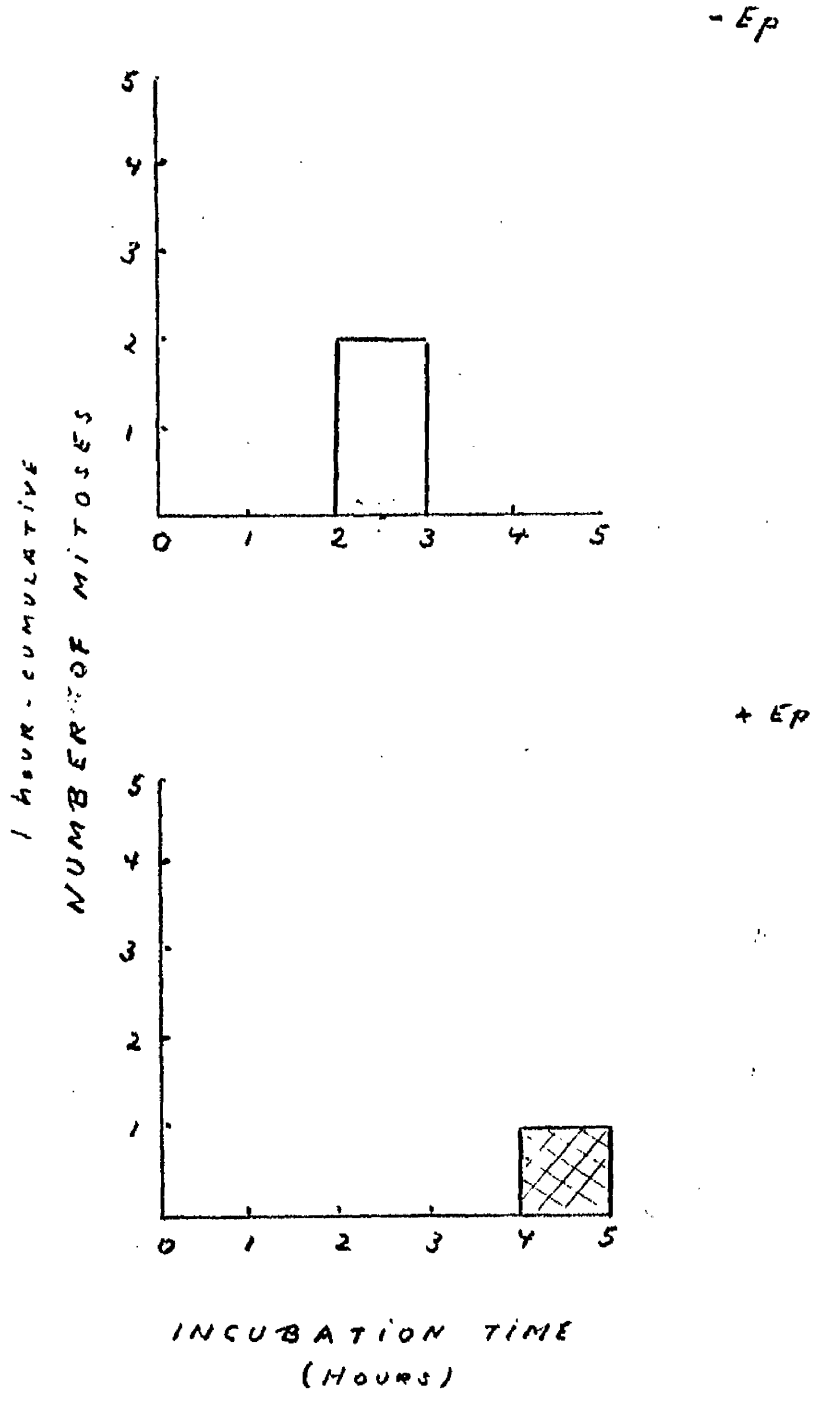

- Ep
+ Ep
1 HOUR - CUMULATIVE NUMBER OF MITOSES
0
1
2
3
4
5
INCUBATION TIME (HOURS)

Figure 63.

Frequency distribution of mitoses in 13-day foetal mouse liver erythroid cells cultured in hypertransfusion-polycythaemic mouse plasma clots.

Technical procedures as in Figure 60.

Experimental conditions in Table 30.

Crossed squares indicate incorporation of $^{3}H$-thymidine.

FIGURE 63

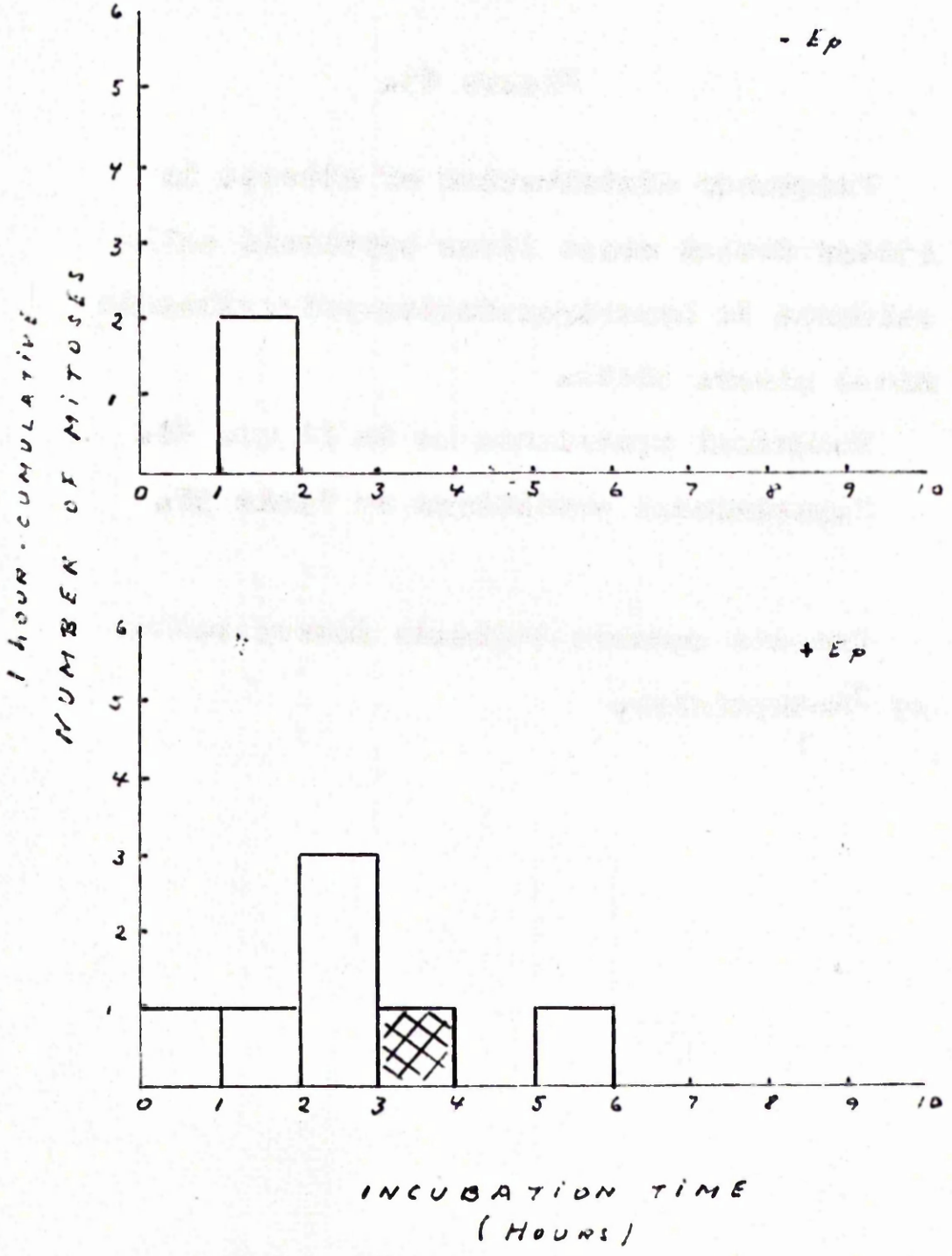

- Ep
+ Ep
1 HOUR - CUMULATIVE NUMBER OF MITOSES
0 1 2 3 4 5 6 7 8 9 10
INCUBATION TIME (HOURS)

Figure 64.

A plot showing whether or not 13-day foetal mouse erythroid daughter cells revealed incorporated $^{3}H$-thymidine in relation to time after exposure of parent to the isotope (5 μc/ml) for 30 minutes.

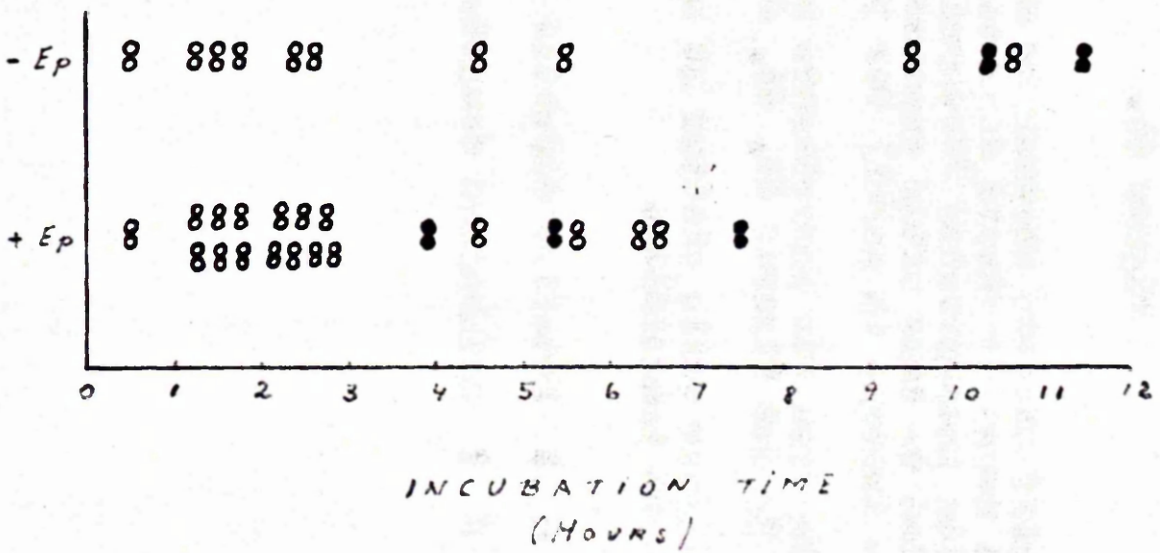
Data from the experiments are shown in Table 30 and Figures 60, 61, 62 and 63.

Daughter cells plotted at anaphase-telophase transition.

● : labelled daughter cells.

○ : unlabelled daughter cells.

FIGURE 64




S⁺ + G2 + ⁺M

Although mitoses occurred in the first hours of incubation, the minimum time of appearance of intranuclear isotope in daughter cells was about 4 hours after the beginning of telophase in erythropoietin-stimulated cells, and during the 10th hour in non-stimulated cells. A considerable proportion of cells which incorporated isotope did not undergo mitosis during incubation. They appeared to belong to the early stages of proerythroblasts, basophilic erythroblasts and polychromatic erythroblasts.

To find the effect of erythropoietin on the post-mitotic phase, cultures of M.F.L. cells were prepared in plasma clots, as described in Methods. After 2 or 4 hours of incubation, the culture medium was changed by perfusing new medium containing 5 μc/ml of $^{3}H$-thymidine and filming resumed (or initiated) for a further 1 to 3 hours. Cultures were then fixed, and autoradiographic and staining procedures carried out as previously described. By relating isotope incorporation into daughter cells, as observed in the autoradiographs, to the time since the previous mitosis of the parent cell, as determined from the film, estimates of the time between the beginning of telophase and S were obtained. Figures 65 and 66 show the conditions and results of these experiments. A shortening of $G_1$ phase appeared to occur

Figure 65.

A plot showing mitotic 13-day foetal mouse liver erythroid cells at anaphase-telophase transition in relation to time of incubation and $^{3}H$-thymidine perfusion, and presence or absence of incorporated isotope in the resultant daughter cells at the end of incubation.

The cells were cultured in clots made up of equal parts of cell suspension in Waymouth's medium supplemented with 10% mouse serum + 50% chicken embryo extract in foetal bovine serum + normal mouse plasma. Cinemicrography was initiated, $^{3}H$-thymidine (5 µc/ml) perfused after 1½ hrs., and filming resumed to the end of incubation. Autoradiography was performed as described in the text.

8 : parent cell at anaphase-telophase transition.

● : labelled daughter cells.

8 : unlabelled daughter cells.

FIGURE 65

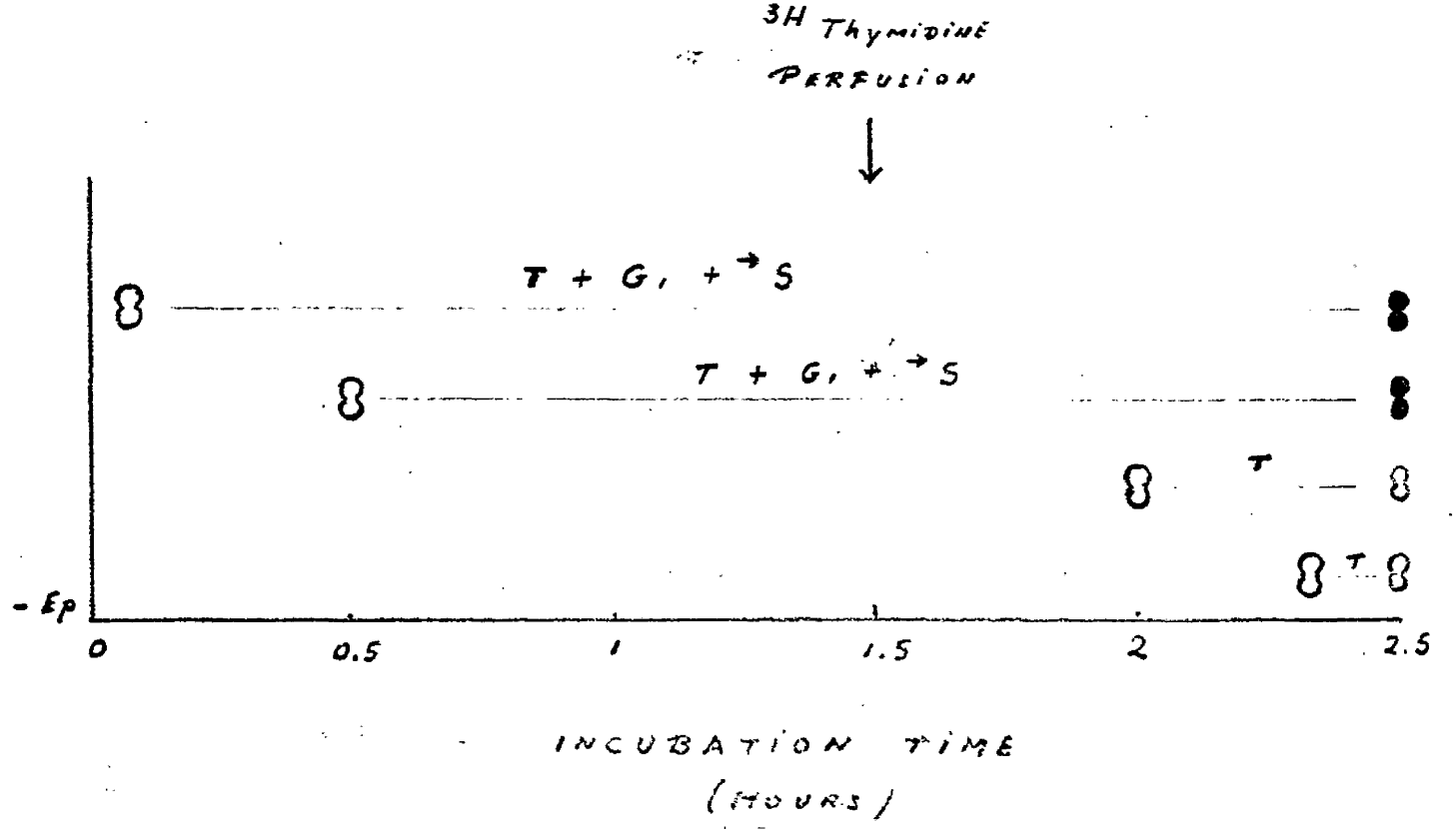

3H Thymidine
Perfusion
T + G1 + → S
T + G1 + → S
T
T
- Ep
0
0.5
1
1.5
2
2.5
INCUBATION TIME
(HOURS)

Figure 66.

A plot showing 13-day foetal mouse liver erythroid cells at anaphase-telophase transition in relation to time after perfusion of $^{3}H$-thymidine, and presence of incorporated isotope in their resultant daughter cells at the end of incubation.

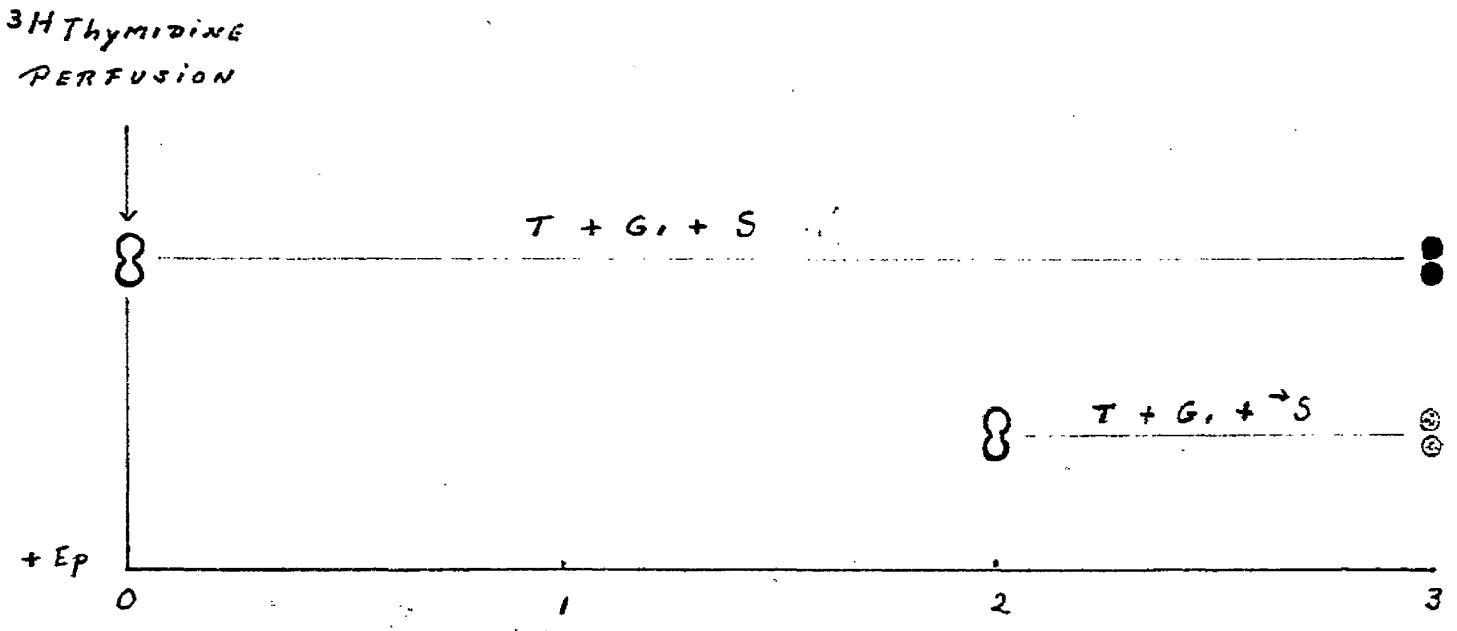
The cells were cultured in similar clots to those described in Figure 65 and stimulated by erythropoietin (0.6 U/ml in the cell suspension medium, 1.2 U/ml in the chamber's medium). $^{3}H$-thymidine (5 $\mu$c/ml) was perfused when a cell was in the process of mitosis. Cinemicrography was then initiated and continued to the end of incubation. Autoradiography was performed as described in the text.

 : parent cell at anaphase-telophase transition.

 : heavily labelled daughter cells.

 : lightly labelled daughter cells.

FIGURE 66


3H Thymidine
PERFUSION
T + G1 + S
T + G1 + →S
+ Ep
0
1
2
3
INCUBATION TIME
AFTER THYMIDINE PERFUSION
(HOURS)

in erythropoietin-stimulated cultures.

Differential counts performed in these experiments were meaningless, due to the high proportion of unrecognized daughters, whether caused by disruption or labelling, and to the small size of the experimental samples.

## III. Discussion and Conclusions.

1 - Evaluation of the experimental system.

2 - Frequency distribution of mitoses in M.F.L. erythroid cell cultures, and
Action of erythropoietin.

3 - Presence of mitoses in different erythroid cell types, and
Cells responding to erythropoietin.

4 - Hypertransfusion-polycythaemic serum effect on mitosis of M.F.L. erythroid cells, and
Presence of erythrocytic mitotic inhibitor.

1. Evaluation of the experimental system:

The culture system was far from ideal, but it was useful for short-term experiments of the type carried out here, although repetition of mitotic cycle was never observed. However, cell behaviour *in vitro* is quite different from cell behaviour *in vivo*, and limitations of some sort are present in any culture system. The most difficult factor to control was pH and inevitably there were small variations. It is perhaps worth while to point out here, that pH 6.6 to 8.0 are the optima for bone marrow cell cultures, and such a broad pH shows that hydrogen-ion concentration is not a factor in the decline of erythropoiesis (Smith and McKinley, 1965). Cultures which showed a marked shift in pH, or evidence of bad culture conditions of any kind, were discarded.

The relatively high mitotic indices obtained are compatible with other data. It is well known that active cell proliferation takes place among erythroblasts and high mitotic indices, up to 40%, have been reported in plasma clot cultures (Astaldi and Maunj, 1949; Berman and Powsner, 1959; Matoth and Ben-Porath, 1959; Matoth, 1968). It is true that total and differential cell counts of this nature are often of doubtful value, because of the difficulty in identifying primitive cell types (Dunn, 1954). In this respect,

following Matoth et al. (1958), it can be said that no claim of accuracy is made for the method, although results appeared to be reproducible and agreed fairly well in repeat experiments, and it was sensitive enough to demonstrate conclusively significant differences.

2. Frequency distribution of mitoses in M.F.L. erythroid cell cultures, and Effect of erythropoietin.

It has been pointed out (Paul and Hunter, 1969) that in the whole animal erythropoietin increases erythrocyte production by stimulating cell maturation of undifferentiated precursors (Filmanowicz and Gurney, 1961; Keighley, Hammond and Lowry, 1964; Schooley, 1965; Gurney and Fried, 1965) and, perhaps, of erythroblasts (Borsook et al., 1968). It has been suggested that in the adult mammal erythropoietin promotes the maturation of the so-called "erythropoietin-responsive cell" (Bruce and McCulloch, 1964) or induces differentiation of stem cells (Lajtha, 1965) and increases the rate of cell proliferation of the proerythroblast precursors, which could result in either decreased maturation time of recognizable cells or increase in total number of mitoses (Blackett, 1969; Tarbutt, 1969; Hanna, Tarbutt and Lamerton, 1969). Increased mitotic indices in bone marrow cells cultured with anaemic serum have been reported (Matoth and Ben-Porath, 1959)

and it has been suggested that erythropoietin acts by stimulating erythroid cells to mitotic division (Matoth and Ben-Porath, 1959).

The present experimental findings demonstrate that erythropoietin increases the total number of mitoses. This confirms the findings of Matoth and Ben-Porath (1959) and the postulations of Blackett (1969), Tarbutt (1969), and Hanna, Tarbutt and Lamerton (1969). However, in spite of the increased mitotic index in erythropoietin-stimulated cultures, there was no evidence that erythropoietin had a mitogenic effect. This is in agreement with the extensive literature which has accumulated along these lines (Bullough and Rytömaa, 1965). They also demonstrate that the presence of erythropoietin was necessary to maintain mitotic activity. This observation is consistent with the findings that withdrawal of erythropoietin produces a fall in Hb., DNA and RNA synthesis (Paul and Hunter, 1969).

No information on the transformation of stem cells or immediate precursors into proerythroblasts was obtained under the conditions of the experiments here reported.

The significant increase in mitoses in the first hour of incubation in erythropoietin-stimulated cells indicates that the effect of erythropoietin is rapid.

This observation is also consistent with the findings of Paul and Hunter (1969) suggesting acceleration of DNA synthesis at about 20 minutes after erythropoietin is added to cultures of M.F.L. It should be pointed out, however, that a great proportion of cells synthesizing DNA, as judged by $^{3}H$-thymidine incorporation, did not enter mitosis. Paul and Hunter (1969) have suggested three explanations of the effect produced by erythropoietin on the normal mouse DNA synthetic cycle 20 minutes after its addition: erythropoietin may accelerate DNA synthesis in cells already in mitosis, precipitate DNA synthesis in cells in late G1, or stimulate a special DNA synthetic phase.

The present experiments by combined time-lapse cinemicrography and autoradiography showed that labelled daughters derived from parents undergoing "anaphase-telophase transition" not less than 4 hours after isotope exposure in erythropoietin-stimulated cells, and at 10 hours in control, non-stimulated cells. These times correspond to part of S + $G_2$ + first part of mitosis up to the beginning of telophase, represented by S $\longrightarrow$ + $G_2$ + $\longrightarrow$ M.

It was not possible to predict the stage of S phase of labelled cells during isotope-exposure, since they all had similar amounts of incorporated label.

Besides, maintaining M.F.L. submerged in trypsin at 4°C did not seem to exert a synchronizing effect on the mitotic cycle, and the range of 4.5 to 5.5 hours estimated for S phase in 13-day M.F.L. erythroblasts in in vivo labelling experiments (Tarbutt and Cole, 1970) would give a wide margin for error. On the other hand, the size of the experimental sample, i.e. the actual number of labelled daughters, was too small to support the randomness of event-appearance. In these circumstances it seems reasonable to conclude that a shortening of $G_2$ takes place in erythropoietin-stimulated cells.

The number of non-labelled daughters derived from mitoses occurring before the times mentioned above indicate that the parents passed S phase before addition of isotope, and were at $G_2$ or the beginning of M, i.e. committed to undergo mitosis, with or without erythropoietin stimulation. Hence the increased number of mitoses seen in erythropoietin-stimulated cultures during the first hours of incubation may be due to erythropoietin permitting continuation of the cell cycle, and/or precipitating the initiation of mitosis, instead of recruiting new cells for mitosis. The results of experiments performed by transfusing $^3$H-thymidine showed that the minimum time-lapse

between "anaphase-telophase transition" and isotope incorporation by the resultant daughters was about 1 hour in erythropoietin-stimulated cells, and about 2 hours in control, non-stimulated cells. These times correspond to telophase + $G_1$ + beginning of S, represented by T + $G_1$ + $\rightarrow$ S.

Tarbutt and Cole (1970) estimated a cell cycle of about 5.6 hours in proerythroblasts and basophilic erythroblasts, and 8 hours in polychromatic erythroblasts of 13-day M.F.L. in vivo, a $G_1$ phase of about 0.4 hour in proerythroblasts and basophilic erythroblasts and 1.5 hours in polychromatic erythroblasts. Duration of mitosis is arbitrarily divided equally between $G_1$ and $G_2$ in this system (Hanna, Tarbutt and Lamerton, 1969). A mitotic time of 45 minutes has been estimated for human erythroid cells (Patt, 1957; Killmann, 1964); and 43 minutes (Dustin, 1959) or about 30 minutes (Roylance, 1966; Lord, 1970) for rat erythroblasts. Hence it is reasonable to conclude that erythropoietin markedly shortened, if it did not abolish, $G_1$ phase. This neither contradicts nor excludes the alternatives suggested by Paul and Hunter (1969), i.e. that erythropoietin either accelerates DNA synthesis in cells already in S, or precipitates DNA synthesis in cells in late $G_1$ phase.

The suggestion of complete or almost complete elimination of $G_1$ phase would lead to the possibility that DNA synthesis re-starts during telophase, perhaps during early telophase. In this regard it is worth while to point out here that DNA synthesis has been reported to begin as early as telophase in other cell types (Mazia, 1961), e.g. cells of the small intestine crypts in the rat (Pasteels and Lison, 1950).

3. Presence of mitoses in different erythroid cell types, and Cells responding to erythropoietin.

Intense DNA synthesis and mitosis have been observed in proerythroblasts, basophilic erythroblasts and polychromatic erythroblasts, and it has been postulated that the acceleration of DNA synthesis which occurs in response to erythropoietin refers mainly to these cells (Paul and Hunter, 1969); but it is not certain which stages of the erythropoietic series are directly affected by erythropoietin (Paul and Hunter, 1969).

Differential counts of erythroid daughters in the present experiments showed that most of them were proerythroblasts and basophilic erythroblasts, in both erythropoietin-stimulated and non-stimulated cultures. Autoradiographic results showed that intense $^3H$-

thymidine incorporation, i.e. DNA synthesis, occurred in early stages, proerythroblasts, basophilic erythroblasts and polychromatic erythroblasts, although the great majority of these cells did not undergo mitosis.

It is very difficult to compare the differential counts of the present studies with those of other workers, since the former refer to daughters derived from a mitotic parent and the latter to total numbers of cells. However, the results presented here seem to be in general agreement with those reported by Djaldetti et al. (1970) who found that the bulk of the cells in younger M.F.L. were immature, while more mature cells predominated in later stages. This agrees with data reported by Tarbutt and Cole (1970) also, but differs somewhat from those of Paul, Conkie and Freshney (1969).

The increased number of mitoses observed in erythropoietin-stimulated cultures appeared in all erythroid cells capable of mitosis. This indicates the general effect of erythropoietin stimulation. However, a shift towards the more mature stages became apparent in cultures with hypertransfusion-polycythaemic plasma. It may be that the erythropoietin level played some role in this situation. The possibility that the erythropoietin level influences

its mode of action has been pointed out by Reissmann and Samorapoompichit (1970). That this was the effect of mitotic inhibitors should not be disregarded completely, although the experimental results - as will be discussed later - do not tend to support this hypothesis.

4. Hypertransfusion-polycythaemic serum effect on mitosis of M.F.L. erythroid cells, and Presence of erythrocytic mitotic inhibitor.

It has been suggested that, besides erythropoietin, there is an additional control mechanism of erythropoiesis mediated by an erythrocytic chalone (Bullough and Rytömaa, 1965)[a,b]. Supporting data on the presence of an inhibitory mechanism in hypertransfusion-induced polycythaemia have been reviewed by Kivilaakso and Rytömaa, 1970). Evidence for the existence of an erythrocytic chalone has been indirect and speculative until now. The observations and results of the present studies, however, provide direct evidence for the existence of a factor inhibiting mitosis of erythroid cells. This factor was present in hypertransfusion-polycythaemic mouse plasma and serum. It was different from erythropoietin, did not prevent its stimulatory effect, and appeared to belong to a different control mechanism from that of erythropoietin. Both factors,

however, mitotic inhibitor and erythropoietin, seemed to affect the same target cells. This suggests that they act on two different stages of the cell cycle, rather than on two different cell populations. Further research is needed to elucidate this situation however.

It might be argued that the inhibitory effect on mitoses in the present experiments was the result of a shortage of erythropoietin, or the presence of toxic factors. However, the fact that polycythaemic serum inhibited mitoses in cultures with normal plasma and serum, i.e. containing adequate levels of erythropoietin, showed that a shortage of erythropoietin was not responsible for this effect. On the other hand, the fact that addition of exogenous erythropoietin still produced increased mitoses, showed that the inhibition was not a non-specific toxic effect. For the same reasons it also appears that this was not a case of erythropoietin inactivation or antagonism.

## SUMMARY.

Studies on erythroid cell maturation were carried out by means of two different experimental approaches leading to the characterization of (a) the haemoglobin type synthesized by spleen colonies produced by transplantation of rat haemopoietic tissue into heavily irradiated mice, and (b) the daughter cells derived from mitoses of earlier haemopoietic precursors, and factors influencing their maturation and proliferation.

Optimal experimental conditions for spleen recolonization by heterologous (rat) transplants were investigated and established through a series of experiments, and methods for preparation and analysis of the type of haemoglobin synthesized by the recolonizing cells were developed.

Investigation of the behaviour of normal and experimental haemoglobins in starch gel electrophoresis and development of assay for their differential characterization were undertaken.

Supralethally irradiated mice (Porton strain) were transplanted with foetal liver and adult bone marrow cells from Wistar rats. After a suitable post-transplantation period, the exogenously recolonized spleens were obtained. The confluent spleen colonies

were isolated and disaggregated, and cell cultures prepared and incubated with $^{59}FeCl_3$. Haemoglobins were extracted, purified by CMC column chromatography, and fractionated by starch gel electrophoresis. The incorporation of $^{59}Fe$ into haemoglobin was determined by cutting the gel strip containing the haemoglobin components into 1mm or 1½ mm thick slices, which were then individually hydrolysed and counted by liquid scintillation system.

It was possible to demonstrate the heterogeneity of rat haemoglobin, and the presence of haemoglobin aggregates. Synthesis of catalase and other non-haemoglobin, haem-containing proteins, along with synthesis of haemoglobin was also demonstrated. This makes the interpretation of assays based on iron incorporation difficult for haemoglobin synthesis studies.

Although recolonization of mouse spleens by rat haemopoietic tissues was achieved in spite of the antigenic disparity between donor and host, the analysis of the type of haemoglobin synthesized by the spleen recolonizing cells was obscured by the presence of endogenous haemoglobin and aggregates. However, there was some indication that the recolonizing cells continued to produce the same type of haemoglobin.

For the <u>in vitro</u> study of behavioural and morpho-

logical characteristics of erythroid cells, and factors influencing their maturation and proliferation, 13-day foetal mouse livers were isolated and disaggregated. Cell cultures were prepared in plasma and fibrin clots, and observed with time-lapse cinemicrography during mitosis. The events recorded on the film were analysed and correlated with the fixed and stained cells.

The effects of different plasma, and erythropoietin stimulation on mitosis of erythroid cells were studied by measuring differences in frequency distribution of mitoses and mitotic indices, and also by autoradiography. The results demonstrated an increased number of mitoses in erythropoietin-stimulated cultures. Erythropoietin appeared to permit the continuation of the cell cycle and to maintain mitotic activity throughout the period of incubation. The results also suggested that erythropoietin-produced shortening of G2 and G1, i.e. precipitated the initiation of mitosis as well as synthesis.

By the use of plasma from mice made polycythaemic by hypertransfusion, and stimulation with erythropoietin, the presence of an erythropoietic mitotic inhibitor was demonstrated.

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## ABBREVIATIONS.

| | | |
|---|---|---|
| AMPB | = | adult mouse periphreal blood. |
| ARBM | = | adult rat bone marrow. |
| ARPB | = | adult rat peripheral blood. |
| CFC | = | colony forming cell. |
| CFU | = | colony forming unit. |
| Ch.EE | = | chicken embryo extract. |
| Ch.S.(P1) | = | chicken serum (plasma). |
| CMC | = | carboxymethyl cellulose. |
| Ep. | = | erythropoietin. |
| FBS | = | foetal bovine serum. |
| FML | = | foetal mouse liver. |
| FRL | = | foetal rat liver. |
| FRPB | = | foetal rat peripheral blood. |
| HPMS (P1) | = | hypertransfusion-polycythaemic mouse serum (plasma). |
| HPVS | = | human polycythaemia vera serum. |
| MFL | = | mouse foetal liver. |
| MPB | = | mouse peripheral blood (adult). |
| MS (P1) | = | mouse serum (plasma), normal, adult. |
| MYS | = | mouse yolk sac. |
| NMS (P1) | = | normal mouse serum (plasma), adult. |
| RFL | = | rat foetal liver. |
| Way's | = | Waymouth's culture medium. |
| U | = | units. |