THE SPLEEN

AND RED CELL DESTRUCTION

Studies in man of:

(a) the role of the spleen in the uptake of red cells altered by heat treatment and by treatment with a sulphydryl inhibitor;

(b) the use of such cells in the measurement of splenic sequestering function;

(c) the effect of noradrenaline on the splenic sequestration of red cells.

THESIS

SUBMITTED FOR THE

DEGREE OF DOCTOR OF MEDICINE

IN THE

UNIVERSITY OF ADELAIDE, SOUTH AUSTRALIA

BY

RICHARD JOHN KIMBER

CONTENTS

ACKNOWLEDGEMENTS

The studies of the role of the spleen in red cell destruction were made in the Department of Medicine, University of Adelaide, during the two years beginning February 1963. During this time I held the position of Lecturer in Medicine in the Department of Medicine.

This study has required the assistance of patients, volunteers and colleagues. I am grateful to the patients of the Royal Adelaide Hospital who permitted me to carry out these investigations and to the many inmates of the Yatala Labor Prison and other volunteers who agreed to act as control subjects on many occasions, and all did so without any assurance that this would be to their personal benefit. I would like to thank the Honorary Physicians and Surgeons of the Royal Adelaide Hospital for allowing me to study patients under their care; Dr. J.A. Bonnin, Director of the Institute of Medical and Veterinary Science, for making available the facilities of his various departments; and to Mr. Allen and the Staff of H.M. Gaols and Prisons for their trouble in arranging the attendance and transport of the volunteers from the Yatala Labor Prison.

I am grateful to Professor H.N. Robson for his interest in this work and to other colleagues in the Department of Medicine for the valuable stimulus of their discussion. I am particularly indebted to Dr. H. Lander, Reader in Medicine, University of Adelaide, who assisted in this project for his encouragement, advice and constructive criticism.

It is also with pleasure that I acknowledge the help of Miss Jean Devaney who undertook the typing of the thesis, Miss Margaret Hall who provided devoted technical assistance and prepared many of the graphs, and Mr. W. Nolan for the photographic reproductions of micrographs and other figures.

I declare that this thesis is my own composition and that it is a true record of research done while working in the Department of Medicine, University of Adelaide.

CHAPTER I

INTRODUCTION

The spleen has, for many years, been implicated in the pathogenesis of anaemia, and the term "splenic anaemia" was well entrenched in the last century. Stanley (1895) used this phrase to cover some cases of splenic hypertrophy of unknown cause and somewhat prophetically suggested that the splenomegaly "may be brought about by an effort on the part of the spleen to eliminate.....morbid products of the blood". Some years previously Murchison (1877) described a family with "hereditary jaundice" affecting a woman, her two sons and several children of the latter. He noted the point that, in contradistinction to obstructive jaundice, the "motions were fairly coloured with bile". Wilson in 1890 gave a very clear description of a family with chronic jaundice and noted the splenic enlargement. He reported six cases, three male and three female in three generations, all of whom showed splenomegaly and sallow icteric complexions. He also noted several features which later were to become well recognised facets of congenital spherocytosis. These included the association of biliary colic, due to the increased propensity to form pigment gall stones, and the occurrence of acute exacerbations, or crises. In two instances he mentioned how the shock of a cold bath turned the complexion of two of his patients to a "daffodil colour". In a follow up of this family in 1893 Wilson and Stanley established anaemia as part of this familial jaundice and implicated the spleen in its causation. They proposed that the rapid progress of the anaemia was dependent upon active red

cell destruction of splenic origin. In this they followed the proposal of William Hunter (1892) who in "Lectures on the Physiology and Pathology of Blood Destruction" claimed for the spleen "the power of arresting red corpuscles". He had shown that cells having received injury "short of their complete disintegration.....are seized on in great numbers by the spleen". Hunter also was probably the first to use the word "haemolytic" when he stated".....the presence of blood pigment within the spleen is an indication of a special haemolytic activity of that organ". It then became established that bilirubin, in abnormally high concentrations, was the cause of jaundice, that haemoglobin was the source of bilirubin and that haemolytic jaundice was due to excessive destruction of red cells and breakdown, in various parts of the body, of the contained haemoglobin. Early work clarifying these factors is reviewed by Gray (1953).

The terms "haemolytic jaundice" and "haemolytic anaemia" then became widely used and accepted as synonymous. Also the word "haemolysis" has been used to cover the rupture of cells with consequent release of haemoglobin in vitro or in the circulating blood in vivo (intravascular haemolysis) as well as the separation of haemoglobin from the effete red cell. The latter probably occurs within the macrophages of the reticulo-endothelial system (extravascular haemolysis) and is not associated with the release of free haemoglobin.

Crosby (1955) drew attention to the different meanings of the

terms "hemolytic disease", "hemolytic anemia" and "hemolytic jaundice" and stated: "They are not equivalent. Hemolytic disease occurs when, in the absence of hemorrhage, the average life span of the circulating red cells is less than normal. Hemolytic anemia occurs when the bone marrow, by increased production of red cells, is unable to compensate completely for the shortened life span. Hemolytic jaundice occurs when such quantities of bilirubin are presented to the plasma that the normal excretory capacity of the liver is unable to maintain a normal concentration of bilirubin in the plasma. Hemolytic disease may exist with no anemia or jaundice." However, due to the fact that the majority of patients with haemolytic disease are indeed anaemic Dacie (1960) feels that the use of the term "haemolytic anaemia" for patients showing a shortened red cell life span, is justified.

Though as mentioned there were early reports of the association of a familial jaundice with anaemia and splenomegaly Minkowski (1900) is credited with the first complete description of congenital haemolytic anaemia. He studied eight cases in three generations and noted the association of chronic jaundice and splenomegaly, while Chauffard (1907) also described several cases and reported that the red cells in this condition were osmotically more fragile than normal. Only congenital haemolytic anaemia was recognised until 1898. At this time Hayem widened the classification of haemolytic disease when he

observed a severe type which was regarded as acquired. Though the first report of such an acquired form is generally attributed to this author, Widal, Abrami and Brule in a series of papers from 1907 to 1912 clearly distinguished this type from the congenital disease. Following this Chauffard and Troisier (1908) described acute non-congenital haemolytic anaemia with a haemolysin in the serum. Similar cases were later described (Lederer, 1925; Giordano and Blum, 1937) associated with autohaemagglutinins and the distinction between an acquired and congenital haemolytic disease became well recognised and clearly established (Dameshek and Schwartz, 1940). At this time these authors classified haemolytic anaemia as

A. Congenital hemolytic anemia
   1. Chronic
   2. Subacute
   3. Acute

B. Acquired hemolytic anemia
   1. Secondary to infections, chemicals, toxic
   2. Symptomatic - in association with certain, usually malignant, diseases
   3. Of uncertain cause - with or without hemolysins in the serum (idiopathic).

An important landmark in the investigation of the symptomatic and idiopathic acquired types was the discovery that cells coated with

antibody could be detected by the use of anti-human globulin serum (Coombs et alii, 1945). The physical characteristics of the various antibodies in these so-called auto-immune haemolytic anaemias are discussed by Dacie (1962).

Progress in the understanding of some congenital types followed the finding of Pauling et alii (1949) that the haemoglobin of normal negroes and that of sickle anaemia sufferers were unlike. This led to a recognition of the importance of molecular abnormalities of haemoglobin in the causation of haemolytic disease and to the discovery of a variety of abnormal haemoglobins. Some of these were found to be associated with a shortening of red cell life. Also the study of red cell metabolism, reviewed by Prankerd (1955) and Carson and Tarlov (1962) threw light on some of the enzymatic deficiencies that were associated with the short survival of erythrocytes in other congenital haemolytic states. In this respect also the action of certain drugs known to cause haemolytic disease was elucidated (Beutler, 1959).

The use of cross transfusion experiments and the estimation of red cell life by this means, and by the use of red cell labelling with radioactive isotopes, made it possible to distinguish haemolytic anaemias due to an intrinsic defect in the red cells from that produced by external factors. Although the ultimate mechanism in some of these syndromes is becoming clearer it is still felt convenient (Dacie, 1962) to group them broadly under congenital and acquired

types, or (Dameshek, 1955) as those due to intrinsic defects of the red cell and those due to extrinsic mechanisms.

The operation of splenectomy and its success in various conditions was reviewed by Spanton (1895) when he reported that the best results were seen in cases of the so-called "hypertrophy" or "splenic anaemia" many of which may well have been congenital haemolytic anaemia. He records that the first splenectomy for such a condition was performed by Zacharelli in Naples in 1549 on a female of 24 with the diagnosis of splenic hypertrophy. The patient did well and the spleen weighed 2 pounds 15 ounces. He also gives credit for the first splenectomy in England to Spencer Wells in 1865. Again the operation was on a female of 34 years with the diagnosis of splenic hypertrophy and the organ weighed 6 pounds 15 ounces. Unfortunately, the patient succumbed with a possible thrombosis on the seventh post-operative day. Lendon (1896) probably performed the first splenectomy for hereditary spherocytosis in this country. He reports the case of a 21 year old female with muddy complexion, leg ulcers (now recognised as a concomitant of this condition) and anaemia. Post-operatively her leg ulcers healed and she was free of anaemia. Interestingly, her family history includes cases of splenomegaly in two sisters, one brother and a niece. Micheli (1911) is credited with the first splenectomy in acquired haemolytic anaemia.

Though splenectomy was found to be an effective form of treatment

in many cases of haemolytic anaemia, the operation was not invariably crowned with success. Not all the red cell changes brought about by the various congenital or acquired factors cause the early removal of the cells from the circulation solely by the spleen. Also despite the discovery of some constant cellular changes in different haemolytic diseases it is not yet possible to accurately predict which particular subject will benefit from removal of the spleen. Cross transfusion studies and study of the site of uptake of red cells utilizing radio-active isotopes provide help in this decision, but frequently equivocal results are obtained by such methods. The growth in knowledge of the relationship between erythrocytes and the spleen in haemolytic states is traced in the following section.

CHAPTER II

LITERATURE REVIEW. THE SPLEEN AND THE REMOVAL OF ERYTHROCYTES FROM THE CIRCULATION IN HAEMOLYTIC DISEASE

Estimation of Red Cell Life Span by Cross Transfusion Experiments

Some clarification of the role of the spleen in red cell destruction followed the successful delineation of red cell life span. Todd and White (1911) described the detection of transfused cells in the circulation of cattle. They used a specific haemolytic antiserum prepared against the cells used for transfusion. Using an adaption of this technique, Ashby in 1919 demonstrated the survival of transfused cells in human subjects. She administered compatible cells, of a different group to the recipient, and subsequently detected these by differential agglutination in vitro, using the appropriate agglutinin. She showed originally in seven subjects (one normal, two with pernicious anaemia, two with haemorrhage and two with other anaemias) that compatible cells survived for longer than 30 days. The full life span of red cells was not measured when Ashby (1921a), in a large series of normal and abnormal subjects, claimed that circulating cellular destruction was periodic and that in females the phasic destruction coincided with menstruation. An approximation of normal cell life was however given by this worker (Ashby, 1921b) when she reported that the cell life varied from 91 to over 100 days in four patients with pernicious anaemia in whom donor cells were followed until complete elimination. From this she concluded that in pernicious anaemia there was no excessive destruction of donated compatible cells and thus no haemolysin was active. She reported short donor red cell

life in two cases of aplastic anaemia, one case of myeloid leukaemia, one case with carcinoma of the kidney and one case of haemolytic jaundice. In the last instance the haemolysis was relieved by splenectomy. Using an adaption of the Ashby technique Wearn et alii (1922) showed that compatible cells survived for periods of from 59 to 113 days (average 83 days) in four patients with pernicious anaemia and in four with anaemia secondary to chronic renal failure. However, although Landsteiner in 1928 detected transfused cells in seven normal subjects for over 50 days, the normal life span was not definitely established until several years later. In 1934 Weiner, in a discussion of methods used for this estimation, reported ten cases in whom red cell elimination was not complete until 120 days.

The Ashby method of estimating red cell life was not widely used until 1940. At this time Mollison and Young utilised it to study the usefulness of stored cells for transfusion in war casualties. They showed the normal life was indeed approximately 100 days and that blood could be stored for periods of up to 18 days before there was any appreciable reduction in survival. Again in 1942 they reported an average red cell life of 109 days in 15 cases of hypochromic anaemia given compatible cells. Callender et alii (1945) confirmed the life of 120 days in three normal males, and in 1947 described the fall in red cell count after transfusion as curvilinear rather than truly linear. Thus they claimed that some cells were destroyed randomly

rather than as a function of age and that this fraction was greater in females than males. They gave the normal red cell life as 90 to 110 days in females and 110 to 120 days in males.

This was followed by many studies of the survival of normal cells in subjects with haemolytic disease, and vice versa, in an endeavour to differentiate conditions with an inherent red cell defect from those in which the cell was secondarily acted upon by an extracellular influence. For example, Dacie and Mollison (1943) followed the survival of normal erythrocytes transfused into six patients with familial haemolytic anaemia (hereditary spherocytosis). In five of these the survival was unaffected and in one slightly diminished. Conversely blood taken, both before and after splenectomy, from one patient disappeared rapidly when transfused into a normal recipient. This, they stated, "supports the generally accepted view that the increased rate of cell destruction is due to an abnormality of the patient's own erythrocytes". Brown et alii (1944) demonstrated an extracorpuscular agent in three cases of acquired haemolytic anaemia. The life of transfused normal red cells was grossly reduced in the three patients and these authors drew attention to the exponential fall in red cell count characteristic of random cellular destruction. Loutit (1946) confirmed this observation, and described eight cases showing the same phenomenon, and extended it by demonstrating normal survival, of cells from four patients with acquired haemolytic

jaundice, in control subjects. The extracorpuscular factor so demonstrated he described as a circulating haemolysin. This worker also showed a reduced survival of cells from a patient with pernicious anaemia in a normal subject but a longer life of donor cells in a patient with this disease. This suggested that the red cells in pernicious anaemia were inherently defective and prone to premature removal from the circulation. Also he confirmed the findings of Dacie and Mollison (1943) with respect to the defect in hereditary spherocytosis. Mollison (1947) showed there was no extracorpuscular factor acting in cases of hypochromic anaemia, chronic sepsis, carcinoma and nocturnal haemoglobinuria, that could reduce the survival of normal cells, but confirmed the rapid elimination of such cells in subjects with acquired haemolytic anaemia. He noted also that normal cells given to patients with familial haemolytic anaemia suffered no change in osmotic fragility during their stay in the circulation. Singer et alii (1948) confirmed Loutit's (1946) finding of a corpuscular defect in three cases of untreated pernicious anaemia and the reversal of this defect in one treated case.

Other studies utilising cross transfusion experiments and Ashby survival studies include those of Evans and Duane (1949) who showed no reduction in the life of normal cells in subjects with Mediterranean anaemia (thalassaemia major) and paroxysmal nocturnal haemoglobinuria. Emerson and Burrows (1949) showed an element of mild haemolysis in

cases of chronic uraemia. However, they concluded that the major causative factor in the anaemia of this condition was bone marrow depression. Kaplan and Zuelzer (1950) showed a corpuscular defect in Mediterranean anaemia by demonstrating a shortened survival of these cells in normal subjects although the life span of cells from patients with the carrier state was not reduced. Some cells from a case of this disease survived longer than others, and the authors suggested that it was the cells with a basic defect of ultrastructure or composition, resulting in an abnormal shape, that were destroyed more rapidly. Hamilton et alii (1950) also analysed Cooley's anaemia (thalassaemia major) by transfusing (a) normal cells into patients with the disease and into those with Cooley's trait (thalassaemia minor), (b) Cooley's trait cells into patients with the anaemia and (c) cells from patients with the overt disease into normal subjects. Only in the latter case was survival reduced and they concluded that their data supported the hypothesis that the disorder in this disease was an intrinsic defect of the erythrocyte which shortened its survival. Chaplin and Mollison (1953) showed with this technique that haemolysis was not a marked feature in chronic renal disease but in five cases of cirrhosis the life span of donor cells was reduced. Other conditions studied in this way included sickle cell disease and paroxysmal nocturnal haemoglobinuria. The former was investigated by Callender et alii (1949) who demonstrated no excessive destruction of normal

cells in patients with sickle cell disease. Sickle cells, but not sickle trait cells, showed reduced survival in normal subjects. They thus concluded that the defect in this disease was an inherent one in the cell and not a function of its age. Dacie and Mollison (1949) showed that cells from a donor with nocturnal haemoglobinuria had a short life in a normal subject. This confirmed the suggestion of Evans and Duane (1949) that the defect in this disease was confined to the cells of the sufferer and was not due to an extracellular agent.

Red Cell Abnormalities in Haemolytic Disease

The haemolytic anaemias were generally classified under the headings of A. Congenital (e.g. congenital spherocytosis) and B. Acquired. In the latter, three subgroups (i) cellular damage by infective or chemical agents, (ii) symptomatic or secondary to such conditions as the leukaemias, Hodgkin's disease and carcinoma, and (iii) unknown aetiology (idiopathic), were generally recognised (Dameshek and Schwartz, 1940). However, in 1947 Haden extended the congenital group with the first clear description of a "hereditary haemolytic jaundice without spherocytosis". He reported eight cases in two families and noted that the red cells were of normal shape and tended to undergo spontaneous haemolysis. Haemoglobinaemia was often seen and thus indicated intravascular lysis. Splenectomy was found not to cure this condition, in contradistinction to congenital spher-

cytosis, and he concluded that the disease was due to an inherited defect of the red cell stroma. Crosby (1950) fully documented this condition and showed that the cells were not abnormally fragile, the condition was transmitted in a family as a Mendelian dominant, cells from patients with the disease were cleared from the circulation of a normal recipient in 12 days, and again splenectomy did not cure the anaemia. Crosby and Akeroyd (1952) performed further cross transfusion experiments in this condition and hereditary spherocytosis, and showed that the cells from both diseases had a short survival in normal patients but that donor cells survived normally in these individuals. Selwyn and Dacie (1954) divided the congenital non-spherocytic anaemias into types I and II on the basis of in vitro auto-haemolysis tests and Robinson et alii (1961) demonstrated a block in the red cell glycolytic cycle in type II. The biochemical abnormalities in red cells in congenital haemolytic anaemias have been reviewed by Carson and Tarlov (1962) and de Gruchy (1963).

An advance was made in the delineation of the acquired forms of haemolytic anaemia when Coombs et alii (1945) introduced the test by which red cells with surface adsorbed antibody globulin could be detected. This antibody was demonstrated in vitro by using an anti-human-globulin serum prepared by the injection of human serum into rabbits. From this developed the concept of auto-immune haemolytic anaemia and Loutit and Mollison (1946) thus proposed that, while in

the familial types there is a hereditary inborn defect of the erythron, in the acquired type there is often an abnormal antibody destroying both autologous and homologous red cells. This was confirmed by these workers by further cross transfusion experiments in which they demonstrated that donor erythrocytes in a patient with acquired haemolytic anaemia were rapidly eliminated, while cells from the patient with the acquired form (Coombs' positive) had a normal or only slightly reduced life in a normal subject. Boorman et alii (1946) described 17 cases of the congenital type in which the cells were not agglutinated by this means and postulated that only the acquired form was due to a form of auto-immunisation. Cross transfusion studies by Owren (1949) confirmed the presence of an extracellular agent, as most of the erythrocytes from a patient with Coombs' positive acquired haemolytic anaemia did have a normal life in a control subject. However, some of the transfused cells were rapidly removed and he postulated that these cells had already been sensitised, and this resulted in a short life even in the normal recipient.

In the acquired haemolytic anaemias the division into three groups: idiopathic, secondary (symptomatic) to some underlying disease, and that due to infective or toxic agents was still followed. The term "auto-immune" was then used to cover those within these groups when there was an associated formation of antibodies against the patient's own erythrocytes. However, not all cases of acquired haemolytic anaemia

were associated with such antibody formation though this was almost invariably found in the idiopathic group. Over recent years the antibodies of acquired haemolytic anaemia have been widely studied and two main types recognised, a "warm" type active at body temperature and a "cold" type active at a few degrees lower. The serological findings of these conditions have been extensively reviewed by Dameshek (1951), Dacie and de Gruchy (1951) and Dacie (1962, 1963).

Later work attempted to correlate these findings with the outcome of splenectomy and in this way to decide which red cell characteristics brought about destruction in the spleen.

Splenectomy in Haemolytic States

Though splenectomy had been performed for over a century in the treatment of haemolytic disease, often with dramatic success, this was not always the case and the role of the spleen in the pathogenesis of the various syndromes was not clear cut. Cross transfusion experiments and the beneficial results of operation seemed to indicate that at least in hereditary spherocytosis, the spleen alone was acting as the site of removal of congenitally abnormal cells. Emerson et alii (1947) investigated the mechanism of blood destruction in this condition by studying red cells recovered from the splenic pulp after operation. In patients with hereditary spherocytosis they found that such cells were more susceptible to osmotic lysis and mechanical trauma compared

to those in the peripheral blood. In five control subjects the cells from the spleen were normal. They thus concluded that when inherently defective cells were present in the circulation the spleen sequestered these cells and caused their rapid deterioration. The erythrocytes then disintegrated or were engulfed by the cells of the reticulo-endothelial system. However, Dameshek and Bloom (1948) postulated a further role for the spleen in this syndrome and described the presence of iso-antibodies in two cases. They claimed that the haemolytic crisis in hereditary spherocytosis was due to an increase in bone marrow inhibition as well as to the phagocytic action of the spleen. They also postulated the presence of a haemolysin, produced by the spleen, that became more active at times of crisis. In support of this they reported a shortened life of transfused compatible normal cells during crisis and the disappearance of the abnormal circulating antibody following splenectomy. Also Owren (1948) claimed that the crisis in hereditary spherocytosis was due to marrow aplasia (as shown by bone marrow smears). Young, Izzo and Platzer (1951) laid down the criteria for the diagnosis of hereditary spherocytosis, and Young et alii (1951) further investigated the role of the spleen. By studying the relative number of spherocytic and normal cells taken up by the spleen they established a definite trapping of spherocytic cells in this organ without any concentration of normal cells. The spherocytic cells were rendered more fragile (osmotically and mechanically) in the spleen while normal cells were not thus affected.

They therefore confirmed that the spleen acted as a filter and trap and as an "incubator" in accelerating the destruction of red corpuscles in patients with this disease.

Wagley et alii (1948) proposed that in Coombs' positive acquired haemolytic anaemia the spleen acted as a source of the antibody and was also the site where stagnation, agglutination and an increase in red cell osmotic and mechanical fragility occurred. Another role for the spleen in the causation of haemolysis was proposed by Wiseman and Doan (1942) when they described the condition of "hypersplenism". They envisaged the condition as one in which the reticulo-endothelial system of the spleen was "on a rampage" of destruction and engulfed platelets and white cells as well as erythrocytes.

Though splenectomy was performed in many instances, it was only in cases of hereditary spherocytosis that this was universally followed by a remission in the haemolytic state. Dameshek and Schwartz in 1940 reported the results of this operation in 23 cases of congenital and acquired forms with success in 20 instances. However, a less hopeful picture was painted by Singer and Dameshek in 1941 when they claimed that splenectomy was often valueless in cases of acute acquired haemolytic disease. Robson (1949) concluded that splenectomy was of value when the spleen was acting predominantly in a destructive capacity. He reported good response in all of four cases of familial haemolytic anaemia and in five out of seven cases of acquired idiopathic

haemolytic anaemia. In the latter the successful ones were those in which the cells preoperatively showed spherocytosis and increased osmotic fragility. Also two cases of the acute form of acquired haemolytic anaemia showed a good response. Thus though no definite indications for the operation could be formulated from this, it appeared that splenectomy was of most benefit when the acquired form most closely resembled the familial form with respect to cellular morphology and fragility.

Also in 1949 Cole et alii reviewed a series of cases and claimed that "it is frequently very difficult to differentiate congenital haemolytic jaundice in which splenectomy is so universally successful from acquired haemolytic jaundice in which poor results are so common". In this series 23 of 28 cases of the congenital type had an excellent response to operation while only one case of five of the acquired form was favourable. Evans and Duane (1949) reported five good and two poor responses to splenectomy in seven cases of the acquired type. In these Ashby survival studies had shown a short life of compatible normal cells. There was no indication from these studies, nor from preoperative antibody titres, as to which cases would respond to the operation. Postoperatively, the antibody titres declined in the successful cases and the authors claimed that the benefit of the operation was due to this. However, post-splenectomy more spherocytes and fragile cells could be shown to be surviving in the circulation.

Thus at least a contribution to the remission was the simple physical absence of the spleen which presumably would have removed such cells.

A review by Welch and Dameshek (1950) of 200 cases of splenectomy in blood dyscrasias reported 100 per cent success in congenital spherocytosis, 50 per cent success in idiopathic acquired haemolytic anaemia and 33 per cent in acquired forms secondary to other disease. In commenting on the indications for the operation they concluded that it should be done firstly, on the diagnosis of hereditary spherocytosis, secondly, in idiopathic acquired forms if haemolysis, a positive Coombs' test and a shortened survival of donated cells were demonstrated, and thirdly, in cases of the secondary acquired type if haemolysis was of a marked degree. However, such indications in the latter two groups did not ensure 100 per cent success. Dreyfus et alii, in 1951, concluded that there was, at the time of writing, no reliable guide to splenectomy in auto-immune haemolytic anaemia and reported three failures out of five cases in whom the operation was performed.

The demonstration that adrenocorticotrophic hormone (ACTH) and cortisone were effective in at least some cases of acquired haemolytic anaemia (Dameshek, 1950; Gardner, 1950) changed the attitude to splenectomy considerably. However, in 1951 Young et alii claimed that the chance of obtaining benefit from splenectomy was good enough to justify the operation in most cases, though it was impossible to predict which ones would respond. They recommended the above hormonal

agents only for the preparation of acutely ill patients for splenectomy. Conversely, as a result of the inconsistent response to splenectomy in acquired haemolytic anaemia, Dameshek and Rosenthal (1951) recommended that the treatment of this condition should be firstly transfusion, followed by ACTH or cortisone and that splenectomy should only be done if other methods were ineffective. Reviewing their own past results of splenectomy in this condition they reported 52 cases of success; namely in half the cases suffering from the idiopathic group and one-third of cases with the symptomatic group. Also Dameshek et alii (1951) reported the results of treatment of five cases of acquired haemolytic anaemia with a positive Coombs' test. In three of these splenectomy was performed without success but four of the five showed an almost complete remission on ACTH.

Though splenectomy was generally considered not indicated in thalassaemia major, Lichtman et alii (1953) considered this operation advisable where an extracorpuscular factor had developed as the result of repeated transfusions. They demonstrated such a factor by cross transfusion studies in all of seven children requiring many transfusions. In five of these subjected to splenectomy transfusion requirements were reduced. This suggested that in such cases the antibody was one that made the erythrocytes susceptible to uptake by the spleen.

Young and Miller (1953) reviewed the long term picture in auto-immune haemolytic anaemia. However, in 23 cases they were unable to

to give indications as to which would most benefit from splenectomy. Also Dausset and Malinvaud (1954) reported 54 cases of acquired haemolytic anaemia (both idiopathic and secondary) with demonstrable antibodies. They claimed that the results of splenectomy were variable and difficult to evaluate and the operation was only completely successful in two cases out of 16 subjected to the operation. de Gruchy (1954) also commented that the indications for splenectomy in this condition were not reliable. Out of a total of 22 cases reviewed, 12 were subjected to splenectomy. Of these five had a complete remission, two a moderate response and five failed to gain any benefit. He concluded that, as it was not possible to predict clinically or serologically which cases would respond to operation, medical treatment was the first line of attack. He also stated that when successful, the benefit of splenectomy was probably due to the removal of the major site of cellular destruction and not to the fact that the spleen was the site for the production of the abnormal antibody. In these cases the Coombs' test did not become negative and antibody titres were not reduced after operation. Rosenthal et alii (1955) claimed that splenectomy should only be performed when hormonal therapy was found to be partially or totally unsuccessful, and Dacie and Chertkow (1955) reported successful operation in only one-third of a group of 28 cases of the auto-immune type. These two authors (Chertkow and Dacie, 1956) in an extension of this study, claimed that there was no

relationship between remission following operation and the red cell spherocytosis and fragility. They also concluded that neither the type of antibody detected ("warm" or "cold") nor the classification into idiopathic or secondary types, enabled one to predict the success of operation, though there was some suggestion that a favourable response was more likely if the spleen was large rather than small. They concluded that there was no reliable clinical, haematological or serological criteria for splenectomy, but there was a suggestion that the results would be less favourable if the patients were young, severely anaemic and if the spleen was small or only slightly enlarged. Crosby and Rappaport (1957) in a review of their experience with 57 cases reported splenectomy in 27 cases of idiopathic auto-immune haemolytic anaemia with good results in 10. In the secondary type they claimed that splenectomy may be of value, but indications and results were difficult to evaluate particularly in those secondary to malignancy.

Thus no constant relationship between the red cell abnormalities and splenic destruction emerged from these studies. Subsequently radioactive isotopes were used in the investigation of this problem.

Red Cell Survival Using Radioactive Isotope Labelling

Though much valuable work was done using the Ashby differential technique, this method suffered from the disadvantage that it was

impossible to follow erythrocyte survival in the patient's own circulation. Several other methods were developed to measure red cell life, including the rate of disappearance of cells labelled with sulphaemoglobin (Jope, 1946) and the determination of the reticulocyte maturation time in vivo (Berlin, 1950). Also, following the introduction of radioactive isotopes into clinical medicine, several of these were employed to label red cells both in vivo and in vitro. With nitrogen-15 labelled glycine which, as a precursor of protoporphyrin, was incorporated into red cell haemoglobin in vivo, Shemin and Rittenberg (1946) estimated the normal red cell span in man as 127 days. Similarly Berlin et alii (1951) utilized carbon-14 labelled methyl glycine to estimate red cell life in cases of chronic leukaemia and polycythaemia vera, while Finch et alii (1949) used the isotopes iron-55 and iron-59 for in vivo labelling of haemoglobin. Phosphorus-32 labelled diisopropylfluorophosphate was also employed following the description, by Cohen and Warringa (1954), of this substance as an erythrocyte labelling agent after injection into humans.

However, the most widely used isotope for the determination of red cell life in the subject's own circulation, and the determination of the sites of red cell destruction by radioactive surface counting, was chromium-51 (^{51}Cr). The introduction of this isotope for these purposes followed the description of Gray and Sterling (1950) of the binding of chromium-51 to red cell haemoglobin when sodium chromate

($Na_2{}^{51}CrO_4$) was added to human red cells in vitro. These authors (Sterling and Gray, 1950) employed this method in the estimation of circulating red cell mass. Ebaugh et alii (1953) first utilized this isotope for the determination of red cell life. These authors standardized a labelling technique and also described the phenomenon of gradual elution of chromium from the cells with time, and examined the rate of this reaction. After correction for this elution they found the life of red cells in normal subjects to be 108 to 115 days. Necheles et alii (1953) reported their findings in 14 normal subjects and recorded a half time of blood radioactivity ranging from 30 to 40 days when no correction was made for elution. They stated that this correction gave a survival curve that corresponded to that given by the Ashby technique, but that it was not necessary to make this calculation in order to obtain a reasonably accurate measurement of red cell life. They thus used "the apparent half survival time" of 30 to 40 days and this mode of expression of red cell life became widely accepted. Sutherland et alii (1954) performed concurrently Ashby and chromium-51 survival studies in 5 normal subjects using compatible red cells of a different group, and in 5 other subjects transfused compatible chromium labelled blood of the same group. They confirmed the curvilinear shape of the chromium survival curve and claimed that this could be due to the random destruction of cells damaged by the radioactive labelling, rather than to elution. However they

considered that this damage was only of a minor degree. Weinstein and Leroy (1953) studied autologous erythrocyte survival in various haematological disorders taking the apparent half life of 30 to 40 days as normal. They described a reduced red cell life in some, but not all, cases of chronic lymphatic leukaemia, myeloid metaplasia, acquired haemolytic anaemia and aplastic anaemia. Talbot et alii (1954) described a method for estimating red cell survival from the decline of activity in the first 48 hours following the infusion of erythrocytes. This agreed well with that calculated from a routine Ashby survival.

Following these early studies the radioactive chromium-51 method of measuring red cell life was widely assessed. Eadie and Brown (1955) considered that the curvilinear shape of the survival curve was definitely due to chromium elution and not to toxic effect on the cells. Read et alii (1954) described an apparent half life of 32.5 days in normals and a short survival in cases of congenital haemolytic anaemia, myeloid metaplasia, thalassaemia minor, hypoplastic anaemia and acquired haemolytic anaemia (secondary to chronic lymphatic leukaemia and uraemia). Weinstein et alii (1954), Sturgeon and Finch (1957), Erlandson et alii (1958), Malamos et alii (1961) and Sitarz et alii (1963) studied the abnormal haemoglobin syndromes. They showed a reduction in the life of cells in sickle cell disease, haemoglobin C disease, sickle cell-haemoglobin C disease and

thalassaemia major but normal survival in sickle cell trait and haemoglobin C trait. Cooper et alii (1955) showed that similar survival curves to those seen in a haemolytic process occurred with blood loss.

The technique was fully evaluated and standardized by Donohue et alii (1955). Ebaugh et alii (1955), Mollison and Veall (1955), Mollison (1959) and Cooper and Owen (1956) gave laboratory criteria for most efficient labelling and reported normal half times ranging from 28 to 37 days, and Hughes Jones and Mollison (1956) described a mathematical method for the estimation of mean cell life with correction for elution. Joske et alii (1956) in a reported study of red cell life in chronic renal disease, stated that "conclusions drawn from radiochromium survival data rest upon the premise that the isotope is eluted from cells in a diseased state at the same rate as in normal persons. This is supported by the normal survival time of renal failure cells after transfusion into healthy recipients." Similarly no difference in elution rates was demonstrated in other haemolytic states. Mollison (1961) studied more closely the phenomenon of chromium elution and reported a rapid early loss of cellular chromium-51 followed by the well recognised slower elution rate.

Dornhorst (1951) exhaustively reviewed the interpretation and mathematical analysis of red cell survival curves obtained in cross transfusion experiments. He postulated that there were two possible

modes of red cell elimination: either that each cell had a determinate life span and the cell number declined in a linear fashion or, secondly, that the cells were subject to the risk of destruction at a steady rate by some external mechanism, for example, the chance that a cell would, in unit time, pass through a certain organ or be exposed to particular physicochemical conditions. This second type would result in an exponential fall in cell numbers. This author, as did Eadie and Brown (1953) drew attention to the fact that cellular destruction could be the result of a combination of random destruction and senescence. Strumia et alii (1955) similarly reviewed the validity of the estimate of red cell life using radioactive chromium-51. They concluded

(a) that auto transfusion of ^{51}Cr tagged cells in a normal individual was a valid method for the measurement of the post transfusion survival of these cells;

(b) that transfusion of ^{51}Cr cells from a patient suspected of having abnormalities of the red cell, affecting their life span, into a normal recipient was a very satisfactory method for determining the existence of alterations of the red cells; and

(c) when auto transfusion of ^{51}Cr tagged red cells was used to determine the life span of red cells in a patient suffering from haemolytic phenomena, corrections of the values obtained must be made according to the variations in the

volume of red cells caused by the rate of new red cell production. The validity of such corrections, by repeated determinations of the circulating red cell mass, was subject to the limitations of the accuracy of the method.

Crawford and de Gruchy (1958) considered that radioactive chromium-51 was a reliable agent for the estimation of red cell mass and red cell survival. The method had the advantages that the cells could be followed in the patient's own circulation, that only small volumes of blood were necessary and patients of all blood groups could be used. These workers gave a normal uncorrected half time of 26 $\pm$ 3 days. Berlin et alii (1959) in an exhaustive review of all available methods of red cell life estimation concluded that chromium-51 was the most convenient and valuable agent with the following reservations. Firstly using this method one had only a relative measure of red cell life unless the isotope content of the cell was followed for the duration of the life span. Secondly, this relative measure was of value only if it were assumed that the elution rate of chromium-51 from intact cells was constant for a given species and furthermore, in the case of disease states, if the elution rate was the same as in the normal. He noted that, due to variations of technique in the hands of various workers, the apparent half time ranged from 23 to 40 days. Hughes Jones (1961) commented on other advantages of this technique, namely that the labelling of red cells with chromium-51 was a simple procedure,

that the gamma rays emitted were easy to detect and that the radiation dose to the subject was small.

Assessment of the Organ Uptake of Red Cells Using Chromium-51

After the use of radioactive chromium had become well established as a reliable method of measuring red cell life, attempts were made to determine the sites of erythrocyte destruction in haemolysis. Thus the rate of accumulation of radioactivity was measured, at the body surface, over various organs particularly the spleen. Two problems were encountered in the quantitation of cellular sequestration by a particular organ. Firstly it was necessary to differentiate the activity due merely to blood circulating through the organ from that due to cells permanently taken up. Secondly, the same quantity sequestered in different organs did not necessarily produce identical surface count rates. For example, Mollison and Hughes Jones (1958) showed that in a cadaver a given amount of radioactivity in a spleen of normal size, gave approximately three times the number of surface counts as the same amount of radioactivity in the normal liver. Various calculations were devised to overcome these inaccuracies.

Huff et alii (1951) had measured the differential organ uptake of cells, by external scintillation counting, during ferrokinetic studies utilizing radioactive iron. In an endeavour to quantitate the uptake of different organs the changes, with time, of the body surface counts

were recorded. It was assumed that the rates over liver, spleen and bone marrow at zero time represented only circulating radioactivity. This circulating background was recalculated, at the times of subsequent tissue surface counts, from the decline in the blood activity and its subtraction from the total surface count rate was considered to give a truer representation of the radioactivity more permanently sequestered in the underlying organ.

Surface counting following the transfusion of chromium-51 labelled red cells was first described by Korst et alii (1955). These authors introduced the measure of the spleen:liver surface count ratio as an index of the relative amounts of chromium-51 labelled red cells in the liver and spleen. In investigations into the fate of cells from a patient with hereditary spherocytosis, after infusion into normal subjects and patients with this condition, they noted a ratio of 4:1. This ratio was 1:9 when normal compatible cells were injected into the two groups of patients. They reported also striking amelioration of the haemolytic process by splenectomy in two patients with acquired haemolytic anaemia with preoperative spleen:liver ratios of 3:5 and 4:3. One patient with a preoperative ratio of 1:7 obtained no benefit from splenectomy. They thus made a major contribution to the selection of patients for operation and claimed that those showing evidence of splenic "trapping" were more likely to obtain benefit.

Motulsky et alii (1956) recorded the mixing time of blood in the

spleen and liver, by external scintillation counting, following the infusion of chromium-51 labelled erythrocytes. They found that mixing in the spleen was completed in a few minutes in normal subjects, those with hereditary non-spherocytic haemolytic disease and polycythaemia vera. However, this took up to 60 minutes in patients with hereditary spherocytosis, autoimmune haemolytic anaemia, thalassaemia, leukaemia and paroxysmal nocturnal haemoglobinuria. Similarly Harris et alii (1958) described investigations demonstrating that activity over the spleen after the injection of labelled cells rose in a simple exponential manner and reached equilibrium in approximately 15 seconds. Equilibrium was achieved under similar conditions over the heart in 20 seconds. This rate of change was said to be due to the mixture of the labelled cells within the closed circulation of the spleen. If a multiple exponential graph was obtained it was postulated that the slower component was due to the equilibrium in a larger open circulation of the organ and that this would occur if the cells were abnormal and trapped in this compartment, or if the pulp space was enlarged in hypersplenic conditions. This slow component, it was claimed, could be associated with subsequent destruction of the cells in this compartment and such studies would be of use in the evaluation of patients for splenectomy.

The determination of the sites of red cell sequestration in haemolytic syndromes by chromium-51 surface counting was fully

evaluated and described by Jandl et alii (1956). They studied as well as the fate of injected cells, the clearance of injected radioactive chromium chloride and labelled haemoglobin. They differentiated between a rapid rise in surface count rate to a high initial level, due purely to increased vascularity of the organ, and a slow continual rise, proportional to the rate in decline in circulating radioactivity, when cells were being actually sequestered and retained. An "Index of Sequestration" was used to correct for this fall in organ count rate due to declining circulating activity. This index was calculated by comparison with the precordial count rate as the latter was assumed to be due primarily to circulating labelled cells. They postulated that values of the index in excess of those of the spleen and liver in normal subjects constituted evidence of red cell uptake at these sites. They showed a short red cell life (less than the mean of 32.8 days, chromium-51 half life) in the following conditions: hereditary spherocytosis (with splenic sequestration of cells), sickle cell disease (liver uptake) Cooley's anaemia (moderate splenic uptake), paroxysmal nocturnal haemoglobinuria (splenic uptake and intravascular haemolysis), haemoglobin-C disease (slight splenic uptake), liver disease (spleen plus slight liver sequestration), pernicious anaemia (moderate splenic uptake) and acquired haemolytic anaemia (splenic removal of cells with a slight hepatic component).

Schloesser et alii (1957) reported their findings in 14 cases of

haemolytic anaemia in whom they used the previously mentioned spleen: liver ratio as the index of differential organ sequestration. They showed a short red cell life in hereditary spherocytosis, paroxysmal nocturnal haemoglobinuria and acquired haemolytic anaemia, and found splenectomy gave good results, in the relief of haemolysis, if the spleen:liver ratio exceeded 3.0 and rose with time.

Other workers followed the fall in surface counts over an organ and compared this to the expected fall, as measured by the decrease in count rate over the heart, as described by Jandl et alii (1956). The changes expected in the liver and spleen surface counts, on any day as they fell in proportion to the heart counts, were estimated by Hughes Jones and Szur (1957) and the difference between the counts expected and those actually obtained were plotted against time as the "excess" counts. This excess count rate was determined on the day when half the chromium-51 activity had left the circulation, i.e. the chromium-51 red cell half life. In normal subjects this did not exceed 300 counts per minute over the spleen and 150 over the liver. Also the spleen:liver ratio was compared on the day of injection and at the chromium-51 half time. Using these criteria the authors examined five normal subjects, three cases of hereditary spherocytosis, four cases of splenomegaly without haemolysis, and five cases of acquired haemolytic anaemia. Splenectomy was claimed to be indicated when excess spleen counts above normal were found at the chromium-51

half time and also if the spleen:liver ratio at the half period exceeded that of day zero. Patients with haemolytic disease were found to fall into three categories utilizing these criteria:

1. Those in whom the accumulation of chromium-51 in both liver and spleen fell in the expected range. Paroxysmal nocturnal haemoglobinuria, in which the haemolysis was intravascular, was cited as an example of this situation.

2. Those with no excess counts in the liver but a marked excess in the spleen. This group included those with hereditary spherocytosis which were cured by splenectomy.

3. Those in which there were excess counts over both liver and spleen. This group constituted those where a rising spleen:liver ratio was used as an indication for splenectomy.

Harris et alii (1957) related the count rate over different organs, to the activity injected, by dividing the surface by the rate recorded by the syringe of chromium-51 labelled blood, measured before injection. The ratio obtained was referred to as relative units of radioactivity for each organ. Preliminary measurements with a dummy spleen and liver showed that 100 per cent uptake in these organs gave relative units of radioactivity of 3.1 and 2.3 respectively. Though dependent on the varying size of the organs these values were claimed to be reasonably accurate for normal subjects. The fractional uptakes in spleen and liver were then given by:

$\frac{C\ spleen}{Cs} \times \frac{1}{3.1}$ and $\frac{C\ liver}{Cs} \times \frac{1}{2.3}$ respectively

where C spleen and C liver = count rate over the organ corrected for decay to the time of injection. Cs = count rate over the syringe of labelled blood.

The normal spleen:liver ratio of Motulsky et alii (1958) was from 0.75 to 1.25 and they considered that a ratio of over 2.0 indicated significant splenic sequestration.

Measurements were carried out as follows:

1. Chromium-51 half time. Less than 20 days was considered to show a significant reduction in red cell life.

2. Splenic mixing time. A longer period than 2 to 4 minutes was taken to indicate sequestration of cells in this organ.

3. Spleen:liver ratio one hour after injection. A value greater than 2 was considered to indicate splenic sequestration.

4. Organ counts. These were carried out on successive days and from the count rate over the organ on any day was subtracted the count rate due to cells still circulating intravascularly. The latter was calculated from the rate of fall of blood activity.

This correction for circulating blood, and the standardization to a common organ count rate of 1000 at day zero, permitted comparison between subjects.

McCurdy and Rath (1958) reported on the prediction of results of splenectomy in 50 patients utilizing chromium-51 surface counting.

They described the procedure of counting over the spleen until equilibrium was reached and then over the liver and heart at day zero, and used, as described previously, the precordial count rate as the measure of blood activity. From this a "Splenic Localization Index" (S.L.I.) was calculated which compared the maximum spleen:heart ratio of 1.5 or greater or in those with a S.L.I. of over 1.0. In their series nine patients who fulfilled these criteria were definitely improved by splenectomy.

In a study of the haemolytic anaemia of cancer patients Ultmann (1958) described the use of spleen:heart and liver:heart ratios at day zero (20 minutes after infusion) and on subsequent days. In normal subjects he found that the spleen:heart and liver:heart ratios at zero time were less than 1 and neither ratio rose significantly with time. A high spleen:heart ratio on day zero was claimed to indicate splenomegaly and increased vascularity. The ratios were also recorded at the times at which they achieved their maximum values and in a number this occurred at the chromium-51 half time.

Lewis et alii (1960) also took the count rate over the heart to reflect circulating activity. The ratio of spleen:liver counts at 30 minutes was designated as 1, and all subsequent measurements of this ratio were related to this value. Particular note was taken of the corrected spleen:liver ratio at the chromium-51 half time. Experience with four different patterns was reported.

1. Excess counts over the spleen, in hereditary spherocytosis and elliptocytosis.

2. Excess counts over the liver, in sickle cell disease.

3. No excess counts over liver and spleen, in hereditary non-spherocytic haemolytic anaemia and paroxysmal nocturnal haemoglobinuria.

4. Excess counts over liver and spleen, in auto-immune acquired haemolytic anaemia. Results of splenectomy in this group were inconsistent.

The different modes of analysis previously described were all devised in an attempt to select patients with haemolytic disease for splenectomy. Such a selection was not difficult in cases where the chromium-51 half life was grossly reduced and there was a rapid and marked splenic uptake of cells compared with that over other organs. The division was more difficult when "borderline" cases were under consideration. In such cases the criteria used did not always provide a definite lead to the outcome of operation. Nakai et alii (1962) reviewed 29 cases of agnogenic myeloid metaplasia and chromium-51 survival studies and organ uptake measurements were performed on 17 of these cases. In an endeavour to select patients who might benefit from splenectomy the sequestration indices were calculated using four methods, namely the Index of Sequestration of Jandl et alii (1956), the spleen:liver ratio of Hughes Jones and Szur (1957), the estimation of

trapped cells by the formula of Motulsky et alii (1958) and the Splenic Localization Index of McCurdy and Rath (1958). The results were, however, disappointing in that it was often impossible to state categorically that a particular index was positive or negative, the results by different methods were sometimes contradictory, and no one method could be claimed to be superior to another. Also, despite such estimations in many haemolytic syndromes, very little information was obtained on the specific erythrocyte characteristics governing splenic sequestration. It was still not possible to predict the outcome of operation from such characteristics as cell morphology, fragility or the nature of any associated antibody, before chromium-51 studies were performed.

Red Cell Characteristics Governing Selective Organ Sequestration

Following its value in detecting the survival of autologous cells in subjects with haemolysis, chromium-51 erythrocyte labelling was adapted to elucidating the fate of erythrocytes, altered in various ways, in human subjects (Table I). Such studies, in conjunction with other clinical and experimental observations, helped to clarify the factors governing selective sequestration in the different organs of the reticulo-endothelial system.

Minkowski (1900) first described the spheroidal shape, and Chauffard (1907) the increased osmotic fragility of erythrocytes in

Table I - Studies of the effect of erythrocyte abnormalities on the site of organ uptake.

Authors	Date	Nature of Cellular Damage	Site of Sequestration
Jandl	1955	Incubation with low concentrations of metallic cations.	Spleen
Jandl	1955	Moderate cellular agglutination by higher concentrations of metallic cations.	Liver
Jandl et alii	1957	Marked cellular agglutination by concentrated solutions of metallic cations.	Lungs and liver
Mollison and Cutbush	1955	Cellular coating with non-complement binding antibodies.	Spleen
Mollison and Cutbush	1955	Cellular coating with complement binding antibodies.	Liver
Jandl	1956	Non-agglutinated reticulocytes.	Spleen
Jandl	1956	Agglutinated reticulocytes.	Liver
Jandl et alii	1957	Low concentrations of non-agglutinating anti D antibodies.	Spleen
Jandl et alii	1957	High concentrations of non-agglutinating anti D antibodies.	Liver
Jandl et alii	1957	ABO agglutinating antibodies.	Liver and spleen
Harris et alii	1957	Cells rendered spherocytic by heat at 49.6°C for 15 minutes.	Spleen
Harris et alii	1957	Metabolic damage with sodium arsenate.	Liver and spleen

continued

Table I - continued

Authors	Date	Nature of Cellular Damage	Site of Sequestration
Harris et alii	1957	Cells haemolysed with distilled water.	Liver
Harris et alii	1957	Cellular surface lipid reduced by treatment with alumina.	Spleen
Jandl and Tomlinson	1958	Sterile incubation at 37°C for 48 hours.	Liver
Jandl and Tomlinson	1958	Acetyl phenylhydrazine 1 per cent for 1 hour at 37°C.	Liver
Jandl and Tomlinson	1958	Cells rendered spherocytic by incubation with lecithin 1 per cent at 25°C for 1 hour.	Spleen
Chaplin	1959	Low concentrations of isoantibodies.	Spleen
Chaplin	1959	High concentrations of isoantibodies.	Liver
Prankerd	1960	Cells of hereditary spherocytosis with reduced ATP regeneration.	Spleen
Jacob and Jandl and Wagner et alii	1962	Low concentrations of sulphydryl inhibitors.	Spleen
Jacob and Jandl and Wagner et alii	1962	High concentrations of sulphydryl inhibitors producing spherocytosis.	Liver
Jacob and Jandl	1962	Arrest of high energy phosphate metabolism with arsenate.	Liver
Jacobs and Jandl	1962	Arrest of anaerobic glycolysis with fluoride.	Nil
Wagner et alii	1962	Heat at 50°C for 1 hour.	Spleen
Wagner et alii	1962	Heat at 50°C for 90 minutes	Spleen and liver
Wagner et alii	1962	Heat at 50°C for 120 minutes.	Liver

hereditary spherocytosis, and subsequently Dacie and Mollison (1943) demonstrated the rapid removal of these cells from the circulation in normal individuals. These facts, together with the well known remission produced by splenectomy in this disease, made it likely that spherocytosis and increased osmotic fragility were the cellular characteristics causing sequestration by the spleen. Later work by Emerson, Shen and Castle (1946), Emerson et alii (1947), Young et alii (1951), Emerson (1954), Emerson et alii (1956) and Weisman et alii (1953) supported this. Also the observations of Evans and Duane (1949) and Robson (1949) suggested that the spleen removed the red cells with these characteristics in cases of acquired haemolytic anaemia. Similarly Rappaport and Crosby (1957) found a positive correlation between spherocytes, with increased osmotic fragility, and splenic cord congestion in a study of the red cell content of spleens, removed at operation, from 50 cases of autoimmune haemolytic anaemia.

However these were not the only cellular factors of importance for Chertkow and Dacie (1956) found no relationship between them and the response to splenectomy in the acquired group. Hayhoe and Whitby (1955) considered that the spleen provided a mechanism for the destruction of cells with intrinsic structural weakness. In some cases only, as in hereditary spherocytosis, this was manifested as a spheroidal shape. Swisher and Young (1954) raised the possibility that the attachment of antibody, in autoimmune haemolytic states,

produced metabolic abnormalities and subsequent loss of viability. Prankerd et alii (1955) reported a biochemical defect in hereditary spherocytosis characterized by an inadequate rate of adenosine triphosphate (ATP) regeneration in the erythrocytes. He subsequently demonstrated that it was this abnormality, rather than shape, that caused splenic sequestration when he showed that normally metabolizing, chromium-51 labelled cells rendered spherocytic by heat treatment, were not rapidly removed from the circulation by the spleen (Prankerd, 1960). Motulsky et alii, in 1958, considered that the cellular factors governing splenic localization were unknown and, though spherocytosis was often a feature of cells sequestered in this organ, this characteristic was not essential for specific splenic uptake.

Different cellular characteristics were apparently responsible for the removal of cells by the liver as Owren (1949) reported failure of splenectomy, in the relief of autoimmune haemolytic anaemia, when the cells were agglutinated by the patient's own serum, and de Gruchy (1954) recorded a poor response to operation in a case of this condition associated with hepatomegaly. The possibility that red cell agglutination resulted in hepatic sequestration had been raised by the findings of Pearce (1904) of hepatic congestion with red cell agglutinates in dogs given intravenous injections of erythrocyte haemagglutinins. This finding was confirmed by Castle et alii (1950) who also produced the phenomenon in dogs' livers in vitro and by Bloch

and Piovella (1956) who observed it in livers of rats and frogs in vivo.

Jandl and his associates in a series of experiments studied the influence of the physical state of the cells on the site of sequestration. It was shown (Jandl, 1955; Jandl and Castle, 1956; and Jandl and Simmons, 1957) that treatment of red cells with low concentrations of metallic cations did not cause cellular agglutination, though it sensitized the cells so that clumping did occur with Coombs' serum and in the presence of other macro-molecular substances. Conversely cells treated with higher concentrations of metallic cations did undergo agglutination in saline. Utilizing chromium labelling of these cells it was demonstrated (Jandl, 1955) that upon reinfusion the moderately agglutinated cells were removed by the liver, coarsely agglutinated cells by the lung and liver, while the non-agglutinated cells were removed by the spleen. The spleen was also shown to be the site of removal of cells coated with, but not agglutinated by, anti D serum. The same phenomenon of agglutinated cells being trapped in the liver and sensitized non-agglutinated cells in the spleen was demonstrated using iron-59 labelled reticulocytes. The release of iron-59 within hours from the sequestering organ indicated a rapidly acting lytic mechanism, within these intact tissues, operative against the sequestered cells (Jandl, 1956).

The role of antibodies, in the production of these cellular

changes and in controlling the sites of subsequent sequestration, was further studied by Jandl, Jones and Castle (1957). They found that cells sensitized in vitro with incomplete anti D sera were sequestered in the spleen while D cells injected into hyperimmunized subjects showed some liver uptake. Also ABO compatible cells, which were promptly agglutinated, were rapidly taken up in the liver. A series of investigations (Mollison and Cutbush, 1955; Hughes Jones, Mollison and Veale, 1957; Cutbush and Mollison, 1958; Mollison and Hughes Jones, 1958; Mollison, 1959a, 1959b; and Mollison, 1962) confirmed these findings, but the ability of the antibody to bind complement, rather than its ability to produce agglutination in vitro, was found to be the important feature governing hepatic uptake. These workers showed that while complement binding antibodies caused the removal of cells in the liver, incomplete antibodies, which did not bind complement, brought about the removal of cells almost completely by the spleen.

The importance of the quantity of available antibody was demonstrated by Chaplin (1959) who studied the survival of incompatible cells in four patients with agammaglobulinaemia and very low iso-haemagglutinin titres. He found that with a small amount of available antibody the cells were removed by the spleen while, with slightly higher titres, both liver and spleen were responsible for sequestration. The same principle was illustrated by Jandl and Kaplan (1960) and

Mollison (1962) as increasing amounts of anti D serum per unit volume of cells changed the site of uptake from spleen to liver. The role of low antibody titres was also illustrated by the description by Jandl and Greenberg (1957) of two patients with splenomegaly in whom there was a normal survival of autologous cells but reduced survival with splenic uptake of serologically compatible homologous cells. The increasingly rapid removal of homologous cells from the circulation, and the relatively normal survival of autologous cells and cells from one donor, suggested the existence of a weak antibody demonstrable only in vivo.

Reticulocytes are also sequestered by the spleen. Berendes (1959) showed in dogs and human subjects an increased proportion of these cells in the spleen and proposed that this was due to increased reticulocyte "stickiness". Jandl (1960) also demonstrated this phenomenon in rats, rabbits and man and believed that an increased tendency to agglutination, and the larger size of these cells were the important factors. He proposed, as did Crosby (1959), that such cells were retained until full maturation occurred, after which the mature cells were released into the circulation. Jandl (1958) and Jandl, Simmons and Castle (1961) proposed that the spleen, when responsible for the uptake of these and other cells, acted as a passive filter. They tested the ability of cells to pass through a millipore filter with a pore size of 5 microns diameter, in an

endeavour to explain why reticulocytes and sensitized cells were trapped in the normal spleen, while agglutinated red cells were sequestered in the liver. They found that sickle cell anaemia erythrocytes at low oxygen tensions did not pass the filter and the more pronounced spherocytic cells from hereditary spherocytosis were also retained. Reticulocytes and cells coated with an incomplete antigen were not trapped, unless previously treated with rouleaux producing agent. Higher perfusion pressures caused these cell clumps to dissociate permitting passage through the pores. Red cells directly agglutinated by complete antibodies passed the filter only under still higher perfusion pressures. Thus the ability of red cell rouleaux or agglutinates to penetrate the filter was a function of the force required to dissociate these aggregates. They concluded that the sites of sequestration of immature, abnormal or agglutinated cells may be determined largely by the pore sizes and perfusion pressures of different vascular filter beds. They claimed that this work raised the possibility, but did not prove, that splenic trapping was dependant on a spheroidal shape. This, however, did not explain the splenic removal of erythrocytes, coated with incomplete antibody, which were not spherocytic in shape, nor the splenic sequestration of reticulocytes.

The Effects of Thermal and Metabolic Damage on Red Cell Survival

Heat Treatment: It was shown, a century ago (Schultze, 1965; von Lesser, 1880 and Silbermann, 1890), that when blood was heated in vitro, to temperatures ranging from 52° - 65°C, the red cells underwent a spheroidal change and fragmentation. This change was also noted in the peripheral blood of experimental animals subjected to burning or scalding (Klebs, 1963; Wertheim, 1868 and von Lesser, 1880), and in these animals von Lesser described the rapid disappearance of the fragmented micro-spherocytes from the peripheral circulation, with the development of haemoglobinaemia and haemoglobinuria. However, no haemolysins or agglutinins could be detected in the plasma or serum of burnt animals (Burkhardt, 1905). Isaacs et alii repeated the in vitro experiments in 1925 and again described spherocytes and micro-spherocytes and also claimed that when the blood was submitted to temperatures of 55°C for 30 minutes, 70 to 80 per cent of the erythrocytes were converted to indistinct "shadow" forms. They also confirmed the increase in osmotic fragility of heated cells which had been described bySpiegler in 1896.

Shen, Ham and Fleming (1943) studied 40 patients who had suffered combined second and third degree burns. They found these same morphological changes in the peripheral blood of the more severely burned patients and noted that the fragmented forms disappeared, in less than half an hour, from the blood of such patients. Osmotically

fragile cells of spherocytic shape survived for many hours however. It was established that heating up to 46°C in vitro produced no change in human erythrocytes while at temperatures between 47°C and 50°C the degree of microspherocyte formation and the increase in osmotic fragility were generally proportional to the time of exposure to the high temperature. When erythrocytes were heated to 53°C and reinjected into dogs the fragments rapidly disappeared from the peripheral blood while the spherocytes persisted for longer periods. This was the same sequence previously observed in the burnt patients. In an extension of the above work Ham et alii (1948) established that in vitro the changes in the red cells occurred in the following sequence: the formation of spherocytes followed by the budding of cell fragments to give microspherocytes and finally the appearance of indistinct "ghost-like" forms. The latter were presumably identical with the "shadow" cells described by Isaacs et alii (1925). It was also established that the increase in osmotic fragility was associated with an increase in mechanical fragility (Ponder, 1948). Ham and his associates therefore postulated that the fall in haemoglobin, in patients suffering burns, was due to the destruction of the mechanically fragile forms by the trauma of circulation, whilst the spheroidal cells were sequestered in the spleen and other organs.

Harris et alii (1957) reinjected chromium-51 labelled cells that had been heated at 49.6°C for 15 minutes. These cells were rapidly

removed from the circulation and surface counting showed uptake predominately in the spleen with slight sequestration by the liver. Utilizing the same techniques Wagner et alii in 1962 postulated that the predominant site of uptake of heated cells was a function of the degree of damage produced at 50°C. They reported that heating for 60 minutes resulted in a predominantly splenic uptake, after 90 minutes the uptake was divided between liver and spleen, while 120 minutes of heating caused anhepatic sequestration.

Chemical and Metabolic Damage: In his experiments, on blood destruction in dogs in 1892, Hunter reported that "..... if pyrogallic acid were injected into the blood, then .....a great enlargement of the spleen occurred.....and fifteen minutes later these altered red cell corpuscles were found enclosed within large spleen cells of the pulp. After death .....many large cells filled with but slightly altered red corpuscles, were found in the spleen, while absent from the liver or from the blood elsewhere." More recently a variety of chemical agents have been used to damage human erythrocytes in vitro and, following transfusion, the site of sequestration of these cells has been studied.

Harris et alii (1957) showed that red cells made metabolically inert by treatment with arsenic were taken up by the liver and spleen, cells with the surface lipid content reduced by incubation with alumina were removed by the spleen, and finally cells haemolysed with

distilled water were sequestered predominantly by the liver on re-injection. Jandl and Tomlinson (1958) subjected red cells to sterile incubation at 37°C for 24 to 48 hours and found that they were removed by the liver while those incubated with 1 per cent acetyl phenyl hydrazine or 1 per cent lecithin solution were taken up by the spleen.

In a variety of haemolytic syndromes it has been shown that the susceptibility of the red cells to destruction is associated with a failure to maintain the sulphydryl compound glutathione (GSH), in the reduced state. For example, this was demonstrated in certain drug induced haemolytic anaemias (Beutler et alii, 1955) and favism (Szeinberg et alii, 1958), associated with glucose-6-phosphate-dehydrogenase (G6PD) deficiency, and in some cases of congenital non-spherocytic haemolytic anaemia (Shahidi and Diamond, 1959; Zinkham and Lenhard, 1959). Consequently, Jacobs and Jandl (1962a, 1962b) studied in detail the effects of the sulphydryl inhibitors N-ethyl maleimide (NEM) and p-mercuric benzoate (PMB) on red cell metabolism, morphology, survival and site of organ uptake in human subjects. They showed that low concentrations of NEM and PMB, which produced no morphological change and slight spherocytosis respectively, resulted in a splenic uptake of the treated erythrocytes. Higher concentrations, producing marked spherocytosis, caused a liver sequestration of the cells. They demonstrated that specific uptake by the spleen did not depend on changes in red cell shape or an increased tendency to

agglutination, but suggested that a loss of membrane sulphydryl activity was the decisive factor. It appeared also that this specific membrane change rather than an effect on intracellular sulphydryl groups, with metabolic arrest, was of greatest importance. Thus the inhibition of high energy phosphate metabolism in red cells by arsenate caused a slow destruction by the liver, while a cessation of anaerobic glycolysis, by exposure to fluoride, had comparatively little effect on survival.

Wagner et alii (1962) postulated that, in the case of chemical sulphydryl inhibition, as for heat, the site of organ uptake was dependent on the degree of damage. They again showed a change in the site of organ uptake from spleen to liver as the cells were exposed to increasing concentrations of such inhibitors.

The results of experiments, relating these various erythrocyte abnormalities to sites of organ sequestration, are summarized in Table I.

Measurement of Splenic Sequestering Function

Following the demonstration that red cells altered in vitro by heat, chemical agents or antibodies were in some instances localized by the spleen, it became possible, by scintillation scanning, to examine the site and size of this organ after the intravenous injection of cells so treated and labelled with chromium-51. Johnson et alii

(1960) utilized Rh positive cells coated with Rh antibody for this purpose, whilst Winkelman et alii (1960), Johnson et alii (1961), Gershon-Cohen et alii (1962), Holzbach et alii, (1962) and Fischer and Wolf (1963) employed heated cells. Both heated cells, and those treated with N-ethyl maleimide, were employed by Wagner, McAfee and Winkleman (1962).

It was recognised that, if the clearance rate of altered red cells was measured, this could provide an estimate of the uptake capacity of the particular organ of the reticulo-endothelial system responsible for the cellular sequestration. For example, Wagner et alii (1962), in the study of the uptake of heated and NEM treated cells, noted a more rapid clearance and splenic uptake in patients with cirrhosis and splenomegaly and a slow clearance in a subject with sickle cell anaemia. In the latter they assumed that the reticulo-endothelial system in the spleen was already engorged with red cells. Jacob and Jandl (1962b) had noted the prolonged survival of cells, mildly damaged with NEM, in patients following splenectomy and the same phenomenon was demonstrated by Gordon and Ultmann in rats. Thus both NEM-treated erythrocytes (Jacob, McDonald and Jandl, 1963) and antibody coated cells (Jandl, 1960) were used to assess the presence or growth of splenic tissue in rats following splenectomy or hemi-splenectomy. Also subcutaneous auto-transplants of splenic tissue were shown, by this means, to be viable and to

proliferate in animals subjected to these operations.

An attempt has been made to quantitate the red cell sequestering function of the spleen in human subjects utilizing heat treated red cells (Holzbach et alii, 1964). These workers showed that the rate of removal of such cells was increased, above that seen in normal subjects, in patients with hepatic cirrhosis and this increase correlated well with the degree of splenomegaly. A similar enhanced splenic sequestering function was seen in another five subjects with splenomegaly, namely, cases of hereditary spherocytosis, chronic lymphatic leukaemia, homozygous haemoglobin-C disease, lymphosarcoma and one subject with splenomegaly of unknown aetiology. However, it was difficult to accurately quantitate uptake function by this method as the authors found it impossible to prepare a uniform population of cells showing the same degree of heat damage. A wide variation between cells, reflected in differing osmotic fragilities, was encountered. All the erythrocytes of this heterogeneous population were not removed solely by the spleen, and the resulting complex curve of decline in circulating activity made absolute calculation of the organ uptake rate difficult. Similar problems were experienced by Crome and Mollison (1964) who found that red cells heated at 50°C for approximately 20 minutes were removed from the blood stream at variable rates. This diversity was considered to be due partly to differences in individual cell susceptibility to heat damage and partly

to variation between subjects. Therefore a reliable and reproducible method of measuring splenic sequestering function could not be developed by this means.

The Use of Corticosteroids in Haemolytic Disease

Dameshek (1950) and Gardner (1950) first drew attention to the beneficial effects of treatment with adrenocorticotrophic hormone (ACTH) in acquired haemolytic anaemia. Dameshek reported two patients, with haemolysis secondary to lymphosarcoma, who showed a marked improvement, with a fall in the level of serum antibody, and two further subjects, suffering from idiopathic type, who improved on treatment with this hormone despite failure to respond to splenectomy. Of the five cases reported by Gardner, three responded well with an associated fall in Coombs' titre and a decrease in cellular osmotic and mechanical fragility, and two showed a less marked amelioration.

Following these reports this form of therapy was widely used and the beneficial effects largely confirmed. Dameshek et alii (1951) reported the frequent fall in antibody titre and suggested that the mode of action was due to dissolution of the lymphoid tissue of the body with a consequent decrease in antibody production, while Gardner et alii (1951) documented a fall in serum gamma globulin levels. Wintrobe et alii (1951) described a beneficial

response in three of four cases of acquired haemolytic anaemia, though in one a positive Coombs' test became even more pronounced. Davidson et alii (1951) assessed the response of congenital haemolytic anaemia to ACTH and found a poor result in both cases on which it was tried, though one subject did show a rapid diminution in the size of the spleen. They confirmed the beneficial effects in one case of the acquired type but did not feel that the mode of action was by the suppression of abnormal red cell antibodies, as these were still demonstrable when haemolysis was controlled.

Ley and Gardner (1951) showed that cortisone acetate, as well as ACTH, was effective in the idiopathic acquired form though the change in the Coombs' reaction was variable. However, the two drugs were apparently not interchangeable as Etess et alii (1951) reported response to ACTH in a case in which cortisone was previously ineffective. A fall in antibody titre was a common but not invariable accompaniment of remission in the haemolytic state and Unger (1951) described a case without change in antibody levels despite marked clinical remission on ACTH, and relapse on discontinuation of hormone therapy. Best et alii (1951) claimed that the beneficial response was due to an increase in the osmotic resistence of the globulin coated cells.

Despite the earlier encouraging results, the Medical Research Council (1952) was guarded in its recommendation of ACTH and cortisone in the treatment of acquired haemolytic anaemia. In a controlled

trial the panel found the results uncertain and unpredictable. However, a second report by the same body (1953) painted a more favourable picture of the response in this condition. The improved results in the second trial were probably due to the higher doses of the drugs employed and possibly confirmed Dameshek's contention (1952) that very high doses were necessary in a proportion of cases.

In 1956 Dameshek and Komninos reviewed their experience in the hormone treatment of 43 patients with autoimmune haemolytic anaemia. They found complete response in 65 per cent and complete failure in 3 percent, and found that prognosis was most favourable in the idiopathic type. They considered that steroid hormones constituted a very effective form of therapy in this disease, and that continuous hormonal therapy, or steroids plus splenectomy, was better than splenectomy alone. Delta cortisone (prednisone) was used in the most recent cases of this series and Dausset and Colombani (1959), after reviewing 128 cases, considered this drug preferable to other hormones. However, they found that in certain cases intravenous ACTH gave spectacular results when cortisone and even delta cortisone were ineffective orally. Dacie (1962) considered that many reports established steroid treatment as a good, but not universally successful, form of therapy in autoimmune haemolytic anaemia. In his experience (1963) prognosis was better in those cases with a warm, rather than a cold antibody type.

The decline in red cell antibody titre, occurring concurrently with remission of the haemolytic state, led to the postulate that ACTH and the adrenocortical steroid hormones acted by reducing antibody production (Dameshek, 1950; Gardner, 1950; Dameshek et alii, 1951; Gardner et alii, 1951; Etess, 1951; and Dameshek, 1952). Also the agglutinin titre was reported to rise at the cessation of therapy (Saint and Gardner, 1952). However a fall, with remission in haemolysis, was not universally recorded in the early studies (Unger, 1951; Best et alii, 1951; Ley and Gardner 1951; and Davidson et alii, 1951) and in one case a rise in antibody titre was recorded (Wintrobe et alii, 1951). Experimental evidence also was conflicting on this point. Vaughan et alii (1950) showed, in ten patients with rheumatoid arthritis, scleroderma or disseminated lupus erythematosus, a decline in gamma globulin levels during ACTH therapy and De Vries (1950) reported a slight decline in antibody nitrogen in experimental animals. Also Kaliss et alii (1956) demonstrated that antibody production was decreased in mice when cortisone was administered with the antigen, though corticosteroids did not reduce the secondary response if cortisone was not given at primary immunization. However, evidence against antibody suppression was provided by the work of Dougherty and White (1947) who showed an increase in gamma globulin during the administration of these hormonal agents. Also no significant reduction in antibody production, in

response to diphtheria toxoid (Havens et alii, 1952) or typhoid antigen (Freedman, 1953) could be detected, under the influence of steroid therapy, in human subjects. Also utilizing experimental haemolytic anaemia in animals Clearkin (1952) and Ecklebe and Sander (1952) were unable to detect a reduced agglutinin production during ACTH therapy.

A second postulated role of ACTH and cortisone in the relief of autoimmune haemolytic anaemia was that of interference with red cell and antibody union (Thorn, 1950), with protection of erythrocyte integrity, as claimed by Best et alii (1951). There was support for this in the work of Ecklebe and Sander (1952), for despite continued agglutinin production, less autoagglutination of the red cells was detected during ACTH administration. Also Davidsohn and Spurrier (1954) reported that, in some cases of the human disease, the Coombs' test became negative though free autoantibodies remained in the circulation at unreduced levels. Thus they felt that ACTH and cortisone inhibited the binding of antibody and red cell antigen. Also hydrocortisone, in concentrations little more than those effective clinically, was shown to be capable of reversing the electrical change wrought on the red cell by antibody action in vitro, though the Coombs' test remained positive (Creger et alii, 1956). This provided one explanation for the finding that, in some cases of autoimmune haemolytic anaemia, steroids were successful in

arresting red cell destruction despite the absence of change in the Coombs' titre. However, Clearkin (1952) failed to demonstrate a change in antigen-antibody union, in vitro or in vivo, in experimental haemolytic anaemia and no benefit could be detected from steroids in erythroblastosis foetalis (Krivit, 1958) where an inhibition of antigen-antibody union was anticipated to be of value.

It was also claimed that steroid hormones in this condition may have acted as a bone marrow stimulant (Heilmeyer, 1952; Thorn, 1953). Hudson et alii (1952) described an increased cellularity in the bone marrow of guinea pigs given ACTH for 7 days. However in the same animals Yoffey et alii (1951) could see no definite evidence of red cell hyperplasia in animals sacrificed 6 hours after ACTH and cortisone administration. Also Baikie and Pirrie (1958) could not demonstrate an increase in the red cell mass when steroid hormones were given to guinea pigs with experimental haemolytic anaemia.

Finally, it was thought that the mode of action of steroids might have been by depression of the phagocytic activity of the tissues of the reticulo-endothelial system, in particular the spleen. If such were the case, it would be anticipated that steroids would cause remission in cases of congenital haemolytic anaemia where the spleen was responsible for removal of cells from the circulation. Davidson et alii (1951) did record a decrease in splenic size in a case of hereditary spherocytosis treated with ACTH and Coleman and Finch

(1956) reported benefit in this condition, as indicated by a reduction in urobilinogen excretion and reticulocyte count. Also red cell fragility improved, and these authors considered that cortisone was interfering with the ability of the spleen to induce spherocytosis and thus decreased its destructive capacity for red cells. Mollison (1962) showed that erythrocytes sensitised by Rh antibody, that is cells preferentially taken up by the spleen, were cleared from the circulation at a reduced rate in subjects treated with steroids. The clearance of agglutinated cells, namely those removed by the liver, was not effected in the same way by such therapy. This reduction in red cell phagocytosis under the influence of ACTH and cortisone has also been demonstrated in vitro. Thus Crepea et alii (1951) and Packer et alii (1960) showed that peripheral leucocytes from steroid treated patients, or such cells exposed to these agents in vitro, had a reduced capacity to engulf sensitized erythrocytes. However, this action of corticosteroids was not generally accepted, and Chertkow and Dacie (1956) considered the finding that some cases of autoimmune haemolytic anaemia responded to steroids, despite unsuccessful splenectomy, made it unlikely that these hormones owed their effectiveness to a suppression of splenic uptake function.

Animal experiments also gave varying results concerning the effect of corticosteroids on splenic architecture and its uptake

capacity. Dougherty and White (1947) showed dissolution of all lymphoid tissue, including that of the spleen in mice treated with ACTH. Though 50 times the human dose of cortisone was needed to produce splenic atrophy in mice (Antopol, 1950), doses comparable to those used in humans were shown to have the same effect by Molomut et alii (1950). As well as a reduction in the concentration of anti-pneumococcal antibody in rabbits, Bjørneboe et alii (1951) showed marked lymphoid tissue atrophy with a reduction in mononuclear cells while Baker et alii (1951) described also degeneration of the reticular connective tissue cells of the spleen. As well as causing splenic atrophy, corticosteroids have been shown to reduce the uptake, by the reticulo-endothelial system, of bacteria and bacterial products (Thomas, 1953), carbon particles (Spain et alii, 1950; Nicol et alii, 1956; Bilbey and Nicol, 1958), colloidal chromium phosphate (Heller, 1953, 1955) and Trypan Blue (Nicol et alii, 1956).

However, these findings are at variance with those of other workers, for Bern (1963) found hypertrophy of the spleen and its reticular phagocytes in fish given deoxycorticosterone and Gordon and Katsch (1949) described an increase in the number of macrophages in the spleen, and an enhanced uptake of thorium dioxide, in rats given adrenal extract. Also Laurie et alii (1951) found an increase in the phagocytosis of carbon particles by the liver and spleen in cortisone treated rabbits. Kaplan and Jandl (1961) studied the

effect of cortisone on the sequestration of antibody coated and incubated red cells in rats. Though they found that the hepatic uptake of such cells was reduced, splenic sequestration was not inhibited by this corticosteroid.

Therefore the means by which steroid hormones produced remission in haemolytic states was not definitely established. Marmont and Fusco (1960) in considering the various possible mechanisms, namely, (a) acceleration of erythropoiesis; (b) protection of red cell integrity; (c) inhibition of antigen-antibody union; (d) inhibition of antibody production; and (e) depression of reticulo-endothelial phagocytosis, believed the last of these the most likely explanation. Baikie and Pirrie (1958) claimed that the widely divergent findings in different animal species must be interpreted with caution. One could not, from such experiments, definitely delineate the role of corticosteroids in human haemolytic states, as the differences between the latter and experimental haemolytic anaemia was too wide to permit close comparison.

Noradrenaline and the Splenic Red Cell Pool

The term "pool" as applied to the spleen firstly implies the presence of a vascular compartment, in communication with the general vascular bed of the body, but characterized by a sluggish circulation. Injected erythrocytes may therefore infiltrate only relatively

gradually into this compartment, in comparison to the rapid equilibration achieved in the general circulation, and also cells may be retained in this pool for varying periods of time. The second feature included in this concept is that erythrocytes, so sequestered, can be expelled back into the general circulation when splenic contraction is produced by adrenaline, noradrenaline or other means.

Such a compartment with the former characteristics has been shown to be present in the spleens of normal sheep (Turner and Hodgetts, 1959) and cats (Toghill and Prichard, 1964). It was demonstrated by recording the times taken for stable count rates to be reached, over the spleen following the intravenous infusion of chromium-51 labelled erythrocytes. In contradistinction to the heart where equilibrium is achieved in two minutes, five to twenty minutes elapsed before a plateau was recorded at the spleen in these animals. No such compartment could be demonstrated in the normal human spleen, utilising the same technique. Harris et alii (1958), Prankerd (1963) and Toghill and Prichard (1964) agree that in man the counts equilibrate rapidly, over this organ, at a rate comparable to that seen over the precordium.

A pool with the second characteristic, that is, the expulsion of erythrocytes on splenic contraction, exists in a variety of mammals. Hodgkin in 1822 demonstrated contraction of the spleen in

cats, on immersion of the limbs in cold water, and described the discharge of blood from the spleen under stress. Barcroft and his associates (1925) showed, radiographically, that the spleen contracted in rats, rabbits and cats during excitement and haemorrhage, and estimated that the reduction in splenic volume was equal to from 6 to 15 per cent of the total blood volume. He (Bancroft, 1925) envisaged the spleen as a reservoir of blood, which was fitted, by its reticulum to detain cells, and by its musculature to expel them when required. Cruickshank (1926) produced splenic contraction in cats and dogs by stimulation of the splanchnic nerves and estimated that from 2.6 to 5.6 per cent of the total blood volume could be so mobilized, and MacKenzie et alii (1941) demonstrated the same phenomenon in mice, rats, rabbits, guinea pigs and cats. Turner and Hodgetts (1959, 1960) showed that whereas the jugular haematocrit of sheep fell during sedation and isolation, it rose during excitement and exercise and on the administration of intravenous adrenaline and noradrenaline. Utilizing external scintillation counting over the spleen following infusion of chromium-51 labelled erythrocytes, as well as by studying splenectomized animals, they established that the changes in jugular haematocrit were due to relaxation and contraction of the spleen. Thus the fall in haematocrit produced by sedation was abolished by splenectomy. Greene et alii (1960) considered that this expulsion of blood by the spleen could be due to passive elastic

recoil of the organ, upon constriction of the afferent arterial sphincters, in such mammals.

Opinion was divided as to whether contraction, with the expulsion of blood, could occur in the normal human spleen. Stukely in 1722 described it as a "sponge" filled with blood which could "upon its contraction throw it all out again into the great channel of the splenic vein". Barcroft (1925) also attributed the same function to the human spleen and believed that the rise in circulating blood volume, which he recorded during exposure to high environmental temperatures, was due to mobilization of cells from this organ. He considered that it was achieved by the contraction of the smooth muscle fibres of the splenic trabeculae. Watson and Paine (1942) similarly interpreted their findings in splenic venous blood at laparotomy. They detected a rise in the haematocrit and red cell numbers when adrenaline was given. The same changes were reported by Chatterjea et alii (1953) but they considered that the reduction in splenic size was due to constriction of the splenic artery and its branches, with resultant passive deflation of the spleen. Shrinkage, judged by diminution in the size of the splenic shadow on X-ray, was demonstrated by Bierman et alii (1953) when noradrenaline was injected into the catheterized splenic artery. At this time the small vessel pattern of the organ was obliterated and there was a marked diminution in the size of the larger arteries.

This arterial constriction and the sparsity of smooth muscle in the spleen, led them to agree that the decrease in the size of the organ was not primarily due to an active constriction of the splenic capsule, trabeculae or other part of the parenchyma, but to a passive shrinkage as the input of blood was reduced. However, the expulsion of an appreciable volume of blood from the human spleen under these circumstances has been denied by Parson et alii (1948). They found no significant change in plasma volume (measured with Evans Blue dye, T-1824) or red cell mass (estimated with phosphorus-32 labelled red cells) when adrenaline was given subcutaneously. Prankerd (1963) and Teghill and Prichard (1964) utilized chromium-51 labelled red cells in their investigations. They considered that the failure of an infusion of noradrenaline, in normal human subjects, to produce an appreciable rise in the venous haematocrit or in the count rate in the peripheral blood, demonstrated no splenic contraction or red cell mobilization.

However in man splenomegaly, due to various causes, can apparently be associated with the formation of a "pool-like" compartment. Motulsky et alii (1956) reported that, when surface scintillation counting was carried out after the injection of radioactive erythrocytes, complete mixing of cells in the splenic vascular bed took up to 60 minutes in cases of hereditary spherocytosis, autoimmune haemolytic anaemia and paroxysmal nocturnal haemoglobinuria,

with splenomegaly. Also Jandl et alii (1956) differentiated between a rapid rise to a high initial count rate over an enlarged spleen, due purely to the plethora of a large vascular bed, from the slower build up indicating active cellular sequestration, presumably in such a pool. Harris et alii (1958) claimed that in such cases the rate of equilibration of the splenic count rate was compared to that seen over the spleens of normal sheep and cats. This finding was confirmed by Bowdler (1962) and Toghill (1964).

Splenic contraction, and consequent expulsion of the blood from this abnormal compartment in the enlarged spleen, has been demonstrated by Miller and Rhoads (1933), Watson (1939) and Wright et alii (1951). Miller and Rhoads and Watson utilized radiographic evidence of splenic shrinkage while Wright and his associates noted the decrease in splenic size when adrenaline was injected into the splenic artery at operation.

Lastly, it has been postulated (Prankerd, 1963) that abnormal cells, when removed from the circulation by the normal spleen in man, are sequestered in a pool in this organ. Red cells from patients suffering from hereditary spherocytosis, cells subjected to heat in vitro, or to mild damage with N-ethyl maleimide and those coated with non-complement fixing antibodies are, as previously mentioned, taken up by the spleen. Thus such cells, labelled with radioactive chromium-51, were employed to test the above hypothesis.

Following the infusion of an aliquot of one or other of these cell preparations, Harris et alii (1958), Prankerd (1963), and Toghill and Prichard (1964) showed that the count rate over the spleen equilibrated at a slower rate than normal. This suggested the collection of the cells in a compartment apart from the main arterio-venous communications. Toghill and Prichard (1964) claimed that the prior injection of noradrenaline prevented the splenic accumulation of these cells, suggesting that this substance closed off the arterial inflow into the compartment. They and Prankerd (1963) also demonstrated that noradrenaline given during, or following, removal of these cells by the spleen caused a rise in the count rate in the peripheral blood and a fall in the surface count rate over the splenic area. This they interpreted as meaning that, not only did nor-adrenaline prevent access of the cells to the pool, but also that it was capable of expelling the trapped cells from this compartment and causing their re-entry into the general circulation.

Opinion is still divided on the nature of the splenic circulation (Björkman, 1947). Thus it may be "open" with all blood filtering through the pulp spaces, "closed" with the cells passing through venous sinuses and being forced into the pulp only under the influence of increased venous pressure, or "divided" with some blood passing through the sinuses and some red cells being trapped in the pulp and released after varying periods of time. Toghill and

Prichard (1964) have presented a hypothetical scheme of the splenic circulation in the light of the above findings. They proposed that there was a free flow of blood through arterio-venous channels, with a proportion of cells passing through the pulp spaces, and that abnormal cells were held up in this pulp. It was postulated that noradrenaline by acting on the smooth muscle trabeculae, caused expulsion of the latter. Also, to explain the effect of noradrenaline in preventing abnormal red cell accumulation, they suggested that arterial inflow sphincters, first described by Schweigger-Seidel (1862), were constricted by this hormone and thus effectively prevented the entry of cells into the pool.

Several of the aforementioned studies employed a rise in the peripheral venous haematocrit as an index of increase in the red cell mass and thus, as evidence that noradrenaline caused red cells to be expelled from a body store, namely the spleen. The validity of this interpretation is open to doubt for Lucia et alii (1937) showed that, in humans, subcutaneous noradrenaline produced an increase in haematocrit and in the peripheral red cell count whether the spleen was present or not. They believed that this haemo-concentration was due to a decrease in plasma volume and not to a rise in the red cell mass. This was confirmed by Ebert and Stead (1941a) when they found that, in normal and splenectomised subjects given subcutaneous adrenaline, the plasma volume fell. This fall

was sufficient to account for the recorded rises in haematocrit, haemoglobin and serum protein levels. Thus, as well as confirming the haemo-concentrating action of adrenaline, they established that a reservoir of blood was not present in the normal human spleen. Kaltreider et alii (1942) performed similar studies in six normal subjects, two patients following splenectomy and two with polycythaemia and splenomegaly. They believed that though a small volume of erythrocytes was possibly expelled from the normal spleen, this was of no consequence in comparison to the total red cell mass. A slightly larger volume of cells was probably discharged in patients with splenomegaly, but in all cases the major change was a fall in plasma volume. They considered that this was brought about by a shift in fluid, poor in protein, from the vascular to the interstitial compartment. Finnerty et alii (1958) obtained essentially the same results when studying the effect of noradrenaline on 10 normal human subjects. As well as the parameters previously assessed, these workers estimated the red cell mass using chromium-51 labelled red cells. Again though the plasma volume fell (the haematocrit rose by an average of 8 per cent) the red cell mass remained constant demonstrating that erythrocytes were not added to the circulation during the infusion. Noradrenaline secreting phaeochromocytomas can also produce a low plasma volume (Brunjes et alii, 1960) and this is at least partly responsible for the shock state which follows

the removal of the tumour (von Euler, 1952). Similar findings were reported in experimental animals for Hahn et alii (1942), using radioactive iron labelled cells, found that adrenaline raised the peripheral haematocrit, without a change in the red cell mass, in dogs and Rose and Freis (1957) showed a fall in total blood volume in these animals given noradrenaline. Also Freeman et alii (1941) produced a marked fall in plasma volume and a state of shock by administering continuous intravenous adrenaline.

The mechanism of the rise in peripheral haematocrit under the influence of vasoconstrictors was considered by Hahn et alii (1942) and Brunjes et alii (1960) to be due to a redistribution of cells and plasma in various parts of the circulation. They suggested that these agents caused many blood vessels to be reduced in calibre and, because in small vessels the ratio of cells to plasma is decreased, pooling of fluid occurred in the peripheral circulation. There is experimental support for this concept as Ebert and Stead (1941b) showed that the haemoglobin concentration in small vessels is from 0.8 to 1.8 grams per 100 ml. lower than that of the venous blood. Gibson et alii (1946) using iron-55 and iron-59 labelled cells and I^{131} labelled albumin, demonstrated that the cell to plasma ratio of the blood in the capillaries is very low and that the ratio of the minute vessel haematocrit to the peripheral haematocrit in the dog is only 0.7. Using similar isotopic methods

Crane et alii (1960) confirmed these findings. In support of this Freis et alii (1949) claimed that laminar flow, with cells in the axial stream and plasma next to the vessel wall, was most pronounced in vessels of small diameter. Therefore as the vessels were constricted more plasma lost velocity through frictional resistance with the vessel wall as the cells moved ahead in the central stream.

However other workers considered a different mechanism, namely a leakage of fluid, out of the vascular compartment, a more likely explanation of the change. Kaltreider et alii (1942) claimed that it was due to a shift in fluid, poor in protein, from the interstitial compartment. Finnerty et alii (1958) agreed with this interpretation and believed that the hypotensive agents, by increasing the hydrostatic pressure in the capillaries, forced fluid out of the circulation. They considered that a loss of protein low fluid was established by their finding of a rise in plasma protein concentration, during the course of the infusion. Also Freeman et alii (1941) found that following the administration of intravenous adrenaline to dogs fluid was indeed lost from the blood. They noted, at post mortem examination, that fluid was distributed throughout the tissue spaces and also that it passed through the mucosa into the lumen of the gastro-intestinal tract.

Therefore a rise in peripheral haematocrit, during the infusion of vasoconstricting agents, cannot simply be interpreted as reflecting

an increase in the red cell mass due to the mobilization of red cells from a storage pool.

Commentary

Clinical observations including the study of the response to splenectomy, the estimation of red cell life utilising cross transfusion experiments and radioactive isotope techniques, as well as the determination of the sites of erythrocyte sequestration using the latter method of investigation, have shown that the spleen is in many cases responsible for the trapping and destruction of red cells in haemolytic states. However these studies, in conjunction with those investigating the fate of erythrocytes altered by treatment with red cell antibodies, or by physical and chemical agents, have not finally solved the problem of which particular cellular changes bring about the sequestration of red cells in the spleen. In particular the role of spherocytosis and increased osmotic fragility is confused. These cellular features are present in some conditions, notably hereditary spherocytosis, in which the spleen is established as the site of red cell uptake. In other situations however, splenic sequestration occurs in the absence of these changes and no consistent pattern, relating morphological characteristics to the site of sequestration in the reticulo-endothelial system, has emerged from such investigations.

Despite this it is well established that the spleen has a definite function in the uptake of certain classes of erythrocytes, a function which is not shared by other organs of the reticulo-endothelial system. Such cells, which are rapidly removed from the blood stream by the spleen, survive in some cases for a prolonged period of time in an otherwise normal individual who has been subjected to splenectomy. However, it has not been possible to measure quantitatively this function with any accuracy despite attempts using heated red cells as a specific reagent. It has been found difficult to prepare, by this means, a uniform cell population all of the members of which are removed only by the spleen following intravenous infusion. There are suggestions, from investigations attempting to quantitate the sequestering action of the spleen, that splenomegaly is associated with an increase in uptake function. Unfortunately, the trapping of more severely damaged cells in extra-splenic sites makes accurate analysis of the cell clearance rate very difficult. Also due to these disadvantages no information is available on whether the spleen, in haemolytic syndromes, shows an increase in sequestering function.

For reasons which are widely debated, corticosteroid hormones often produce a remission in haemolytic states. One possible explanation is that they act by reducing the ability of the spleen to trap red cells. A technique, making possible the accurate

quantitation of splenic uptake function, would allow one to assess the effect of these hormones on this parameter.

Opinions are divided on the presence, or characteristics, of a red cell pool in the spleen of normal human subjects and of those with splenomegaly. Conclusions concerning this aspect of splenic structure often rest on evidence that noradrenaline causes the expulsion of erythrocytes from the spleen, as judged by a rise in the haematocrit and red cell concentration of the peripheral venous blood. Such studies have often ignored the effects of such vasoconstrictors on plasma volume and thus investigation of a red cell pool demands evaluation taking into account such secondary effects.

CHAPTER III

MATERIALS AND METHODS

Purposes of the Present Study

The work on which this thesis is based was designed to investigate a number of the aspects of the relationship between red cell destruction and the spleen. These include attempts to:

(a) examine the effect of heat at 50°C and of a sulphydryl inhibitor N-ethyl maleimide (NEM), in vitro, on the morphology and osmotic fragility of human erythrocytes.

(b) examine the fate and site of organ uptake of such treated cells, labelled with radioactive chromium-51, after reinfusion into normal subjects and otherwise normal individuals following splenectomy. Also by this means investigate the influence that such cellular changes have on sequestration in the various organs of the reticulo-endothelial system, particularly the spleen.

(c) prepare an erythrocyte population, which is removed from the circulation only by the spleen, and using this reagent measure qualitatively splenic sequestering function by a study of the rates of clearance and organ accumulation of radioactive labelled cells.

(d) establish the range of uptake function of the spleen in normal subjects and compare this to the values obtained in a group of patients with splenomegaly, and demonstrate to what extent uptake is a function of splenic size.

(e) compare the rates of uptake and sites of removal of such altered cells to those of autologous chromium-51 labelled erythrocytes in haemolytic states and thus examine the usefulness of such a study in the selection of patients for splenectomy in the aleviation of haemolysis.

(f) examine the effect of noradrenaline infusion on the peripheral haematocrit and red cell count and in this way to critically examine the postulate that the normal human spleen expels red cells under the influence of vasoconstrictors and also the proposal that reversible "pooling" of abnormal cells occurs in the human spleen.

MATERIAL

In all 107 studies were carried out on the following groups of patients. The procedures were fully explained to all subjects before commencing the investigations.

Control Studies. The effect of heat on red cell morphology, osmotic fragility and survival was studied in 16 normal males. Twelve of these were volunteers from the Yatala Labor Prison, Northfield, South Australia, who were fit men housed in minimum security wards. They performed light manual work on the prison farm and were given a normal diet. The 4 remaining volunteers were males from the wards of the Royal Adelaide Hospital and were

admitted with the diagnoses of hysteria, duodenal ulceration, dyspepsia and one was convalescing after an uneventful appendicectomy. None of the 16 had a past history of haematological disease and all were normal on clinical examination and had normal blood pictures. The clearance of chromium-51 labelled haemoglobin was studied in 2 subjects, one a volunteer from Yatala and the other a patient with quiescent duodenal ulceration. This group is summarized in Appendix A I.

Similarly the effect of N-ethyl maleimide (NEM) on erythrocyte morphology, fragility and survival was studied in 14 normal males. Of these 12 were from Yatala and 2 were convalescing male patients from the Royal Adelaide Hospital. One patient had been operated on for appendicitis and the other was recovering from an episode of pancreatitis (Appendix A II).

In addition the effects of increasing periods of heat and increasing concentrations of NEM on erythrocyte fragility were studied on blood taken from a further 12 normal subjects. These were volunteers from the staff of the Department of Medicine or convalescing patients from the wards of the Royal Adelaide Hospital (Appendix A III).

Subjects Following Splenectomy. The records of the hospital were searched for patients who had splenectomy for traumatic rupture of the spleen within the previous 3 years. To 8 of these subjects

a letter (Appendix B) was sent requesting their participation in the study. Answers expressing willingness to take part in the investigation were received from 4 of these and also 2 other subjects were found by chance among the volunteers from Yatala. Also 3 other former inpatients of the hospital who had been subjected to the operation for various reasons also agreed to be included in the study. On these 9 subjects 11 experiments were undertaken utilising heated cells, NEM-treated cells or noradrenaline infusion (Appendix A IV).

Subjects with Haematological Disease. 28 studies of the survival of heated or NEM-treated cells were carried out in 25 patients with a variety of haematological disorders. All but 2 of these subjects, in whom heated cells were utilized early in the investigation, had palpable splenomegaly. These subjects were all inpatients or outpatients at the Royal Adelaide Hospital with the exception of 3 cases of thalassaemia minor who were discovered by chance among the volunteers from the Yatala Labor Prison, and one case of autoimmune haemolytic anaemia in a child referred from the Adelaide Children's Hospital. The diagnoses were in all cases confirmed by routine haematological investigations, biopsy and biochemical investigations where indicated and are summarized in Appendix A V).

Autologous Chromium-51 Survival Studies. In 11 subjects of the preceding group the survival and organ uptake of untreated autologous chromium-51 labelled erythrocytes were studied (Appendix A VI). In this way the fate of treated and autologous cells could be compared.

Noradrenaline Studies. Finally 13 investigations utilizing noradrenaline infusions were carried out in 12 subjects. Eight of these were in individuals with no detectable haematological disease. Two suffered from duodenal ulceration, two were normal volunteers from Yatala, one had a resolving lung disease, one was recovering from an episode of incipient delerium tremens, one had hysterical paralysis and one was referred to the Department of Medicine for the investigation of weakness and found to be normal. Of the remaining four one had been subjected to splenectomy and three had haematological disorders already itemized (Appendix A VII).

METHODS

1. Chromium-51 Erythrocyte Labelling and Heat Treatment

30 to 40 ml. of whole blood were collected from each subject into a flask containing 5 to 10 ml. of a sterile solution of acid-citrate dextrose (Formula A). The specimen was centrifuged at 1500 r.p.m. for 15 minutes in a refrigerated centrifuge, the supernatant removed and the cells labelled with 100 to 200 microcuries of radio-chromium ($Na_2\ ^{51}Cr\ O_4$, specific activity = 22 to 110 microcuries per

microgram) by the method of Mollison and Veall (1955). After washing, the cells were suspended in their original volume of plasma buffered isotonic saline and treated in a water bath at 50°C ± 0.5°C for periods of 10, 20, 30, 60, 90 or 120 minutes. The cells were then cooled at 20°C for 30 minutes, washed once, and again made up to the original volume. Aliquots of the final suspension were removed to serve as standards and for morphological and osmotic fragility studies. A known volume of the remainder, usually 20 ml. containing between 75 and 160 microcuries, was injected intravenously into the donor over a period of 5 to 10 seconds.

2. Chromium-51 Labelling and NEM Treatment

20 ml. of whole blood were collected and labelled as described above and the cells washed in plasma buffered normal saline. A solution of N-ethyl maleimide (NEM, Sigma Chemical Company, St. Louis 18, Missouri U.S.A.) was firstly prepared in sterile isotonic saline to a final concentration of 4 micromoles per ml. This solution was further sterilized by Seitz filtration and added to the chromium-51 labelled cells to final concentrations of 8, 10 or 20 micromoles of NEM per ml. of red cells. The suspension was incubated at 37°C for 45 minutes and then centrifuged and the cells washed twice in plasma buffered normal saline. It was then made up to a final volume of 25 ml. in this solution. Aliquots were removed to serve as standards and for morphological and fragility studies. 20 ml.

were infused intravenously as above. The amount of radiochromate injected ranged from 85 to 175 microcuries.

3. Cellular Morphology

Changes in erythrocyte morphology were assessed by the study of smears treated with Jenner-Giemsa stain (Dacie, 1956a) and also by the study of cells suspended in plasma buffered normal saline by a "hanging-drop" technique using a tissue culture "well" slide. The latter preparation was used to avoid possible cellular distortion caused by the smearing and staining procedure. In all cases the cells were examined before and after treatment with heat or NEM. Spherocytes were judged to be present by the criteria of Shen and his associates (1943).

4. Red Cell Osmotic Fragility Determinations

These were performed both before and after heating or NEM treatment of erythrocytes by the method of Dacie (1956b) using phosphate buffered solutions of sodium chloride in concentrations ranging from 0.1 to 1.2 grams per 100 ml. These solutions are made by serial dilutions of 10% NaCl containing NaCl 180 gm., NaH_2PO_4 27.31 gm. and $NaH_2PO_4.2H_2O$ 4.86 gm. made up to 2 litres. Aliquots of the blood to be tested were added to 5 ml. volumes of the range of saline solutions using a glass automatic pipette calibrated to deliver 0.05 ml. of blood. After standing for 30 minutes the solutions were

re-mixed and centrifuged at 2000 r.p.m. The degree of haemolysis in each tube was compared with that in the 100 per cent lysis tube (0.1 per cent NaCl) by means of a photoelectric colorimeter (Evans electroselenium) with an OGRI yellow green filter. Fragility curves were constructed by plotting the percentage haemolysis in each tube against the concentration of the saline in that tube: the occurrence of lysis in isotonic saline was estimated by measuring the concentration of haemoglobin released in 0.85 per cent NaCl. From the fragility curves the median corpuscular fragility (M.C.F.) was calculated. This was recorded as the concentration of saline causing 50 per cent lysis of the particular blood sample.

5. Preparation of Chromium-51 Labelled Haemoglobin

Blood was collected and labelled with chromium-51 as described above. After this the cells were washed in isotonic saline and then subjected to osmotic rupture by the addition of sterile distilled water. The solution was then subjected to Seitz filtration to remove red cell stroma. It was shown by Gray and Sterling (1950) that when red cells are labelled with chromium-51, in the form of $Na_2Cr^{51}O_4$, that at least 97 per cent of the of the radioactivity was in the haemoglobin fraction. Pearson and Vertrees (1961) showed that chromium-51 is attached largely to the beta chains of the globin and Malcolm et alii (1963) confirmed that the beta chains have a greater affinity for chromium than the alpha chains. Therefore a

chromium labelled haemoglobin solution, prepared as described, was injected intravenously in studies of the clearance rate and site of uptake of haemoglobin in normal subjects.

6. Collection and Preparation of Blood Samples

20 ml. samples of blood were rapidly collected by means of an indwelling intravenous catheter (Bardic type 1814) in the medial cubital vein of the subject (Figure 1). The blood was placed into Universal containers containing disodium ethylenediaminetetraacetic acid crystals (Na_2EDTA) as anticoagulant. Sampling was commenced 30 seconds after the completion of the infusion of labelled cells and was continued at increasing intervals ranging from 30 seconds up to 15 minutes, for 2 to 3 hours. Further samples were collected at 24 hours and, when clearance was slow, daily for up to one week. Immediately after the collection of samples, red cell haematocrit measurements were performed and 4 ml. aliquots were removed for whole blood radioactivity determinations. The remainder was centrifuged at 1500 r.p.m. for 20 minutes and a 4 ml. sample of the supernatant plasma collected and its radioactivity determined.

7. Haematocrit Determination

All haematocrit estimations were carried out in duplicate on each specimen using a Hawksley micro-haematocrit centrifuge developing 12,000 times gravity. Each specimen was centrifuged for

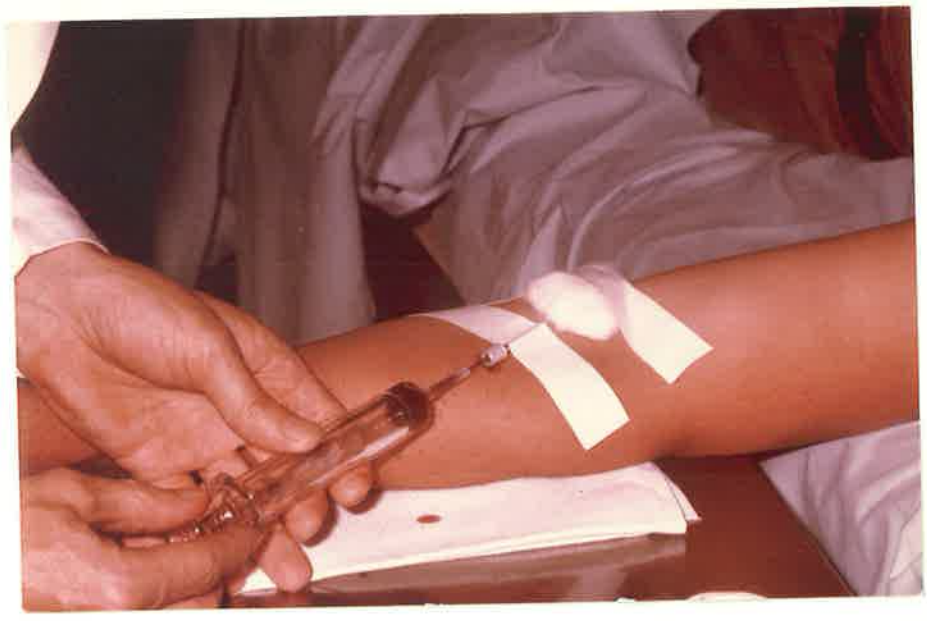

Figure 1: Collection of blood using an indwelling intravenous catheter.

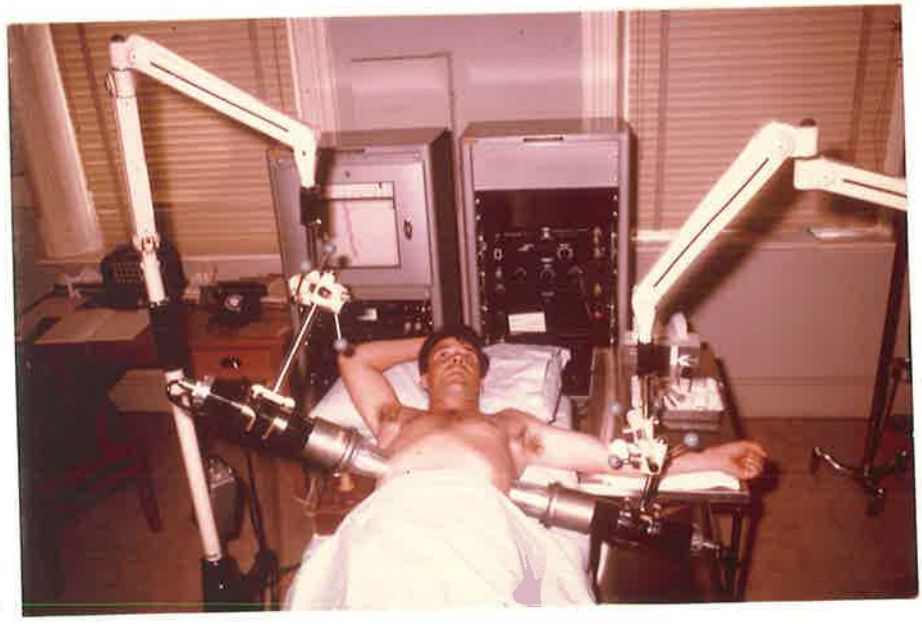

Figure 2: Recording organ count rates with shielded scintillation probes over the surface markings of liver and spleen.

5 minutes. This obviated the necessity of making corrections for trapped plasma (Shils et alii, 1952; Strumia et alii, 1954; and Dacie, 1956e).

8. Determination of Whole Blood and Plasma Radioactivity

4 ml. samples of whole blood were treated with saponin to produce cellular lysis and the activity in these and the 4 ml. plasma samples measured. This was done with a scintillation detector (EKCO N664A) and a well-type sodium iodide crystal (EKCO N597) adapted to a Philips Universal gamma spectrometer with facilities for pulse height analysis. In this system a count rate of 130 to 140 counts per second per microcurie of chromium-51 was obtained with a background of 2 to 3 counts per second. The presence of radioactivity in the plasma fraction was used as an indication of intravascular lysis and haemoglobin release into the circulation (Mollison, 1962). From these values the degree of intravascular lysis was determined by comparing the amount of plasma activity to the red cell activity. The count rates due to red cell activity were graphed and analysed to give an estimate of the rate of removal of red cells from the circulation in each subject.

9. Organ Surface Counting

Radioactivity over the precordium, spleen and liver was measured simultaneously by means of three shielded scintillation probes

(Philips PW 4119) fitted with 2" x 1¾" crystals and wide angled collimators. Pulses were counted on Philips Universal spectrometers utilizing their facilities for optimal "channel" counting. Continuous recording was carried out at each site for at least 60 minutes (Figure 2). Subsequently each of the above sites, together with the lungs and sacrum, were counted in turn by one probe for a further 3 to 4 hours. Surface counting was repeated, with the same probe, 24 hours later and daily for up to one week if the blood clearance of activity was found to be slow. For purposes of comparison, the actual count rates recorded with two of the units were standardized with those of the third. In these studies the count rates over the various organs, showing red cell uptake, rose to a steady level after a variable period of time depending on the rate of accumulation of active cells. For purposes of recording and comparison, the criteria of Holzbach and his associates (1962) were followed, and the asymptotic level towards which the highest organ count rate tended has been designated as 100 per cent uptake, for that particular organ.

10. Mathematical Analysis of Blood Clearance and Organ Uptake

The mechanism and rate of removal of treated cells from the circulation are analysed by plotting the logarithmof the red cell activity in the peripheral blood against time. If the curve obtained is a straight line it demonstrates that the decline in

activity follows an exponential function. Thus the rate at which cells are removed from the circulation is proportional to the amount remaining.

This is expressed by the equation

$$\log_e \frac{C}{Co} = -\lambda t$$

$$\text{i.e. } \frac{C}{Co} = e^{-\lambda t} \quad \text{or} \quad C = Co.e^{-\lambda t}$$

where C = red cell activity in the circulation at time t

Co = red cell activity in the circulation at time O

e = the base of the Natural system of logarithms and has the value 2.71828, and

λ = the rate constant, the fraction removed in unit time.

The exponent is negative as the amount of activity in the blood is declining.

When such a straight line relationship on the semilogarithmic scale is obtained the curve can as well be specified by using the half period, ($T\frac{1}{2}$), which is the time taken for the value to fall to half its initial value.

By substituting $\frac{C}{Co} = \frac{1}{2}$ in the equation $\log_e \frac{C}{Co} = -\lambda t$

$$\log_e 2 - \log_e 1 = \lambda t$$

$$= \frac{\log_e 2}{T\frac{1}{2}}$$

$$= \frac{0.693}{T\frac{1}{2}} \text{ (minutes)}$$

The units of λ (the fraction removed in unit time) are reciprocal

time units, for example, if K = 0.5 $min.^{-1}$, the cells are removed at the rate of 50 per cent per minute.

If the graph obtained on plotting the cellular activity in the circulation on a logarithmic scale against time yields a curve with a straight terminal portion it demonstrates that the decline in activity follows a combination of exponential functions, reflecting the removal of more than one population of cells at different rates. The terminal linear portion of the curve demonstrates that at this stage the rapid component, or components, are of negligible effect. If this straight portion of the wave is extrapolated back to zero time from this slope the half time and rate constant can be calculated for the slow component. Values obtained from subtracting the count rates on the extrapolated portion of the curve from the observed values are graphed on the same scale. If this in turn yields a straight line it establishes that the complex wave is compounded of two components only, a rapid and a slow. This can be expressed by the equation

$$C = C_A e^{-\lambda at} + C_B e^{-\lambda bt}$$

where C = red cell activity in the circulation at time t

C_A and C_B = the contributions to the initial count rate due to each population at time t = 0, and

λa and λb = the corresponding rate contents for the two populations.

As before each component can be specified by the appropriate half period ($T\frac{1}{2}$) or by calculation of the fractions removed in unit time from the formula $\lambda = \frac{0.693}{T\frac{1}{2}\ (\text{minutes})}$ for each part of the curve (Veall and Vetter, 1958a).

In the analysis of the rate of organ uptake of red cells the value $\frac{C_A - C}{C_A}$ is plotted on a logarithmic scale against time.

where C = count rate over the organ at time t

C_A = the asymptotic limit towards which the organ count rate tended.

Again if the wave obtained is a straight line it demonstrates that the uptake by the organ is exponential and expressed by the equation

$$\frac{C_A - C}{C_A} = e^{-\lambda t}$$

i.e. $C = C_A (1 - e^{-\lambda t})$ (Veall and Vetter, 1958b).

where in this case is the fraction of active cells removed by the organ in unit time.

In these studies the uptake curves have again also been specified by the half period ($T\frac{1}{2}$). The symbols λ S and λ L have been used to designate the functional uptake over the spleen and liver respectively.

11. Organ Size

The size of the spleen and liver was recorded in centimetres as the maximum distance to which the lower border extended on inspiration below the left and right costal margins respectively (Toghill, 1964).

12. Noradrenaline Infusion

Noradrenaline in normal saline containing ascorbic acid as a reducing agent was infused via an indwelling intravenous catheter (Bardic 1814) in the antecubital vein using a power driven constant infusion pump (Figure 3). The dose rate ranged from 12 to 16 micrograms per minute.

13. Autologous Red Cell Survival and Organ Uptake Studies

20 ml. of whole blood were collected and labelled with 100 microcuries of radiochromium ($Na_2{}^{51}CrO_4$) as previously described. After washing and centrifuging the cells were resuspended in plasma buffered normal saline and 20 ml. of the labelled red cell suspension were injected intravenously. Several ml. were retained for the preparation of the standard. Samples were then taken by venepuncture from the opposite median cubital vein 15 and 30 minutes after the time of injection as the zero (Day 0) specimens.

Red Cell Survival. Following the above procedure blood specimens were collected every day for 4 or 5 days and then 3 times per week for 3 further weeks. The mean of the count rate of the 15

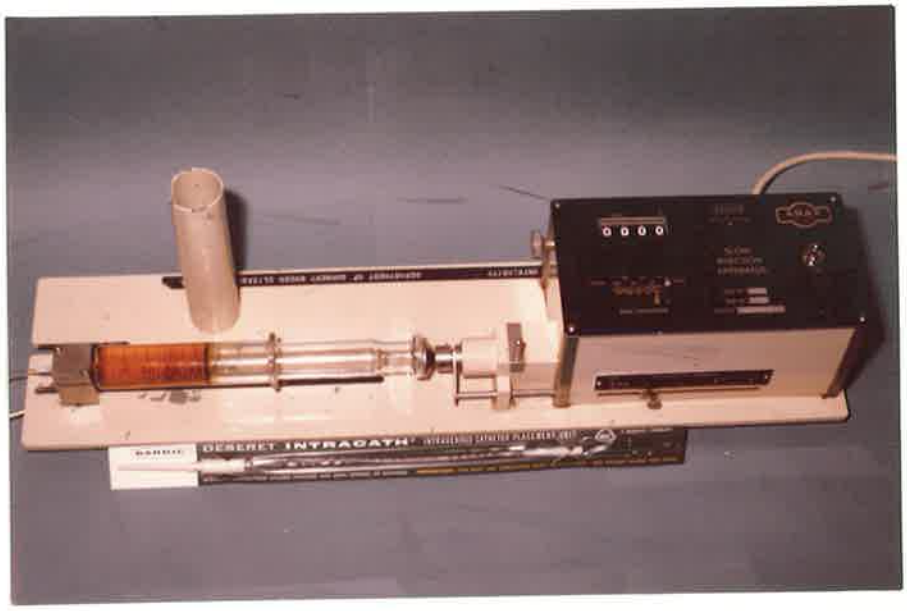

Figure 3: Constant infusion pump and indwelling intravenous catheter for noradrenaline injection.

and 30 minute specimens as described was taken as the 100 per cent value and red cell survival at any time was taken as a percentage of the injected chromium-51 which remained in the circulating blood. In this laboratory red cell life is considered to be normal when at least 50 per cent of the injected radioactivity remains in the circulation after 24 days. This figure is comparable to that reported by other workers (Ebaugh, Emerson and Ross, 1953; Necheles, Weinstein and Leroy, 1953; Read, 1954; Read, Wilson and Gardner, 1954; Sutherland et alii, 1954; Mollison and Veall, 1955; and Crawford and de Gruchy, 1958). The radioactivity in the 4 ml. samples of haemolysed whole blood was counted in a well-type sodium iodide crystal as previously described.

Organ Uptake Studies. Using a shielded scintillation probe, radioactivity was recorded over the precordium, liver, spleen, sacrum and lung. Counting was started over each organ site 30 minutes after the initial injection and then each site was counted daily for 4 to 5 days and then 3 times per week for the next 3 weeks. The precordial count rate 30 minutes after the injection of the labelled cells was corrected to 1000 by multiplying by $\frac{1000}{N}$ (where N = the actual count rate). All subsequent determinations at this and all other sites were multiplied by this factor. Also as an index of the relative amounts of chromium-51 deposited in the liver and spleen the ratio of spleen counts to liver counts was calculated.

This value, the spleen:liver ratio, was compared on the day of injection and at the chromium-51 half time in the circulation (Hughes Jones and Szur, 1957). Also according to the criteria of Jandl et alii (1956) a slow continual rise in the organ count rate, proportional to the decline in circulating radioactivity, was taken to indicate that cells were being sequestered and retained in the organ. The correction for the decline in organ count rate due to the reduction in circulating radioactivity was made by comparison with the precordial count rate as the latter was assumed to be due primarily to circulating labelled cells. In practice therefore, uptake was taken to be occurring when the organ:heart ratio at the chromium-51 half time in the circulation exceeded that at Day O.

The following symbols have been used to describe the degree of organ uptake: marked accumulation +++; moderate accumulation ++; detectable accumulation +.

14. Statistical Methods

Results were analysed according to the statistical methods outlined by Bailey (1959) and Documenta Geigy Scientific Tables, 5th edition (1956).

List of symbols used in statistical formulae:

n, n_1, n_2 = numbers of observations in samples.

p = significance level achieved by data.

s^2 = variance.

s = estimated standard deviation.

Σ = summation symbol.

t = deviation, "student's" t.

x, x_1 = observed measurements, independent variables in regression.

$\bar{x}$ = mean of sample of measurements x.

y, y_1 = observed measurements, dependent variables in regression.

$\bar{y}$ = mean of sample of measurements y.

b = estimated regression coefficient.

r = estimated correlation coefficient.

Statistical Formulae:

1. Calculations of the mean, variance and standard deviation

$$\text{mean, } \bar{x} = \frac{1}{n} \Sigma x$$

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

$$\text{standard deviation, } s = \sqrt{\frac{\Sigma(x^2) - \bar{x}\,\Sigma(x)}{n-1}}$$

2. Testing the significance of the mean of differences, that is whether the mean differs significantly from zero.

variance s^2 as above

$$\text{variance of the mean, } s^2_{\bar{x}} = \frac{s^2}{n}$$

$$\text{standard deviation of the mean, } s_{\bar{x}} = \frac{s}{\sqrt{n}}$$

$$t = \frac{\bar{x}}{s_{\bar{x}}}$$

3. Testing the significance of the difference of two means.

variance of the two samples, $s^2_{x\,x_1}$ =

$$\frac{\Sigma(x^2) - \bar{x}\ \Sigma(x) + \Sigma(x_1^2) - \bar{x}_1\ \Sigma(x_1)}{n + n_1 - 2}$$

standard deviation of the differences of the means,

$$s_d = \sqrt{\frac{s^2_{x\,x_1}}{n} + \frac{s^2_{x\,x_1}}{n_1}}$$

$$t = \frac{\bar{x} - \bar{x}_1}{s_d}$$

4. Calculation of regression coefficient.

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

constant $a = \bar{y} - b\,\bar{x}$

filled regression line, $y = a + b\,x$

5. Calculation of correlation coefficient

$$r = \frac{\Sigma(x - \bar{x})\ (y - \bar{y})}{\sqrt{\Sigma(x - \bar{x})^2 \cdot \Sigma(y - \bar{y})^2}}$$

significance test for correlation coefficients

$$t = \frac{r\sqrt{n - 2}}{\sqrt{1 - r^2}}$$

The significance levels for the various tests were obtained from published tables (Bailey, 1959). The following notations have been used.

$p > 0.05$	not significant
$p < 0.05$	almost significant
$p < 0.01$	significant
$p < 0.001$	highly significant

CHAPTER IV

THE EFFECT OF HEAT ON RED CELL MORPHOLOGY, OSMOTIC FRAGILITY AND SUBSEQUENT SURVIVAL IN VIVO

Aims of the Investigation

The general purpose of the study was outlined in Chapter III. In this section the effect of heat on erythrocyte structure and survival was investigated and an endeavour made, by this means, to prepare a cellular reagent that could be used in the measurement of splenic uptake function in normal and abnormal subjects. Factors, in the mechanism of anaemia in cases of severe burning, were also thus explored.

Subjects Studied

The total composition of the subjects admitted to the investigation has been set out in Chapter III. In this section the examinations, 37 in all, were grouped as follows.

A. 27 studies of the effect of heat on cell morphology, fragility and in vivo survival.

Group 1: Normal. 16 healthy male volunteers.

Group 2: Splenectomy. 7 subjects who had been submitted to the operation from 1 to 3 years previously.

Group 3: Haematological disease. 4 subjects; 2 with auto-immune haemolytic anaemia, one with myelofibrosis and one with thalassaemia minor.

B. Morphological and osmotic fragility studies were carried out on heated cells taken from a further 6 normal subjects.

C. The rate and site of uptake of chromium-51 labelled haemoglobin was studied in 2 normal subjects.

D. The survival and organ sequestration of autologous untreated cells was studied in 2 of the subjects with autoimmune haemolytic anaemia and the patient with myelofibrosis.

RESULTS

Morphology: Heating red cells for periods of from 10 to 15 minutes as described caused a change from the normal biconcave discoid shape (Figure 4) to a uniform spherocytic configuration (Figure 5).

When the heating was prolonged for 20 to 30 minutes, as well as the spherocytic change, microspherocytes and other fragmented forms were produced by "budding" from the larger spherocytes (Figure 6). In many cases these fragments could be seen to be attached to the parent cell by an intercellular "bridge" (Figure 7).

After heating for up to 60 minutes the fragmentation became more widespread and cells, which stained less densely, tended to agglutinate in small clumps (Figure 8).

Heating for up to 120 minutes produced as well as the very marked fragmentation a higher proportion of the indistinct agglutinated forms (Figure 9).

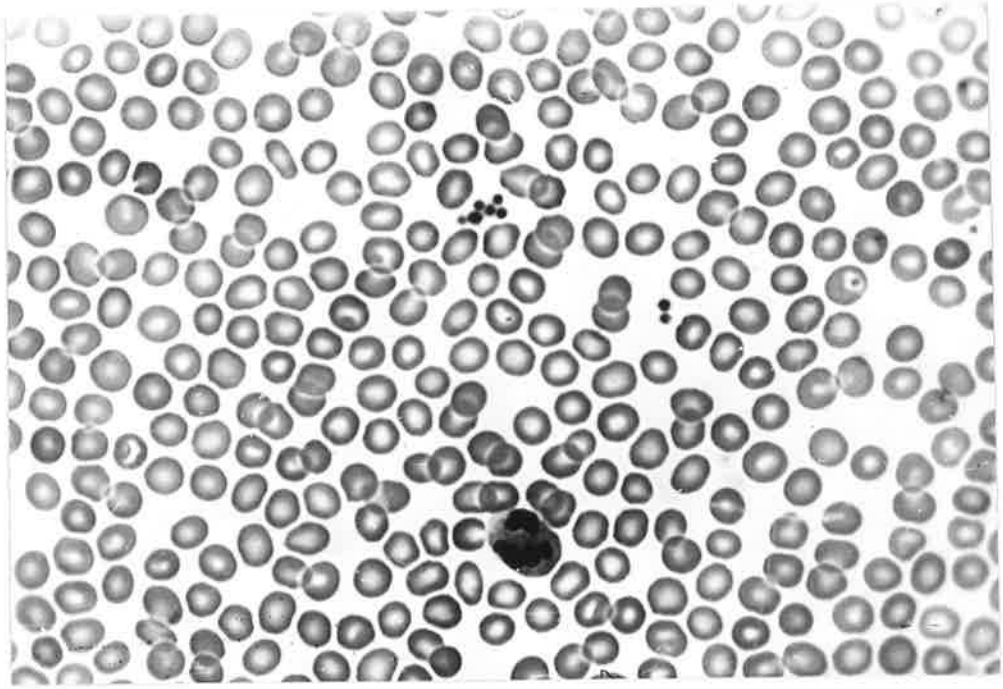

Figure 4: Normal erythrocytes before heat treatment showing biconcave discs. (x 670).

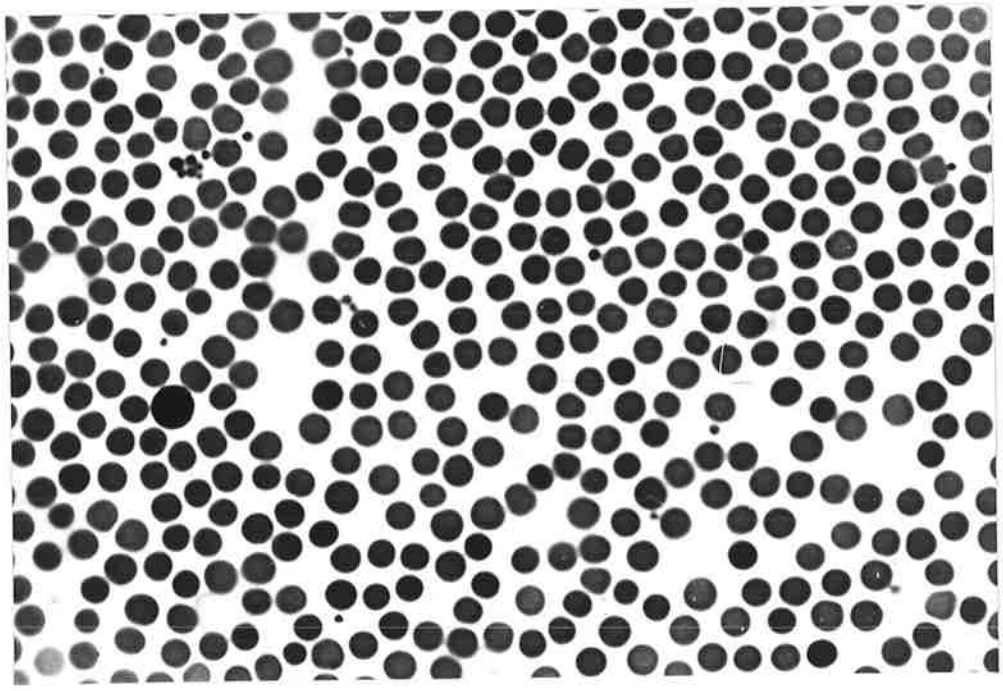

Figure 5: Erythrocytes following heat treatment, 10 mins. at 50°C, showing spherocytes. (x 670).

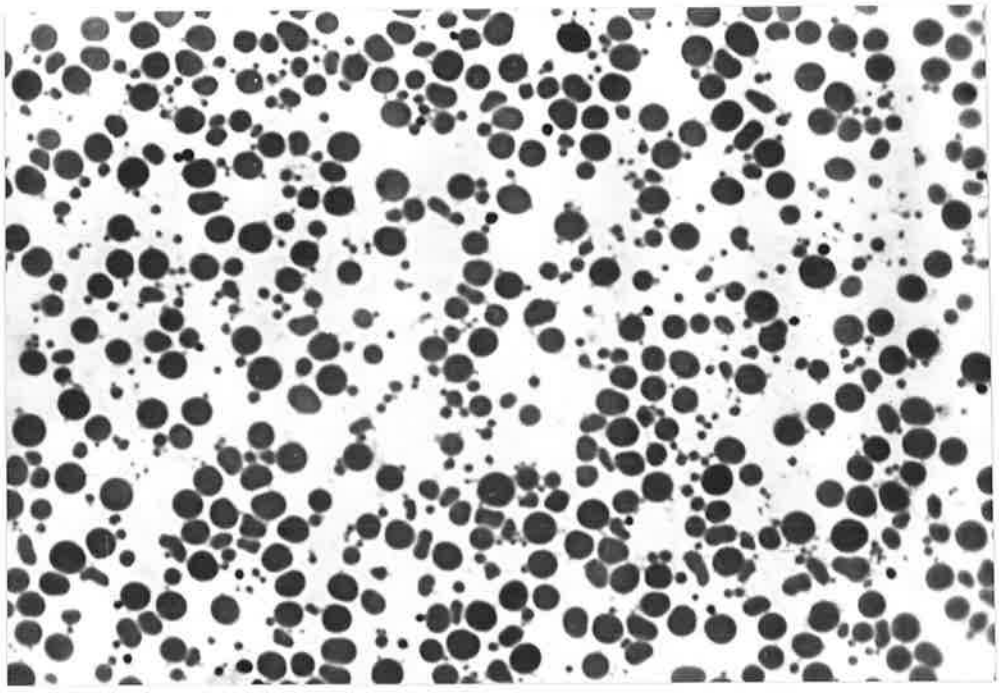

Figure 6: Erythrocytes following heat treatment, 20 mins. at 50°C, showing fragmentation. (x 670).

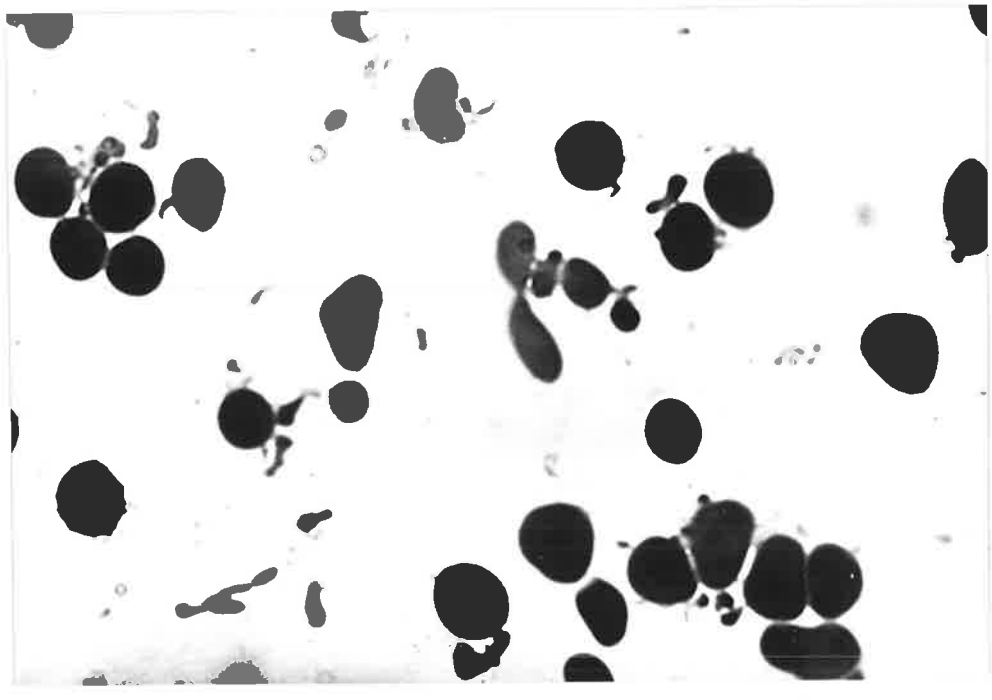

Figure 7: Higher power of the above cells illustrating the formation of microspherocytes by budding (x 1580).

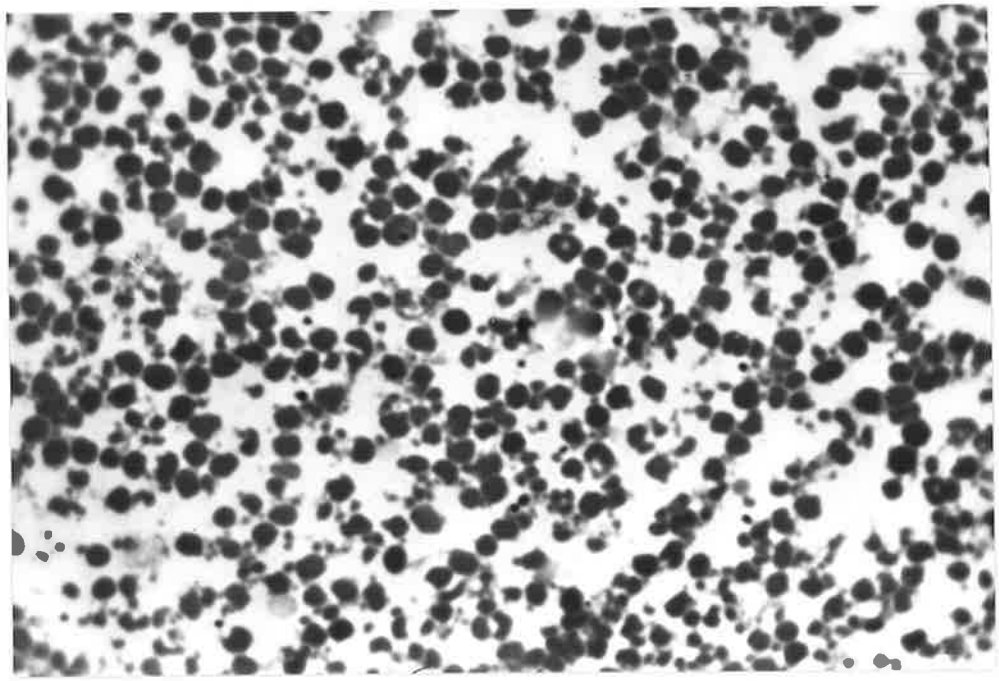

Figure 8: Erythrocytes following heat treatment, 90 mins. at 50°C, showing agglutination. (x 670).

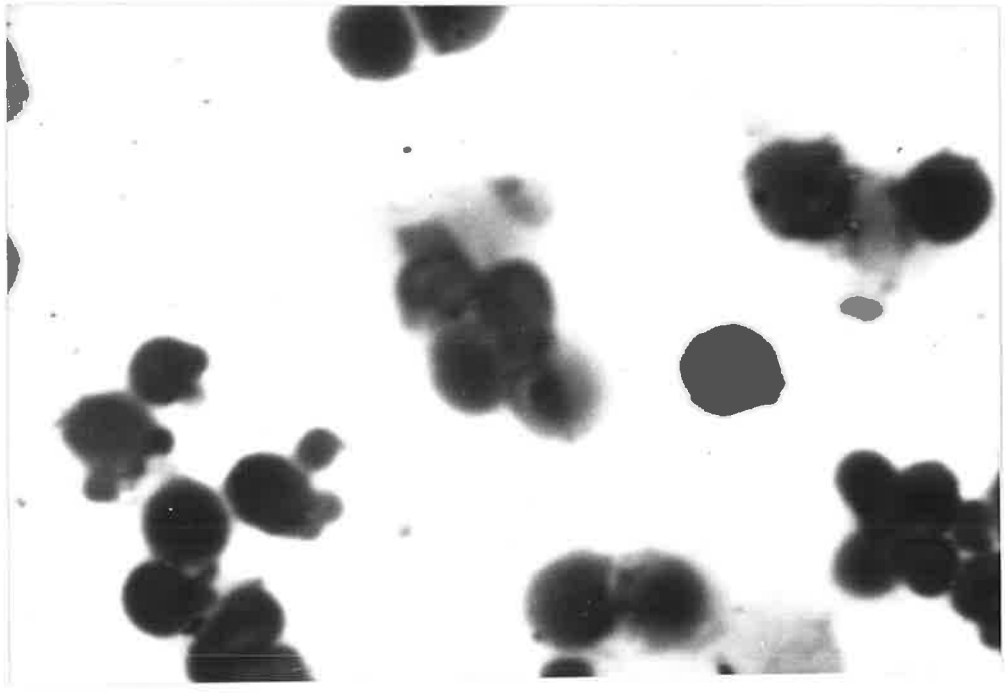

Figure 9: Higher power of severely heat damaged agglutinated cells. (x 1580).

Table II - The effect of heat at 50°C on median cellular fragility (M.C.F.) in 26 subjects.

Study	Median Cellular Fragility Before Treatment	After Treatment	Difference	Analysis
		A. HEAT 10-15 MINUTES		
1 J.O.	0.47	0.43	- 0.04	Significant decrease in M.C.F. $p < 0.01$
2 J.K.	0.45	0.44	- 0.01	
17 C.M.	0.43	0.37	- 0.05	
23 K.E.	0.44	0.41	- 0.03	
25 E.C.	0.37	0.34	- 0.03	
26 L.D.	0.43	0.42	- 0.01	
31 N.S.	0.40	0.38	- 0.02	
		B. HEAT 20-30 MINUTES		
13 F.M.	0.45	0.53	+ 0.08	Significant increase in M.C.F. $p < 0.001$
16 M.A.	0.46	0.55	+ 0.09	
18 W.B.	0.42	0.48	+ 0.06	
20 F.S.	0.43	0.48	+ 0.05	
21 M.S.	0.43	0.48	+ 0.05	
22 L.H.	0.43	0.50	+ 0.07	
24 R.Ka	0.42	0.43	+ 0.01	
29 A.F.	0.48	0.58	+ 0.10	
30 E.B.	0.47	0.53	+ 0.06	
		C. HEAT 60 MINUTES		
3 D.C.	0.45	0.60	+ 0.05	Significant increase in M.C.F. $p < 0.001$
8 R.Kr	0.47	0.55	+ 0.08	
9 A.D.	0.43	0.63	+ 0.20	
10 R.R.	0.46	0.58	+ 0.12	
11 C.H.	0.46	0.65	+ 0.19	
27 N.H.	0.45	0.48	+ 0.03	
28 C.C.	0.45	0.72	+ 0.27	
		D. HEAT 90-120 MINUTES		
4 P.C.	0.45	0.52	+ 0.07	Not Analysed
5 D.P.	0.43	0.65	+ 0.22	
6 M.H.	0.45	0.57	+ 0.12	

Table III - The effect of increasing periods of heat at 50°C on the median cellular fragility (M.C.F.) of erythrocytes of normal subjects.

Study		Median Cellular Fragility					
	Period of Heating (mins.)	0	10	20	60	90	120
96	R.Ki	0.42	0.41	0.47	0.66	0.70	0.78
97	C.Ki	0.45	0.42	0.62	0.80	0.82	0.84
98	H.L.	0.46	0.42	0.53	0.57	0.68	0.70
99	M.D.	0.47	0.44	0.54	0.58	0.64	0.71
100	W.H.	0.47	0.46	0.56	0.60	0.65	0.74
101	J.H.	0.47	0.43	0.74	0.77	0.78	0.79

Analysis of Mean of Differences

(1) 10 minutes, significant decrease ($p < 0.01$) in M.C.F. compared to unheated cells.

(2) 20 minutes, significant increase ($p < 0.001$) in M.C.F. compared to unheated cells.

(3) 60 minutes, probably significant increase ($p < 0.05$) in M.C.F. compared to 20 minutes' heat.

(4) 90 minutes, significant increase ($p < 0.02$) in M.C.F. compared to 60 minutes' heat.

(5) 120 minutes, significant increase ($p < 0.02$) in M.C.F. compared to 90 minutes' heat.

Osmotic fragility: The osmotic fragility change produced in the cells of the 26 subjects originally studied is shown in Table II. This table includes the findings seen in the erythrocytes taken from the subjects with splenectomy and haematological disorders as well as the normal group. Thus heat at 50°C for 10 to 15 minutes produces as well as spherocytes a decrease in osmotic fragility. This decrease in fragility was found to be significant (0.001 p 0.01) by analysis of the mean of the differences. Heating for 20 to 30 minutes produced a significant increase in osmotic fragility (P 0.001) as did heating for 60 minutes (p 0.001). As the group in which the cells were heated for 90 to 110 minutes consisted of blood from only 3 subjects, and also as the overall group contained some individuals who had been subjected to splenectomy or had haematological disorders, the effect of heat on normal red cells was further studied. 36 osmotic fragility estimations were carried out on the blood from 6 normal subjects heated to 50°C (Table III). Aliquots of blood were removed from the water bath at intervals of 10, 20, 60, 90 and 120 minutes. Analysis of this study by determining the significance of the mean of the differences confirmed the fact that heat for 10 minutes at 50°C causes a fall in osmotic fragility (0.001 p 0.01). It was also established that the increase in osmotic fragility produced by heating for 120 minutes was significantly greater than that produced by 90 minutes (0.01 p 0.02), that

that produced by 90 minutes of heating was significantly greater than that produced by 60 minutes ($.01 < p < .02$), and that produced by 60 minutes was probably significantly greater than that produced by 20 minutes ($.02 < p < .05$). These findings are represented graphically in Figure 10 showing the long "tails" of particularly vulnerable cells produced by increasing heat treatment. Also it is shown that periods of 20 minutes or more produced an increasing degree of cellular breakdown in isotonic saline as measured by the concentration of haemoglobin released in 0.85 per cent saline.

Therefore in summary increasing periods of heat from 10 to 120 minutes at 50°C produces the following series of changes. Firstly a spherocytic change with a decrease is osmotic fragility, secondly a fragmentation of cells by budding to form microspherocytes with an increase in osmotic fragility and some cellular lysis in isotonic saline, and thirdly an agglutination of cells with a further increase in osmotic fragility and lysis in isotonic saline.

Survival and organ uptake in normal subjects. Cells heated for 10 to 15 minutes, that is those of spherocytic shape and reduced osmotic fragility, were following reinfusion removed from the circulation slowly in an exponential manner. In the three studies performed the average half time was approximately 10 hours, the maximum being 12 hours and the minimum 9 hours (Table IV). This relatively slow rate of removal of the damaged cells from the

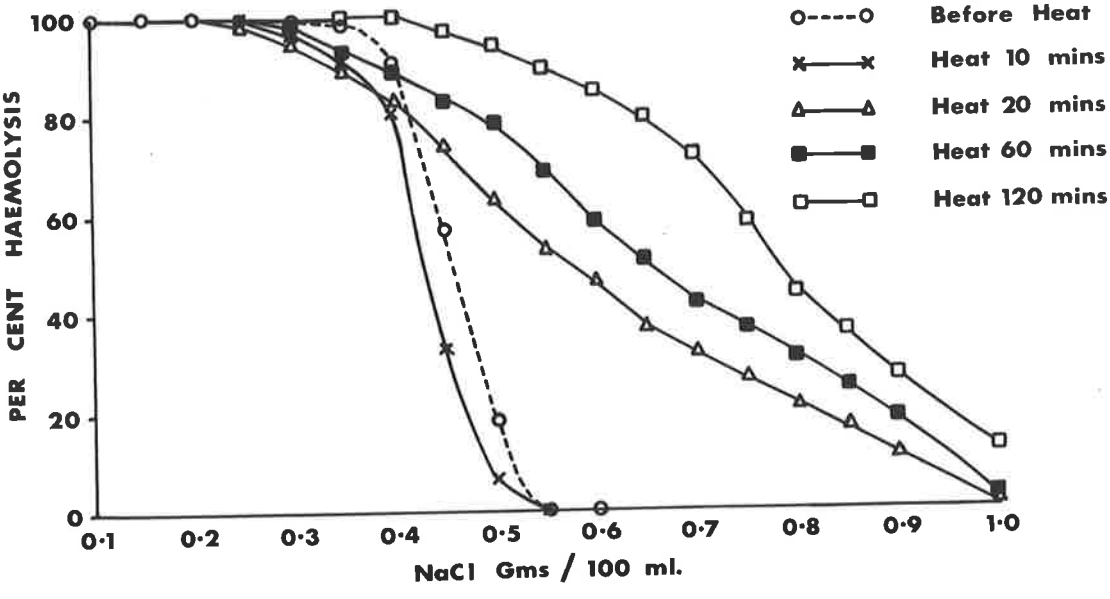

Figure 10: The effect of increasing periods of heat at 50°C on red cell osmotic fragility.

Table IV - The effect of increasing periods of heat at 50°C on the rate of clearance of erythrocytes from the circulation, site of sequestration and degree of intravascular lysis.

Study	Volume of cells injected (ml./kg.)	Clearance Rate - Slow Component $T_{\frac{1}{2}}$	Clearance Rate - Slow Component λ min.$^{-1}$	Clearance Rate - Rapid Component $T_{\frac{1}{2}}$ min.	Clearance Rate - Rapid Component λ min.$^{-1}$	Organ Uptake Spleen	Organ Uptake Liver	Per cent intra-vascular lysis
HEAT 10-15 MINUTES								
1 T.H.	0.061	9 hrs.				+++	0	0
2 I.V.	0.074	12 hrs.				+++	0	0
26 L.D.	0.065	10 hrs.				+++	0	0
MEAN		10 hrs.						
HEAT 20-30 MINUTES								
13 F.M.	0.057	70 min.	0.0099	7	0.099	+++	+	15
21 M.S.	0.063	100 min.	0.0069	4	0.173	+++	+	18
22 L.H.	0.030	100 min.	0.0069	8	0.087	+++	+	16
MEANS		90 min.	0.0079	6	0.120			16.3
HEAT 60 MINUTES								
3 D.C.	0.126			5	0.139	+++	++	45
8 R.Kr	0.088			6	0.116	+++	++	10
9 A.D.	0.071			5	0.139	+++	++	25
10 R.R.	0.066			5	0.139	+++	++	14
11 C.H.	0.059			6	0.116	+++	++	18
27 N.H.	0.080			8	0.087	+++	++	9
28 C.C.	0.072			7.5	0.092	+++	++	11
MEANS				6.1	0.102			18.9
HEAT 90 - 120 MINUTES								
4 P.C.	0.082			4	0.173	++	+++	30
5 D.P.	0.063			4	0.173	++	+++	77
6 M.H.	0.078			5	0.139	++	+++	23
MEANS				4.3	0.162			43

circulation was associated with a gradual accumulation of radioactivity over the spleen, most marked at 24 hours. There was no evidence of any accumulation of activity in the liver or elsewhere under these circumstances. The pattern of events in one of these studies is shown in Figure 11. In no case was activity detected in the plasma fraction showing that no intravascular lysis had occurred.

Cells heated for 20 to 30 minutes (showing fragmentation and increased osmotic fragility) showed a biphasic fall in circulating red cell activity and this could be demonstrated as reflecting two exponential components. In three subjects the half times of the rapid component were 4, 7 and 8 minutes with a mean therefore of 6 minutes. These corresponded to clearance rates of 0.173, 0.999 and 0.087 $min.^{-1}$ and a mean of 0.120 $min.^{-1}$ (Table IV). The slow components showed half periods of 70, 100 and 100 minutes and thus an average of 90 minutes corresponding to a mean clearance rate of 0.0079 $min.^{-1}$ This biphasic pattern of cell removal from the circulation was associated not only with a marked splenic uptake of radioactivity but also with a detectable rising count rate over the liver. The activity over both organs stabilized within 1 to 2 hours after reinfusion in each subject (Figure 12). The removal of such cells from the circulation was in part due to intravascular lysis. From the radioactive content of the plasma fraction it was

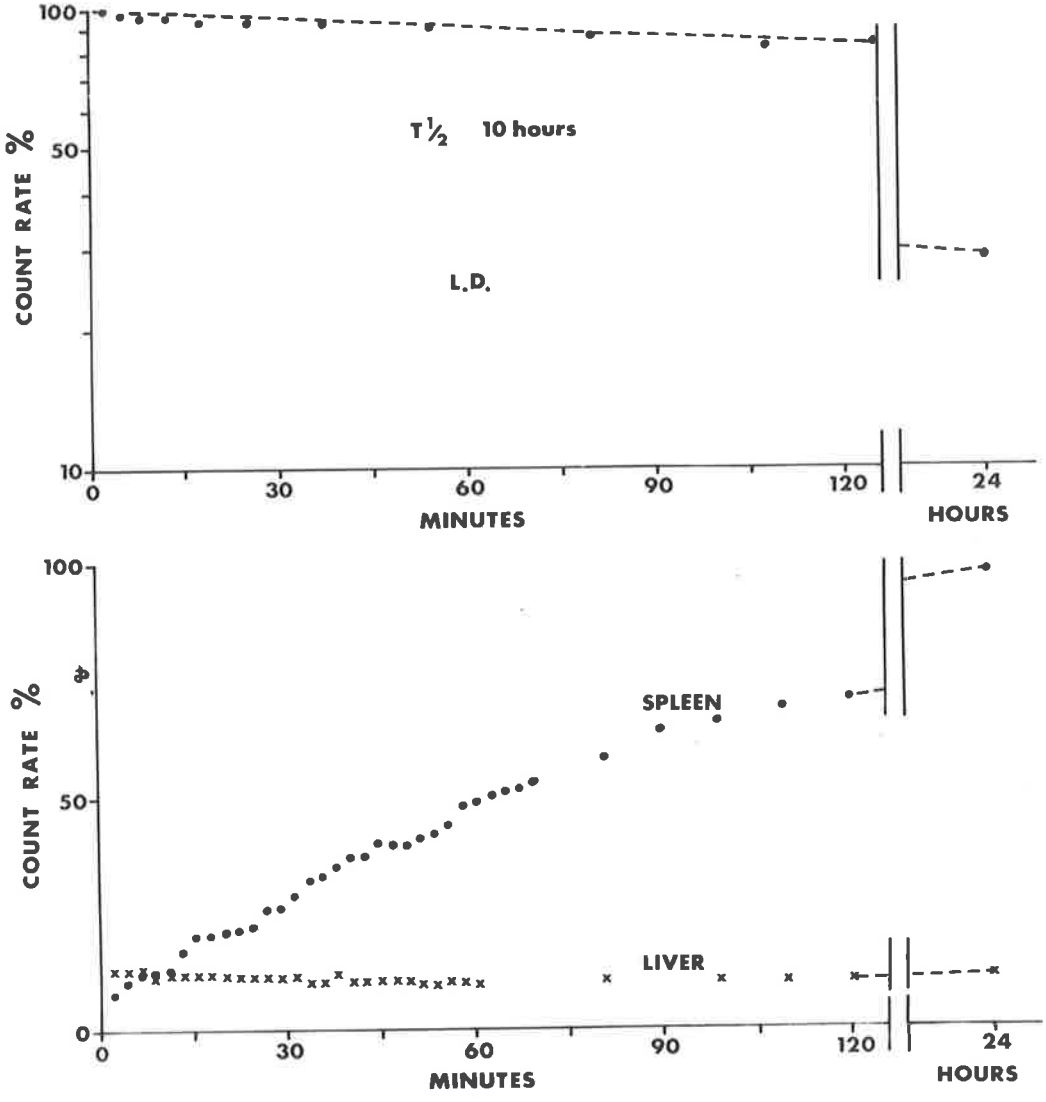

Figure 11: The clearance from the blood (above) and organ uptake (below) of red cells heated for 10 minutes at 50°C (Study No. 26).

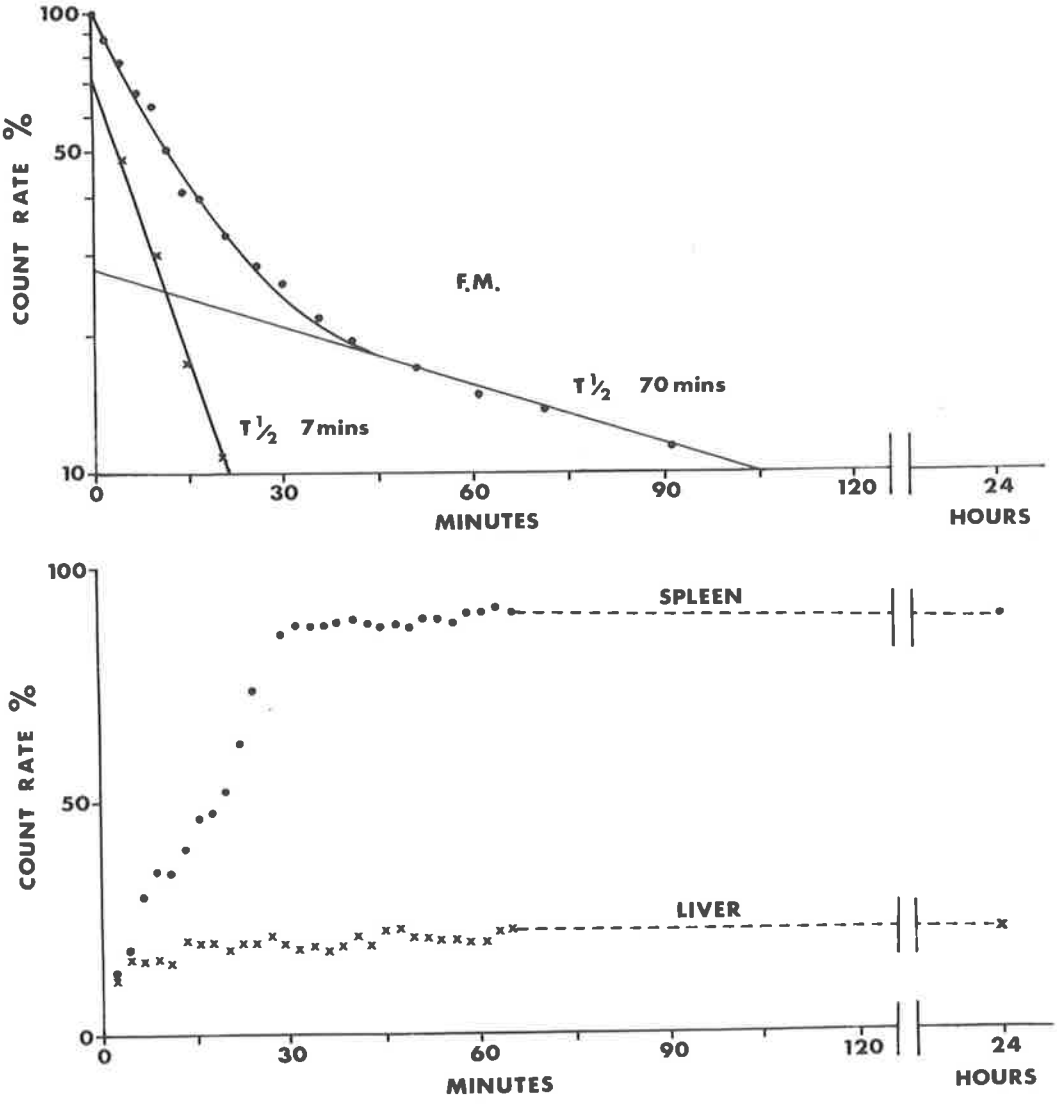

Figure 12: The clearance from the blood (above) and organ uptake (below) of red cells heated for 20 minutes at 50°C (Study No. 13).

calculated that from 15 to 18 per cent of the cells suffered this fate (Table IV).

Cells heated for 60 minutes (fragmentation, agglutination and increased osmotic fragility) were cleared rapidly from the circulation of 7 subjects in an exponential manner. The mean half time of clearance was 6 minutes (mean clearance rate 0.102 $min.^{-1}$) the minimum being 5 minutes (clearance rate 0.139 $min.^{-1}$) and the maximum 8 minutes (clearance rate 0.087 $min.^{-1}$). (Table IV). In each instance, clearance of the damaged cells from the circulation was associated with a marked splenic and also a moderate hepatic uptake of radioactivity (Figure 13). Cellular destruction by intravascular lysis was again evident and the maximum amount of radioactivity in the plasma accounted for the destruction of from 9 to 45 per cent of cells by this means, the mean being 19 per cent (Table IV).

Cells heated for 90 to 120 minutes (increased agglutination and osmotic fragility) also were rapidly removed in an exponential manner in 3 subjects with half periods of 4 minutes (0.173 $min.^{-1}$), 4 minutes and 5 minutes (0.139 $min.^{-1}$) respectively (Table IV). In each subject relatively more activity accumulated in the liver than in the spleen (Figure 14). Cellular destruction by intravascular lysis was pronounced and the maximum plasma activity demonstrated that from 23 per cent to 70 per cent of the cells were so destroyed (Table IV).

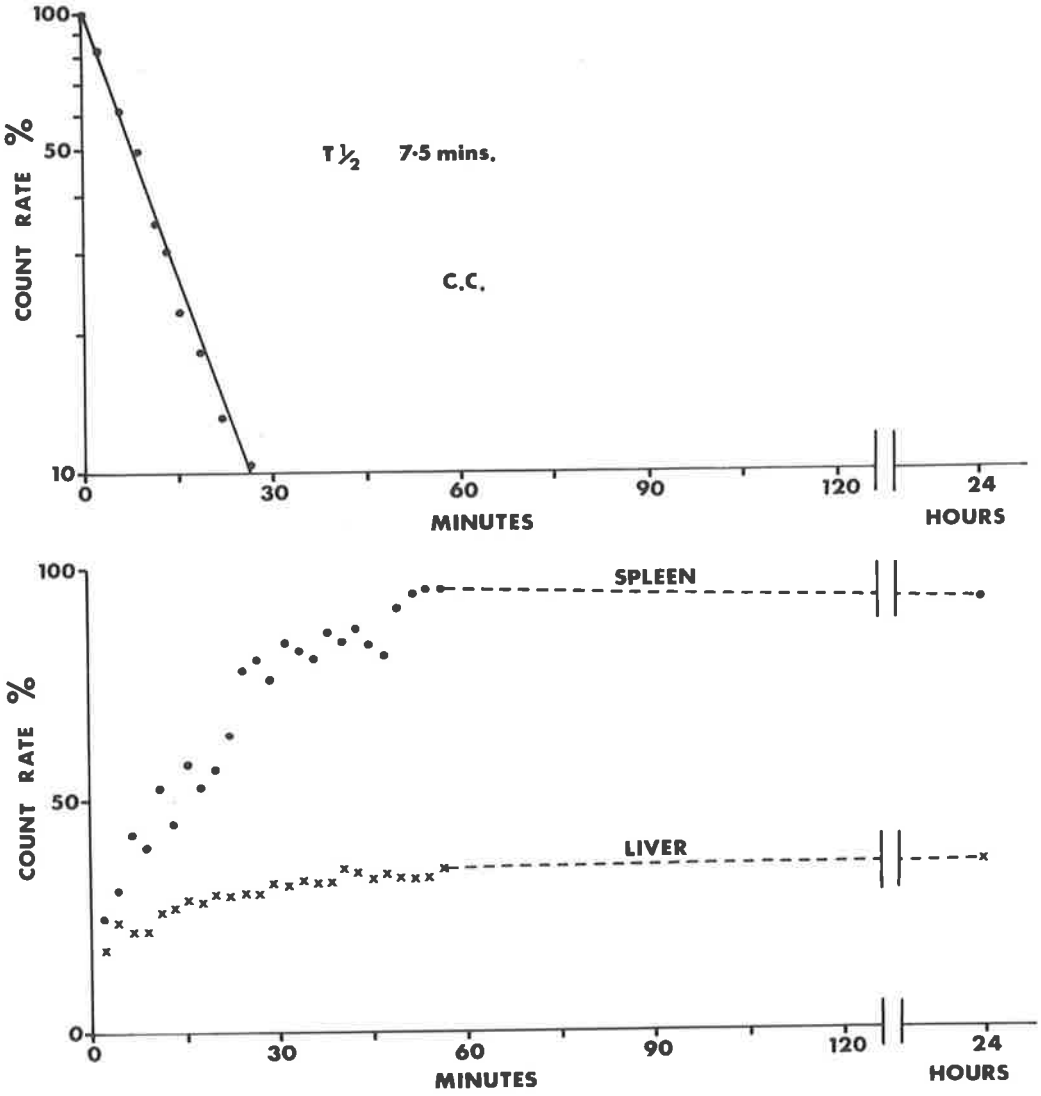

Figure 13: The clearance from the blood (above) and organ uptake (below) of red cells heated for 60 minutes at 50°C (Study No. 28).

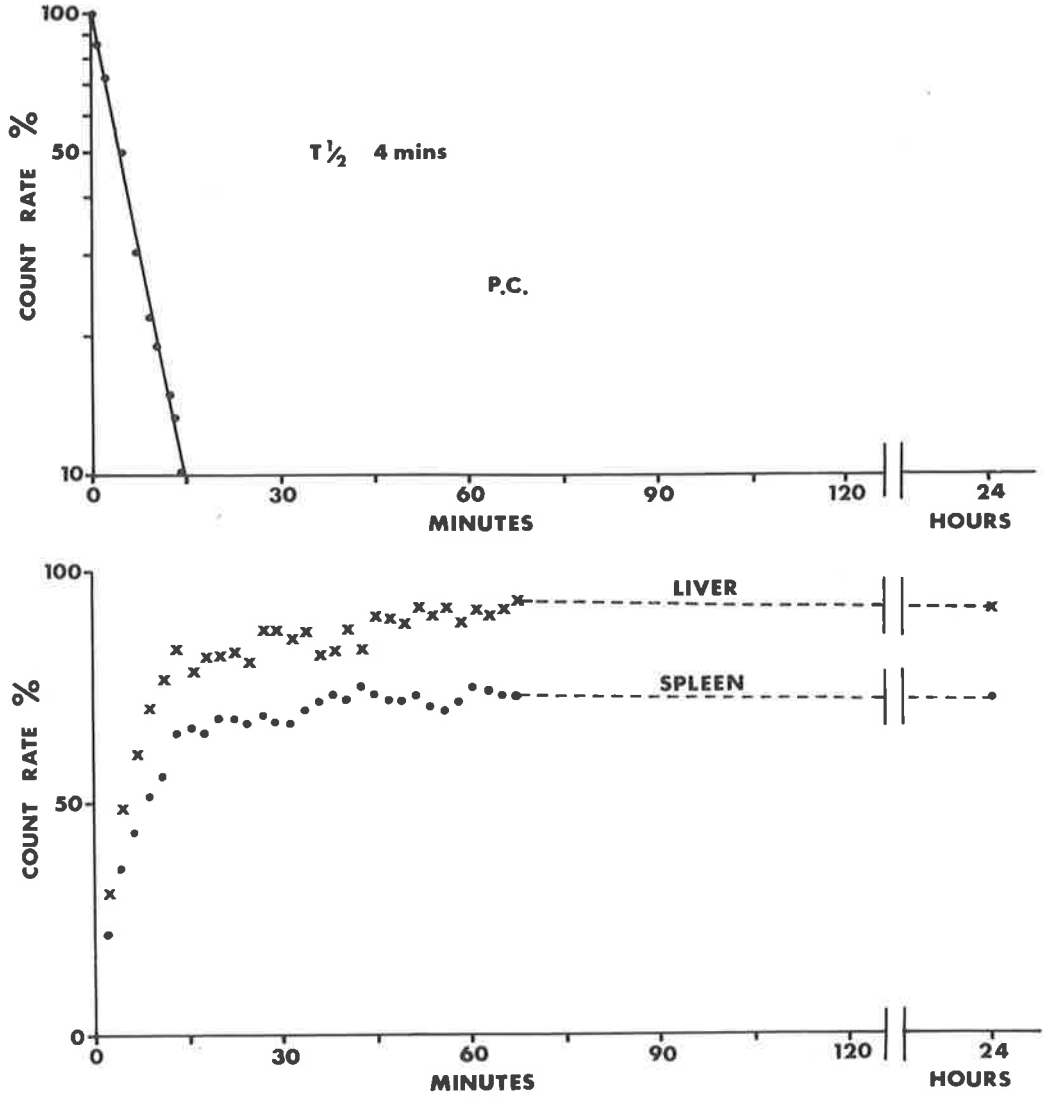

Figure 14: The clearance from the blood (above) and the organ uptake (below) of red cells heated for 120 minutes at 50°C (Study No. 4).

Table V - The effect of increasing periods of heat at 50°C on the rate of clearance of erythrocytes, site of sequestration and degree of intravascular lysis in patients following splenectomy.

Study	Volume of cells injected (ml./kg.)	Clearance Rate - Slow Component $T\frac{1}{2}$	Clearance Rate - Rapid Component $T\frac{1}{2}$ min.	Clearance Rate - Rapid Component λ min.$^{-1}$	Organ Uptake in liver	Per cent intra-vascular lysis
HEAT 10-15 MINUTES						
17 C.M.	0.067	3 days			+	0
23 K.E.	0.047	3 days			+	0
HEAT 20-30 MINUTES						
16 M.A.	0.055	14 hrs.	25	0.028	++	10
18 W.B.	0.084	11 hrs.	10	0.069	++	14
20 F.S.	0.059	9 hrs.	10	0.069	++	19
24 R.Ka	0.081	18 hrs.	18	0.039	++	6
29 A.F.	0.056	24 hrs.	15	0.046	++	9
MEANS		15 hrs.	16	0.050		11.5

Survival and organ uptake in the absence of the spleen. Cells heated for 10 to 15 minutes when reinfused into two donors who had undergone splenectomy for traumatic rupture of the spleen were removed relatively slowly from the circulation with a half time of approximately 3 days (Figure 15). There was little evidence of accumulation of activity in the liver or other areas during the first 24 hours, but within 3 days some hepatic and sacral accumulation had occurred in both cases. None of the cells were removed by intravascular lysis as no activity was detected in the plasma fraction (Table V).

Cells heated for 20 to 30 minutes were cleared in a biphasic manner in 4 subjects who had undergone splenectomy for traumatic rupture, and in one patient who had been subjected to the operation in the course of treatment of hereditary spherocytosis. In this group the half time of the slow component ranged from 9 to 24 hours with a mean of 15 hours while that of the rapid component ranged from 10 to 25 minutes with a mean of 16 minutes. This corresponded to a clearance of from 0.069 $min.^{-1}$ to 0.028 $min.^{-1}$ with a mean of 0.050 $min.^{-1}$ (Table V). Associated with this pattern of cell clearance there was a steady moderate accumulation of activity in the liver (Figure 16). Destruction of cells by intravascular lysis occurred in these subjects and ranged from 6 to 19 per cent of the cells injected, the mean being 11.5 per cent (Table V).

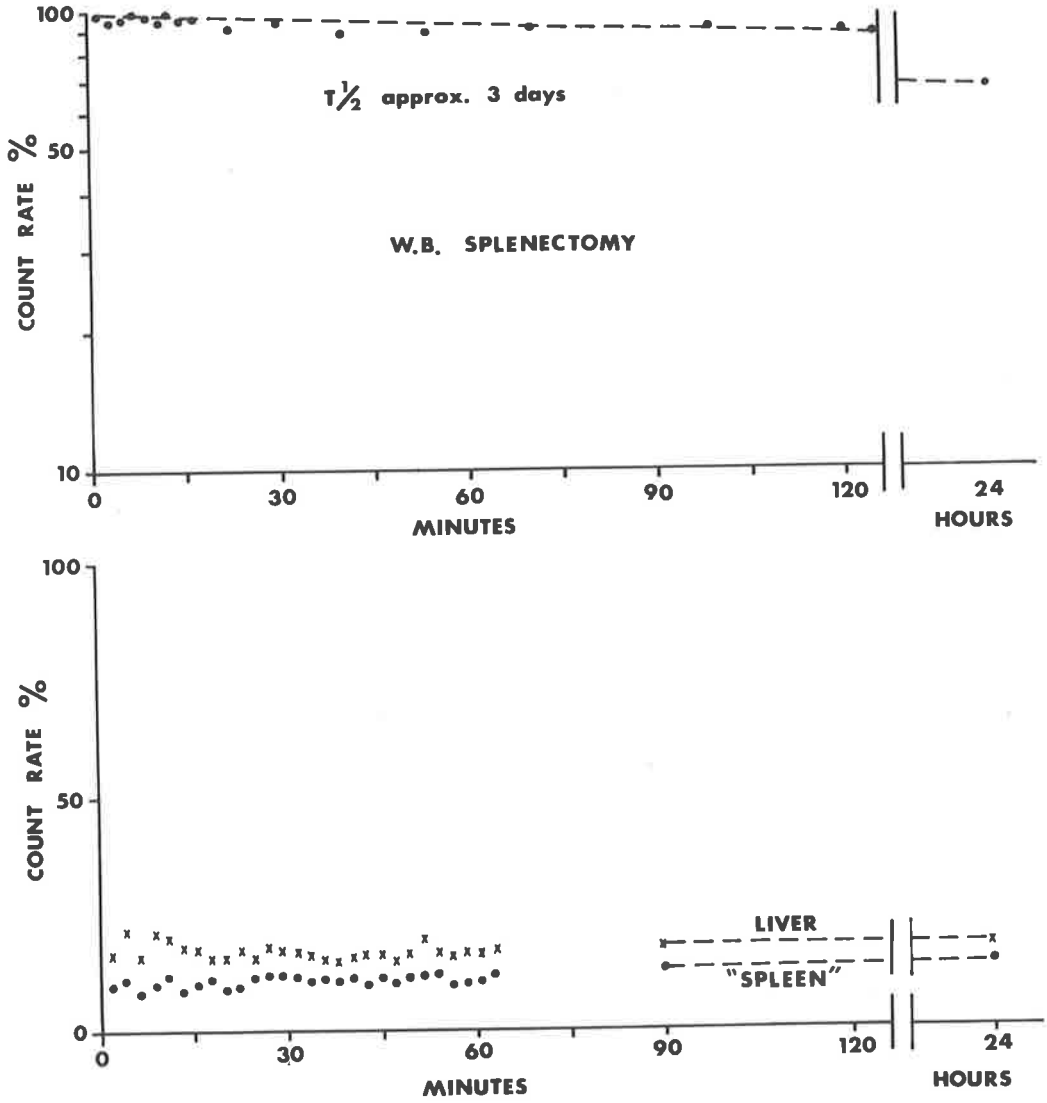

Figure 15: The clearance from the blood (above) and organ uptake (below) of red cells heated for 10 minutes at 50°C (splenectomized subject, Study No. 17).

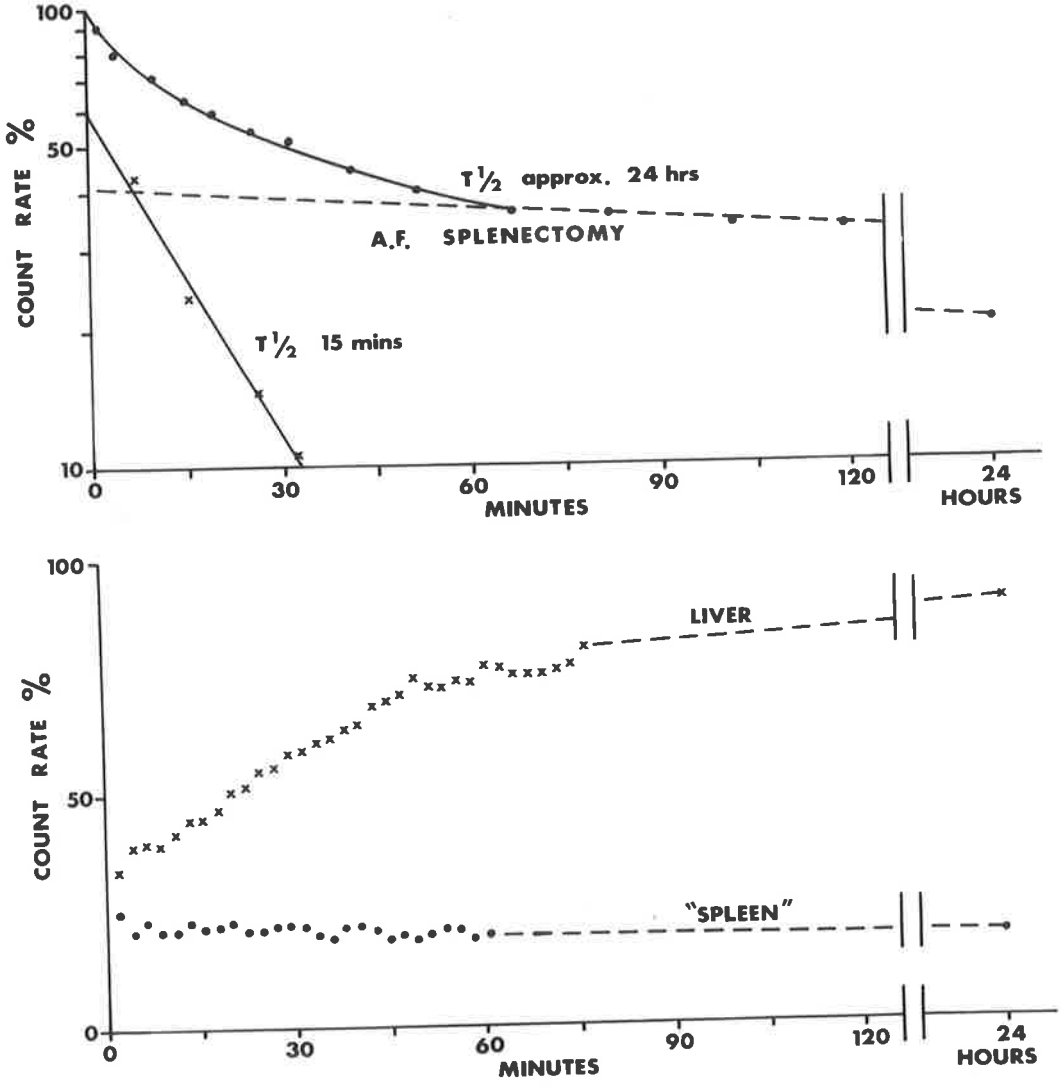

Figure 16: The clearance from the blood (above) and organ uptake (below) of red cells heated for 20 minutes at 50°C (splenectomized subject, Study No. 29).

Table VI - The fate of heated cells in 4 subjects with haematological disorders and the rate of clearance and site of uptake of labelled haemoglobin in 2 normal subjects.

Study	Diagnosis	Period of heat treatment (mins.)	Splenic Size (cm. below left costal margin)	Clearance Rate - Slow Component $T\frac{1}{2}$	λ min.$^{-1}$	Clearance Rate - Rapid Component $T\frac{1}{2}$ min.	λ min.$^{-1}$	Organ uptake Spleen	Liver	Per cent intra-vascular lysis
				HEATED CELLS IN ABNORMAL SUBJECTS						
25 E.C.	Thalassaemia minor	15	1	24 hrs				+++	0	2
30 E.B.	Autoimmune haemolytic anaemia	22	0	70 min	0.0099	5	0.139	+++	+	15
31 N.S.	Myelofibrosis	15	13	5 days				+++	0	1.5
33 P.S.	Autoimmune haemolytic anaemia	25	0			7	0.099	++	+	Not estimated

(Survival studies of autologous untreated chromium-51 labelled cells in N.S. showed a half life of 10 days with marked splenic uptake and in P.S. a half life of 8.5 days and moderate uptake in spleen and liver.)

Study	Conc. Hb. injected (Gm./100 ml.)	Volume Injected (ml.)	Total Hb. injected (Gm.)	Clearance Rate $T\frac{1}{2}$ hrs.	Organ Uptake Spleen	Liver
	CLEARANCE OF LABELLED HAEMOGLOBIN					
7 D.S.	3.9	15	0.59	3.5	0	++
12 W.B.	4.5	12.5	0.56	4	0	++

Thus in summary, heat at 50°C produces firstly spherocytic cells, of reduced osmotic fragility, removed from the circulation solely by the spleen. This is confirmed by the prolonged survival of such cells in patients following splenectomy. Longer periods of heating cause a progressive cellular fragmentation and agglutination of cells associated with an increase in osmotic fragility and a gradual change in the site of sequestration from the spleen to the liver. Also with the longer periods of heating intravascular lysis plays an increasing role in the removal of cells from the circulation.

Clearance of chromium-51 labelled haemoglobin. In two normal subjects the rate of clearance and organ uptake of labelled haemoglobin was studied. In these the half times of clearance were 3.5 and 4 hours and the liver was responsible for the accumulation of the haemoglobin (Table VI).

Studies in patients with haematological disease. One of the aims of the present study was, as mentioned, to prepare by means of heat treatment a uniform population of cells prone to rapid removal from the circulation by the spleen only. Using such a preparation it was planned to assess splenic sequestering function in abnormal subjects. However, following the study of the effect of heat on red cells it was found impossible to prepare such a population.

Cells heated from 10 to 15 minutes were removed by the spleen but not at a rapid rate showing that some additional conditioning in the circulation was necessary before their sequestration. Heating for longer periods caused rapid removal, but a complex population of the cells was produced and many were taken up by the liver or reticulo-endothelial system in general. Therefore, following the analysis of the data which illustrated these factors, this means of producing such a population was abandoned. However, four studies of the fate of heated cells in such abnormal subjects were concurrently performed and the findings tabulated in Table VI.

In subject F.C. who was suffering from thalassaemia minor with splenomegaly, autologous cells heated for 15 minutes were cleared from the circulation with a half time of approximately 24 hours and sequestered by the spleen. This showed a longer survival than that of similarly treated cells in a normal subject where as reported above the half time ranged from 9 to 12 hours. Thus it suggested a reduced sequestration capacity despite splenomegaly in this case, or an erythrocyte resistance to thermal damage.

Subject E.B., with auto-immune haemolytic anaemia in remission, showed a biphasic exponential clearance of autologous cells heated for 22 minutes. The half times of the slow and rapid components were 70 and 5 minutes respectively. The organ activity was of a marked degree in the spleen with detectable liver uptake (Table VI).

These findings are within the range found in normal subjects in whom cells heated for 20 to 30 minutes were infused (Table IV). This suggested no abnormality in splenic sequestering function in this subject.

Subject N.S. was suffering from myelofibrosis and haemolytic anaemia at the time of study. Autologous red cell survival of untreated cells was studied and showed a reduced red cell life ($T\frac{1}{2}$ 10 days) with a marked splenic uptake of cells, that is haemolytic anaemia with splenic sequestration. Autologous cells after heating for 15 minutes were removed very slowly with a half time of approximately 5 days with a slow but marked splenic accumulation of activity (Table VI). The life of these heated cells was much longer than that seen in normal spleen intact subjects given such mildly damaged cells (Table IV) and was more comparable with that seen in splenectomized subjects (Table V). This suggested therefore that despite splenomegaly this patient with myelofibrosis showed a reduced splenic sequestration capacity.

P.S., a child with autoimmune haemolytic anaemia, had a markedly reduced red cell life (half time 8.5 days) and a moderate uptake of cells in both liver and spleen when autologous untreated red cell life was studied. When erythrocytes heated for 25 minutes were infused they were rapidly and exponentially cleared from the circulation with a half time of 7 minutes with activity accumulating

moderately in spleen and liver (Table VI). Cells heated for this period of time and reinfused into normal subjects (Table IV) were cleared in a biphasic exponential manner with marked splenic and slight liver accumulation. Thus it appeared that in this subject the cells were more susceptible to damage by heat, or that the liver had an increased affinity for damaged cells.

Little positive conclusions could be drawn from these few isolated studies, but the results were of interest in the light of later results, to be recorded, of the fate of cells subjected to chemical damage in patients with splenomegaly and haemolytic disease.

CHAPTER V

THE EFFECT OF N-ETHYL MALEIMIDE ON RED CELL MORPHOLOGY, OSMOTIC FRAGILITY AND SUBSEQUENT SURVIVAL IN VIVO

Aim of the Investigation

In accordance with the objects discussed, (Chapter III), the sulphydryl inhibitor N-ethyl maleimide (NEM) was used in a study of the erythrocyte factors causing splenic and liver uptake of red cells. Again an attempt was made to produce cells which could be used in the quantitation of splenic sequestering function. Having achieved this object, the fate and site of uptake of chemically damaged cells was compared with those of autologous untreated cells, in cases of haemolytic disease and splenomegaly. Lastly using this reagent the effect of corticosteroids on splenic uptake function was studied.

Subjects Studied

A total of 57 studies were carried out on a group of 53 subjects from the total admitted to the investigation (Chapter III).

A. 41 studies of the effect of NEM on cell morphology, fragility and survival in 38 individuals as follows:

Group 1. Normal: 14 healthy male volunteers.

Group 2. Splenectomy: 3 subjects operated on over one year previously.

Group 3. Splenomegaly: 16 subjects with a variety of disorders (see Tables X and XII); one, with haemolytic anaemia and myelofibrosis, was studied both before and after splenectomy.

Group 4. Steroid therapy: The 5 subjects in this group (see Table XIII) all had enlarged spleens and were being treated with prednisolone.

B. Morphology and osmotic fragility studies, following treatment with various concentrations of NEM, were carried out on red cells from 6 further normal subjects.

C. The survival and organ uptake of autologous untreated cells were studied in 9 of the subjects, with splenomegaly, from section A (see Table XIV).

Results

Morphology: The treatment of cells with 8 micromoles of NEM per ml. of red cells produced no detectable morphological change. The biconcave discoid shape of the untreated cells (Figure 17) was retained in the cells subjected to this treatment (Figure 18). When the cells were incubated with 10 micromoles of NEM per ml. of cells a spherocytic change was visible in a proportion of the erythrocytes. Some of the cells however retained a discoid shape and probable transition between these two forms was seen as cellular crenation (Figure 19). Increase to a concentration of 20 micromoles NEM per ml. of cells caused an overall spherocytic change in the erythrocytes (Figure 20).

Osmotic fragility: Red cell osmotic fragility was consistently

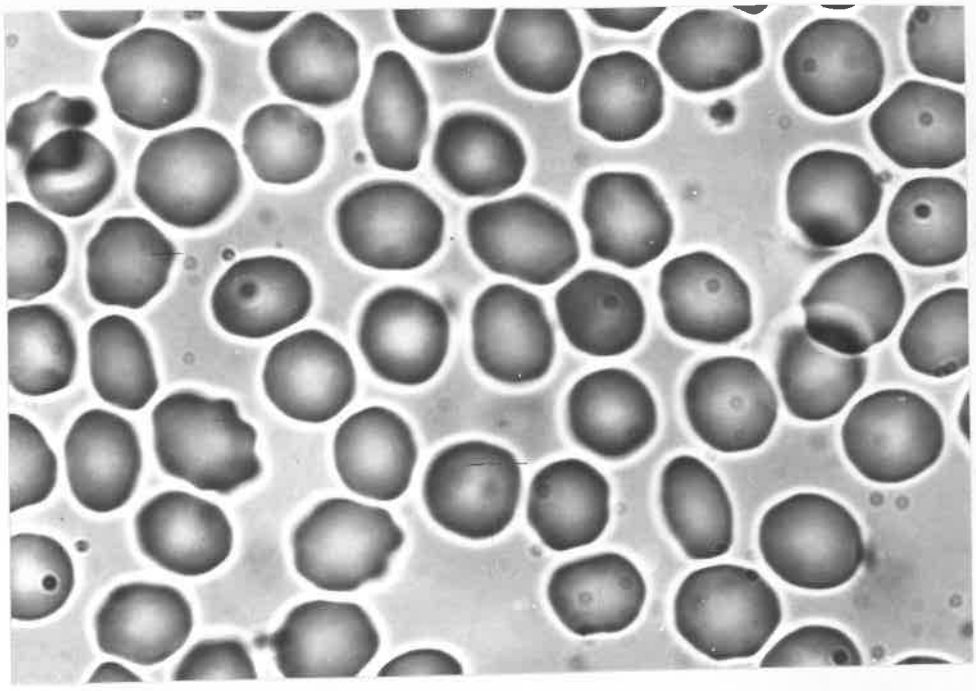

Figure 17: Normal erythrocytes before treatment with NEM showing biconcave discs (x 1580).

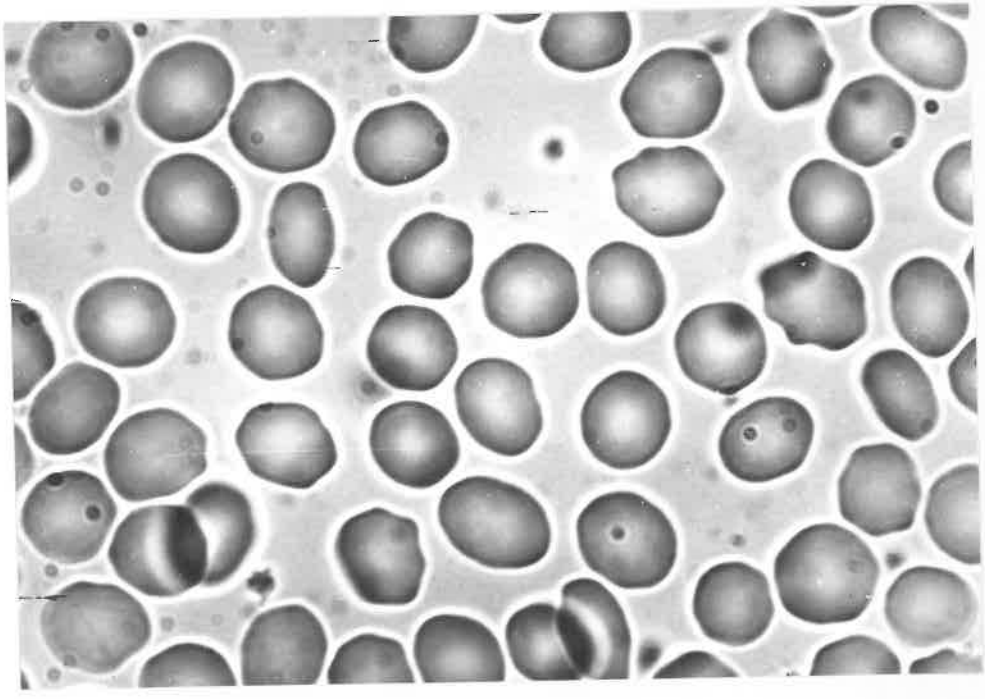

Figure 18: Erythrocytes following treatment with 8 micromoles NEM showing the maintenance of normal morphology (x 1580).

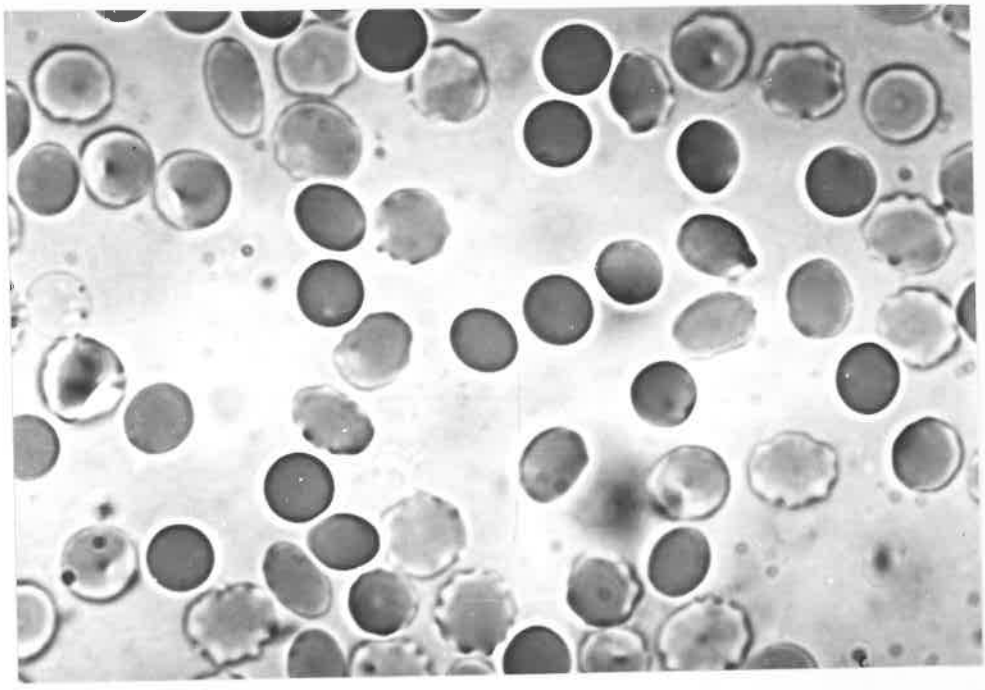

Figure 19: Erythrocytes following treatment with 10 micromoles NEM, showing transition to spherocytes (x 1580).

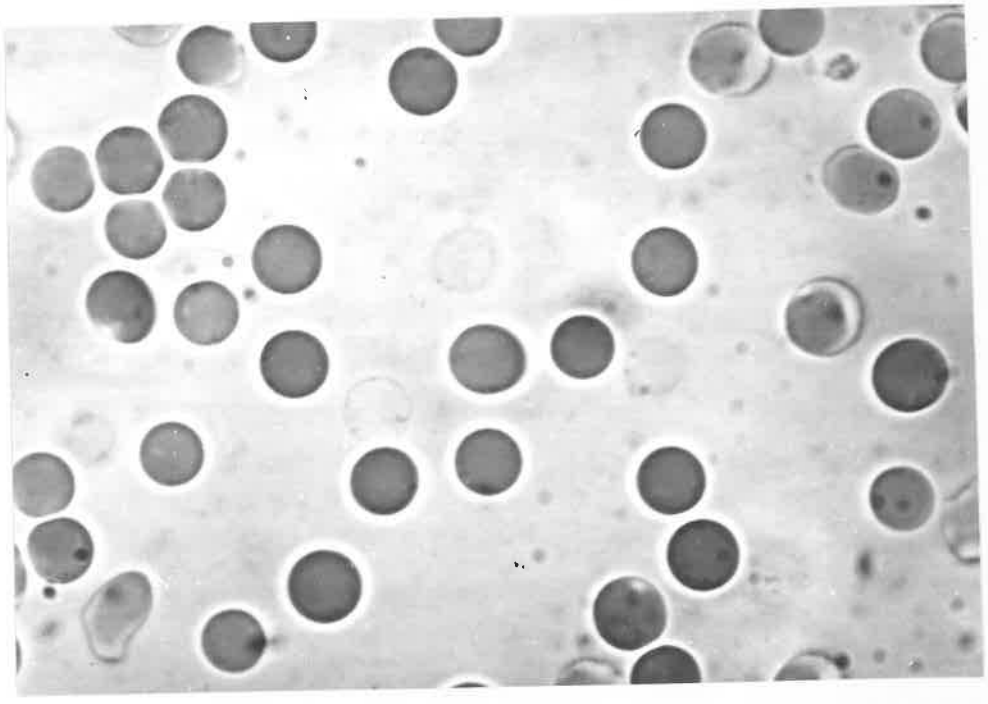

Figure 20: Erythrocytes following treatment with 20 micromoles NEM, showing uniform spherocytic change (x 1580).

Table VII - The effect of treatment with 8 micromoles NEM per ml. red cells on median cellular fragility (M.C.F.)

Study		Median Cellular Fragility Before Treatment	After Treatment	Difference	Analysis
		NORMAL SUBJECTS			
15	M.S.	0.41	0.32	- 0.09	
36	L.C.	0.42	0.38	- 0.04	
39	F.H.	0.44	0.38	- 0.06	
42	B.C.	0.41	0.37	- 0.04	Significant decrease in M.C.F. $p < 0.001$
46	D.Ma	0.47	0.44	- 0.03	
48	K.J.	0.43	0.36	- 0.07	
49	R.B.	0.41	0.30	- 0.11	
52	J.McM.	0.43	0.36	- 0.07	
59	D.Me	0.44	0.37	- 0.07	
60	J.M.	0.39	0.32	- 0.07	
		SUBJECTS WITH HAEMATOLOGICAL DISEASE			
43	G.K.	0.28	0.21	- 0.07	
35	A.F.	0.43	0.39	- 0.04	
37	V.R.	0.40	0.21	- 0.19	
50	E.Ri	0.42	0.34	- 0.08	
58	M.H.	0.43	0.39	- 0.04	Significant decrease in M.C.F. $p < 0.001$
53	M.T.	0.43	0.37	- 0.06	
55	A.W.	0.47	0.38	- 0.09	
57	J.C.	0.45	0.40	- 0.05	
45	A.S.	0.45	0.34	- 0.11	
54	L.S.	0.51	0.43	- 0.08	
56	B.H.	0.48	0.47	- 0.01	
66	R.C.	0.54	0.43	- 0.11	
44	M.R.	0.53	0.50	- 0.03	
51	J.McK	0.50	0.40	- 0.10	

Table VIII - The effect of increasing concentrations of NEM on the median cellular fragility (M.C.F.) of erythrocytes of normal subjects.

Study		Median Cellular Fragility			
	NEM, micromoles per ml. red cells.	0	8	10	20
102	W.H.	0.42	0.37	0.25	0.52
103	P.N.	0.44	0.32	0.23	0.47
104	L.U.	0.43	0.34	0.30	0.56
105	B.T.	0.45	0.39	0.27	0.55
106	I.E.	0.46	0.40	0.31	0.60
107	T.D.	0.43	0.36	0.28	0.48

Analysis of Mean of Differences

(1) 8 micromoles NEM, significant decrease ($p < 0.001$) in M.C.F.

(2) 10 micromoles NEM, significant decrease ($p < 0.001$) in M.C.F. compared to 8 micromoles.

(3) 20 micromoles NEM, significant increase ($p < 0.001$) in M.C.F.

reduced by treatment with 8 micromoles of NEM per ml. and the comparison between median cellular fragility (M.C.F.) before and after such treatment in normal subjects and those with haematological disorders is shown in Table VII. This decrease in M.C.F. was significant in both groups as shown by analysis of the mean of the differences ($p < 0.001$ in each case).

This phenomenon, and the effect of higher concentrations of NEM on erythrocyte osmotic fragility, were further investigated (Table VIII) and the findings are represented graphically in Figure 21. 8 micromoles NEM produce, despite the absence of morphological change, a significant decrease in M.C.F. ($p < 0.001$). Treatment with 10 micromoles NEM, which produced a mixture of discoid and spherocytic cells, gave an even more marked and significant decrease in M.C.F. ($p < 0.001$). Despite this the graphical analysis showed a "tail" of cells of increased fragility and therefore the double morphological population was associated with a mixture of cells with enhanced or reduced resistance to osmotic rupture. 20 micromoles NEM, which resulted in generalized spherocytosis, gave an overall and significant increase ($p < 0.001$) in medial cell fragility, compared both to the normal state and to cells treated with lower concentrations of the sulphydryl inhibiter.

Survival and organ uptake in normal subjects: Cells treated with 8 micromoles NEM per ml. of red cells (referred to as "mildly"

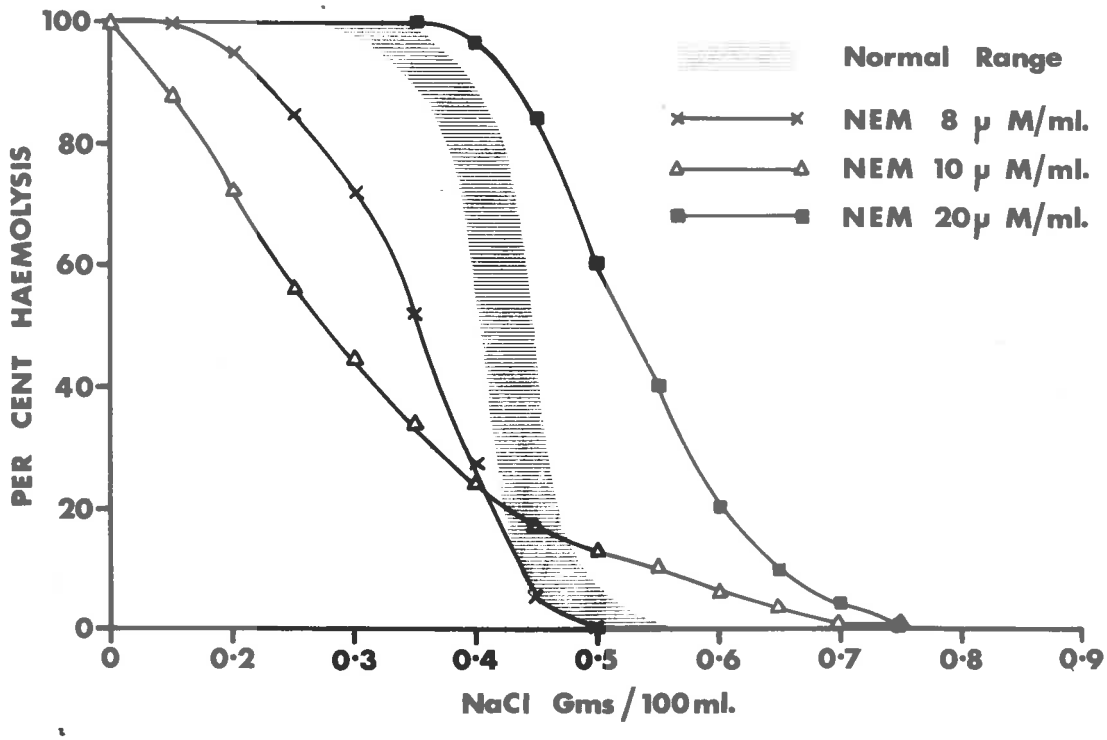

Figure 21: The effect of increasing concentrations of NEM on red cell osmotic fragility.

Table IX - The effect of increasing concentrations of NEM on the rate of clearance of erythrocytes from the circulation and the site of sequestration.

Study	Volume of cells infused (ml./kg.)	Clearance Rate - Slow Component $T_{\frac{1}{2}}$ min.	λ min.$^{-1}$	Spleen Uptake Rate $T_{\frac{1}{2}}$ min.	λ_s min.$^{-1}$	Clearance Rate - Rapid Component $T_{\frac{1}{2}}$ min.	λ min.$^{-1}$	Liver Uptake Rate $T_{\frac{1}{2}}$ min.	λ_L min.$^{-1}$
8 MICROMOLES NEM PER ML.									
15 M.S.	0.070	115	0.0060	112	0.0062				
36 L.C.	0.073	120	0.0058	95	0.0073				
39 F.H.	0.054	95	0.0073	75	0.0092				
42 B.C.	0.052	110	0.0063	90	0.0077				
46 D.Ma	0.063	125	0.0055	75	0.0092				
48 K.J.	0.070	112	0.0062	115	0.0060				
49 R.B.	0.061	80	0.0081	65	0.0106				
52 J.McM.	0.057	75	0.0092	70	0.0099				
59 D.Mc	0.064	100	0.0069	82	0.0085				
60 J.M.	0.069	75	0.0092	70	0.0099				
MEAN ± 2 S.D.		100 ± 37.5		85 ± 35.4					
10 MICROMOLES NEM PER ML.									
38 F.W.	0.054	65	0.0107	75	0.0092	5	0.139	2	0.347
40 D.B.	0.052	115	0.0060	80	0.0087	4	0.173	3.5	0.198
20 MICROMOLES NEM PER ML.									
14 B.N.	0.046					3	0.231	2	0.347
34 M.G.	0.034					5	0.139	2	0.347

damaged cells: of normal morphology and reduced osmotic fragility) were removed from the circulation in an exponential manner in the 10 subjects studied. The half time of clearance ranged from 75 to 120 minutes with a mean of 100 minutes. This corresponded to clearance rates ranging from 0.0092 $min.^{-1}$ to 0.0055 $min.^{-1}$ (Table IX). Removal of these cells from the circulation was associated with an accumulation of activity solely in the spleen. The splenic uptake in these cases was also exponential in nature with half times varying from 70 to 115 minutes with a mean of 85 minutes, corresponding to uptake rates of from 0.0099 $min.^{-1}$ to 0.0060 $min.^{-1}$. The pattern of events in such a study is illustrated in Figure 22. The mean blood clearance rate did not differ significantly from the mean splenic uptake rate (0.8 p 0.9). Also there was a close correlation between the clearance rate and splenic uptake rate in the individual cases as shown in the correlation plot (Figure 23). These factors confirmed the impression, from the surface counting studies, that the spleen was indeed solely responsible for the uptake of such mildly damaged cells in normal subjects.

No plasma activity was detected in any of the specimens showing that the removal of cells from the circulation was not, in any part, due to intravascular breakdown.

Cells incubated with 10 micromoles NEM per ml. (that is that concentration which produced a mixed population of discoid and

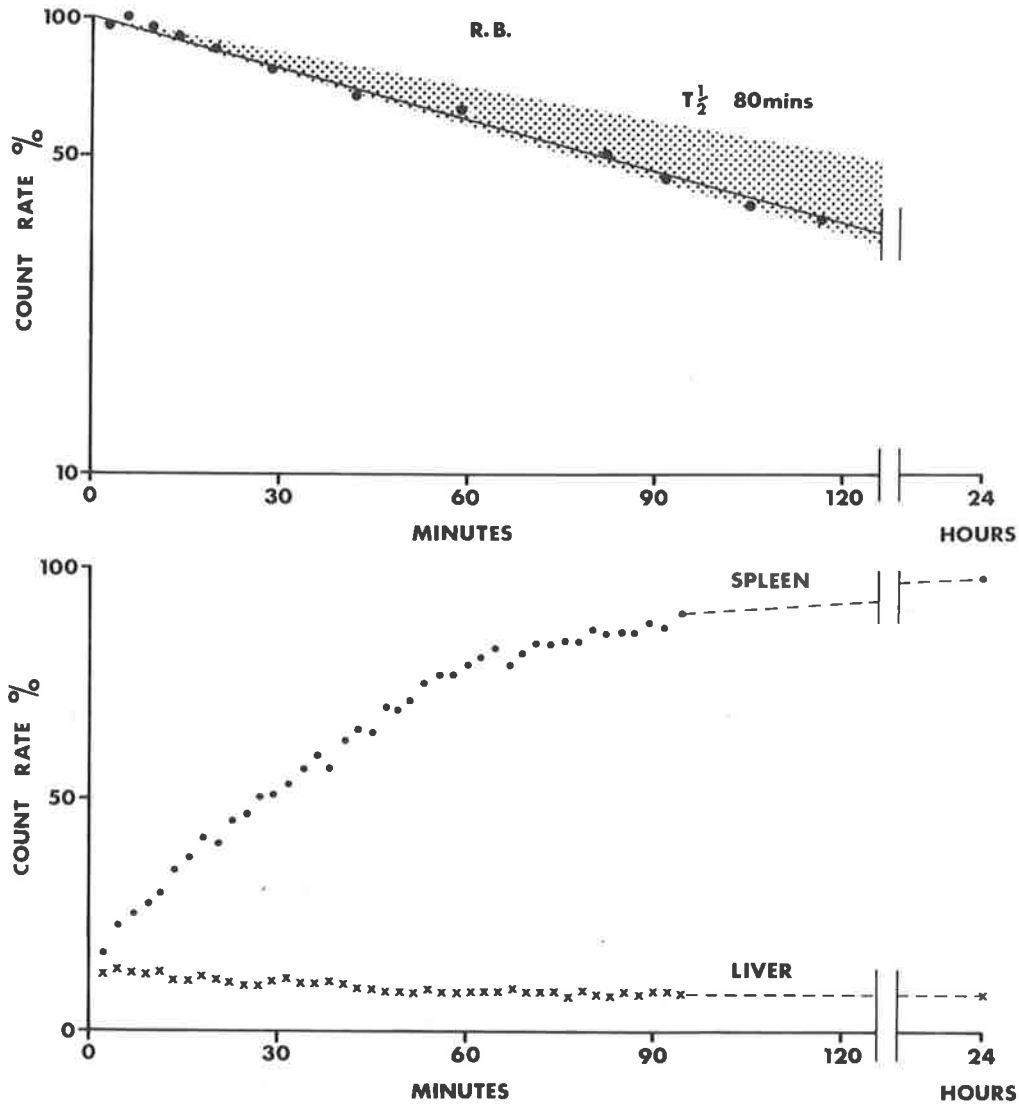

Figure 22: The clearance from the blood (above) and organ uptake (below) of red cells treated with 8 micromoles NEM (Study No. 49). Shaded area - range in 10 normal subjects.

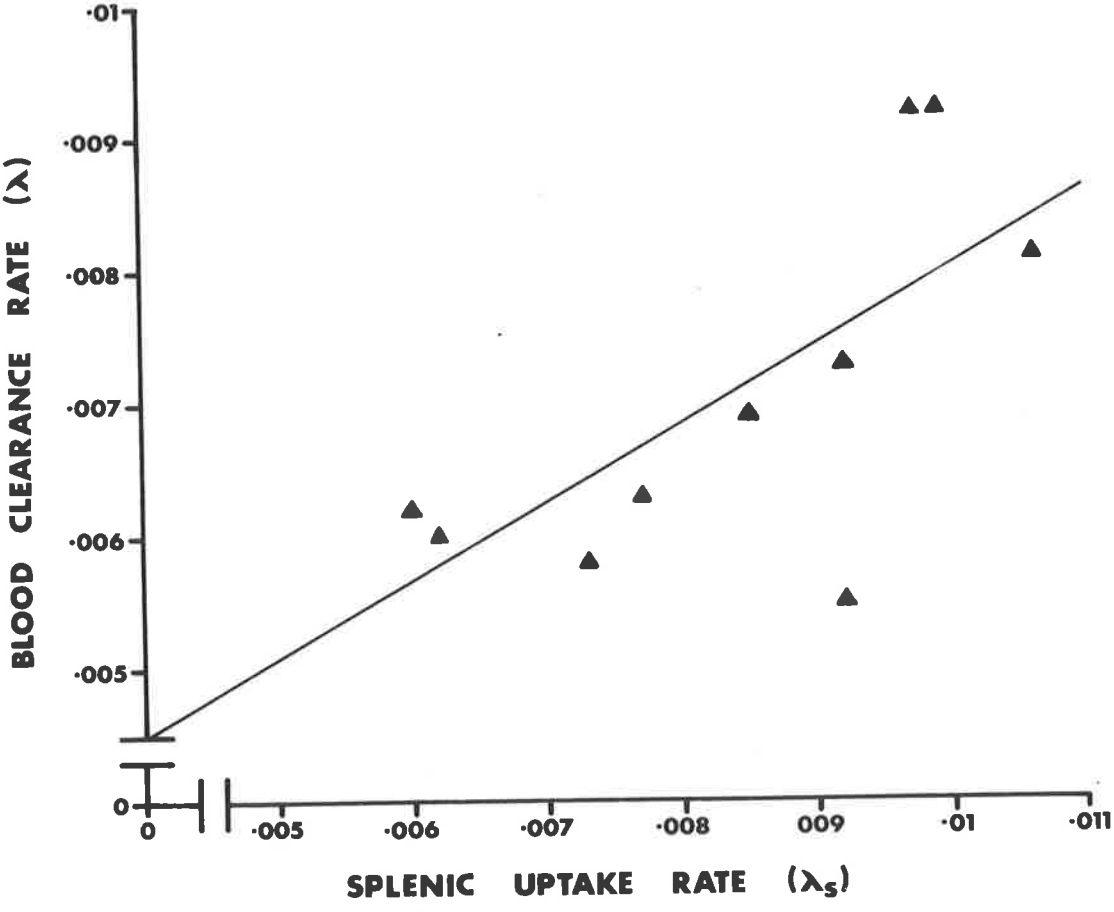

Figure 23: Correlation of blood clearance and splenic uptake rates in normal subjects.

regression coefficient, b = 0.6
regression equation, y = 0.002 + 0.6 x
correlation coefficient, r = 0.71, $p < 0.01$

spherocytic cells of reduced and increased osmotic fragilities) were cleared in a manner found to consist of the sum of two exponents. The slow components showed half times of 65 and 115 minutes corresponding to clearance rates of 0.0107 $min.^{-1}$ and 0.0060 $min.^{-1}$ respectively. The rapid components had half periods of 5 and 4 minutes representing clearance rates of 0.139 $min.^{-1}$ and 0.173 $min.^{-1}$ (Table IX).

This biphasic pattern of cell disappearance from the circulation was associated with a marked but gradual accumulation of activity over the spleen and a very rapid rise over the liver (Figure 24). Analysis of the uptake in these two subjects showed them to be exponential in nature with half times of 75 and 80 minutes over the spleen (clearance rates 0.0092 and 0.0087 $min.^{-1}$) and half times of 2 and 3.5 minutes over the liver (clearance rates 0.0347 and 0.198 $min.^{-1}$) (Table IX). No plasma activity could be detected in these studies.

Cells incubated with 20 micromoles per ml. (producing a spherocytic change and increased osmotic fragility), were cleared exponentially rapidly from the circulation. The half times were 3 and 5 minutes (clearance rates 0.231 and 0.139 $min.^{-1}$) (Table IX). This was associated with a marked rise in activity over the liver with a relatively small rise in the splenic count rate (Figure 25). The exponential uptakes over the liver showed half times of 2 minutes

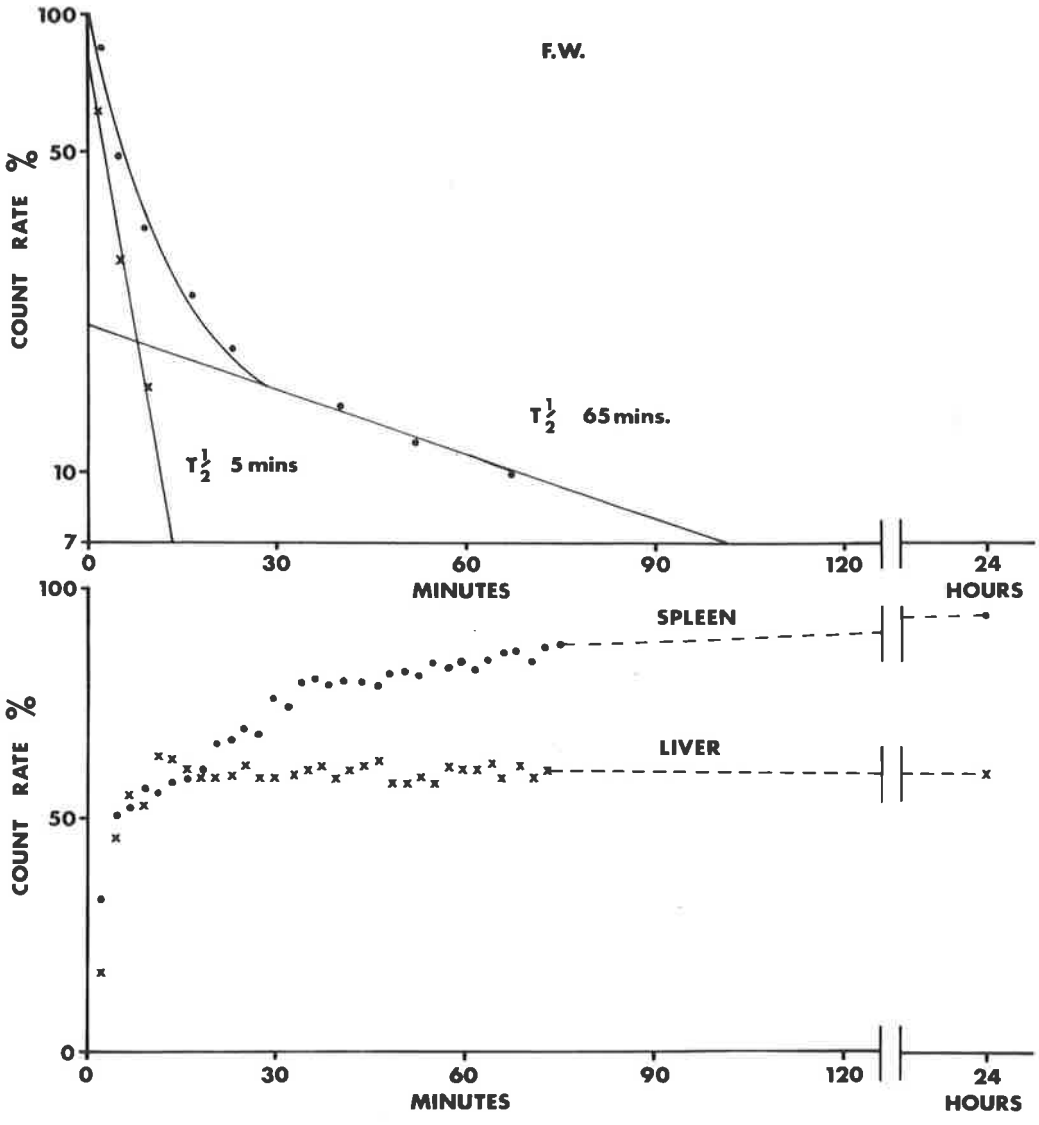

Figure 24: The clearance from the blood (above) and organ uptake (below) of red cells treated with 10 micromoles NEM (Study No. 38).

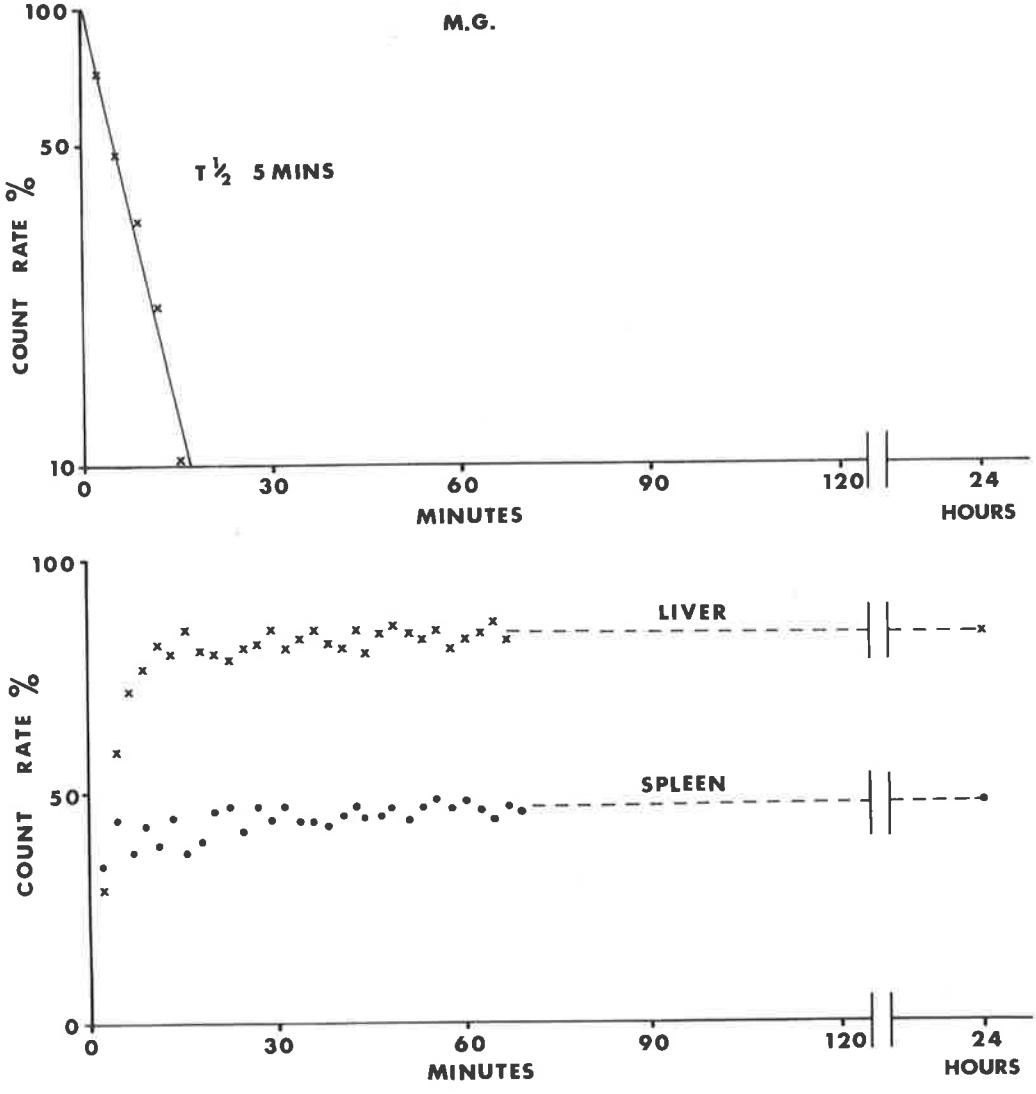

Figure 25: The clearance from the blood (above) and organ uptake (below) of red cells treated with 20 micromoles NEM (Study No. 34).

Table X - The clearance rates and organ uptake rates of mildly NEM-damaged cells in patients with splenomegaly.

Study	Diagnosis	Spleen size (cm. below L. costal margin)	Liver size (cm. below R. costal margin)	Volume of cells infused (ml./kg.)	Clearance rate $T_{\frac{1}{2}}$ min.	Clearance rate λ $min.^{-1}$	Spleen uptake rate $T_{\frac{1}{2}}$ min.	Spleen uptake rate λ_S $min.^{-1}$
43 G.K.	Thalassaemia minor	2.0	-	0.065	38	0.0182	36	0.0193
35 A.F.	Thalassaemia minor	0.5	-	0.057	48	0.0144	50	0.0139
37 V.R.	Chronic lymphatic leukaemia	1.0	1.0	0.073	52	0.0133	50	0.0139
50 E.Ri	Cirrhosis of liver	0.5	4.0	0.054	45	0.0154	35	0.0198
58 M.H.	Hodgkin's disease	4.0	-	0.128	20	0.0347	18	0.0385
53 M.T.	Felty's syndrome	3.0	-	0.082	55	0.0126	55	0.0126
66 C.W.	Idiopathic thrombocytopenia	0.5	-	0.035	65	0.0167	50	0.0139
68 E.Ra	Pancreatic pseudo-cyst	0.5	-	0.074	50	0.0139	40	0.0173
65 M.P.	Chronic myeloid leukaemia	20.0	-	0.085	50	0.0139	50	0.0139
73 L.T.	Acute leukaemia	11.0	-	0.055	45	0.0154	36	0.0193
55 A.W.	Unknown aetiology	3.0	-	0.093	20	0.0347	20	0.0347
	MEAN $\pm$ 2 S.D.				44 $\pm$ 27.6		40 $\pm$ 25.0	

							Organ uptake Spleen	Organ uptake Liver
57 J.C.	Hodgkin's disease	1.0	2.0	0.067	65	0.0107	+++	+
45 A.S.	Cirrhosis of liver	0.5	1.0	0.044	64	0.0108	+++	+

in both cases (clearance rate 0.0347 min.$^{-1}$) (Table IX). Again no activity was detected in the plasma fraction.

As the results in this section demonstrated that cells treated with NEM in a final concentration of 8 micromoles per ml. of erythrocytes were removed solely by the spleen in normal subjects, red cells from each of the other cases listed were treated with this concentration of NEM and reinfused into the donors. The findings in these were grouped as follows:

(a) those showing rapid blood clearance rate

(b) those showing slowed blood clearance rate

(c) those showing liver uptake of mildly damaged cells

(d) those illustrating the effect of corticosteroids on splenic sequestration

(e) those in which a comparison is made between survival and site of uptake of NEM treated cells and of autologous untreated cells

(f) those illustrating the effect of the volume of cells administered on blood clearance rate.

Increased clearance rate of treated red cells: The mildly damaged cells were removed more rapidly than normal in 13 subjects all of whom had enlarged spleens and 4 of whom had palpable hepatomegaly (Table X). In 11 of these the half times of clearance ranged from 20 to 65 minutes (clearance rates 0.0347 min.$^{-1}$ to

Table XI - The clearance and splenic uptake rates of mildly NEM-damaged cells in normal subjects compared to those in patients with splenomegaly.

Normal (10)	Splenomegaly (11)	Significance of difference between means
Clearance rate (λ min.$^{-1}$)	Clearance rate (λ min.$^{-1}$)	
Mean $\pm$ S.D.	Mean $\pm$ S.D.	$p < 0.001$
0.0071 ± 0.0014	0.0185 ± 0.0082	
Splenic uptake rate (λ_s min.$^{-1}$)	Splenic uptake rate (λ_s min.$^{-1}$)	
Mean $\pm$ S.D.	Mean $\pm$ S.D.	$p < 0.001$
0.0085 ± 0.0016	0.0197 ± 0.0088	

0.0167 $min.^{-1}$) and only splenic accumulation of cells could be detected on surface counting. The half periods of the splenic uptake in these cases ranged from 18 to 55 minutes (clearance rates 0.0385 $min.^{-1}$ to 0.0126 $min.^{-1}$). Results of 3 such studies are illustrated graphically in Figures 26, 27 and 28.

Confirming that the spleen was solely responsible for the removal of the cells, there was no significant difference between the mean blood clearance rates and the mean splenic uptake rates listed in Table X ($0.8 < p < 0.9$) and there was a close correlation between the blood clearance rate and splenic uptake rate, in individual cases, shown in the correlation plot (Figure 29). In several cases the two rates were identical and one graph illustrating such an example is represented in Figure 30.

In this group with splenomegaly the more rapid removal of mildly damaged cells by this organ is confirmed by the finding of a significant increase in the mean blood clearance rate compared with that in the normal subjects given the same preparation of cells ($p < 0.001$). There was similarly a significant increase in the liver splenic uptake rate in comparison with that in the normal subjects ($p < 0.001$). These findings are summarized in Table XI. Despite this there was no positive correlation between splenic size and blood clearance rate (Figure 31). Thus the greater uptake in these cases was not purely related to the degree of splenomegaly.

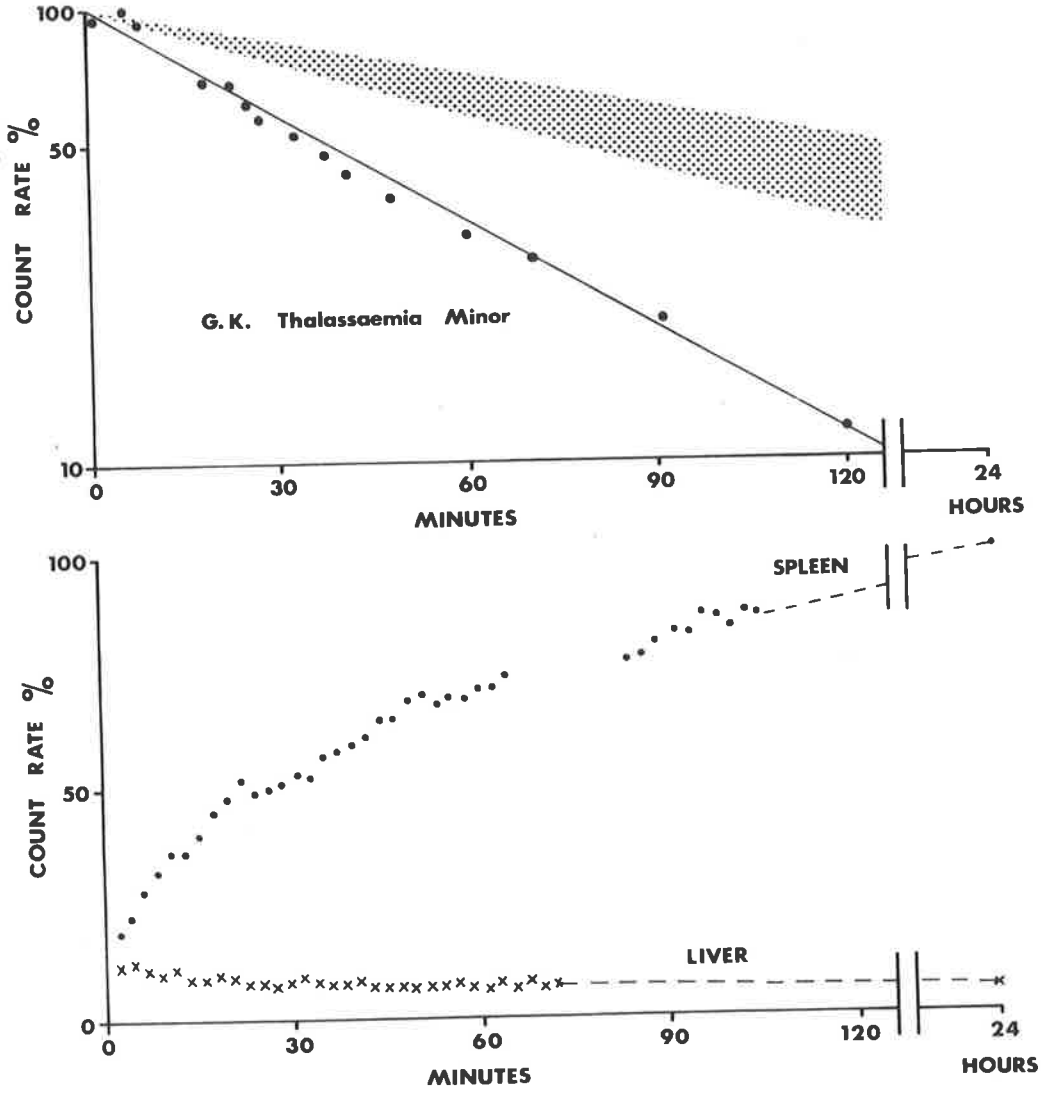

Figure 26: The clearance from the blood (above) and organ uptake (below) of mildly NEM-damaged red cells in a patient with thalassaemia minor and splenomegaly (Study No. 43). Shaded area - normal range.

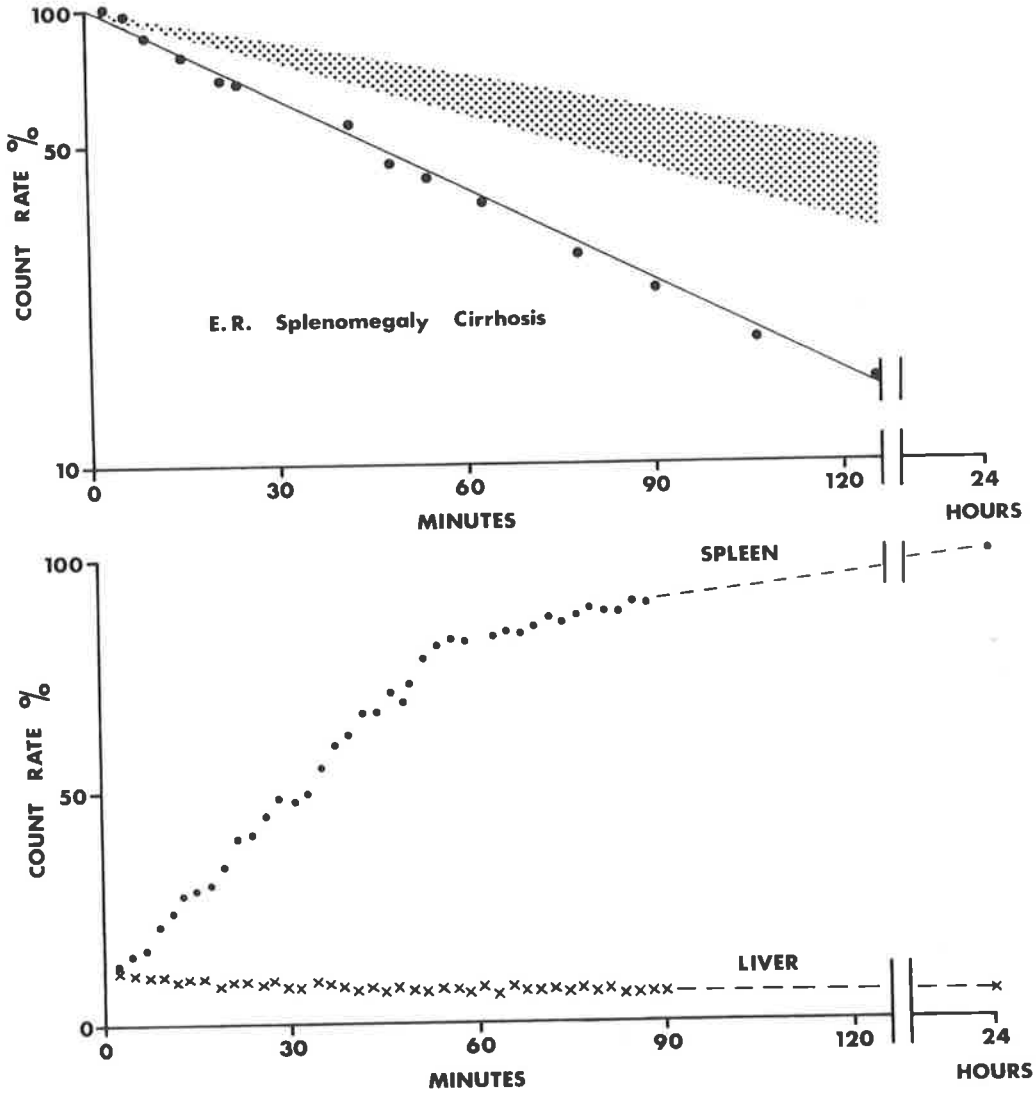

Figure 27: The clearance from the blood (above) and organ uptake (below) of mildly NEM-damaged cells in a patient with cirrhosis of the liver and splenomegaly (Study No. 50). Shaded area - normal range.

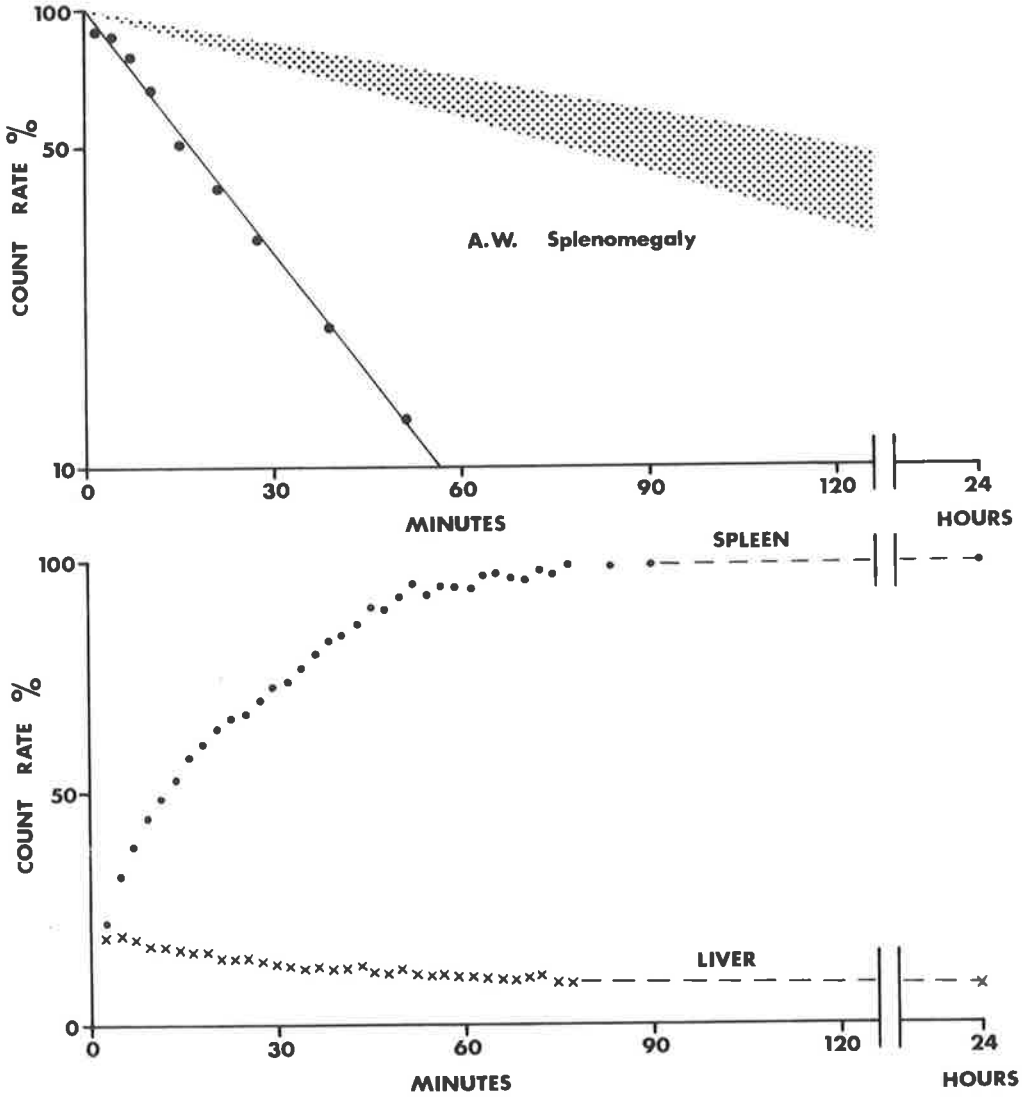

Figure 28: The clearance from the blood (above) and organ uptake (below) of mildly NEM-damaged cells in a patient with splenomegaly of unknown aetiology (Study No. 55). Shaded area - normal range.

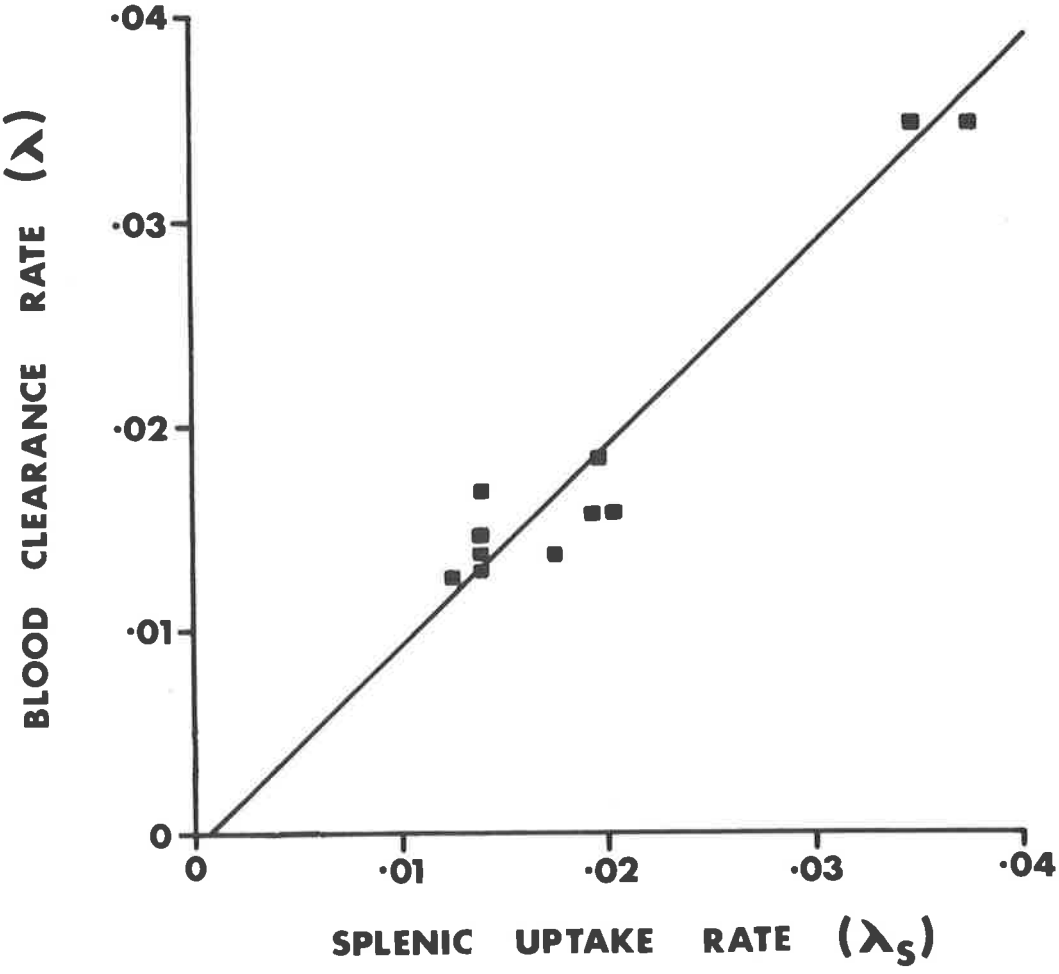

Figure 29: Correlation of blood clearance and splenic uptake rates in 11 subjects with splenomegaly.

regression coefficient, b = 0.9
regression equation, y = 0.0005 + 0.9 x
correlation coefficient, r = 0.97; $p < 0.001$

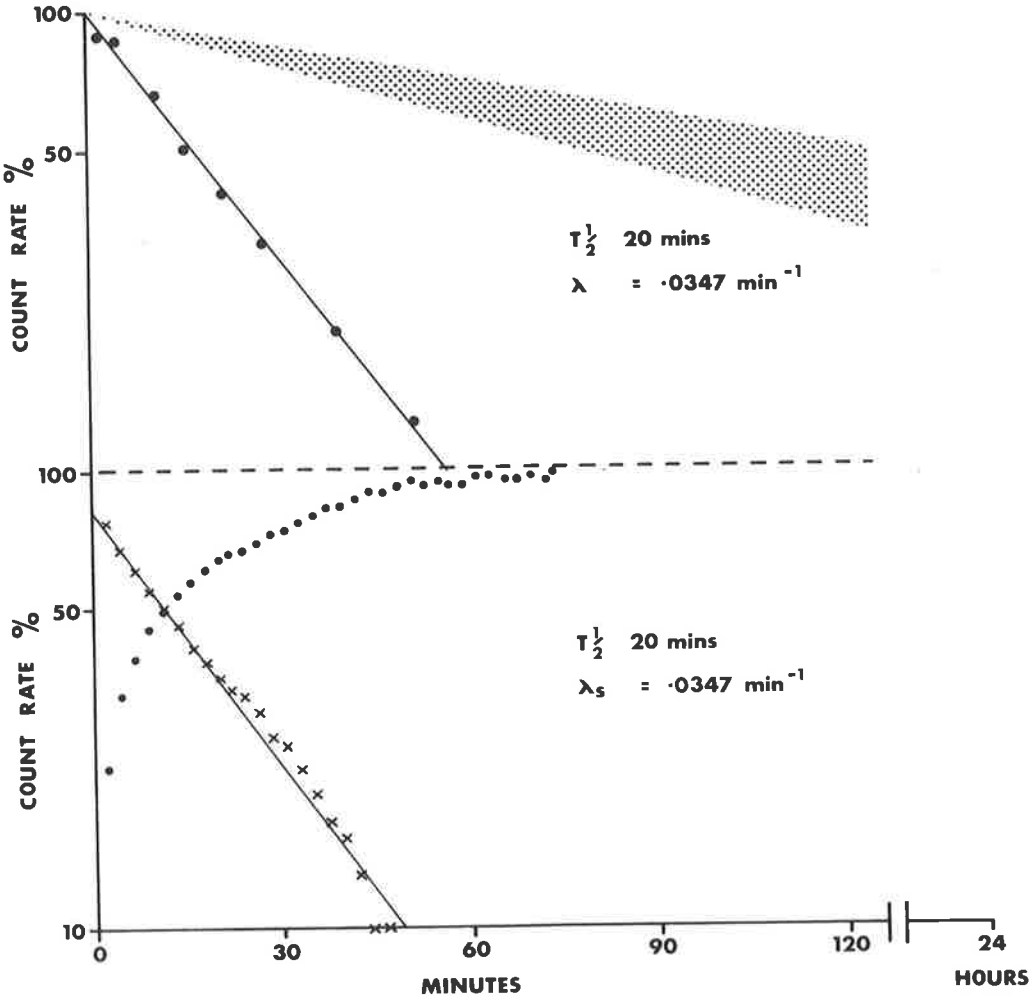

Figure 30: Showing correspondence of the rates of blood clearance (above) and splenic uptake (below) of mildly NEM-damaged cells in a patient with splenomegaly (Study No. 55). Shaded area - range in normal subjects.

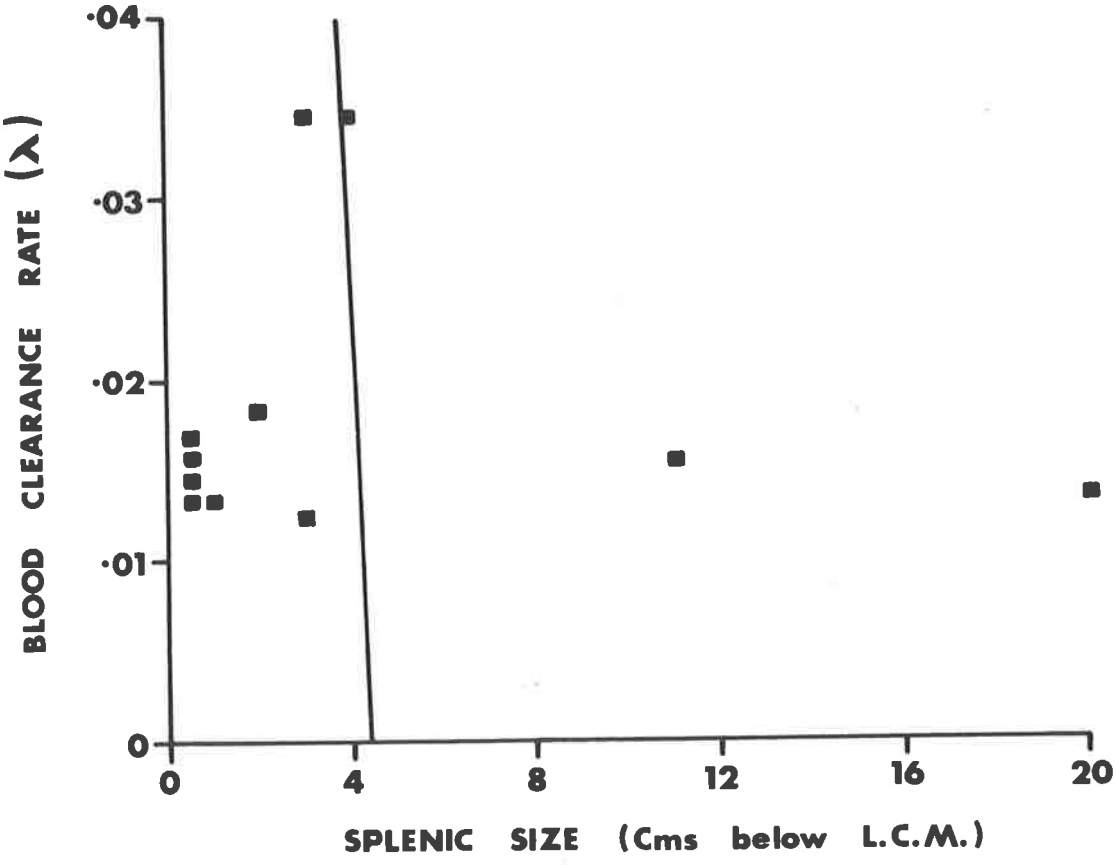

Figure 31: Correlation between spleen size and clearance rate in 11 subjects with splenomegaly.

regression coefficient, b = - 1.13
regression equation, y = 0.0185 + (- 1.13 x 4.2 x)
correlation coefficient, r = 0.084, p > 0.8.

Table XII - Reduced clearance rates of mildly NEM-damaged cells in 5 subjects.

Study	Diagnosis	Spleen size (cm. below L. costal margin)	Liver size (cm. below R. costal margin)	Clearance rate $T_{\frac{1}{2}}$ min.	Clearance rate λ min.$^{-1}$	Organ uptake
47 R.Ka	Splenectomy	-	-	negligible		slight liver after 24 hours
71 W.S.	Splenectomy	-	-	negligible		slight liver after 24 hours
62 F.C.	Splenectomy	-	-	negligible		slight liver after 24 hours
54 L.S.	Myelofibrosis - splenomegaly	8.0	-	slow		spleen
56 B.H.(a)	Myelofibrosis - gross splenomegaly	15.0	4.0	slow		liver
69 (b)	Myelofibrosis - post splenectomy	-	6.0	180	0.0039	liver
63 L.P.	Hydatid cyst of spleen	11.0	-	230	0.0030	spleen

In two subjects, one of whom (J.C.) suffered from Hodgkin's disease and the other (A.S.) from cirrhosis of the liver (Table X) a rapid clearance of mildly damaged cells was associated not only with marked uptake of radioactivity in the spleen but also with evidence of sequestration in the liver. One case is illustrated in Figure 32. Both patients had palpable hepatomegaly.

Reduced clearance rate of treated red cells: A slower rate of clearance than normal was observed in 3 subjects without spleens, who were otherwise normal, and in 3 patients despite splenomegaly (Table (XII). In the absence of the spleen, very few cells were removed from the circulation and only after 24 hours was some activity detected in the liver (Figure 33), and to a lesser extent in the bone marrow. Two of the remaining three subjects in this group suffered from myelofibrosis and the third had a large hydatid cyst in the spleen. In two of these subjects (L.S. and L.P.) the accumulation that did occur took place in the spleen, but in the other subject (B.H.) a slow hepatic uptake was evident with no definite splenic sequestration. Figures 34 and 35 illustrate the findings in the two subjects with myelofibrosis. The patient B.H. was subjected to splenectomy for the treatment of haemolytic anaemia and repetition of the study following this operation again showed hepatic accumulation of the cells. The rate of clearance was more rapid than that seen prior to operation, but was still below normal.

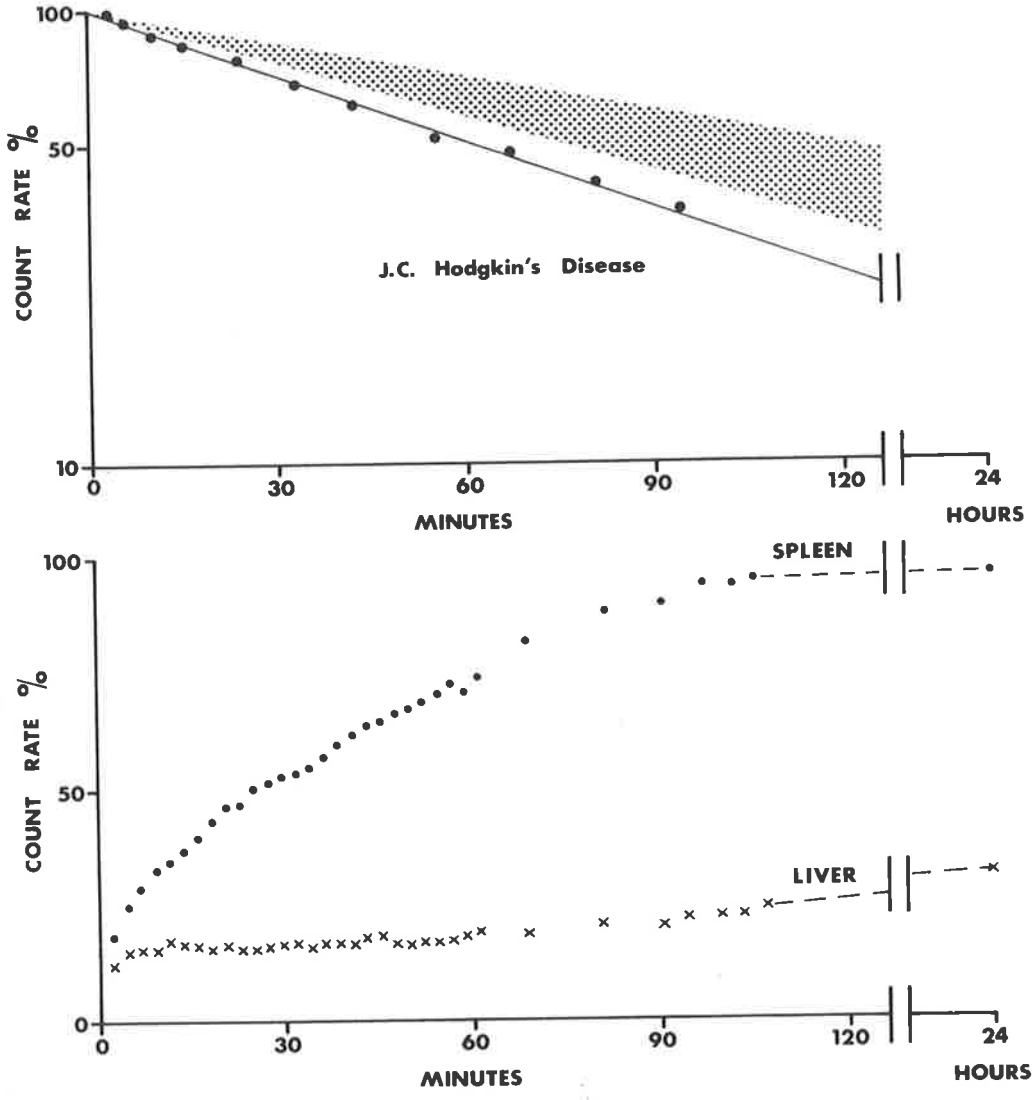

Figure 32: Some hepatic uptake of mildly NEM-damaged cells in a patient with Hodgkin's disease and hepatosplenomegaly (Study No. 57). Shaded area - range in normal subjects.

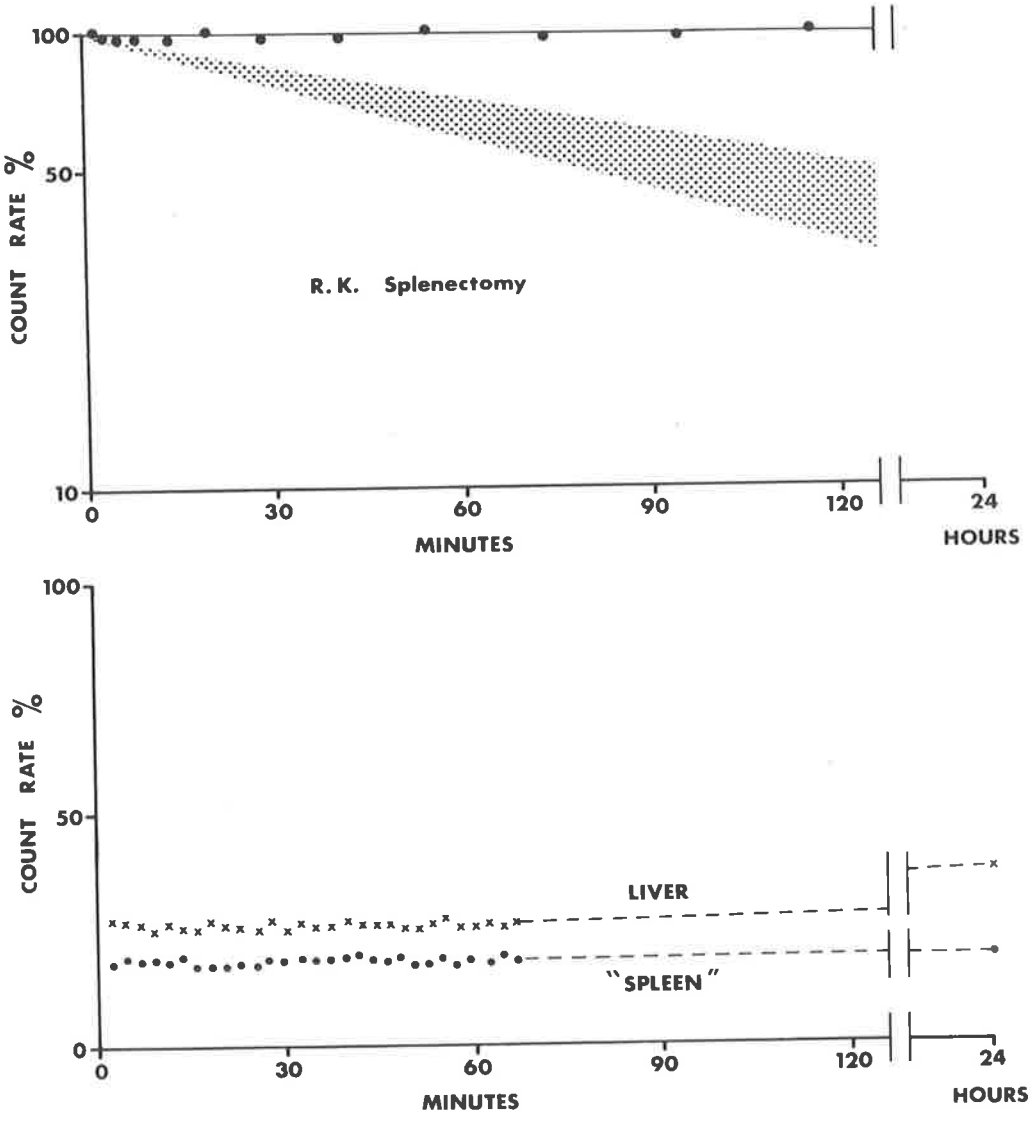

Figure 33: The clearance from the blood (above) and organ uptake (below) of mildly NEM-damaged cells in the absence of the spleen (Study No. 47). Shaded area - range in normal subjects.

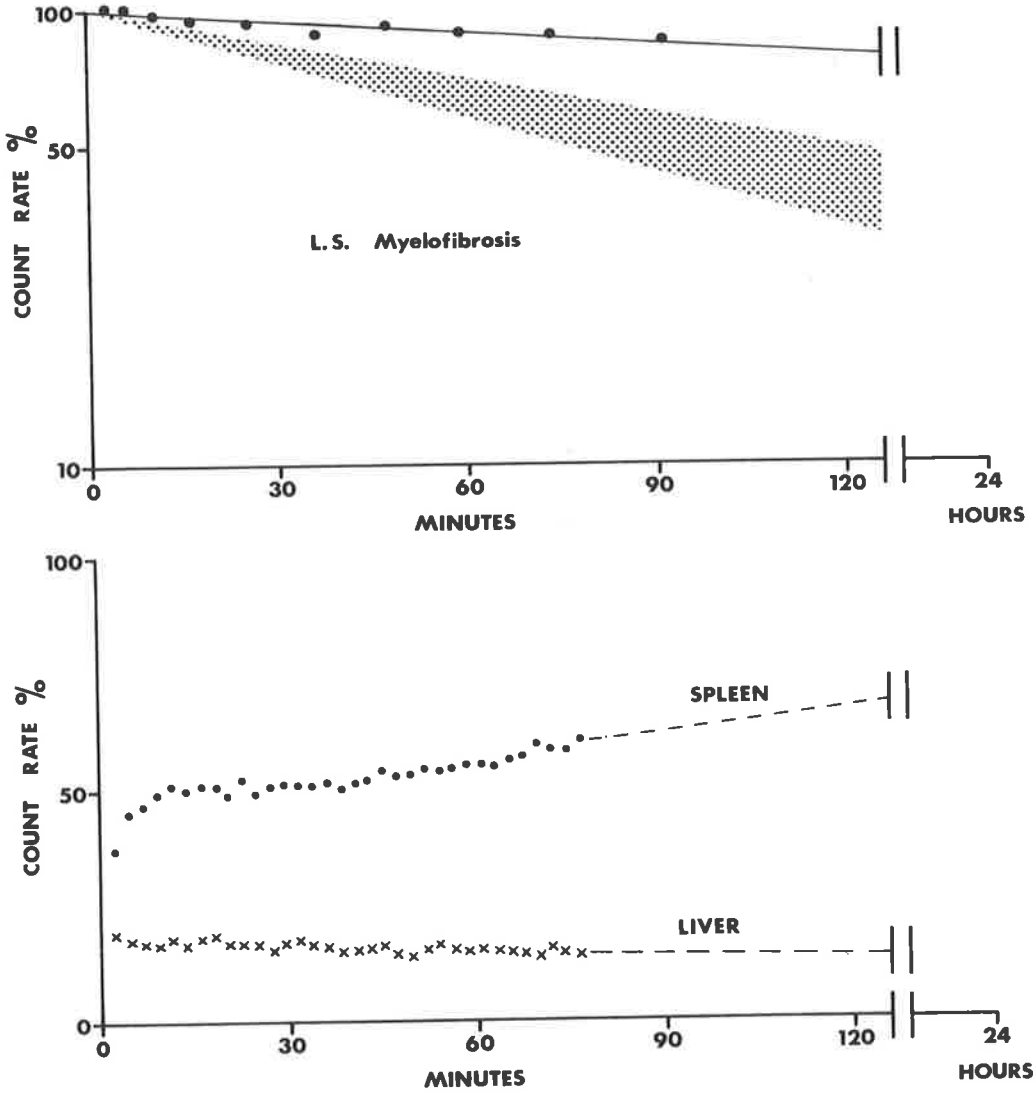

Figure 34: The clearance from the blood (above) and organ uptake (below) of mildly NEM-damaged cells in a patient with myelofibrosis (Study No. 54). Shaded area - range in normal subjects.

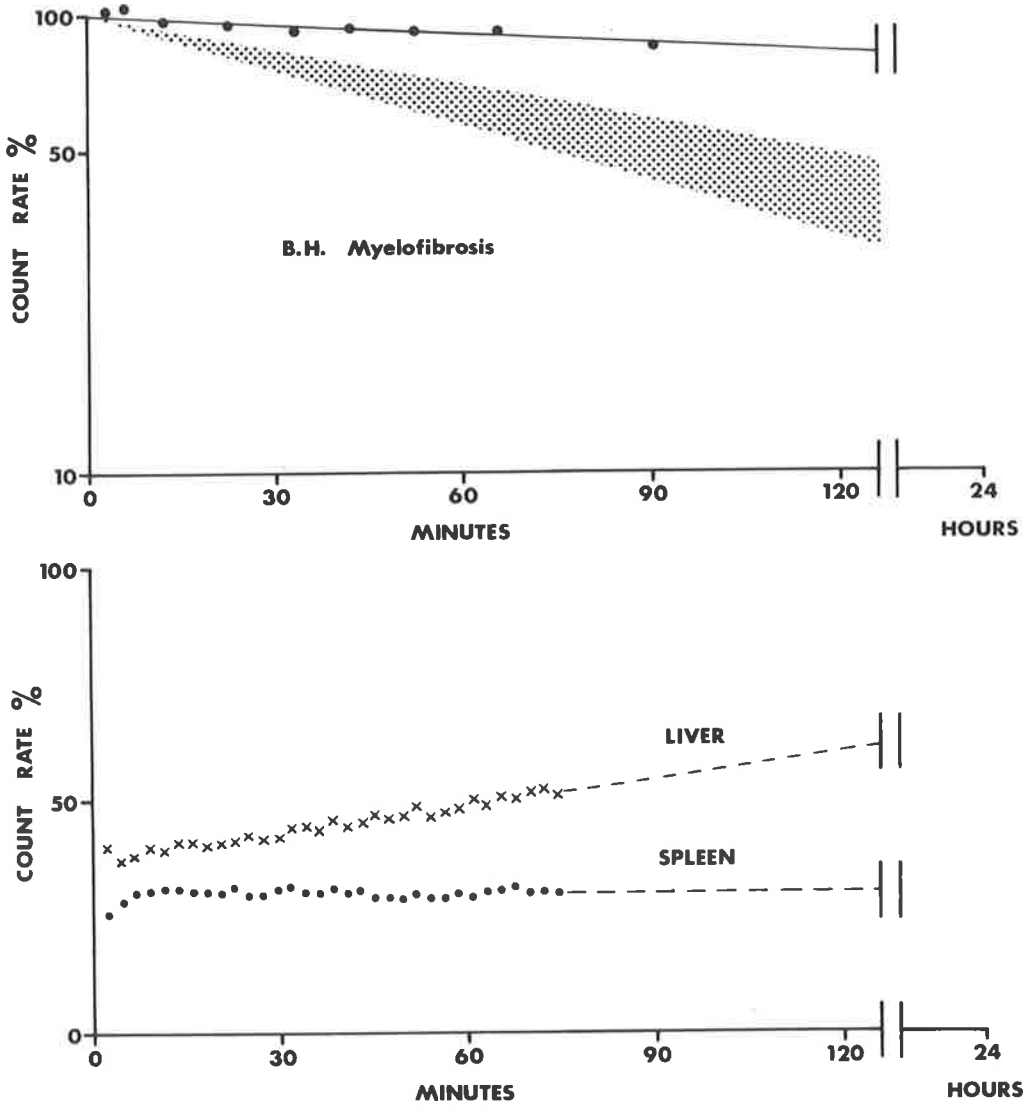

Figure 35: The clearance from the blood (above) and organ uptake (below) of mildly NEM-damaged cells in a patient with myelofibrosis (Study No. 56). Shaded area - range in normal subjects.

Table XIII - The effect of steroid therapy on the uptake rate of mildly NEM-damaged cells in 5 subjects.

Study		Diagnosis	Spleen size (cm. below L. costal margin)	Liver size (cm. below R. costal margin)	Prednisolone (mg./day)	Clearance rate $T_{\frac{1}{2}}$ min.	Clearance rate λ $min.^{-1}$	Organ uptake
70	K.W. (a)	Chronic lymphatic leukaemia	4.0	4.0	-	70	0.0099	spleen
72	(b)	" " "	1.0	4.0	40	95	0.0073	spleen
64	E.D. (a)	Lymphosarcoma	1.0	2.0	-	80	0.0087	spleen
67	(b)	"	0	2.0	40	300	0.0023	spleen
61	R.C.	Auto-immune haemolytic anaemia	1.0	3.0	40	180	0.0039	spleen
44	M.R.	Auto-immune haemolytic anaemia	0.5	1.0	20	200	0.0035	spleen
51	J.McK.	Lymphosarcoma	4.0	4.0	20	120	0.0058	spleen

At the time of the second study haemolytic anaemia had recurred (Table XII and Figure 36).

Studies illustrating liver sequestration: Significant hepatic uptake of mildly damaged cells was seen in 7 instances already mentioned; Firstly in the 3 normal subjects who had had their spleens removed; secondly in 2 subjects (J.C. and A.S.) suffering from Hodgkin's disease and cirrhosis of the liver; thirdly in subject B.H. with myelofibrosis both before and after splenectomy had been performed in the course of treatment of haemolytic anaemia.

The effect of corticosteroids on splenic sequestration: Seven studies of the effect of prednisolone therapy on the rate of clearance of these NEM treated cells, were carried out in 5 subjects who had received this form of treatment for periods ranging from 2 to 3 weeks. All had enlarged spleens and palpable hepatomegaly (Table XIII). In 2 subjects studied prior to and during this therapy (K.W. and E.D.) prednisolone caused a slowing of cellular clearance. The half times increased from 70 to 95 and from 80 to 300 minutes, that is the clearance rates decreased from 0.0099 to 0.0073 $min.^{-1}$ and from 0.0087 to 0.0023 $min.^{-1}$ respectively. This effect is illustrated in Figures 37 and 38.

Also the clearance rate in the 5 subjects during therapy with steroids was prolonged beyond that seen in the group of cases with

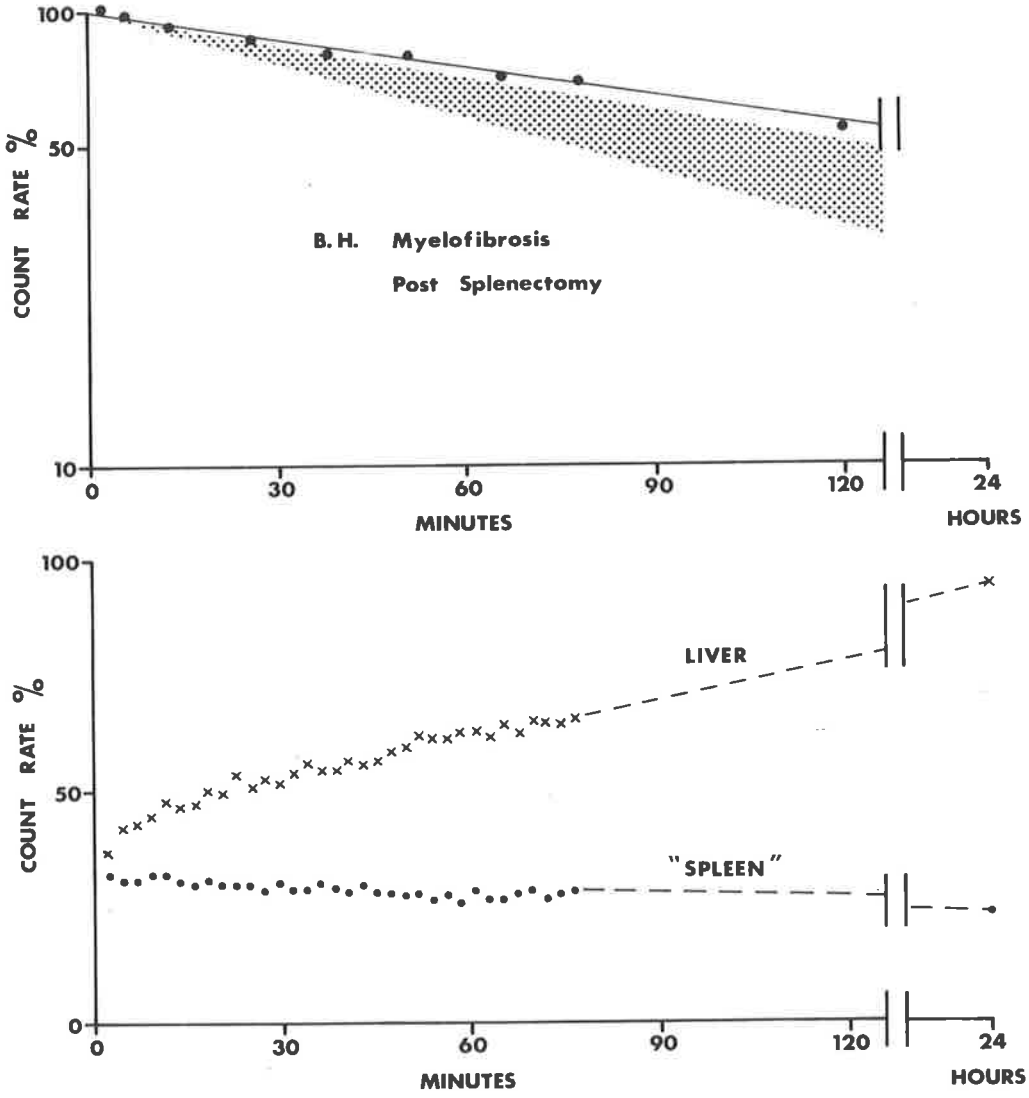

Figure 36: The clearance from the blood (above) and organ uptake (below) of mildly NEM-damaged cells following splenectomy in a patient with myelofibrosis (Study No. 69). Shaded area - range in normal subjects.

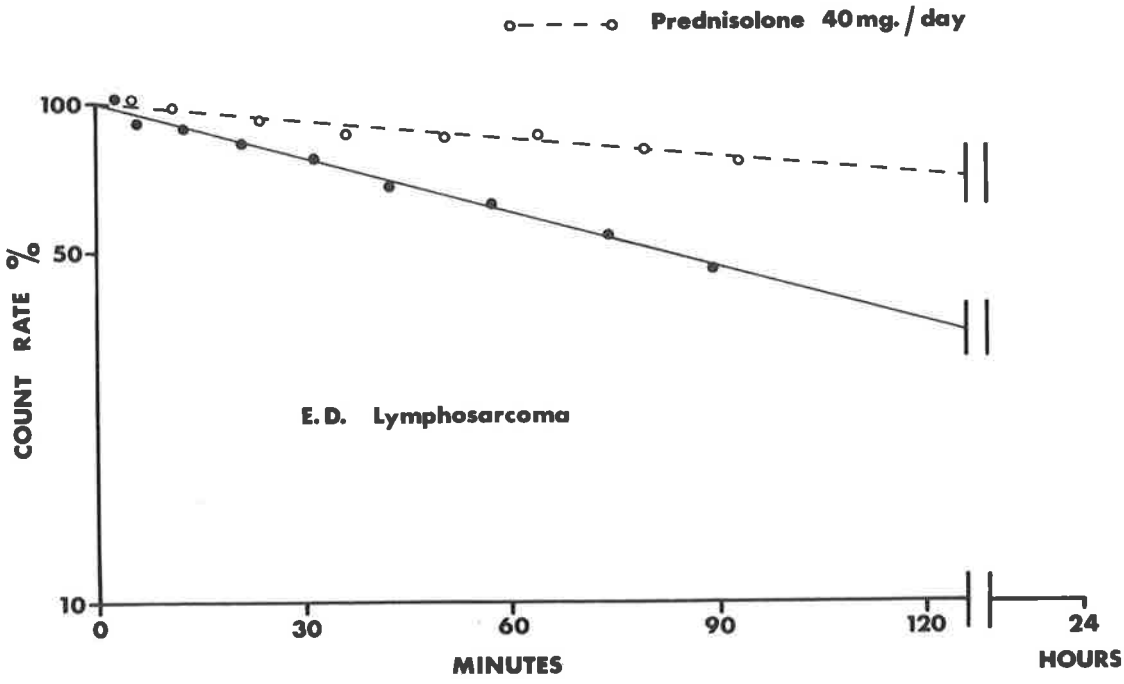

Figure 37: Reduction in the clearance rate of mildly NEM-damaged cells produced by corticosteroids (Studies No. 64 and 67).

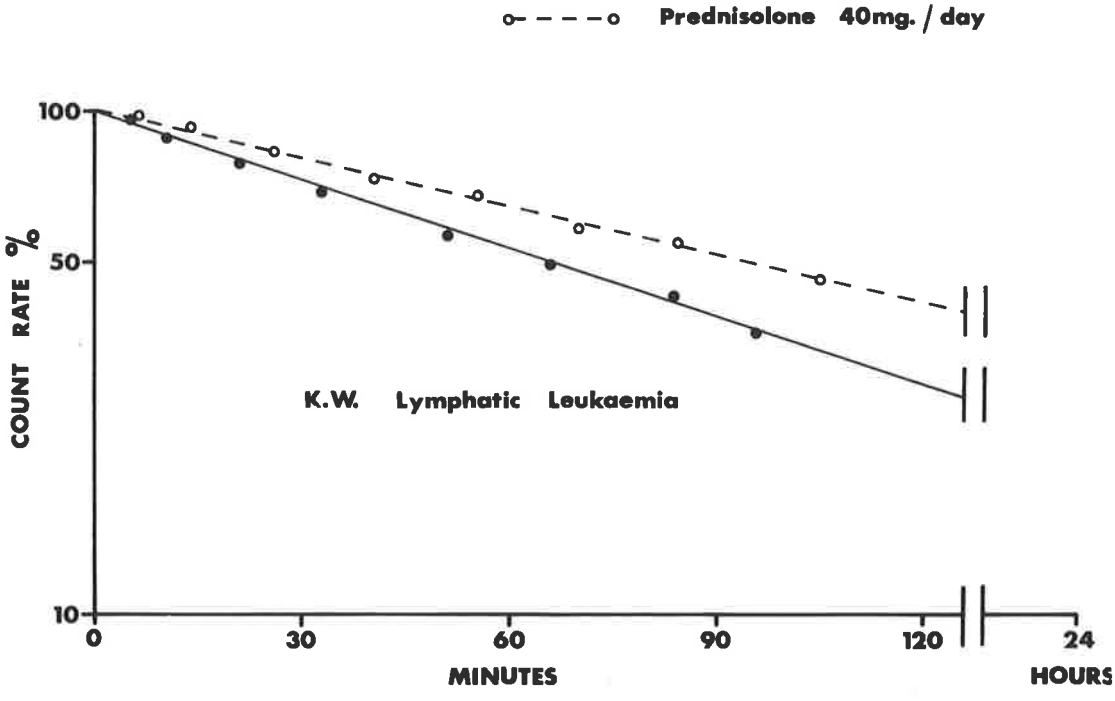

Figure 38: Reduction in the clearance rate of mildly NEM-damaged cells produced by corticosteroids (Studies No. 70 and 72).

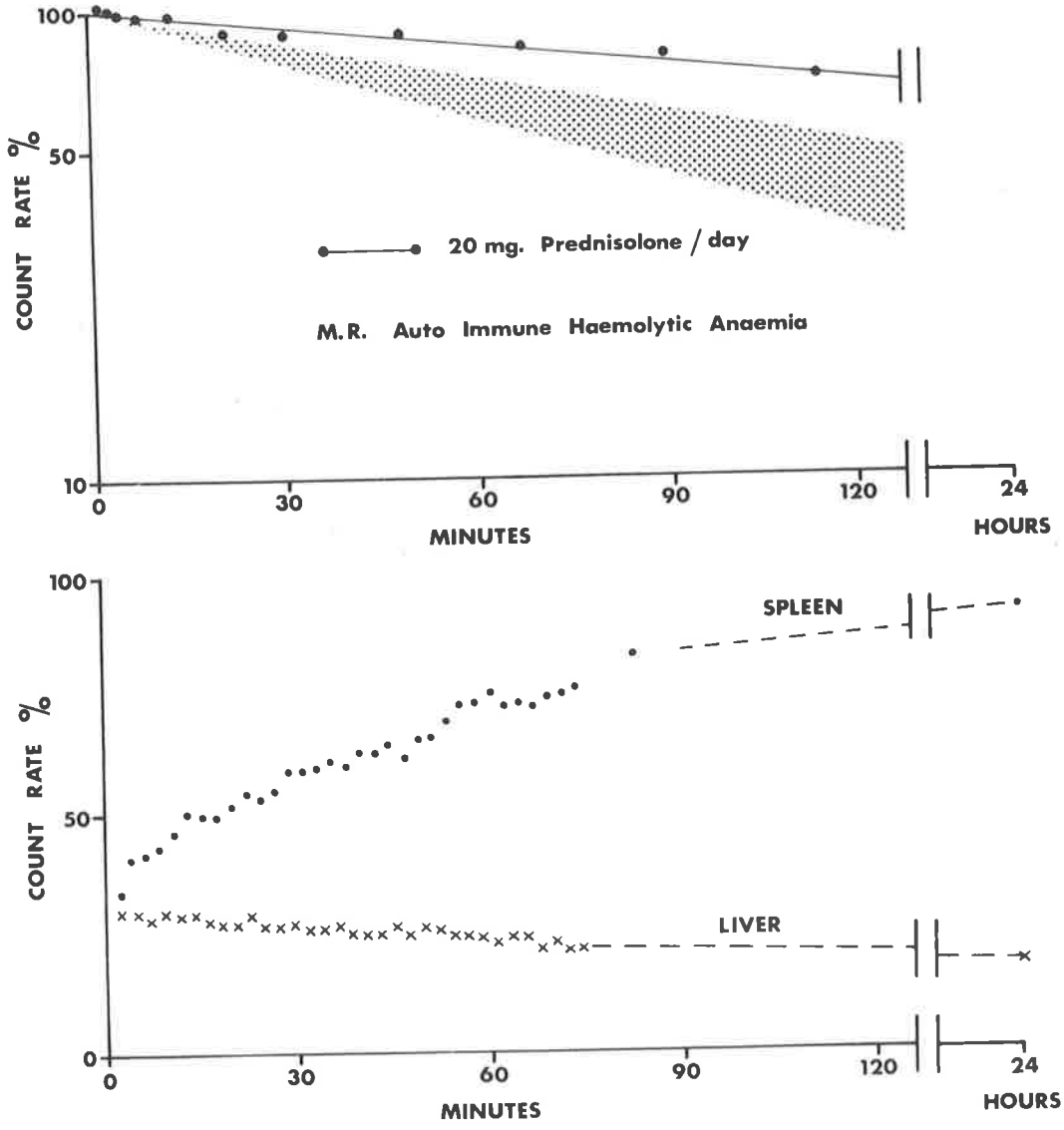

Figure 39: The reduced clearance rate of mildly NEM-damaged cells during treatment with corticosteroids (Study No. 44).

Table XIV - The survival and organ uptake of autologous untreated cells compared to that of mildly NEM-damaged erythrocytes.

Study	Diagnosis	Autologous Red Cell Survival		NEM-Treated Cell Survival		Remarks
		$T\frac{1}{2}$ days (Normal over 25)	Organ uptake	$T\frac{1}{2}$ Mins. (Normal 75-125)	Organ uptake	
87	V.R. Chronic lymphatic leukaemia	25	-	52	spleen	No relation between NEM and autologous red cell life. Organ uptake coincides.
88	M.H. Hodgkin's disease	9	spleen	20	spleen	NEM and autologous cell life both reduced. Organ uptake coincides.
89	M.P. Chronic myeloid leukaemia	16	spleen	50	spleen	NEM and autologous cell life both reduced. Organ uptake coincides.
90	L.S. Myelofibrosis	26	-	slow clearance	spleen	No relation between NEM and autologous red cell life. Organ uptake coincides.
91	B.H. Myelofibrosis	7	liver and spleen	slow clearance	liver and spleen	No relation between NEM and autologous red cell life. Organ uptake coincides. Haemolysis not relieved by splenectomy.

continued

Table XIV - continued

Study	Diagnosis	Autologous Red Cell Survival $T_{\frac{1}{2}}$ days (Normal over 25)	Organ uptake	NEM-Treated Cell Survival $T_{\frac{1}{2}}$ Mins. (Normal 75-125)	Organ uptake	Remarks
92 E.D.	Lymphosarcoma	25	-	80	spleen	NEM and autologous cell life both normal. Organ uptake coincides.
93 R.C.	Autoimmune haemolytic anaemia	3	spleen	180 (on steroids)	spleen	Unable to compare NEM and autologous cell life due to steroid therapy. Organ uptake coincides. Haemolysis relieved by splenectomy.
94 M.R.	Autoimmune haemolytic anaemia	13	spleen	200 (on steroids)	spleen	Unable to compare NEM and autologous cell life due to steroid therapy. Organ uptake coincides.
95 J.McK.	Lymphosarcoma	25	-	120 (on steroids)	spleen	Unable to compare NEM and autologous cell life due to steroid therapy. Organ uptake coincides.

splenomegaly not being so treated. The clearance rates ranged from 0.0023 $min.^{-1}$ to 0.0073 $min.^{-1}$ (mean 0.0047 $min.^{-1}$) in the treated subjects (Table XIII) compared with a range of 0.0126 $min.^{-1}$ to 0.0347 $min.^{-1}$ (mean 0.0203 $min.^{-1}$) in the untreated group with splenomegaly (Table X). The difference between these means was significant ($0.001 < p < 0.01$) showing that prednisolone caused a definite reduction in the splenic uptake rate of mildly damaged cells. One such case is illustrated in Figure 39.

In none of these subjects was there any evidence of hepatic uptake of cells despite liver enlargement.

Comparison between autologous and treated red cell survival: In 9 of the subjects with various haematological disorders the rate and site of removal of autologous untreated red cells were compared with those of cells mildly damaged with NEM (Table XIV). There was no constant relationship between the life of autologous and treated cells for rapid, slow and normal rates of distribution of NEM cells were found in patients with a normal autologous red cell life, as seen in subjects V.R., L.S. and E.D. It was not possible to compare these parameters in 3 subjects in whom steroid therapy was considered to have reduced the rate of removal of NEM treated cells (R.C., M.R. and J.McK.) Only in 2 subjects (M.H. and M.P.) did a shortened autologous red cell life coincide with a reduced life span of the NEM treated cells. In one subject (B.H.) a short autologous red

cell life was associated with a prolonged survival of the treated cells. In all the subjects with haemolytic anaemia so studied however the organ primarily responsible for the removal of both types of cells coincided (M.H., M.P., B.H., R.C. and M.R.) In 2 subjects subsequently subjected to splenectomy for the treatment of the haemolytic state, this relationship was confirmed. This in R.C. where both studies had indicated the spleen as the site of cell destruction, the haemolysis was relieved by the operation. In B.H. where the studies pointed to the liver being at least partly the organ of cell destruction, the operation did not relieve the haemolysis and subsequent study with NEM cells showed an increase in the degree of hepatic sequestration (Table XII and Figures 35 and 36).

The effect of the dose of cells on splenic uptake: The influence of the volume of infused cells on the rate of uptake of cells by the spleen was examined in the 21 subjects (10 normal and 11 with splenomegaly) who demonstrated a pure splenic uptake of mildly damaged cells. The volume of cells was expressed as ml. per kilogram body weight and the dose administered ranged from 0.0035 to 0.128 ml. per kg. Over this range no influence of the volume of cells given on the role of splenic uptake could be demonstrated, as shown by the absence of correlation between these parameters illustrated in the correlation plot, Figure 40.

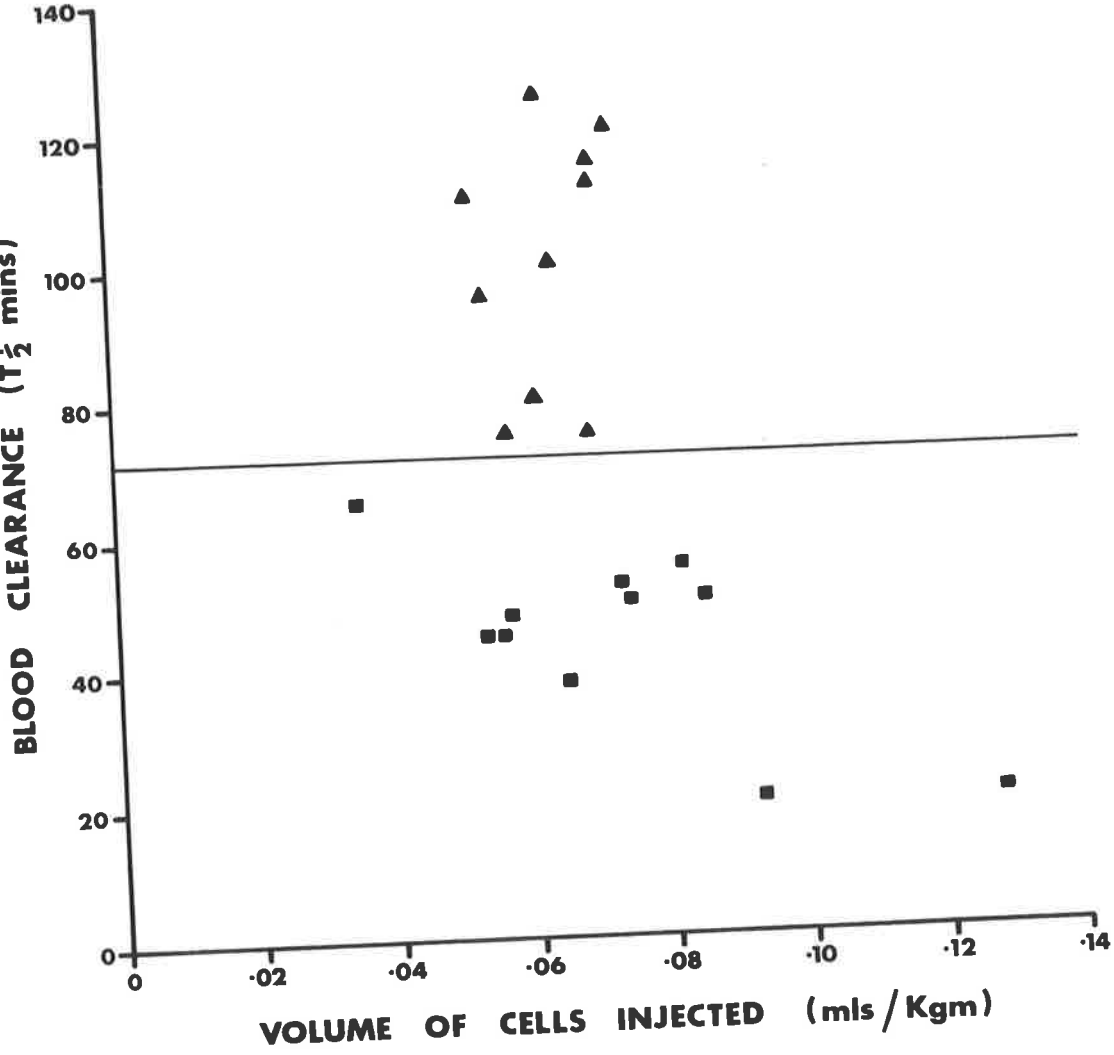

Figure 40: Correlation between the volume injected and the clearance rate of mildly NEM-damaged cells in 21 subjects (■ - normal, ▲ - splenomegaly).

regression coefficient, $b = -0.69$
regression equation, $y = 71 + (-0.69 \times 0.068\ x)$
correlation coefficient, $r = 0.04$, $p > 0.4$

CHAPTER VI

NORADRENALINE AND THE SPLENIC RED CELL POOL

Aims of the Investigation

To examine the postulate that "pooling" of abnormal erythrocytes occurs in the normal human spleen and that these cells can be expelled from such a pool under the influence of noradrenaline.

Subjects Studied

The effects of noradrenaline infusion on the peripheral venous haematocrit, the whole blood radioactivity and the splenic surface count rate were studied in 13 subjects as follows (Table XV).

A. 4 normal subjects in whom autologous, untreated, chromium-51 labelled cells were circulating.

B. A splenectomized subject in whom untreated, labelled cells were circulating.

C. A case of haemolytic anaemia with splenomegaly following the infusion of untreated, labelled cells.

D. 3 normal subjects, at varying intervals following the infusion of cells labelled with chromium-51 and mildly damaged by treatment with NEM.

E. 2 patients with splenomegaly, at varying intervals following the infusion of labelled, NEM-treated cells. One such study was performed at the time of splenectomy.

F. A subject in whom noradrenaline infusion was commenced before the injection of labelled NEM treated cells.

Table XV - The effect of noradrenaline on the peripheral haematocrit, blood count rate and splenic surface count rate.

Study	Time between infusion of cells and noradrenaline	Period of infusion (mins.)	Rise in haematocrit (per cent)	Rise in peripheral blood count rate (per cent)	Change in splenic surface counts
		UNTREATED AUTOLOGOUS CELLS			
74 A.J.	16 min.	12	4.4	6.0	Fall
78 H.T.	24 min.	21	6.4	5.2	Fall
85 B.L.	20 min.	22	9.4	8.3	Fall
77 E.D.	17 min.	19	7.2	8.3	Fall
82 W.S.	13 min.	25	5.9	9.1	Nil
76 R.C. (a)	29 min.	21	21.6	11.2	Fall
		NEM-TREATED AUTOLOGOUS CELLS			
81 D.M.	58 min.	28	5.6	No significant rise	Fall
86 J.M.	110 min.	13	3.3	9.6	Fall
79 I.P.	5 hrs.	22	4.2	5.4	Fall
80 J.R.	20 hrs.	20	3.7	No counts detected	Fall
75 M.T.	48 hrs.	12	10.1	No counts detected	Rise
83 R.C. (b)	23 hrs.	5	11.1	No counts detected	Not estimated
84 L.T.	5 min. prior	21	7.5	Exponential fall	Gradual rise

RESULTS

A. Untreated cells in normal subjects. In these cases the following pattern of events was seen. After noradrenaline infusion there was a rise in the peripheral haematocrit ranging from 4.4 to 9.4 per cent and an elevation in circulating whole blood activity from 5.2 to 8.3 per cent (Table XV). Associated with this there was a marked fall in the count rate recorded over the spleen. Two examples are illustrated in Figures 41 and 42. There was no change in the count rate over the liver. In one subject, B.L. (Figure 43) it was noted that deep inspiration, after noradrenaline had produced a fall in the splenic surface count rate, caused the counts to rise almost to the preinfusion level. Also after the infusion when the surface counts had returned to the original state, deep inspiration gave a detectable but less marked rise in splenic counts.

B. Untreated cells in a splenectomized subject. In this case a rise in the peripheral haematocrit of 5.9 per cent was associated with an elevation in whole blood radioactivity of 9.1 per cent (Table XV). There was no change over the "splenic" site or liver at this time (Figure 44).

C. Untreated cells in a patient with autoimmune haemolytic anaemia and splenomegaly. Following the injection of cells in this case the count rate over the spleen rose gradually to reach a steady count rate in approximately 12 minutes. This, compared with stabilisation

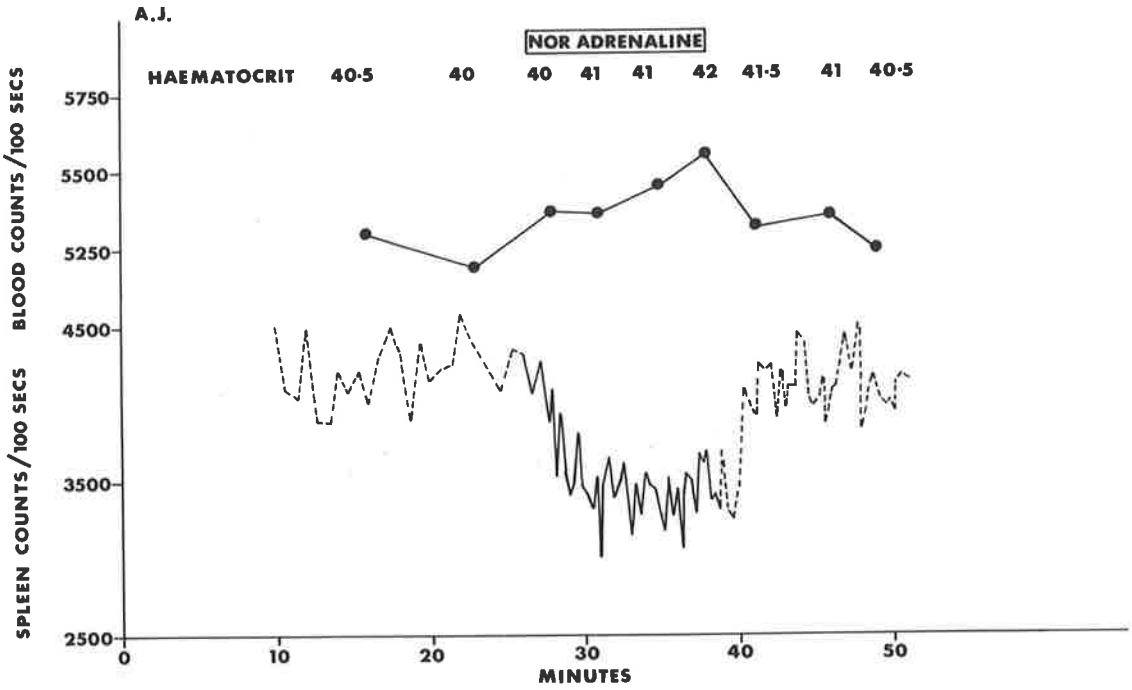

Figure 41: The effect of noradrenaline on the venous haematocrit, blood count rate and splenic surface count rate (Study No. 74).

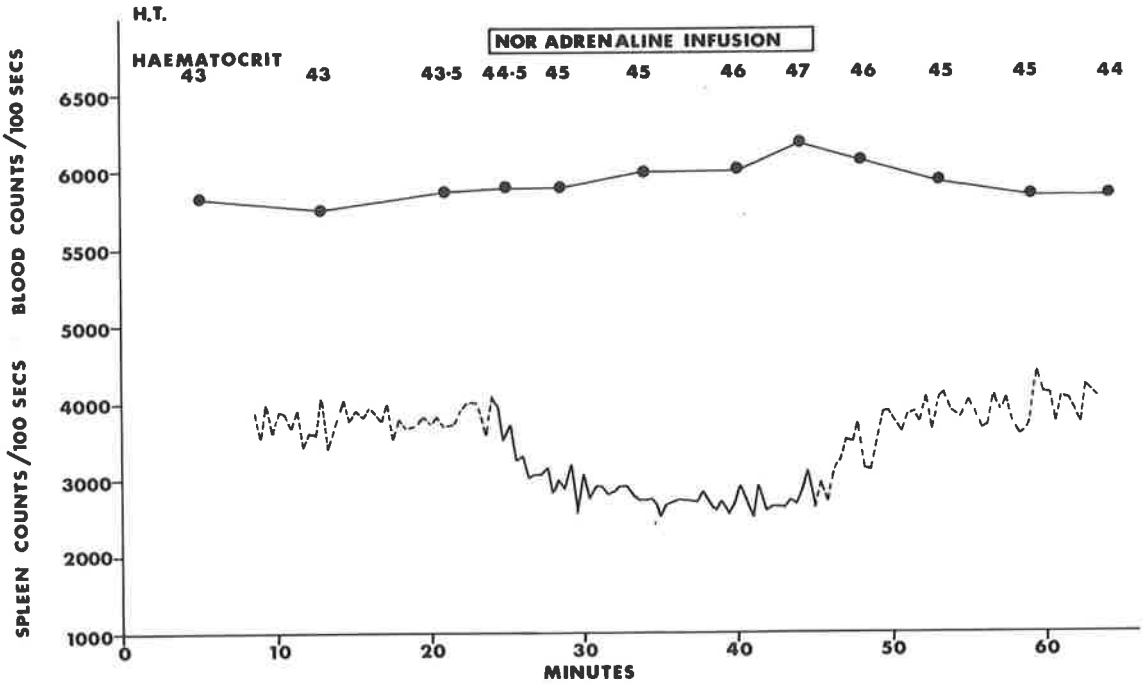

Figure 42: The effect of noradrenaline on the venous haematocrit, blood count rate and splenic surface count rate (Study No. 78).

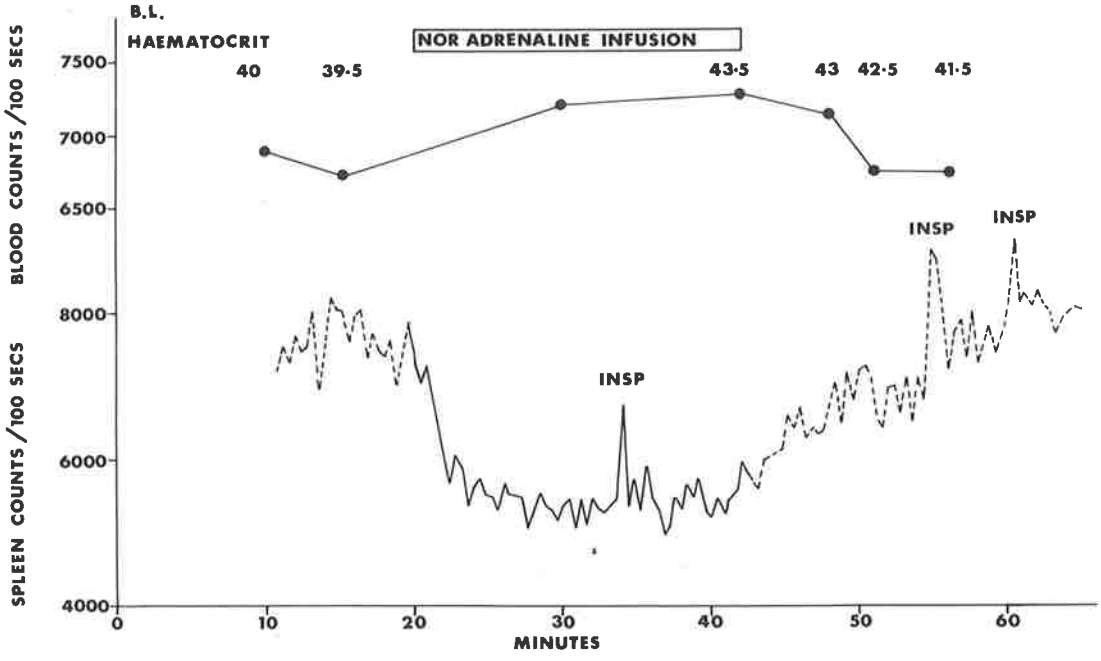

Figure 43: The effect of deep inspiration (INSP) on the splenic surface count rate during noradrenaline infusion (Study No. 85).

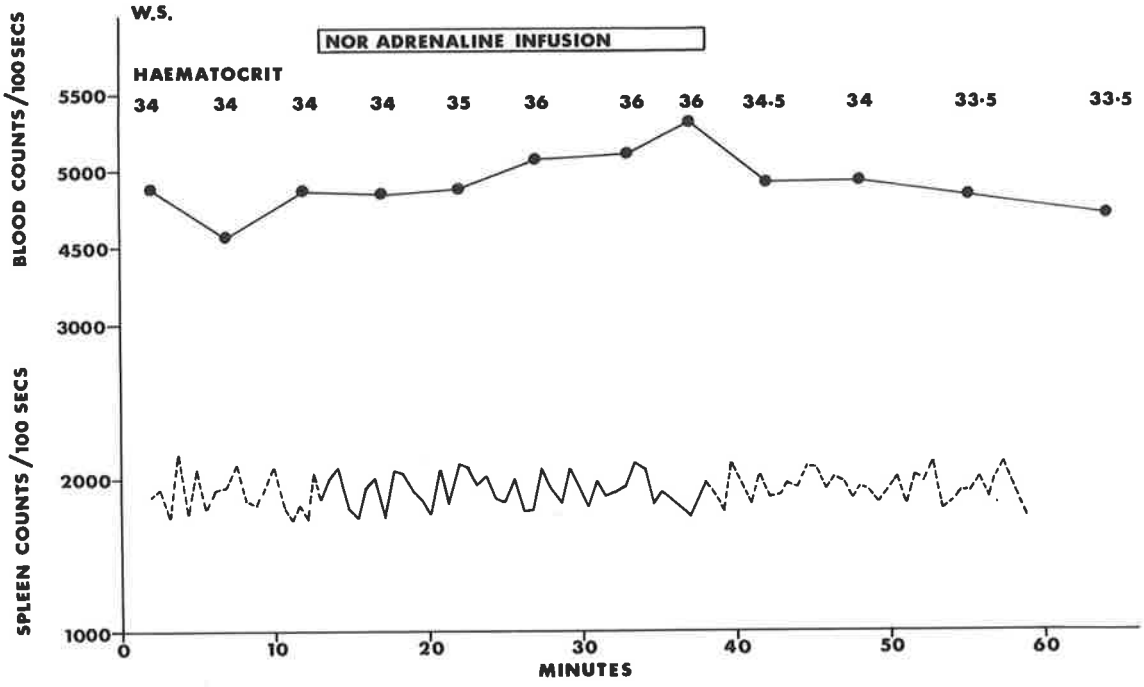

Figure 44: The effect of noradrenaline infusion on venous haematocrit, blood count rate and surface counts following splenectomy (Study No. 82).

in approximately 15 seconds over the normal spleen, suggested the presence of a splenic pool. Noradrenaline infusion produced a rise in the peripheral haematocrit of 21.6 per cent and a rise in blood radioactivity of 11.2 per cent (Table XV). Again there was a marked fall in count rate over the spleen (Figure 45) and no change over the liver.

D. Treated cells in normal subjects. In these 4 cases noradrenaline infusion was commenced at intervals of 58 minutes, 100 minutes, 5 hours and 20 hours after the NEM cells were administered. There were rises in peripheral haematocrit of 5.6 per cent, 3.3 per cent, 4.2 per cent and 3.7 per cent respectively (Table XV).

In the first case (Figure 46) prior to the infusion of noradrenaline, the peripheral blood radioactivity followed an exponential decline as the treated cells were removed from the circulation by the spleen. Noradrenaline, though it did not cause an absolute rise in counts in the blood specimens, interrupted this exponential fall. At the conclusion of the infusion the original rate of decline was resumed. Also there was a fall in splenic surface activity which rose again when the noradrenaline was discontinued.

In the second instance noradrenaline, given 100 minutes after the treated cells, again interrupted the exponential decline in the peripheral blood count rate and in fact caused it to rise by 9.6 per cent. There was again a fall in splenic surface activity. The

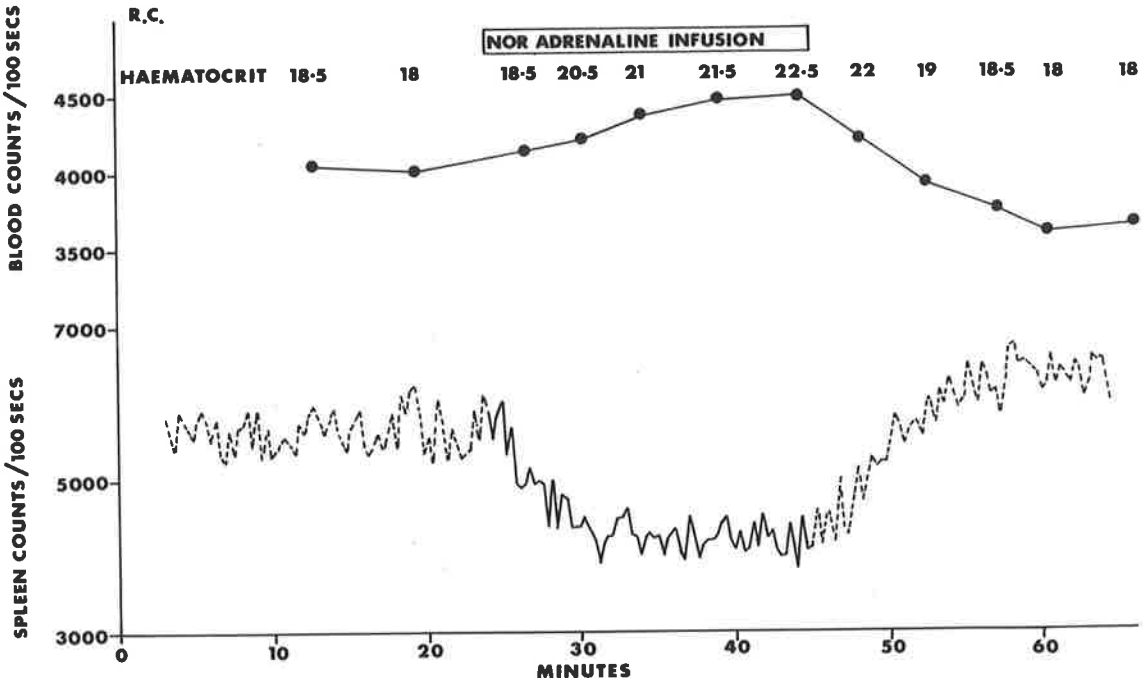

Figure 45: The effect of noradrenaline infusion on the venous haematocrit, blood count rate and splenic surface count rate in a subject with autoimmune haemolytic anaemia and splenomegaly (Study No. 76).

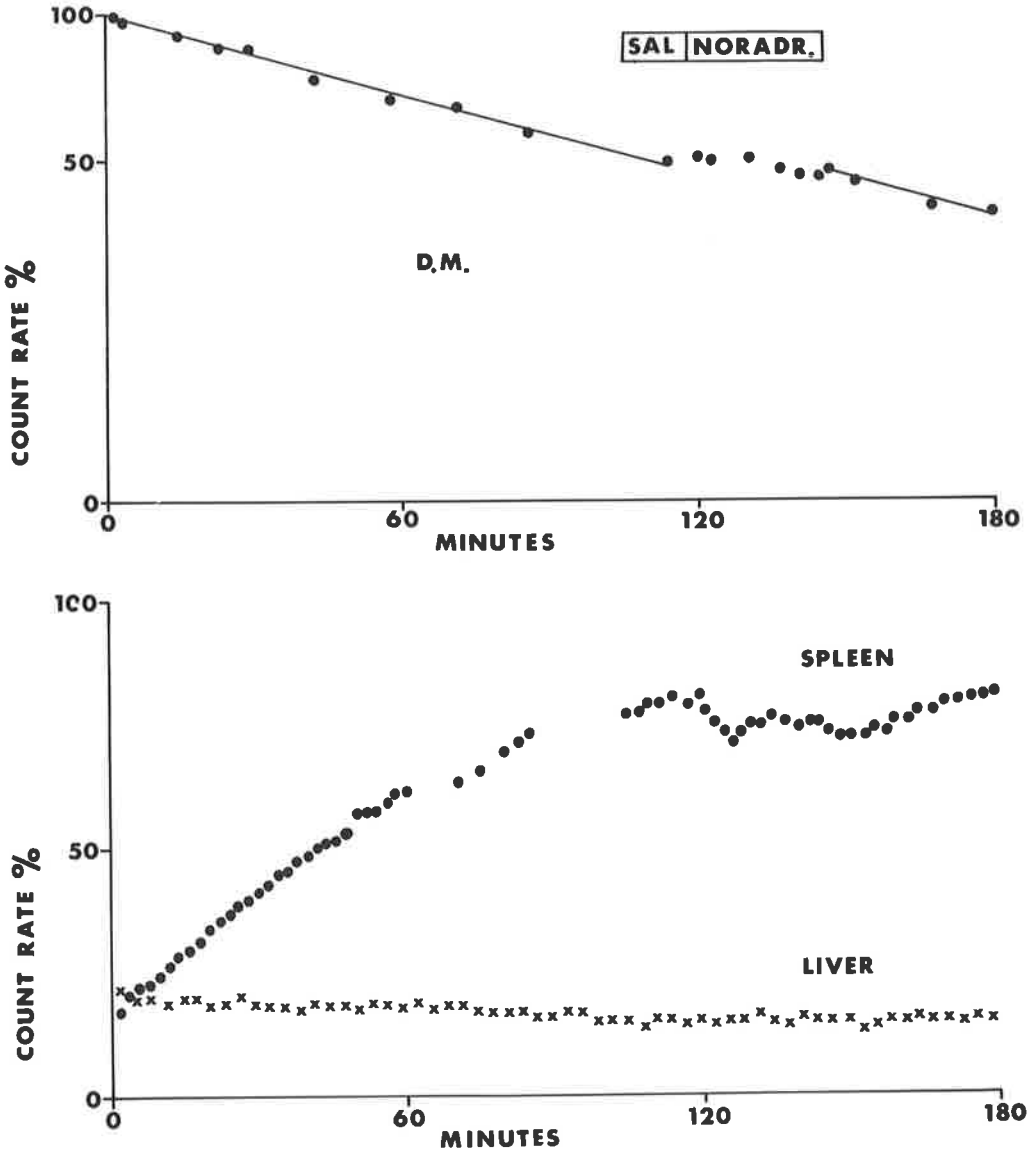

Figure 46: The effect of noradrenaline on the blood clearance (above) and splenic uptake (below) of mildly NEM-damaged cells (Study No. 81).

exponential fall in blood activity and the rise in splenic count rate resumed at the completion of the infusion (Figure 47).

In the third study in this group noradrenaline, given 5 hours after the injection of mildly damaged NEM cells, again interrupted the decline in circulating radioactivity and produced a 5.4 per cent rise in the count rate in the peripheral blood. There was a fall in splenic surface activity.

At the time that noradrenaline was given in the fourth case (20 hours after the cellular infusion) no activity was detectable in the peripheral blood. After this interval of time all the mildly damaged cells had been removed from the circulation and sequestered in the spleen. The count rate over this organ was thus at a high and stable level. Noradrenaline did not cause the appearance of detectable radioactivity in the peripheral blood though there was again a distinct fall in splenic surface activity (Figure 48).

E. Treated cells in patients with splenomegaly. In the first of these, a patient with Felty's syndrome, noradrenaline infusion was given 48 hours after the administration of the erythrocytes. At this time no activity was detectable in the peripheral blood. Noradrenaline caused a rise in the peripheral haematocrit of 10.1 per cent but this was not associated with the appearance of activity in the peripheral blood. Thus no labelled cells were expelled from

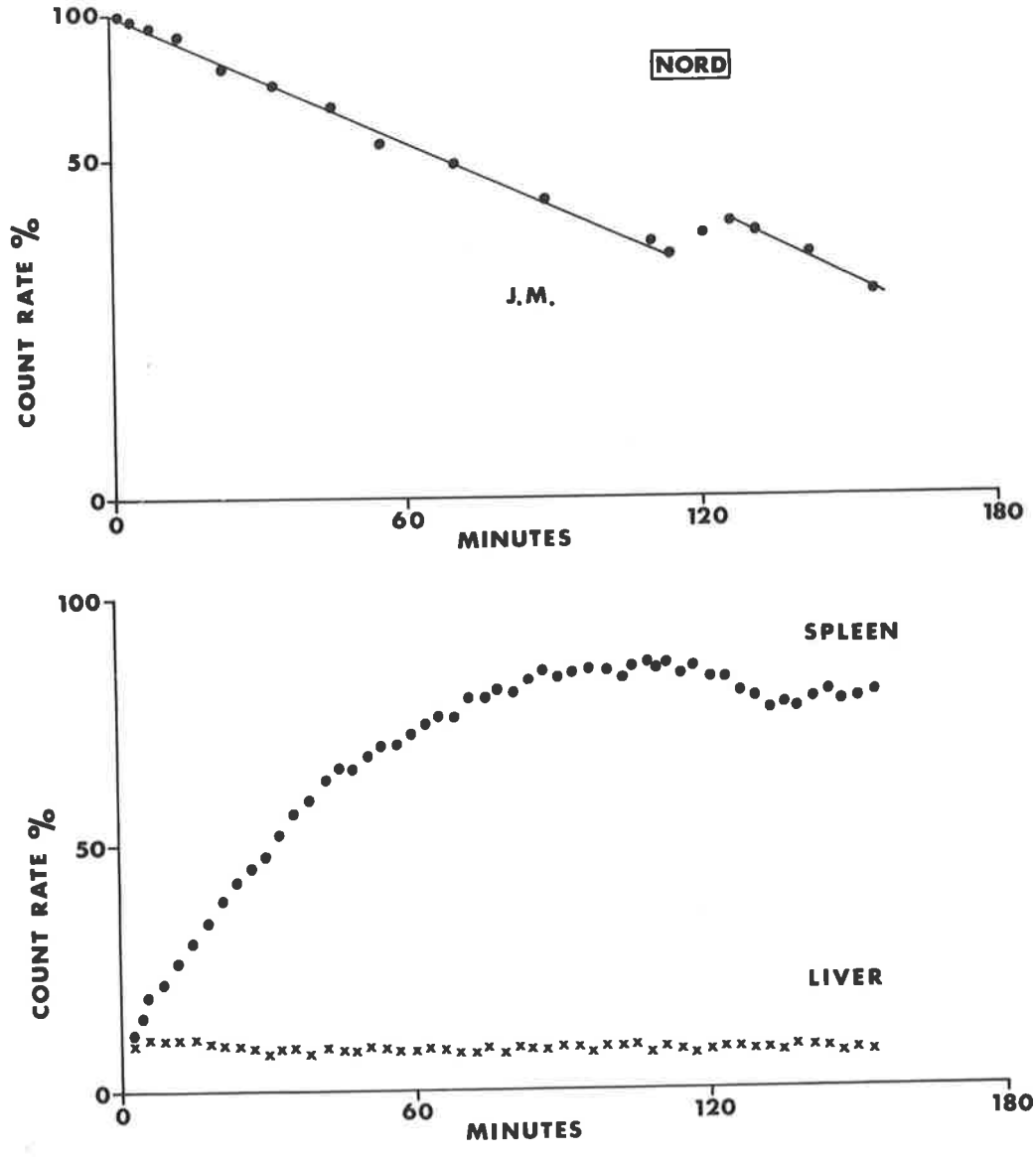

Figure 47: The effect of noradrenaline on the blood clearance (above) and splenic uptake (below) of mildly MEM-damaged cells (Study No. 86).

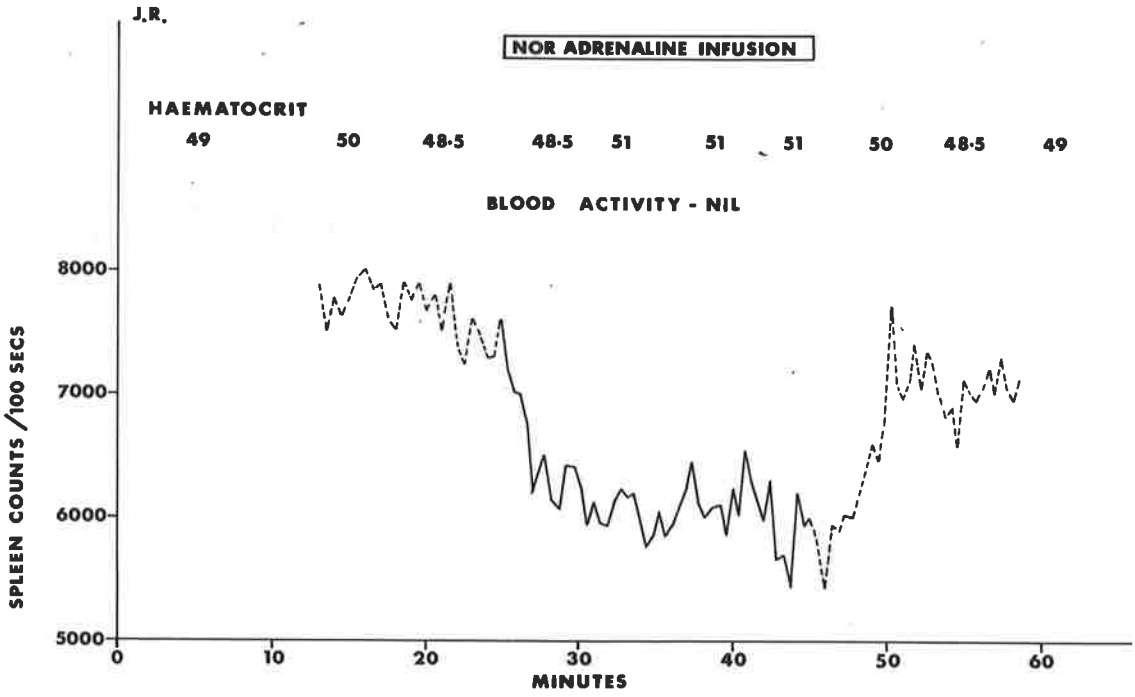

Figure 48: The effect of noradrenaline on the venous haematocrit, blood radioactivity and splenic surface count rate following the splenic sequestration of mildly NEM-damaged cells (Study No. 80).

the spleen. In this case however, the count rate measured over the spleen rose during the period of the infusion (Figure 49).

In the second subject in this group NEM cells were given 20 hours before splenectomy was performed for autoimmune haemolytic anaemia. At the time of operation blood was sampled from the splenic vein before, during and after noradrenaline was given intravenously. The haematocrit rose 11.1 per cent under the influence of the infusion but at no time was activity detected in the splenic venous blood (Table XV).

F. Treated cells following the commencement of noradrenaline infusion. The noradrenaline did not prevent the normal exponential decline in blood radioactivity nor the splenic uptake that occurs when NEM cells are cleared from the circulation. Also there was no marked change in these parameters at the conclusion of the infusion. However the noradrenaline did produce a rise in the peripheral haematocrit of 7.5 per cent, comparable to that seen in the other subjects (Figure 50).

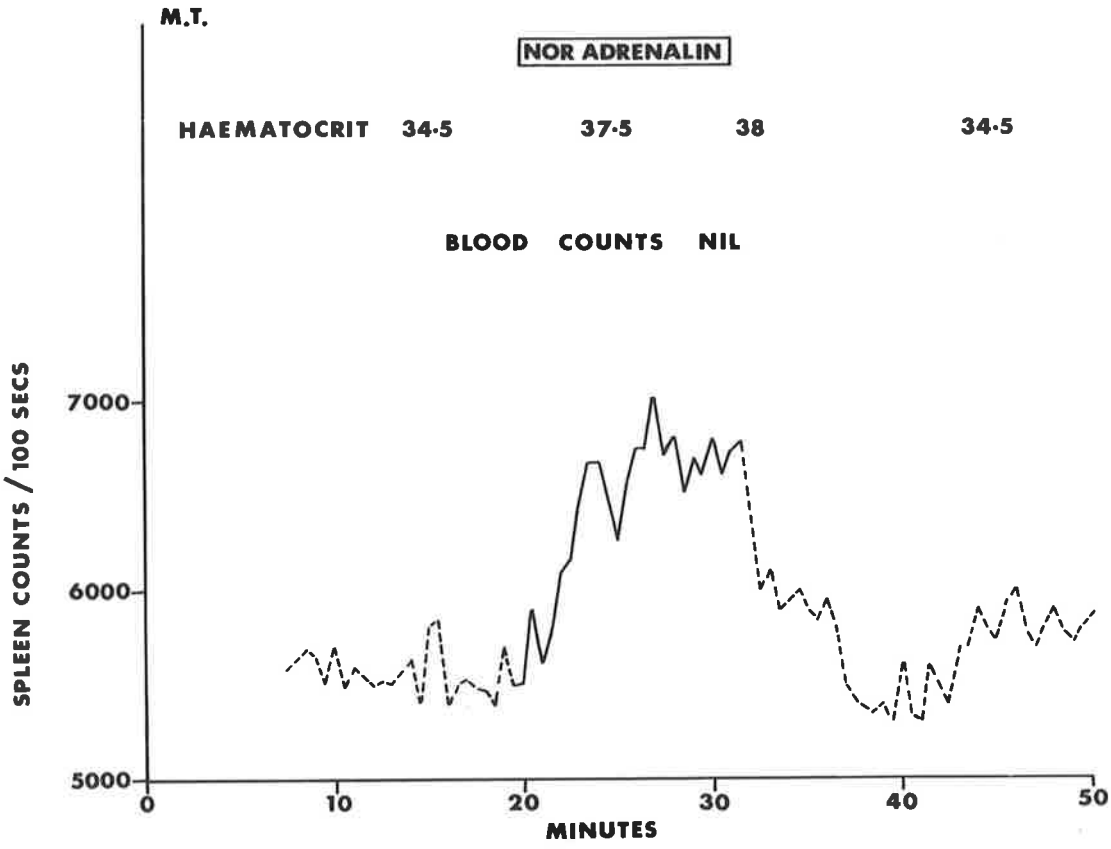

Figure 49: The effect of noradrenaline on the venous haematocrit, blood radioactivity on splenic surface count rate following splenic sequestration of mildly NEM-damaged cells (Study No. 75).

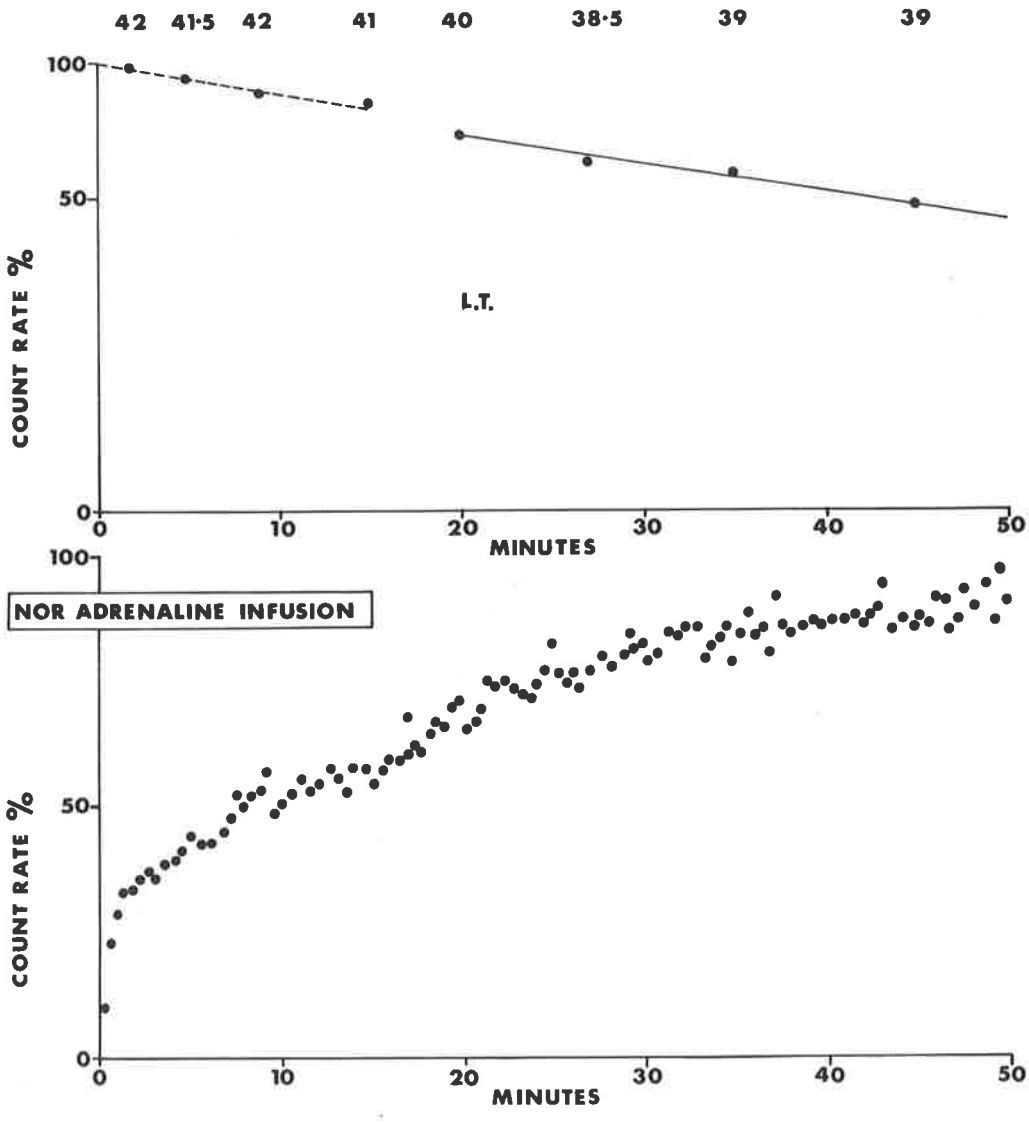

Figure 50: The failure of noradrenaline infusion to prevent the blood clearance (above) and splenic uptake (below) of mildly NEM-damaged cells (Study No. 84).

CHAPTER VII

DISCUSSION

A simple exponential fall in circulating radioactivity indicates the disappearance of a fixed proportion of a single population of labelled cells in unit time. These cells may be removed by one or more organs. To establish that only one site is responsible for the uptake it is necessary to demonstrate either that the organ in question actually contains 100 per cent of the injected radioactivity, or that the blood clearance rate and the organ uptake rate are identical. The demonstration of 100 per cent uptake, for example by the spleen, is not possible unless splenectomy is subsequently performed but an acceptable measurement of the uptake rate is practicable in many cases, as demonstrated in this study.

When the pattern of cell clearance can be shown to be compounded of two exponential components, it establishes a double erythrocyte population. If the liver and spleen uptake rates correspond to the two clearance components it demonstrates selection of different cells by the two organs.

Rapid simple exponential clearance may be seen when one cellular population is removed by a single organ but will also occur when such a population is being taken up at two or more sites. Thus, for example, if the liver and spleen are both removing non-viable cells at a rate proportional to their concentration in the peripheral blood, the rate of clearance will be characterized by the exponential constant $\lambda_1 + \lambda_2$, where λ_1 and λ_2 are the fractions

of cells cleared by the liver and spleen respectively in unit time.

It is possible that cells could be removed from the blood stream in a single passage through a particular organ. It has been estimated by Hughes Jones and his associates (1957) that in a person with a total blood volume of 4.5 litres, 150 ml., representing approximately 3.5 per cent of the total, will pass through the spleen per minute. Then if the spleen removes all the cells passing through it the half time of circulating radioactivity will be approximately 20 minutes,

$$\text{for the fraction removed} = \frac{0.693}{T\frac{1}{2}\ (\text{mins.})}$$

$$T\tfrac{1}{2} = \frac{0.693}{0.035}$$

$$= 20 \text{ minutes.}$$

Similarly if the liver removes all incompatible cells in a single passage, the half time of circulating activity will be approximately 2 to 3 minutes assuming that the liver blood flow is 25 per cent of the total blood volume per minute. These theoretical considerations are of importance in the interpretation of the results recorded here.

It is demonstrated that when human red cells are mildly damaged by heating at 50°C for 10 to 15 minutes, they undergo spheroidal transformation and become significantly less, rather than more, susceptible to osmotic rupture. The spleen plays a specific role in the removal of these cells from the circulation as shown by the

uptake of radioactivity, only in this organ, following intravenous infusion. The prolonged erythrocyte life in patients without a spleen confirms this. However though cell deposition is confined to this site, the rate of removal is relatively slow and each cell is not taken up upon first passing through the spleen. As mentioned previously, if such were the case half the damaged cells would be cleared from the circulation within about 20 minutes of administration. Therefore progressive degeneration within the blood stream must occur until the erythrocytes reach a state which results in specific splenic trapping.

When cells are more severely damaged, by heating for 20 to 30 minutes, a double population of spherocytes and fragmented forms is produced and in this case there is an increase in osmotic fragility. This double population, on morphological criteria, is confirmed by the biphasic exponential clearance of the cells after reinfusion. There is associated with this a marked splenic and also a hepatic accumulation of radioactivity. The studies suggest that the slow component reflects the preference of the spleen for the spherocytes and that the rapid component is due to fragments being cleared rapidly by the liver and possibly other parts of the reticulo-endothelial system. This interpretation is again supported by the findings in subjects following splenectomy. Here also the fall in activity, composed of two exponents, confirms a mixture of cell types.

It seems probable that in these circumstances the slower component of the curve reflects the removal of the spherocytes taking place at a greatly reduced rate due to the absence of splenic tissue. The fast clearance of the fragmented forms, in these patients, is again less than in the spleen-intact subjects. This seems likely to be due to the absence of splenic tissue which would otherwise materially assist, non-specifically, in the removal of such fragments.

The occurrence of an initial slight increase in plasma radio-activity reflects an element of intravascular lysis, presumably due to an increased mechanical fragility of that proportion of the fragmented forms in the circulation which escapes sequestration. The fragmented forms would therefore appear to be characterized by an increased osmotic fragility, some susceptibility to disruption by the trauma of circulation, and by a propensity to be sequestered and destroyed by reticulo-endothelial tissue not only in the spleen, but also in the liver and elsewhere in the body. This interpretation has support from the observations of Ham et alii (1948) that cell particles are detectable in the peripheral blood for only a few minutes, while large spherocytes remain in the circulation for some hours.

More marked erythrocyte damage by heating at 50°C for 60 minutes results in a considerable reduction in the number of spherocytes, but a great increase in the proportion of fragments. In addition, more

severely damaged cells tend to agglutinate in small masses. In this case spontaneous lysis in isotonic saline is easily detectable. Reinfusion of these cells into the normal donors is followed by a rapid, simple exponential decline in circulating cellular activity. In contrast to the studies in which the cells are heated for shorter periods, proportionately less activity is bound in the spleen and more in the liver. It is suggested that this more pronounced hepatic accumulation probably is due not only to the removal of some of the fragmented forms by the liver, but also to a preferential affinity on the part of this organ for the agglutinated masses. Verifying this, a very marked hepatic uptake is seen when erythrocytes are heated for 90 to 120 minutes, when a majority of the cells and fragments form such aggregates. The splenic activity found suggests the non-specific uptake of some of the cells and the absence of a slow clearance component is consistent with the paucity of spherocytes susceptible to removal only by the spleen.

It might be thought that much of the radioactivity which accumulates in the liver soon after the infusion of moderately and severely heat-damaged cells, could be due merely to the removal by the liver of labelled haemoglobin released from cells broken down in the circulation. This is unlikely however, for like Jandl et alii (1956) it is shown that the hepatic uptake of chromium-51 labelled haemoglobin, injected intravenously, occurs at a rate very much slower

than that discussed here. Haemoglobin is cleared with a half time of 3.5 and 4 hours compared to that of less than 10 minutes in the case of the cells under consideration.

Thus although there is broad agreement with Wagner and his colleagues (1962) who found that there is a "shift" in the predominant organ of red cell uptake from spleen to liver with increasing degrees of heat damage, the present results differ in a number of important respects. These authors did not detect a biphasic clearance of moderately heat-damaged cells reflecting a double population nor did they apparently find evidence of intravascular lysis following more pronounced injury. In addition, with severely damaged cells there is, in contradistinction to the findings of Wagner et alii, always some evidence of splenic uptake. In no instance is there "pure" hepatic sequestration in a spleen-intact subject. The spleen always plays a part in the removal of even the most severely affected cells despite an apparent hepatic "preference" for these forms.

Thus this study, as well as clarifying the morphological factors that govern the site of organ uptake of heat damaged cells, helps to define the mechanism of anaemia in cases of severe thermal burns. Although intravascular haemolysis may occur in the severely burned patient, it seems likely that trapping of cells damaged at the site of thermal injury, by the various organs of the reticulo-endothelial system, is the main cause of the rapid and marked fall in haemoglobin

concentration in such cases.

Erythrocytes treated with low concentrations of NEM, 8 micromoles per ml. of red cells, so called "mildly" NEM-damaged cells, do not undergo any detectable morphological change although they become significantly less susceptible to osmotic lysis. This treatment produces a uniform population of cells removed from the circulation solely by the spleen. This is demonstrated by the positive correlation between the single exponential blood clearance and splenic uptake rates, and also by the absence of intravascular lysis and liver accumulation of radioactivity. Results in the splenectomized subjects support this as in the absence of the spleen, survival of such cells is greatly prolonged and only after an extended period of time is any cellular sequestration evident in the liver and bone marrow.

Just as in the case of minor heat damage, the cells passing through the spleen are not removed in a single passage of this organ, for the half period of activity exceeds 20 minutes. However the half time does not vary widely from a mean of 100 minutes in these normal individuals and this, with the consistent exponential decline in activity, shows that a constant proportion of a single population is sequestered. The variation, around 100 minutes, is presumably due to a difference in the size of the vascular bed of the spleen from person to person.

Slightly more pronounced, or "moderate" red cell damage, using 10 micromoles per ml., produces a mixed group as judged by morphological, osmotic fragility and survival criteria. Discoid and spherocytic cells are associated with a fragility spectrum embracing increased resistance to osmotic rupture with a proportion of more fragile forms. The clearance pattern confirms this separation and the slow component, presumably due to the discoid cells, corresponds to the uptake rate measured over the spleen. Conversely the rapid segment, that of the spherocytes, is comparable though not identical in rate to the uptake over the liver. Thus the overall picture can be interpreted as illustrating discoid cells being removed by the spleen while spheroidal fragile cells are sequestered primarily in the liver.

"Severe" damage, incubation with 20 micromoles of NEM, produces cells that are uniformly spherocytic and osmotically more fragile than normal. The single exponential clearance confirms that these indeed constitute a single population. The blood clearance and liver uptake rates are comparable but not identical and this with the high level of counts reached over the liver suggests that this organ is removing the majority of the cells. However the spleen does play some part as there is an early but easily detectable rise in the count rate over this organ.

Intravascular lysis does not contribute to the destruction of

cells treated with the various concentrations of the sulphydryl inhibitor for in no case does activity appear in the plasma fraction. It is not possible that haemoglobin released into the circulation is removed rapidly and escapes detection. Radioactive haemoglobin is cleared slowly and would be easily identified in blood samples taken at frequent intervals.

Therefore it is confirmed that increasing damage by NEM in vitro results in a change in the site of sequestration of erythrocytes from the spleen to the liver (Jacob and Jandl, 1962b and Wagner et alii, 1962). Also the morphological and osmotic fragility factors associated with this change are established. As in the case of heated cells, despite a severe degree of damage the spleen still removes a proportion of such cells.

The specific affinity of the spleen for the heated spherocytic cells recalls the well known sequestration by the spleen of the osmotically fragile spherocyte in hereditary spherocytosis. Furthermore, just as the removal of the spleen in patients suffering from this condition results in a cessation of the haemolytic process, the infusion of heated spherocytes, in the absence of the spleen, is associated with their prolonged survival in the circulation. This would suggest that the spleen acts as a "filter" for abnormally shaped cells. Thus splenic uptake has been demonstrated, as well as in hereditary spherocytosis, in the case of sickle cells (Weisman et

alii, 1953) and with red cells rendered spherocytic by treatment with lecithin (Jandl and Tomlinson, 1958). In vitro support for this concept was supplied by the description of Jandl, Simmons and Castle (1961) of the inability of such cells to pass a millipore filter.

However morphology cannot be the major factor governing selection by the spleen as, despite striking changes in shape, the erythrocytes in acanthrocytosis show no gross or constant reduction in life span (Simon and Ways, 1964). Conversely, cells treated in a variety of ways, as well as by incubation with NEM, are sequestered by the spleen despite the absence of detectable morphological alteration. These include erythrocytes treated with low concentrations of metallic cations (Jandl, 1955), non-agglutinating non-complement fixing antibodies (Jandl, Jones and Castle, 1957, and Mollison and Cutbush, 1955) and low concentrations of isoantibody (Chaplin, 1959). Also reticulocytes (Berenedes, 1959), erythrocytes with reduced surface lipid (Jacob and Jandl, 1962) and those incubated with acetyl phenylhydrazine (Jandl and Tomlinson, 1958) are trapped by the spleen, though the cells are of normal shape. It has been shown that it is not arrest of cell glycolysis that governs organ selection in such cases for production of this effect by arsenite and fluoride (Jacob and Jandl, 1962b) does not bring about the same rapid splenic uptake as does treatment with NEM.

Further consideration of the relationship between cellular morphology and fragility provides evidence that neither factor is alone responsible for splenic sequestration. It is often assumed that an increase in osmotic fragility reflects a spheroidal erythrocyte shape, and vice versa. This belief was strengthened in 1934 when Haden demonstrated that in hereditary spherocytosis the red cells behaved as perfect osmometers, and Castle and Daland (1937) claimed that the differences in susceptibility, of various types of red cells to haemolysis by hypotonic saline, were due to differences in form. However Guest (1948) showed that sickle cells and those from a patient with thalassaemia suffered less than the expected degree of swelling in a series of hypotonic solutions.

Ponder (1948), commenting on the fact that spherocytosis was often a deduction from the finding of increased osmotic fragility, claimed that a disc to sphere transformation could be brought about by an agent reducing the surface area of a red cell enclosing an unchanged cell volume. He postulated that if this membrane change was associated with an increase in cell surface "toughness" decrease in fragility would be associated with spherocytosis. Also Crosby (1952), in a comprehensive discussion of cellular morphology, postulated that this could be the mode of action of certain "sphering" agents including some red cell antibodies and lysolecithin prepared from plasma lipids. It had been shown by Aub et alii (1925) that

such a change did indeed occur when red cells were treated with lead chloride ($PbCl_2$). This was confirmed by Blackburn (1949) and Harris and Greenberg (1954) and the former also reported a loss of intracellular potassium associated with the spheroidal shape and increased resistence to osmotic lysis, produced by this heavy metal salt.

Joyce et alii (1954) and Passow and Tillman (1955) demonstrated a loss of intracellular potassium ions following treatment of erythrocytes with the salts of various heavy metals. Vincent and Blackburn (1958) and Vincent (1959a, 1959b) following further similar work, postulated that the heavy metals were bound to sulphydryl groups on the cell surface and in this way interfered with mechanisms basic to the maintenance of cell shape and potassium content. Weed et alii (1961) therefore considered that the effect of metals in causing an initial decrease in osmotic fragility was due to a state of intravascular hypotonicity produced by an efflux of potassium without an associated influx of sodium ions. A similar interpretation was propounded by Teitel (1960) when he showed an efflux of potassium and a decrease in osmotic fragility when red cells were incubated for 4 to 6 days in acid citrate dextrose at 37°C in the absence of metallic cations. Also Haut, Cartwright and Wintrobe (1960) demonstrated that aseptic incubation of normal erythrocytes and those from patients with hereditary spherocytosis, thalassaemia minor

and iron deficient anaemia, produced a reduction in osmotic fragility, though preceded by a phase of increased fragility.

Jacob and Jandl (1962a) studied the effects on the erythrocyte of low concentrations of the sulphydryl inhibitor N-ethyl maleimide and parachloromercuric benzoate. The former, which penetrated into the cell disrupted membrane ionic transfer and caused a leakage of potassium and a decrease in osmotic fragility without morphological change. The latter, a surface active agent which does not penetrate into the cell, caused progressive spherocytosis and increased osmotic fragility but no potassium leakage.

These studies demonstrate that erythrocyte fragility and shape are not directly related and that the state of the cell membrane must be considered in correlating the two. Thus a membrane alteration leading to either changes in intracellular tonicity, shrinkage of the cellular envelope or to alteration in the "toughness" of the cell wall can cause various associations between shape and resistance to osmotic lysis. Examples of such variations are seen in the spherocytes of hereditary spherocytosis which show a marked increase in fragility, the mildly heat damaged spherocytes and the discoid NEM treated cells both of which have a decrease in fragility. All are removed specifically by the spleen. Therefore an erythrocyte membrane change may be the governing factor. Such an alteration could produce the various combinations of morphology and fragility

encountered and yet render the cell particularly liable to uptake by the reticulo-endothelial cells of the spleen.

Different erythrocyte factors govern sequestration in other parts of the reticulo-endothelial system. For example the normal liver removes some of the cells that are fragmented by heat treatment. However these particles are not selectively trapped at this site for an appreciable number are taken up by the spleen. There is evidence that erythrocyte agglutination results in hepatic sequestration, and aggregation produced by heat, metallic cations (Jandl, 1955) or iso-antibodies (Jandl, Jones and Castle, 1957) results in the majority of such cells being removed by the liver. This physical change is not essential however for, in the absence of agglutination, erythrocytes treated with 20 micromoles of NEM and complement fixing non-agglutinating antibodies (Mollison and Cutbush, 1955; Mollison, 1962) show a largely hepatic uptake. Therefore if selective uptake by the liver does occur it also, as in the case of the spleen, is not dependant on a gross and obvious change in cell morphology.

One important factor must be kept in mind when assessing specific hepatic trapping. It is difficult to decide, when a single population of cells is rapidly removed from the circulation, if the marked liver uptake of radioactivity truly reflects a specific selection of erythrocytes. If cells are rapidly and indiscriminately taken up by all areas of the reticulo-endothelial system there will be an apparent localisation

of activity in the liver with a lesser rise in the surface count rate over the spleen. This will arise because liver blood flow, approximately 25 per cent of the total blood volume per minute, far exceeds that of any other localised part of the reticulo-endothelial system. To establish a selective action on the part of the liver, it is necessary to demonstrate a correspondence between blood clearance and organ uptake rates, and the absence of any accumulation of activity in the spleen. These criteria are not wholly fulfilled in any of the cases studied despite the fact that very few cells agglutinated by heat are retained by the spleen.

The following conclusions therefore seem justified. The normal spleen is unique in that it is an organ which has a specific function and removes certain slightly altered cells which are not sequestered in any other organ. This is presumably due to its particular vascular architecture or to special characteristics of the contained reticulo-endothelial cells. Though there is some evidence suggesting that the liver selectively traps different types of erythrocytes, particularly those agglutinated by various means, the most critical assessment of the phenomena described makes it more likely that this organ normally acts only as a part of the general reticulo-endothelial system. The apparent selection of the severely damaged cells is probably only a reflection of its copious blood supply.

The demonstration that erythrocytes mildly damaged with NEM are

removed only by the spleen in normal subjects establishes them as a satisfactory agent for the measurement of splenic uptake function. This can be quantitated with reference to the clearance half time or fraction of the cells removed in unit time.

In the early part of the study an endeavour was made to prepare a uniform population of cells by heating at 50°C. Though treatment for periods of from 10 to 15 minutes resulted in only splenic sequestration, it was obvious that the relatively prolonged survival of such cells in the circulation made it difficult to accurately measure the half period in each case. Also a slight prolongation in the period of heating produced a mixed population of cells not all of which were trapped by the spleen, and an error was introducted by the tendency of some cells to undergo lysis in the circulation. This difficulty, in the production of uniform cellular damage, was also experienced by Crome and Mollison (1964) and Holzbach et alii (1964). Though the latter workers did assess splenic uptake function using such cells, the analysis of their data was complicated by these variable factors.

Therefore in this study the attempt to measure sequestration using heated cells was abandoned after only two studies in patients with splenomegaly, one with thalassaemia minor and one suffering from myelofibrosis. In both of these the survival of mildly heat-damaged cells was prolonged beyond that seen in normal subjects. In the light of subsequent work, utilising NEM cells as the agent for this

measurement, the gross prolongation of heated cell life, half time 5 days, in the patient with myelofibrosis, compared with the half life in normal subjects of from 9 to 12 hours, is probably significant.

Using the NEM treated red cells, free of these variations, splenic sequestration was quantitated in the subjects with splenomegaly, as described. In the majority of these cases splenic enlargement was associated with an increase in uptake function and it is most likely that this is due to an increase in the bulk of the vascular reticulo-endothelial tissue of the organ. However in contradistinction to the findings of Holsbach et alii (1964), in this group the capacity of the spleen was not directly proportional to the size of the organ. Therefore although splenomegaly is often associated with an increase in the reticulo-endothelial tissue responsible for sequestration, this cannot be directly related only to splenic size. In fact splenomegaly may be accompanied by a reduced capacity to remove such cells, that is a large spleen can be one with a reduced uptake function. This state of affairs is striking in both the patients with myelo-fibrosis and also in one subject with a large hydatid cyst in the spleen. This suggests a replacement or disruption of the normal vascular reticulo-endothelial structure. The form of such replacement is obvious in the case of hydatid cyst, but the unexpected finding in cases of myelofibrosis deserves special consideration. In this condition, where the gross degree of splenomegaly would lead one to

expect a greatly enhanced sequestration function, it could well be that the establishment of a large mass of tissue subserving the function of extramedullary haemopoiesis is responsible. Such tissue therefore does not retain the normal splenic structure and function of selectively removing mildly damaged cells. This finding probably accounts for the striking prolongation of the life of cells, damaged by heating, in the one subject with myelofibrosis studied using this reagent.

It might be argued that the phenomenon of rapid uptake of altered cells in cases of splenomegaly is due, not only to an increased sequestering capacity of the spleen, but also to an erythrocyte abnormality present before treatment with NEM and resulting in more severe damage than the constant degree produced in normal cells. There are factors against this interpretation however. Firstly, if such were the case the added erythrocyte abnormality would presumably cause a degree of damage bringing about removal of cells in many sites of the reticulo-endothelial system. However the single exponential splenic removal establishes that this is not the case and therefore splenic uptake only is being assessed. Secondly, in cases discussed later where autologous untreated cell survival is studied, despite the presence or absence of an inherent red cell defect leading to clinical haemolytic anaemia, only a single exponential removal of treated cells is encountered. Thus in abnormal as well as normal subjects treatment

of cells with 8 micromoles of NEM produces a single cell population which can be utilized for quantitating splenic sequestration.

The liver shared in the uptake of mildly NEM damaged cells in 6 subjects. Three were patients who had been subjected to splenectomy and in these the hepatic accumulation of activity was delayed and not measurable until the day following the infusion. Thus the liver did not show any immediate affinity for these cells and only shared in the uptake, the bone marrow also being partly responsible, after the cells had been circulating for some hours. During this period the erythrocytes presumably underwent further degeneration which caused sequestration generally throughout the body. This highlights a basic difference in the tissue responsible for red cell uptake in liver and spleen. Also, as the spleens had been removed from these subjects for periods of up to 3 years, it demonstrates that in the normal subject the liver does not undergo a radical change following splenectomy and take over the function which is subserved by the spleen when present.

The other occasions, when a significant early uptake by the liver was detected, were in 3 of the patients with splenomegaly, namely, one each with Hodgkin's disease, cirrhosis of the liver and myelofibrosis. Liver uptake in the last was even more marked following splenectomy for a haemolytic state. These results show a hepatic alteration in such instances as the reagent used, the NEM treated cells, passes

without restriction through the liver in normal subjects. The change in the liver is apparently one involving the vascular and reticulo-endothelial architecture. This must be induced by the particular pathological process affecting the organ for as described above, it is not brought about by removing the spleen. Also it is not only the result of liver enlargement for although the three subjects under discussion did have hepatomegaly, in seven others no liver uptake of mildly damaged cells could be detected despite often marked increase in hepatic size.

The sequestration of cells in the liver in these cases cannot be explained on the grounds of unrecognized, more severe damage to a proportion of the erythrocytes. If such were the case the clearance of radioactivity would be characterized by a biphasic exponential clearance reflecting the presence of a double population of cells. The clearance and uptake by the liver would also show a half time of only a few minutes rather than that of at least an hour seen in these cases.

Corticosteroid hormones, in the form of prednisolone, caused a reduction in the sequestering function of the spleen in cases with splenomegaly, as measured by the rate of removal of cells mildly damaged with NEM. Various mechanisms have been postulated, and investigated, to explain the beneficial effects of such agents in patients with haemolytic disease. These exclude an action as a bone

marrow stimulant, a reduction in antibody production in cases of auto-immune haemolytic anaemia, an interference with antigen-antibody union with protection of red cell integrity, or a depression in the ability of the reticulo-endothelial system to remove abnormal cells. The results in these studies show that the last of these may well be at least partly responsible for the response in such cases. Steroids lead to a reduction in the phagocytic efficiency of the reticulo-endothelial system of the spleen. This is most probably brought about by a disruption in the normal splenic tissue for steroids have been shown to produce splenic atrophy in mice (Molomut et alii, 1958) and rabbits (Bjørneboe et alii, 1951; Baker et alii, 1951). That this is the probable mode of action is also supported by the finding that steroid treatment produced a reduction in the size of the spleen. This effect was marked in the two cases studied before and after steroid therapy. In one instance the spleen shrank from extending 4 centimetres to being palpable only 1 centimetre below the left costal margin. In the second case the spleen became impalpable during the course of drug treatment. Coleman and Finch in 1956 reported a reduction in the rate of haemolysis in hereditary spherocytosis during corticosteroid therapy. This they attributed to the fact that the steroids interfered with red cell destruction by the spleen.

The clearance and site of sequestration of NEM-treated cells was

compared with those of autologous untreated erythrocytes to examine two possible relationships. Firstly, to see if the survival time of altered cells prone to splenic removal bore any constant relation to the life of autologous untreated cells in haemolytic disease; secondly, to decide if the site of uptake of such altered cells corresponded to that of autologous cells in such syndromes. Only two such studies were carried out utilizing heat treated cells. Because a homogeneous population of cells could not be consistently prepared this investigation was not continued. The results of the two studies were shown in Table VI but it was not possible to draw any conclusions along the lines indicated above from these results.

In 9 subjects however autologous and NEM cell survival studies were compared (Table XIV). There was no correlation between autologous cell life and NEM cell survival time in these studies. In 5 patients haemolytic anaemia was present as indicated by a shortened erythrocyte life span. However in only 2 of these was the half time of the NEM treated cells also reduced. In one with myelofibrosis the NEM cell life was prolonged above normal. The significance of this has been previously discussed. In two others treatment with steroids was probably responsible for an NEM cell life longer than that seen in normal subjects. Also in the remaining 4 subjects where haemolytic anaemia was not present (autologous red cell life was normal) there was no correlation between the survival of the two cell

types. Both a prolonged and a shortened NEM cell life was experienced in these cases.

Therefore it can be concluded that the utilisation of cells such as these treated by low concentrations of NEM are of no value in the prediction of the autologous red cell life in haemolytic syndromes. This finding is probably to be anticipated for the rate of clearance of treated cells, that are removed by the spleen, could not be expected to reflect that of autologous cells that are being destroyed in different parts of the reticulo-endothelial system, or by intra-vascular lysis.

However there is a definite relationship between the site of uptake of NEM and autologous cells in haemolytic states for in the 5 cases with haemolysis these sites corresponded. The accuracy of this prediction could be confirmed in two subjected to splenectomy. Thus in one (B.H.) both autologous and NEM cell studies pointed to the liver and spleen as the areas of red cell destruction. Splenectomy confirmed this for following operation the haemolysis continued and a later study using NEM cells showed an even more pronounced hepatic uptake. In the second (R.C.) where NEM and autologous cells were both removed only by the spleen, splenectomy brought about a dramatic remission in the haemolysis.

Thus in a patient with haemolytic anaemia a study of the survival of mildly NEM damaged cells will not in most cases provide an estimate

of autologous red cell life but may provide information leading to the detection of the most important site of red cell destruction.

When red cells or foreign particles are removed from the circulation a factor which may influence the clearance rate is the number of particles administered. When colloids are cleared exponentially by the reticulo-endothelial system, while with small volumes the clearance rate is constant, with increasingly larger doses the rate declines as the immediate uptake capacity of the reticulo-endothelial system is exceeded. Similarly, when erythrocytes are used to measure splenic sequestration, it is possible that an error could be introduced if large volumes of cells are injected.

Dobson and Jones (1952) studied the clearance of colloidal chromium phosphate which is largely removed by the liver. The rate of uptake of small doses was constant and dependent on the magnitude of liver blood flow. The slower clearance rate of large quantities was presumably due to the fact that there were too many particles for the phagocytes of the liver to handle. A similar finding was reported by Benacerraf et alii (1957) when they showed that the rate of clearance was inversely proportional to the dose of colloid injected. Bioszi et alii (1958) used heat denatured, radioactive iodine labelled human serum albumin to measure liver blood flow in man and found that the clearance rate was exponential and reproducible below a certain dose. Only above this dose did the clearance rate vary inversely with the

amount of colloid injected. Ilo and Wagner (1963) confirmed these findings in dog and man and termed the maximal rate, before a decline due to an increasing dose became apparent, the "phagocytic capacity of the reticulo-endothelial system" in respect of the particular colloid used.

A similar phenomenon has been demonstrated in the case of the clearance of erythrocytes in experimental animals for Miescher (1956) showed that guinea pig red cells, reduced non-viable by storage, were cleared at a rate which was inversely proportional to the volume of cells given. The same principles applied in the clearance of formaldehyde treated cells in these animals (Miescher, 1957). Also when pigeon red cells were injected into mice most of the cells were taken up in the liver and the rate of clearance declined with increasing doses (Halpern et alii, 1957). Similar results are reported in man and Noyes et alii (1960) studied the clearance of non-viable (stored) erythrocytes utilising radioactive iron labelling. They gave doses of 0.01 to 1.5 ml. per kilogram body weight and, though these studies did not define the maximal capacity for the disposal of such cells, they demonstrated that with the higher doses there was the same inverse relationship between the dose of cells and the rate of clearance. These cells were shown by surface counting to be removed by the liver and spleen. Bowman et alii (1961) injected a potent incomplete antibody into a 79 kg. man and found that 421 ml.

of red cells (5.3 ml. per kilogram) were destroyed in 24 hours. As the antibody was given in excess and all the red cells were thus coated this destruction rate presumably reflects the maximal uptake capacity of the reticulo-endothelial system for this type of abnormal cell. The site of sequestration of the cells in this case was not reported.

In animal experiments (Mollison, 1962) it has been shown that small doses of red cells, approximately 0.03 ml. per kg., are completely removed from the circulation of rabbits in a few minutes, presumably by the total reticulo-endothelial system. When large volumes of up to 10 ml. were given to these animals approximately 2.5 ml. were quickly cleared. Mollison estimated that as this is equivalent to approximately 50 ml. of red cells in man, about 0.7 ml. per kilogram can be rapidly removed from the circulation in humans. Also Mollison (1961) describes volumes of 22 ml. and 1 ml. of incompatible blood disappearing with a half time of 2 to 4 minutes, and he further calculates that the maximum capacity of the reticulo-endothelial system in humans is of the order of 300 to 400 ml. of red cells per day.

None of these studies however define the maximal uptake capacity of the spleen as the cells given are not removed only by this organ and the half times of 2 to 4 minutes mentioned above are those recorded when the liver and the reticulo-endothelial system as a whole are

responsible for the uptake. Thus although volumes of cells of the order of 20 to 50 ml. can be rapidly removed without saturation of the immediate uptake capacity of the general reticulo-endothelial system, presumably much smaller volumes would saturate the capacity if only the spleen is responsible for the sequestration of cells.

The present study shows that there is no correlation between the rate of splenic uptake and the dose of cells given with volumes between 0.052 and 0.073 ml. per kiligram in normal subjects and between 0.035 and 0.128 ml. per kilogram in patients with splenomegaly. Thus no error has been introduced by the use of volumes of cells which exceed the immediate uptake capacity of the spleen. The phagocytic capabilities of the spleen could be defined by further work utilizing larger volumes of mildly NEM-damaged cells.

When chromium-51 labelled erythrocytes are circulating in man and noradrenaline is administered, rises in the peripheral haematocrit and blood radioactivity have been used as indicators of an increase in red cell mass. Thus such changes are claimed to show mobilization of red cells from areas in the body not in direct continuity with the peripheral blood. Also an associated fall in surface activity over the spleen is used as proof that such cells are expelled from a "pool" in the spleen in cases of splenomegaly (Prankerd, 1963; Toghill, 1964).

However, the findings reported in this study in normal subjects demonstrate that such changes cannot be always so interpretated.

Firstly, increases in these parameters cannot entirely be due to splenic expulsion of blood as they occur in the absence of the spleen. Secondly, the magnitude of the changes seen in spleen intact normal subjects far exceeds that possible due to the splenic expulsion of red cells. The spleen, in normal subjects, only contains 20 ml. of red cells (Motulsky, 1958; Prankerd, 1964) and therefore even if all the cells were expelled into the general circulation this would not cause a rise in haematocrit of even 1 per cent, the total red cell mass being approximately 2200 to 2300 ml. (Gibson et alii, 1946; Gray and Sterling, 1953). As the rise in haematocrit in normal subjects with and without the spleen ranged from 4.4 to 9.4 per cent another mechanism apart from the splenic expulsion of cells must be operating. An explanation of this phenomenon is however provided by the finding that noradrenaline produces a reduction in plasma volume (Lucia et alii, 1937; Ebert and Stead, 1941a; Kaltreider et alii, 1942). Finnerty et alii (1958) found that this reduction in plasma volume caused a rise in haematocrit of 8 per cent in humans, a magnitude of alteration comparable with that seen in these studies. Thus these factors must be taken into consideration in relating such changes to splenic expulsion of erythrocytes.

Also the fall in count rate over the spleen cannot always be assumed to reflect the expulsion of active cells from this organ. The fall in activity over the spleen when noradrenaline is given to normal

subjects in whom labelled normal red cells are circulating may be partly due to this phenomenon. Thus the expulsion of some of the contained 20 ml. of blood would lower the count rate to a degree but such a fall is also seen where no activity is expelled into the general circulation. When the spleen has sequestered labelled NEM treated cells, the surface activity falls despite the fact that no activity, reflecting cellular expulsion, is detectable in the peripheral blood. If the ejection of red cells is not the mechanism of the fall in surface count rate in these cases it is presumably due to a movement of the spleen away from the detecting probe. The postulate that splenic movement is the cause of this change is supported by the finding of a rise in the splenic count rate on deep inspiration despite continuing noradrenaline infusion. This manoeuvre, known to cause descent of the spleen, caused the counts to rise almost to the pre-infusion level. Also after the infusion a similar deep breath caused only a slight increase. This demonstrates that movement of this organ can markedly alter the apparent activity, as measured by surface counting, and that this is the mechanism of such a change in many cases when noradrenaline is given. This explanation also accounts for the finding in one subject where noradrenaline caused a rise in spleen surface activity. This rise could not have been due to a further uptake of labelled cells under the influence of noradrenaline as at the time of the experiment all active NEM-treated cells were sequestered in the spleen and no circulating cells were

available for further trapping. The rise must therefore have been due to splenic movement causing a repositioning of the organ more directly beneath the detecting probe.

Therefore these studies demonstrate that in the assessment of the expulsion of cells from the spleen under the influence of noradrenaline, account must be taken of the haematocrit change and splenic movement produced by this agent. Not all the rise in peripheral haematocrit can be interpreted as indicating a rise in red cell mass and neither can a fall in splenic count rate be looked upon as reflecting only the expulsion of labelled cells from the spleen.

In certain cases of splenomegaly (Prankerd, 1964) the spleen does contain much larger volumes of blood a significant proportion of which can be expelled. This was demonstrated in the case in this series where noradrenaline produced a rise in the haematocrit of 21.6 per cent, far in excess of that due to the reduction in plasma volume in the normal subjects. Also at operation in this subject noradrenaline gave a rise in the haematocrit in the splenic vein of 11 per cent and this was associated with visible splenic contraction.

The findings utilizing mildly damaged NEM cells do not support the claims of Prankerd (1963) and Toghill (1964) that abnormal cells when removed from the circulation by the spleen are sequestered in a pool from which they can be expelled again into the general circulation by noradrenaline. Thus if noradrenaline was given to subjects while such cells were being cleared, the rise in haematocrit and blood

activity was only of such a magnitude as could be accounted for by the reduction in plasma volume discussed above. If in such cases active cells were being squeezed out of the spleen, one would expect a far greater increase in activity than that actually experienced. Also for the reasons given the fall in splenic count rate in these cases could not be interpreted as being solely due to such expulsion. Even more conclusively, it was found that if sufficient time was permitted to elapse so that all the active cells had been taken up in the spleen, no activity at all was mobilized into the peripheral blood. This was demonstrated in one case at the time of splenectomy. Also it was not possible to confirm, as claimed by Toghill (1964) that prior injection of noradrenaline would eradicate such a potential pool. In these studies the infusion did not prevent the subsequent specific removal by the spleen of the treated cells. It must be concluded therefore that while "pooling" of erythrocytes may occur in certain cases of splenomegaly, abnormal cells are not sequestered in such a compartment in the normal spleen and that once removed, they cannot be mobilized into the circulation by noradrenaline.

CHAPTER VIII

SUMMARY

In this study answers were sought to certain questions pertaining to the destruction of red cells by the spleen in man. These problems and the conclusions reached are broadly summarised below.

Do erythrocytes sequestered by the spleen manifest either a constant morphological appearance or a constant alteration in osmotic fragility? The investigations performed using heat or sulphydryl inhibition as the means of inducing red cell change establish that no such simple relationship exists. The spherocytic less fragile cells produced by heat treatment and the discoid less fragile type resulting from sulphydryl inhibition are, like the fragile spherocytic cells of hereditary spherocytosis, trapped in the spleen. Therefore a more subtle alteration in the erythrocyte membrane is probably the governing factor. Although red cell agglutination may predispose to hepatic trapping a similar less obvious surface change is probably operative when the liver is the major site of sequestration. As the heated spherocyte is more, and not less, resistant to osmotic rupture these studies also highlight the fact that erythrocyte morphology cannot be predicted merely from an examination of cellular osmotic fragility. With respect to the anaemia in cases of severe burns, it is suggested that erythrocytes are damaged at the site of thermal injury and subsequently trapped in the organs of the reticulo-endothelial system. This sequence probably accounts for the fall in haemoglobin in such instances.

Is it possible to measure the sequestering function of the spleen by studying the clearance rate of slightly altered erythrocytes? These investigations show that this is indeed possible using cells treated with a sulphydryl inhibitor though an accurate quantitation is not possible if heat damaged erythrocytes are utilized. With the former preparation the normal uptake function of the spleen has been delineated and it is shown that, whereas in most cases splenomegaly is associated with an increase in this parameter, in some cases, most strikingly in the condition of myelofibrosis, an enlarged spleen may be one with a reduced red cell sequestering function. Also even when increased this is not directly related to splenic size alone. The liver may in some cases manifest a "spleen-like" action and remove a proportion of the mildly damaged cells but the hepatic alteration in such cases is not merely brought about by an increase in the size of the organ, for in several instances hepatomegaly is not accompanied by the trapping of the cellular reagent. Results in this section relating to the dose of cells administered to the rate of splenic uptake in normal subjects show that with volumes up to 0.073 ml. red cells per kilogram body weight, there is no decline in the sequestering ability of the spleen. Therefore the immediate uptake capacity of the spleen is in excess of this quantity and larger volumes of such cells would have to be used to define the maximal phagocytic capabilities of the organ.

Can the measurement of splenic sequestration by such means be used to predict the value of splenectomy in subjects with haemolytic disease? There is no correlation between the life span of altered cells and that of autologous untreated cells in a particular subject and therefore the former preparation cannot be used to establish the presence of haemolysis. However the sites of sequestration of the two cellular preparations do correspond and the results in the cases reported make it likely that such an investigation may permit one to detect the major site of red cell destruction in a haemolytic state and predict more accurately the outcome of splenectomy.

In what way do corticosteroid hormones affect splenic sequestering function? The present investigations establish that these agents reduce the uptake capacity of the spleen for altered red cells. Though other actions of such steroids were not studied it is suggested that at least part of the benefit in patients with haemolytic disease is due to this role in depressing the ability of the reticulo-endothelial system to remove abnormal cells.

Are abnormal cells, when taken up in the spleen, retained in a "pool" from which they can be expelled by vasoconstrictor agents? It is conclusively demonstrated that the infusion of a vasoconstrictor, noradrenaline, does not prevent the splenic uptake of abnormal cells which are normally sequestered in this organ. Also though "pooling"

of untreated cells may take place in some cases associated with splenomegaly, abnormal cells are not trapped in such a compartment in the normal subject. Once taken up by the spleen the subsequent injection of noradrenaline will not bring about their reappearance in the general circulation.

This summary embraces the areas of the present study where it is believed a contribution has been made to present knowledge. As outlined previously certain problems raised in the course of the investigation are still unsolved. Amongst these are the ultimate nature of the erythrocyte surface changes governing organ selection of cells, the nature of the splenic and hepatic alterations that bring about enhanced sequestration, the magnitude of the maximal uptake capacity of the spleen as well as confirmation of the reliability in predicting the value of splenectomy by the study of the site of uptake of altered cells. It is hoped that there will be an opportunity to further study some of these problems in the future.

APPENDICES

APPENDIX A

Subjects Studied

I. Control subjects who underwent studies of the survival of heated red cells.

Study No.	Name	Sex	Age	Diagnosis	Source*
1	Humphreys T.	M	28	Normal	YLP
2	Virgo I.	M	16	Appendicectomy	RAH (No. 013158)
3	Chadwick D.	M	20	Normal	YLP
4	Cook P.	M	18	Normal	YLP
5	Pearce D.	M	30	Normal	YLP
6	Howard M.	M	21	Normal	YLP
8	Krants R	M	22	Normal	YLP
9	Dimitrescu A.	M	42	Normal	YLP
10	Ryan R.	M	21	Normal	YLP
11	Hill C.	M	30	Hysteria	RAH (No. 011010)
13	Morgan F.	M	37	Dyspepsia	RAH (No. 011931)
21	Stepney M.	M	20	Normal	YLP
22	Haynes L.	M	22	Peptic ulcer	RAH (No. 002362)
26	Donnelly L.	M	29	Normal	YLP
27	Harrison N.	M	30	Normal	YLP

*Abbreviations: YLP = Yatala Labor Prison
RAH = Royal Adelaide Hospital

28	Cowan C.	M	24	Normal	YLP

II. Control subjects who underwent studies of the clearance of labelled haemoglobin.

7	Sherrif D.	M	29	Normal	YLP
12	Buffet W.	M	54	Peptic ulcer	RAH (No. 000612)

III. Control subjects who underwent studies of the survival of NEM-treated red cells.

14	Neville B.	M	29	Appendicectomy	RAH (No. 010693)
15	Stockman M.	M	47	Dyspepsia	RAH (No. 010016)
34	Geesing M.	M	30	Normal	YLP
36	Carson L.	M	49	Normal	YLP
38	Wellburn F.	M	22	Normal	YLP
39	Heeps F.	M	44	Normal	YLP
40	Burgess D.	M	33	Normal	YLP
42	Chapman B.	M	18	Normal	YLP
46	Maynard D.	M	19	Normal	YLP
48	Joseph K.	M	25	Normal	YLP
49	Bebb R.	M	27	Normal	YLP
52	McMonagle J.	M	27	Normal	YLP
59	Merrifield D.	M	27	Normal	YLP
60	Mulvihill J.	M	18	Normal	YLP

IV. Control subjects on whose blood additional morphological and osmotic fragility studies were performed.

Study No.	Name	Sex	Age	Diagnosis*	Source
96	Kimber R.	M	34	Normal	Dept. of Med.
97	Kimber C.	F	26	Normal	Dept. of Med.
98	Lander H.	M	36	Normal	Dept. of Med.
99	Davey M.	M	27	Normal	Dept. of Med.
100	Hannam W.	F	54	Post-op.	RAH (No. 014994)
101	Howarth J.	M	56	Post-op.	RAH (No. 031669)
102	Hay P.	M	20	Post-op.	RAH (No. 058577)
103	Nedelcev T.	M	46	Post-op.	RAH (No. 000690)
104	Underwood L.	M	68	Post-op.	RAH (No. 007812)
105	Thorpe H.	M	70	Post-op.	RAH (No. 001838)
106	Evans I.	M	16	Post-op.	RAH (No. 013199)
107	De Palma T.	M	20	Post-op.	RAH (No. 005783)

*Abbreviation: Post-op. = convalescing following minor operations.

V. Subjects who had been submitted to splenectomy.

Study No.	Name	Sex	Age	Reason for Operation	Source
16	Atkinson M.	M	24	Trauma	Volunteer
17	Milera C.	M	49	Trauma	Volunteer
18	Beveridge W.	M	29	Trauma	Volunteer
20	Schoeroft F.	F	49	Trauma	Volunteer
23	Eusden K.	M	42	Trauma	YLP
24, 47	Kalnins R.	M	21	Trauma	YLP
29	Fahey A.	M	54	H.S.*	RAH (No. 015064)
62	Christie F.	F	49	D.L.E.*	RAH (No. 010823)
71	Stephens W.	M	74	Lymphosarcoma	RAH (No. 001416)

---

*Abbreviations:

H.S. = Hereditary spherocytosis.

D.L.E. = Disseminated lupus erythematosus and thrombocytopenia.

VI. Subjects with haematological disease.

Study No.	Name	Sex	Age	Diagnosis	Source
25	Cavallaro E.	M	21	Thalassaemia minor	YLP
30	Bartley E.	F	54	AHA (in remission)*	RAH (No.003220)
31	Sleader N.	M	66	Myelofibrosis	RAH (No.009927)
33	Spezzano P.	M	2	AHA (idiopathic)*	ACH
35	Ferrone A.	M	41	Thalassaemia minor	YLP
37	Rovadetti V.	M	62	Chr. lymph. leuk.	RAH (No.002373)
43	Ktisti G.	M	22	Thalassaemia minor	YLP
44	Ringwood M.	F	72	AHA (idiopathic)*	RAH (No.017657)
45	Seidel A.	M	35	Cirrhosis of liver	RAH (No.021336)
50	Richards E.	M	52	Cirrhosis of liver	RAH (No.024406)
51	McKay J.	M	52	Lymphosarcoma	RAH (No.023729)
53	Thomson M.	F	48	Felty's syndrome	RAH (No.024355)
54	Saint, L.	F	56	Myelofibrosis	RAH (NO.023366)
55	Whittington A.	M	61	Splenomegaly	RAH (No.026750)
56,59	Howe B.	F	50	Myelofibrosis	RAH (No.002374)
57	Carosi J.	M	24	Hodgkin's disease	RAH (No.027180)
58	Holman M.	F	68	Hodgkin's disease	RAH (No.023767)
61	Clugstone R.	M	68	AHA (idiopathic)*	RAH (No.011678)
63	Piercy L.	F	74	Hydatid cyst spleen	RAH (No.030335)
64,67	D'Andrea E.	M	56	Lymphosarcoma	RAH (No.007591)
65	Paraskavopolous	M	32	Chr. my. leuk*	RAH (No.022953)
66	Wedding C.	M	24	I.T.*	RAH (No.020303)
68	Randall E.	F	65	Pancreatic cyst	RAH (No.033701)
70, 72	Ward K.	F	57	Chr. lymph. leuk.*	RAH (No.017014)
73	Trainor L.	M	51	Acute leukaemia	RAH (No.038218)

*Abbreviations: A.C.H. = Adelaide Children's Hospital
A.H.A. = autoimmune haemolytic anaemia
Chr. lymph. leuk. = chronic lymphatic leukaemia
Chr. my. leuk. = chronic myeloid leukaemia
I.T. = idiopathic thrombocytopenia

VII. Subjects with haematological disease in whom autologous red cell life span was measured.

Study No.	Name	Haemolytic Disease
19	Sleader N.	Present
32	Spessane P.	Present
87	Revadetti V.	Absent
88	Helman M.	Present
89	Paraskevopelous M.	Present
90	Saint L.	Absent
91	Howe B.	Present
92	D'Andrea E.	Absent
93	Clugstone R.	Present
94	Ringwood M.	Present
95	McKay J.	Absent

VIII. Subjects on whom noradrenaline infusion studies were performed.

Study No.	Name	Sex	Age	Diagnosis	Source
74	Johnson A.	M	36	Peptic ulcer	RAH (No.039062)
75	Thomson M.	F	48	Felty's syndrome	RAH (No.024355)
76,83	Clugstone R.	M	68	A.H.A.	RAH (no.011678)
77	D'Andrea E.	M	56	Lymphosarcoma	RAH (No.007591)
78	Taylor H.	M	35	Lung abscess	RAH (No.026053)
79	Pawlowski I.	M	43	Peptic ulcer	RAH (No.043679)
80	Rankine J.	M	40	Alcoholism	RAH (No. 044406)
81	Merrifield D.	M	27	Normal	YLP
82	Stephens W.	M	74	Splenectomy	RAH (No. 001416)
84	Trainor L.	M	51	Acute leukaemia	RAH (No. 038218)
85	Lewis B.	F	37	Normal	Dept. Med.
86	Mulvihill J.	M	18	Normal	YLP

APPENDIX B

The University of Adelaide

Department of Medicine

Telephone
8-3211

May 7, 1963

Dear

The University Department of Medicine at the Royal Adelaide Hospital is at present conducting research into anaemia and we are anxious to perform certain tests on normal people who have had their spleens removed. The records at the hospital have your name listed as one who has had this operation performed and I should like to discuss with you the possibility of your participation in these tests.

The procedure is not long or involved and would necessitate spending only part of a day at this department with several brief visits, during which blood samples are taken, over the succeeding few days. Expenses incurred by you in travelling would be paid by the University.

I would appreciate it very much if you could let me know, either by ringing me at the University Department of Medicine, 8 3211 extension 425, or by writing to me at this address, if you are willing

to take part. I could then arrange for you to come and see me and I could then explain more fully what these tests entail.

Yours sincerely,

Richard J. Kimber,
Lecturer in Medicine.

REFERENCES

Antopol, W. (1950). Anatomic changes produced in mice treated with excessive doses of cortisone. Proc. Soc. exp. Biol. & Med. 73, 262.

Ashby, W. (1919). The determination of the length of life of transfused blood corpuscles in man. J. exp. Med. 29, 267.

Ashby, W. (1921a). Studies of transfused blood. I. The periodicity in elimination activity shown by the organism. J. Exp. Med. 34, 127.

Ashby, W. (1921b). Studies of transfused blood. II. Blood destruction in pernicious anaemia. J. exp. Med. 35, 147.

Aub, J.C., Fairhall, L.T., Minot, A.S. and Reznikoff, P. (1925) Lead poisoning. Medicine 4, 1.

Baikie, A.G. and Pirie R. (1958). The effect of ACTH and cortisone in experimental haemolytic anaemias in guinea pigs. Studies on anaemias due to heterologous anti-red cell serum and on the anaemia of chronic lead poisoning. Scot. med. J. 3, 264.

Bailey, N.T.J. Statistical Methods in Biology. The English Universities Press Ltd. London. 1959.

Baker, D.L., Ingle, D.J. and Li, C.H. (1951). The histology of the lymphoid organs of rats treated with adrenocorticotrophin. Am. J. Anat. 88, 313.

Barcroft, J. (1925). Recent knowledge of the spleen. Lancet 1, 319.

Barcroft, J., Harris, H.A., Orahovats, D. and Weiss, R. (1925). A contribution to the physiology of the spleen. J. Physiol. (Lond.) 60, 443.

Benacerraf, B., Biozzi, Halpern, B.N. and Stiffel, C. Physio-pathology of the Reticulo-endothelial System (a symposium). Blackwell Scientific Publications. Oxford, 1957, p.52.

Berendes, M. (1959). The proportion of reticulocytes in the erythrocytes of the spleen as compared with those of the circulating blood, with special reference to hemolytic states. Blood 14, 558.

Berlin, N.I., Lawrence, J.H. and Lee, H.C. (1951). The life span of the red blood cell in chronic leukaemia and polycythaemia. Science 114, 385.

Berlin, N.I., Waldmann, T.A. and Weissman, S.M. (1959). Life span of the red blood cell. Physiol. Rev. 39, 577.

Berlin, R. (1950). Estimation of life span of erythrocytes by determination of reticulocyte maturation time in vivo. Scandinav. J. Clin. and Lab. Invest. 2, 27.

Bern, H.A. (1963). Effects of corticoids on the spleen of the Cichlid fish, Tilapia mossambica. Proc. Soc. exp. Biol. & Med. 112, 805.

Best, W.R., Limarzi, L.R. and Poncher, H.G. (1951). Acquired hemolytic anemia treated with corticotrophin. J. Amer. med. Ass. 147, 827.

Beutler, E. (1959). The hemolytic effect of Primaquine and related compounds: A review. Blood 14, 103.

Beutler, E., Dern, R.J., Flannagan, C.L. and Alving, A.S. (1955). The hemolytic effect of Primaquine. VII. Biochemical studies of drug-sensitive erythrocytes. J. Lab. clin. Med. 45, 286.

Bierman, H.R., Byron, R.L. and Kelly, K.H. (1953). Role of the spleen in leukocytosis following intra-arterial administration of epinephrine. Blood 8, 153.

Bilbey, D.L.J. and Nicol, J. (1958). Effects of various natural steroids on the phagocytic activity of the reticulo-endothelial system. Nature (Lond.) 182, 674.

Biozzi, G., Benacerraf, B., Halpern, B.N., Stiffel, C. and Hillemand, B. (1958). Exploration of the phagocytic function of the reticulo-endothelial system with heat-denatured human serum albumin labeled with I^{131} and application to the measurement of liver blood flow in normal man and in some pathological conditions. J. lab. clin. Med. 51, 230.

Björkman, S.E. (1947). The splenic circulation. Acta Med. Scandinav. Supplement CXCI, 128.

Bjørneboe, M. Fischel, E.E. and Stoerk, H.C. (1951). The effect of cortisone and adrenocorticotrophic hormone on the concentration of circulating antibody. J. exp. Med. 93, 37.

Blackburn, C.R.B. (1949). The role of lead in the reduction of the erythrocyte sedimentation rate by hyaluronidase. J. biol. Chem. 178, 855.

Bloch, E.H. and Piovella, C. (1956). Microscopic observations of the acute effects on the extra and intra-hepatic circulation produced by anti-red cell sera. Anat. Rec. 121, 459.

Boorman, K.E., Dodd, B.E. and Loutit, J.F. (1946). Haemolytic icterus (acholuric jaundice), congenital and acquired. Lancet 1, 812.

Bowdler, A.J. (1962). Theoretical considerations concerning measurement of the splenic red cell pool. Clin. Science 23, 181.

Bowman, H.S., Brason, F.W., Mohn, J.F. and Lambert, R.M. (1961). Experimental transfusion of donor plasma containing blood group antibodies into incompatible normal human recipients. II. Induction of iso-immune haemolytic anaemia by a transfusion of plasma containing exceptional anti-CD antibodies. Brit. J. Haemat. 7, 130.

Brown, G.M., Hayward,O.C., Powell, E.O. and Witts, L.J. (1944). The destruction of transfused erythrocytes in anaemia. J. Path. Bact. 56, 81.

Burkhardt (1905) cited by Shen, S.C., Ham, T.H. and Fleming, E.M. (1943). New. Engl. J. Med. 229, 701.

Callender, S.T.E., Nickel, J.F., Moore, C.V. and Powell, E.O. (1949). Sickle cell disease: studied by measuring the survival of transfused red blood cells. J. lab. clin. Med. 34, 90.

Callender, S.T.E., Powell, E.O. and Witts, L.J. (1945). The life span of the red blood cell in man. J. Path. Bact. 57, 129.

Callender, S.T.E., Powell, E.O. and Witts, L.J. (1947). Normal red cell survival in men and women. J. Path. Bact. 59, 519.

Carson, P.E. and Tarlov, A.R. (1962). Biochemistry of hemolysis. Ann. Rev. Med. 13, 105.

Castle, W.B. and Daland, G.A. (1937). Susceptibility of erythrocytes to hypotonic hemolysis as a function of discoid form. Amer. J. Physiol. 120, 371.

Castle, W.B., Ham, T.H. and Shen, S.C. (1950). Observations on the mechanism of hemolytic transfusion reactions occurring without demonstrable hemolysis. Trans. Assoc. Amer. Physic. 63, 161.

Chaplin, H. Jr. (1959). Studies on the survival of incompatible cells in patients with hypogammaglobulinaemia. Blood 14, 24.

Chaplin, H. Jr. and Mollison, P.L. (1953). Red-cell life span in nephritis and in hepatic cirrhosis. Clin. Science 12, 351.

Chatterjea, J.B., Dameshek, W. and Stefanini, M. (1953). The adrenaline (epinephrine) test as applied to hematologic disorders. Blood 8, 211.

Chauffard, M.A. (1907) cited by Dameshek, W. and Schwartz, S.O. (1940). Medicine 19, 231.

Chauffard, M.A. and Troisier, J. (1908) cited by Dameshek, W. and Schwartz, S.O. (1940) Medicine 19, 231.

Chertkow, G. and Dacie, J.V. (1956). Results of splenectomy in auto-immune haemolytic anaemia. Brit. J. Haemat. 2, 237.

Clearkin, K.P. (1952). Effect of adrenocorticotrophic hormone on clinical and experimental anaemia. Lancet 1, 183.

Cohen, J.A. and Warringa, M.G.P.J. (1954). Fate of P^{32} labeled diisopropylfluorophosphonate in the human body and its use as a

labeling agent in study of turnover of blood plasma and red cells. J. clin. Invest. 33, 459.

Cole, W.H., Walter, L. and Limarzo, L.R. (1949). Indications and results of splenectomy. Ann. Surg. 129, 702.

Coleman, H.D. and Finch, C.A. (1956). Effect of adrenal steroids in hereditary spherocytic anemia. J. lab. clin. Med. 47, 602.

Coombs, R.R.A., Mourant, A.E. and Race, R.R. (1945). A new test for the detection of weak and "incomplete" Rh agglutinins. Brit. J. exp. Path. 26, 255.

Cooper, M. and Owen, C.A. Jr. (1956). Labeling human erythrocytes with radiochromium. J. lab. clin. Med. 47, 65.

Cooper, M. Stroebel C.F., Stickney, J.M. and Pwen, C.A. Jr. (1955). Radiochromium in the study of red cell survival and gastrointestinal bleeding. Fed. Proc. 14, 31.

Crane, M.G., Hollaway, J.E., Adams, R.M. and Woodward, I.C. (1960). The relative flow rates of red cells and plasma - peripheral and central circulation studies in dogs. Internat. J. appl. Radiation Isotopes 7, 23.

Crawford, H. and de Gruchy, G.C. (1958). The use of radioactive chromium (^{51}Cr) in clinical medicine. Med. J. Aust. 1, 657.

Creger, W.P., Tulley, E.H. and Hansen, D.G. (1956). A note on the effect of hydrocortisone on the microelectrophoretic characteristics of human red cell antibody unions. J. lab. clin. Med. 47, 686.

Crepea, S.B., Magnin, G.E. and Seastone, C.V. (1951). Effect of ACTH and cortisone on phagocytosis. Proc. Soc. exp. Biol. Med. 77, 704.

Crome, P. and Mollison, P.L. (1964). Splenic destruction of Rh-sensitized and of heated red cells. Brit. J. Haemat. 10, 137.

Crosby, W.H. (1950). Hereditary non-spherocytic hemolytic anemia. Blood 5, 233.

Crosby, W.H. (1952). The pathogenesis of spherocytes and leptocytes (target cells). Blood 7, 261.

Crosby, W.H. (1955). The metabolism of hemoglobin and bile pigments in hemolytic disease. Amer. J. Med. 18, 112.

Crosby, W.H. (1959). Normal functions of the spleen relative to red blood cells. A review. Blood 14, 399.

Crosby, W.H. and Akeroyd, J.H. (1952). The limit of hemoglobin synthesis in hereditary hemolytic anemia. Its relation to the excretion of bile pigment. Amer. J. Med. 13, 273.

Crosby, W.H. and Rappaport, H. (1957). Auto immune hemolytic anemia. I. Analysis of hematologic observations with particular reference to their prognostic value. A survey of 57 cases. Blood 12, 42.

Cruickshank, E.W.H. (1926). On the output of haemoglobin and blood by the spleen. J. Physiol. (Lond.) 61, 455.

Cutbush, M. and Mollison, P.L. (1958). Relation between characteristics of blood-group antibodies in vitro and associated patterns of red cell destruction in vivo. Brit. J. Haemat. 4, 115.

Dacie, J.V. Practical Haematology. J. & A. Churchill Ltd. London, second edition, 1956, (a) p. 38, (b) p. 94 and (c) p. 33.

Dacie, J.V. The Haemolytic Anaemias. Congenital and Acquired. Part I The Congenital Anaemias. J. & A. Churchill Ltd. London, second edition, 1960.

Dacie, J.V. The Haemolytic Anaemias. Congenital and Acquired. Part II The Auto-immune Haemolytic Anaemias. J. & A. Churchill Ltd. London, second edition, 1962.

Dacie, J.V. (1963). Prognosis in auto-immune haemolytic anaemia. Austral. Ann. Med. 12, 11.

Dacie, J.V. and Chertkow, G. (1955). Splenectomy in auto-immune haemolytic anaemia. Trans. Assoc. Amer. Phys. 68, 126.

Dacie, J.V. and de Gruchy, G.C. (1951). Autoantibodies in acquired haemolytic anaemia. J. clin. Path. 4, 253.

Dacie, J.V. and Mollison, P.L. (1943). Survival of normal erythrocytes after transfusion to patients with familial haemolytic anaemia. Lancet 1, 550.

Dacie, J.V. and Mollison, P.L. (1949). Survival of transfused erythrocytes from a donor with nocturnal haemoglobinuria. Lancet 1, 390.

Dameshek, W. Acquired Hemolytic Anemia. Physiopathology with particular reference to autoimmunization and therapy. In Proc. 3rd internat. Congress internat. Soc. Hemat. Cambridge, England. Heinemann, London, 1950, p. 120.

Dameshek, W. (1950). ACTH in leukemia. Blood 5, 791.

Dameshek, W. (1952). Haematological applications of ACTH and cortisone. Brit. med. J. 2, 612.

Dameshek, W. (1955). Hemolytic anemia. Direct and indirect indications, pathogenic mechanisms and classifications. Amer. J. Med. 18, 315.

Dameshek, W. and Bloom, M.L. (1948). The events in the hemolytic crisis of hereditary spherocytosis with particular reference to the reticulocytopenia, pancytopenia and an abnormal splenic mechanism. Blood 3, 1381.

Dameshek, W. and Komninos, Z.D. (1956). The present status of treatment of autoimmune hemolytic anemia with ACTH and cortisone. Blood 11, 648.

Dameshek, W. and Rosenthal, M.C. (1951). The treatment of acquired hemolytic anemia. With a note on the relationship of periarteritis nodosa to hemolytic anemia. Med. Clin. N. Amer. 35, 1423.

Dameshek, W., Rosenthal, M.C. and Schwartz, L.I. (1951). The treatment of acquired hemolytic anemia with adrenocorticotrophic hormone (ACTH). New Engl. J. Med. 244, 117.

Dameshek, W. and Schwartz, S.O. (1940). Acute hemolytic anemia (acquired hemolytic icterus, acute type). Medicine (Baltimore) 19, 231.

Dausset, J. and Colombani, J. (1959). The serology and the prognosis of 128 cases of autoimmune hemolytic anemia. Blood 14, 1280.

Dausset, J. and Malinvaud, G. (1954). Les anémies hémolytiques acquises avec anti-corps. Evolution, pronostic et traitment d'après l'étude de 54 cas. Sem. Hôp. Paris 30, 3130.

Davidsohn, I. and Spurrier, W. (1954). Immunohematological studies in hemolytic anemia. J. Amer. med. Assoc. 143, 818.

Davidson, L.S.P., Duthie, J.J.R., Girdwood, R.H. and Sinclair, R.J.G. (1951). Clinical trials in haemolytic anaemia. Brit. med. J. 1, 657.

de Gruchy, G.C. (1954). The diagnosis and management of acquired haemolytic anaemia. Austral. Ann. Med. 3, 106.

de Gruchy, G.C. (1963). Red cell metabolism in congenital haemolytic anaemias. Austral. Ann. Med. 12, 6.

de Vries, J.A. (1950). The effect of adrenocorticotrophin hormone on circulating antibody levels. J. Immunol. 65, 1.

Dobson, E.L. and Jones, H.B. (1952). The behaviour of intravenously particulate matter. Acta med. Scandinav. Supplement 273, 1.

Documenta Geigy Scientific Tables. J.R. Geigy S.A., Basle. Fifth edition 1956.

Donohue, D.M., Motulsky, A.G., Giblett, E.R., Prizio-Biroli, G. Viramuvatti, V. and Finch, C.A. (1955). The use of chromium as a red cell tag. Brit. J. Haemat. 1, 249.

Dornhorst, A.C. (1951). The interpretation of red cell survival curves. Blood 6, 1284.

Dougherty, T.F. and White, A. (1947). An evaluation of alterations produced in lymphoid tissue by pituitary-adrenal cortical secretions. J. lab. clin. Med. 32, 584.

Dreyfus, B., Dausset, J. and Vidal, G. (1951). Étude clinique et hématologique de douze cas d'anémia hémolytique acquise avec anticorps. Rev. Hemat. 6, 349.

Eadie, G.S. and Brown, I.W. Jr. (1953). Red blood cell survival studies. Blood 8, 1110.

Eadie, G.S. and Brown, I.W. Jr. (1955). The potential life span and ultimate survival of fresh red blood cells in normal healthy recipients as studied by simultaneous Cr^{51} tagging and differential hemolysis. J. clin. Invest. 34, 629.

Ebaugh, F.G., Emerson, C.P. and Rodman, G.P. (1955). An evaluation of Na $^{51}Cr\ O_4$ as an agent for the determination of the erythrocyte life span, in vivo, in various hemolytic states. J. clin. Invest. 34, 931.

Ebaugh, F.G., Emerson, C.P. and Ross, J.F. (1953). The use of radioactive chromium-51 as an erythrocyte tagging agent for the determination of red cell survival in vivo. J. clin. Invest. 32, 1260.

Ebert, R.V. and Stead, E.A. Jr. (1941a). Demonstration that in normal man no reserves of blood are mobilized by exercise, epinephrine and hemorrhage. Amer. J. med. Sci. 201, 655.

Ebert, R.V. and Stead, E.A. Jr. (1941b). Demonstration that the red cell plasma ratio of blood contained in minute vessels is lower than that of venous blood. J. clin. Invest. 20, 317.

Ecklebe, G. and Sander, K. (1952). Der Ablauf der Immunoreaktionen unter ACTH bei experimenteller hämolytischer Anämie. Z. ges. exp. Med. 119, 388.

Emerson, C.P. (1954). The influence of the spleen on the osmotic behaviour and the longevity of red cells in hereditary spherocytosis (congenital hemolytic anemia). A case study. Bostom Med. Quart. 5, 65.

Emerson, C.P. Jr. and Burrows, B.A. (1949). The mechanism of anemia and its influence on renal function in chronic uremia. J. clin. Invest. 28, 779.

Emerson, C.P. Jr., Shen, S.C. and Castle, W.B. (1946). The osmotic fragility of the red cells of the peripheral and splenic blood in patients with congenital hemolytic jaundice transfused with normal red cells. J. clin. Invest. 25, 922.

Emerson, C.P. Jr., Shen, S.C., Ham, T.H. and Castle, W.B. (1947). The mechanism of blood destruction in congenital hemolytic jaundice. J. clin. Invest. 26, 1180.

Emerson, C.P. Jr., Shen, S.C., Ham, T.H., Fleming, E.M. and Castle W.B. (1956). Studies on the destruction of red blood cells. IX. Quantitative methods for determining the osmotic and mechanical fragility of red cells in the peripheral blood and splenic pulp; the mechanism of increased hemolysis in hereditary spherocytosis (congenital hemolytic jaundice) as related to the functions of the spleen. Arch. intern. Med. 97, 1.

Erlandson, M.E., Schulman, I., Stern, G. and Smith, C.H. (1958). Studies of congenital hemolytic syndromes. I. Rates of destruction and production of erythrocytes in thalassemia. Pediatrics 22, 910.

Etess, A.D., Bassen, F., Litwins, J. and Sussman, L.N. (1951). Acquired hemolytic anemia treated with ACTH prior to splenectomy. Acta Haemat. 6, 105.

Evans, R.S. and Duane, R. (1949a). Hemolytic anemias. Recent advances in diagnosis and treatment. California Med. 70, 244.

Evans, R.S. and Duane, R.T. (1949b). Acquired hemolytic anemia. I. The relation of erythrocyte antibody production to activity of the disease. II. The significance of thrombocytopenia and leukopenia. Blood 4, 1196.

Finch, C.A., Wolff, J.A., Rath, C.E. and Fluharty, R.G. (1949). Iron metabolism. Erythrocyte iron turnover. J. lab. clin. Med. 34, 1480.

Finnerty, F.A. Jr., Buchols, J.H. and Guillaudev, R.L. (1958). The blood volume and plasma protein during levarterenol-induced hypertension. J. clin. Invest. 37, 425.

Fischer, J. and Wolf, R. (1963). Scintillation scanning of the spleen using radiochromium. German med. Monthly 8, 89.

Frank, H. and Gray, S.J. (1953). The determination of plasma volume in man with radioactive chromic chloride. J. clin. Invest. 32, 991.

Freedman, H.T. (1953). The influence of cortisone and hydrocortisone on the production of circulating antibody in human beings. J. Allergy 24, 342.

Freeman, N.E., Freedman, H. and Miller, C.C. (1941). The production of shock by the prolonged continuous injection of adrenalin in unanesthetized dogs. Amer. J. Physiol. 131, 545.

Freis, E.D., Stanton, J.R. and Emerson, C.P. (1949). Estimation of the relative velocities of plasma and red cells in the circulation of man. Amer. J. Physiol. 157, 153.

Gardner, F. (1950). ACTH in leukemia. Blood 5, 791.

Gardner, F.H., McElfresh, A.E., Harris, J.W. and Diamond, L.K. (1951). The effect of adrenocorticotrophic hormone (ACTH) in idiopathic acquired hemolytic anemia as related to the hemolytic mechanisms. J. lab. clin. Med. 37, 444.

Gershon-Cohen, J., McClendon, J.F. and Skaroff, D.M. (1962). Contrast roentgenography versus radioisotope scanning of liver and spleen. Amer. J. Roentgenol. Rad. Ther. Nuc. Med. 88, 304.

Gibson, J.G. II, Seligman, A.M., Peacock, W.C., Aub, J.C., Fine, J. and Evans, R.D. (1946). The distribution of red cells and plasma in large and minute vessels of the normal dog, determined by radioactive isotopes of iron and iodine. J. clin. Invest. 25, 848.

Gibson, J.G., Weiss, S., Evans, R.D., Peacock, W.C., Irvine, J.W. Jr., Good, W.M. and Kip, A.F. (1946). The measurement of the circulating red cell volume by means of two radioactive isotopes of iron. J. clin. Invest. 25, 616.

Giordano, A.S. and Blum, L.L. (1937). Acute hemolytic anemia (Lederer type). Amer. J. med. Sci. 194, 311.

Gordon, A.S. and Katsch, G.F. (1949). The relation of the adrenal cortex to structure and phagocytic activity of the macrophage system. Ann. N.Y. Acad. Sci. 52, 1.

Gordon, A.S. and Ultmann, J.E. (1963). Removal of N-ethyl maleimide treated erythrocytes from the circulation in rats. Fed. Proc. 22, 602.

Gray, C.H. The Bile Pigments. Methuen's Monographs on Biochemical Subjects. Methuen and Co. London. 1953.

Gray, S.J. and Sterling, K. (1950). The tagging of red cells and plasma proteins with radioactive chromium. J. clin. Invest. 29, 1604.

Green, H.D., Ottis, K. and Kitchen, T. (1960). Autonomic stimulation and blockade on canine splenic inflow, outflow and weight. Amer. J. Physiol. 198, 424.

Guest, G.M. (1942). Osmometric behaviour of normal human erythrocytes. J. clin. Invest. 21, 257.

Haden, R.L. (1934). The mechanism of the increased fragility of the erythrocytes in congenital hemolytic jaundice. Amer. J. med. Sci. 188, 441.

Haden, R.L. (1947). A new type of hereditary, hemolytic jaundice without spherocytosis. Amer. J. med. Sci. 214, 255.

Hahn, P.F., Bale, W.F. and Bonner, J.F. (1942). Mechanism of the effect of epinephrine on the venous hematocrit value of the normal unanesthetised dog. Amer. J. Physiol. 137, 717.

Halpern, B.N., Biozzi, G. Bennacerraf, B. and Stiffel, C. (1957). Phagocytosis of foreign red blood cells by the reticulo-endothelial system. Amer. J. Physiol. 189, 520.

Ham, T.H., Shen, S.C., Fleming, E.M. and Castle, W.B. (1948). Studies on the destruction of red blood cells. IV. Thermal injury: action of heat in causing increased spheroidicity, osmotic and mechanical fragilities and hemolysis of erythrocytes; observations on the mechanisms of destruction of such erythrocytes in dogs and in a patient with a fatal thermal burn. Blood 3, 373.

Hamilton, H.E., Sheets, R.F. and Degowin, E.L. (1950). Studies with inagglutinable erythrocyte counts. II. Analysis of the mechanism of Cooley's anemia. J. clin. Invest. 29, 714.

Harris, I.M., McAlister, J.M. and Prankerd, T.A.J. (1957). The relationship of abnormal red cells to the normal spleen. Clin. Sci. 16, 223.

Harris, I.M., McAlister, J.M. and Prankerd, T.A.J. (1958). Splenomegaly and the circulating red cell. Brit. J. Haemat. 4, 97.

Harris, J.W. and Greenberg, M.S. (1954). Erythrocyte fragilities in plumbism. Clin. Res. Proc. 2, 55.

Haut, A., Cartwright, G.E. and Wintrobe, M.M. (1960). Cyclic variation of osmotic fragility during incubation of red blood cells. Clin. Res. 8, 102.

Havens, W.P. Jr., Shaffer, J.M. and Hopke, C.J. Jr. (1952). The effect of ACTH and cortisone on the concentration of circulating diphtheria antitoxin. J. Immunol. 68, 389.

Hayem, G. (1898) cited by Loutit, J.F. and Mollison, P.L. (1946). J. Path. Bact. 58, 711.

Hayhoe, F.G.J. and Whitby, L. (1955). Splenic function. Quart. J. Med. N.S. 24, 365.

Heilmeyer, L. (1952). ACTH und Cortison therapie bei nichtleukämischen Blutenkrankungen. Acta Haemat. (Basel) 7, 206.

Heller, J.H. (1953). Effect of cortisone on the function, capacity and activity of the reticuloendothelial system. Fed. Proc. 12, 65.

Heller, J.H. (1955). Cortisone and phagocytosis. Endocrinology, 56, 80.

Hodgkin, T. (1822) cited by Prankerd, T.A.J. (1963). Brit. med. J. 2, 517.

Holzbach, R.T., Clark, R.E., Shipley, R.A., Kent, W.B. and Lindsay, G.E. (1962). Evaluation of spleen size by radioactive scanning. J. lab. clin. Med. 60, 902.

Holzbach, R.T., Shipley, R.A., Clark, R.E. and Chudzik, E.B. (1964). Influence of spleen size and portal pressure on erythrocyte sequestration. J. clin. Invest. 43, 1125.

Hudson, G., Herdan, A. and Yoffey, J.M. (1952). Effect of repeated injections of ACTH upon the bone marrow. Brit. med. J. 1, 999.

Huff, R.L., Emlinger, P.J., Garcia, J.F., Oda, J.F., Cookrell M.C. and Lawrence, J.H. (1951). Ferrokinetics in normal persons and in patients having various erythropoietic disorders. J. clin. Invest. 30, 1512.

Hughes Jones, N.C. (1961). The life of the red blood cell using chromium-51 (^{51}Cr) as a tracer. Proc. Roy. Soc. Med. 55, 139.

Hughes Jones, N.C. and Mollison, P.L. (1956). The interpretation of measurements with ^{51}Cr-labelled red cells. Clin. Sci. 15, 207.

Hughes Jones, N.C., Mollison, P.L. and Veall, N. (1957). Removal of incompatible red cells by the spleen. Brit. J. Haemat. 3, 125.

Hughes Jones, N.C. and Szur, L. (1957). Determination of the sites of red cell destruction using ^{51}Cr-labelled cells. Brit. J. Haemat. 3, 320.

Hunter, W. (1892). Lectures on the physiology and pathology of blood destruction. Lancet 2, 1209.

Isaacs, R., Brock, B. and Minot, G.R. (1925). The resistence of immature erythrocytes to heat. J. clin. Invest. 1, 425.

Ito, M., Wagner, H.N. Jr. (1963). Studies of the reticuloendothelial system (RES). I. Measure of the phagocytic capacity of the RES in man and dog. J. clin. Invest. 42, 417.

Jacob, H.S. and Jandl, J.H. (1962a). Effects of sulfhydryl inhibition on red blood cells. I. Mechanisms of hemolysis. J. clin. Invest. 41, 779.

Jacob, H.S. and Jandl, J.H. (1962b). Effects of sulfhydryl inhibition on red blood cells. II. Studies in vivo. J. clin. Invest. 41, 1514.

Jacob, H.S., MacDonald, R.A. and Jandl, J.H. (1963). Regulation of spleen growth and sequestering function. J. clin. Invest. 42, 1476.

Jandl, J.H. (1955). Sequestration by the spleen of red cells sensitized with incomplete antibody and with metallo-protein complexes. J. clin. Invest. 34, 912.

Jandl, J.H. (1956). The rapid destruction of sequestered red cells as determined with Fe^{59}-labelled human reticulocytes. Clin. Res. Proc. 4, 81.

Jandl, J.H. (1958). Sequestration of reticulocytes and of abnormal red cells by filtration at low pressures. J. clin. Invest. 37, 905.

Jandl, J.H. (1960). The agglutination and sequestration of immature red cells. J. lab. clin. Med. 55, 663.

Jandl, J.H. and Castle, W.B. (1956). Agglutination of sensitized red cells by large anisometric molecules. J. lab. clin. Med. 47, 669.

Jandl, J.H. and Greenberg, M.S. (1957). The selective destruction of transfused "compatible" normal red cells in two patients with splenomegaly. J. lab. clin. Med. 49, 233.

Jandl, J.H., Greenberg, M.S., Yonemoto, R.H. and Castle, W.B. (1956). Clinical determination of the sites of red cell sequestration in hemolytic anemias. J. clin. Invest. 35, 842.

Jandl, J.H., Jones, A.R. and Castle, W.B. (1957). The destruction of red cells by antibodies in man. I. Observations on the sequestration and lysis of red cells by immune mechanisms. J. clin. Invest. 36, 1428.

Jandl, J.H. and Kaplan, M.E. (1960). The destruction of red cells by antibodies in man. III. Quantitative factors influencing the patterns of hemolysis in vivo. J. clin. Invest. 39, 1145.

Jandl, J.H. and Simmons, R.L. (1957). The agglutination and sensitization of red cells by metallic cations: interactions between multivalent metals and the red cell membrane. Brit. J. Haemat. 3, 19.

Jandl, J.H., Simmons, R.L. and Castle, W.B. (1961). Red cell filtration and the pathogenesis of certain hemolytic anemias. Blood 18, 133.

Jandl, J.H. and Tomlinson, A.S. (1958). The destruction of red cells by antibodies in man. II. Pyrogenic, leukocytic and dermal responses to immune hemolysis. J. clin. Invest. 37, 1958.

Johnson, P.M., Herion, J.C. and Mooring, S.L. (1960). Scintillation scanning of the normal human spleen utilizing radioactive erythrocytes. Radiology 74, 99.

Johnson, P.M., Wood, E.H. and Mooring, S.L. (1961). Splenic scintillation scanning. Amer. J. Roentgenol. 86, 757.

Jope, E.M. (1946). Disappearance of sulphaemoglobin from the blood of TNT workers in relation to dynamics of red cell destruction.

Brit. J. indust. Med. 3, 136.

Joske, R.A., McAllister, J.M. and Prankerd, T.A.J. (1956). Isotope investigations of red cell production and destruction in chronic renal disease. Clin. Sci. 15, 511.

Joyce, C.R.B., Moore, H. and Weatherall, M. (1954). The effects of lead, mercury and gold on the potassium turnover of rabbit blood cells. Brit. J. Pharmacol. 9, 463.

Kaliss, N., Hoecker, G. and Bryant, B.F. (1956). The effect of cortisone on isohaemagglutinin production in mice. J. Immunol. 76, 83.

Kaltreider, N.J., Meneely, G.R. and Allen, J.R. (1942). The effect of epinephrine on the volume of the blood. J. Clin. Invest. 21, 339.

Kaplan, M.E. and Jandl, J.H. (1961). Inhibition of red cell sequestration by cortisone. J. exp. Med. 114, 921.

Kaplan, M.E. and Zeulzer, W.W. (1950). Erythrocyte survival studies in childhood. II. Studies in Mediterranean anemia. J. lab. clin. Med. 36, 517.

Klebs (1863) cited by Isaacs, R., Brock, B. and Minot, G.R. (1925). J. clin. Invest. 1, 425.

Korst, D.R., Clatanoff, D.V. and Schilling, R.F. (1955). External scintillation counting over the liver and spleen after the transfusion of radioactive erythrocytes. Clin. Res. Proc. 3, 195.

Krivit, W. (1958). The effect of cortisone and ACTH on chromium-51 red blood cell survival in erythroblastosis foetalis. Amer. med. Assoc. J. Dis. Child. 96, 529.

Landsteiner, K., Levine, P. and Janes, M.L. (1928). On the development of isoagglutinins following transfusions. Proc. Soc. exp. Biol. Med. 25, 672.

Laurie, M.B., Zappasodi, P., Dannenberg, A.M. and Swartz, I.B. (1951). Constitutional factors in resistance to infection: the effect of cortisone on the pathogenesis of tuberculosis. Science 113, 234.

Lederer, M. (1925). A form of acute hemolytic anemia probably of infectious origin. Amer. J. med. Sci. 170, 500.

Lendon, A.A. (1896). Clinical lecture on splenectomy. Intercol. med. J. Aust. 1, 329.

Lewis, S.M., Szur, L. and Dacie, J.V. (1960). The pattern of erythrocyte destruction in haemolytic anaemia as studied with radioactive chromium. Brit. J. Haemat. 6, 122.

Ley, A.B. and Gardner, F.H. (1951). The effect of cortisone acetate on idiopathic acquired hemolytic anemia. J. clin. Invest. 30, 656.

Lichtman, H.C., Watson, R.J., Feldman, F., Ginsberg, V. and Robinson, J. (1953). Studies in thalassemia. Part I. An extracorpuscular defect in thalassemia major. Part II. The effect of splenectomy in thalassemia major with an associated acquired hemolytic anemia. J. clin. Invest. 32, 1229.

Loutit, J.F. (1946). Discussion on the life and death of the red blood corpuscle. Proc. Roy. Soc. Med. 39, 757.

Loutit, J.F. and Mollison, P.L. (1946). Haemolytic icterus (acholuric jaundice) congenital and acquired. J. Path. Bact. 58, 711.

Lucia, S.P., Aggeler, P.M., Husser, G.D. and Leonard, M.E. (1937). Effect of epinephrine on blood count and haematocrit value. Proc. Soc. exp. Biol. Med. 36, 582.

McCurdy, P.R. and Rath, C.E. (1958). Splenectomy in hemolytic anemia. Results predicted by body scanning after injection of Cr^{51}-tagged red cells. New Engl. J. Med. 259, 459.

MacKenzie, O.W., Whipple, A.O. and Wintersteiner, M.P. (1941). Studies on the microscopic anatomy and physiology of living trans-illuminated mammalian spleens. Amer. J. Anat. 68, 397.

Malamos, B. Belcher, E.H., Gyftaki, E. and Binopoulos, D. (1961). Simultaneous radioactive tracer studies of erythropoiesis and red-cell destruction in thalassaemia. Brit. J. Haemat. 7, 411.

Malcolm, D., Ranney, H.M. and Jacobs, A.S. (1963). Association of radioactive chromium with various components of hemoglobin. Blood 21, 8.

Marmont, A.M. and Fusco, F.A. in "Reticuloendothelial Structure and Function" edited by John H. Heller. The Ronald Press Company, New York, 1960. p. 373.

M.R.C. Haematology Panel (1952). The treatment of blood disorders with ACTH and cortisone. A preliminary report to the Medical Research Council by the panel on the haematological applications of ACTH and cortisone. Brit. med. J. 1, 1261.

M.R.C. Haematology Panel (1953). Treatment of blood disorders with ACTH and cortisone. Second report to the Medical Research Council

by the panel on the haematological applications of ACTH and cortisone. Brit. med. J. 2, 1400.

Micheli (1911) cited by Chertkow, G. and Dacie, J.V. (1956). Brit. J. Haemat. 2, 237.

Miescher, P. (1956). Le mécaniame de l'erythroclasie a l'état normal. Rev. Hémat. 11, 248.

Miescher, P. in "Physiopathology of the Reticuloendothelial System" edited by B.N. Halpern. Blackwell Scientific Publications, Oxford 1957. p. 147.

Miller, D.K. and Rhoads, C.P. (1933). The effect of splenic contraction on the formed elements of the blood in a case of anemia and splenomegaly. J. clin. Invest. 12, 1009.

Minkowski, O. (1900) cited by Emerson, C.P. Jr., Shen, S.C., Ham, T.H., Fleming, E.M. and Castle, W.B. (1956). Arch. int. med. 97, 1.

Mollison, P.L. (1947). The survival of transfused erythrocytes with special reference to cases of acquired haemolytic anaemia. Clin. Sci. 6, 137.

Mollison, P.L. (1959). Blood group antibodies and red-cell destruction. Brit. med. J. 2, 1035.

Mollison, P.L. in "Proceedings 7th Congress Int. Soc. Blood Transfusion" edited by L. Holländer. Basel and New York, 1959. p. 495.

Mollison, P.L. "Blood Transfusion in Clinical Medicine". Blackwell Scientific Publications Ltd. Oxford. 3rd edition, 1961.

Mollison, P.L. (1961). Further observations on the normal survival curve of ^{51}Cr-labelled red cells. Clin. Sci. 21, 21.

Mollison, P.L. (1962). The reticulo-endothelial system and red cell destruction. Proc. Roy. Soc. Med. 55, 915.

Mollison, P.L. and Cutbush, M. (1955). The use of isotope-labelled red cells to demonstrate incompatibility in vivo. Lancet, 1, 1290.

Mollison, P.L. and Hughes Jones, N.C. (1958). Sites of removal of incompatible red cells from the circulation. Vox Sang (Basel) 3, 243.

Mollison, P.L. and Veall, N. (1955). The use of the isotope ^{51}Cr as a label for red cells. Brit. J. Haemat. 1, 62.

Mollison, P.L. and Young, I.M. (1940). On the survival of the transfused erythrocytes of stored blood. Quart. J. exp. Physiol. 30, 313.

Mollison, P.L. and Young, I.M. (1942). In vivo survival in the human subject of transfused erythrocytes after storage in various preservative solutions. Quart. J. exp. Physiol. 31, 359.

Molcmut, N., Spain, D.M. and Haber, A. (1950). The effect of cortisone on the spleen in mice. Proc. Soc. exp. Biol. Med. 73, 416.

Motulsky, A.G., Casserd, F. and Giblett, E. (1956). In vivo measurements of splenic circulation: rapid method for demonstration of splenic red cell sequestration. J. clin. Invest. 35, 725.

Motulsky, A.G., Casserd, F., Fiblett, E., Broun, G.O. and Finch, C.A. (1958). Anemia and the spleen. New Engl. J. Med. 259, 1164.

Murchison, C. "Clinical Lectures on Diseases of the Liver, Jaundice and abdominal Dropsy". Longmans, Green & Co. London, 2nd edition,1877.

Nakai, G.S., Craddock, C.G. and Figueroa, M.D. (1962). Agnogenic myeloid metaplasia. A survey of twentynine cases and a review of the literature. Ann. int. Med. 57, 419.

Necheles, T.F., Weinstein, I.M. and Leroy, G.V. (1953). Radioactive sodium chromate for the study of the survival of red blood cells. I. The effect of radioactive sodium chromate on the red cells. J. lab. clin. Med. 42, 358.

Nicol, T., Snell, R.S. and Bilbey, D.L.J. (1956). Effect of cortisone on the defence mechanisms of the body. Brit. med. J. 2, 800.

Noyes, W.D., Bothwell, T.H. and Finch, C.A. (1960). The role of the reticulo-endothelial cell in iron metabolism. Brit. J. Haemat. 6, 43.

Owren, P.A. (1948). Congenital hemolytic jaundice. The pathogenesis of the "hemolytic crisis". Blood, 3, 231.

Owren, P.A. (1949). Acquired hemolytic jaundice. Scandinav. J. clin. lab. Invest. 1, 41.

Packer, J.T., Greendyke, R.M. and Swisher, S.N. (1960). The inhibition of erythrophagocytosis in vitro by corticosteroids. Trans. Assoc. Amer. Phys. 73, 93.

Parson, W., Mayerson, H.S., Lyons, C. Porter, B. and Trautman, W.V. (1948). Effect of the administration of adrenaline on the circulating red cell volume. Amer. J. Physiol. 155, 239.

Passow, N. and Tillman, V. (1955). Untersuchungen über den Kalium-verlust bleivergifteter Menschenerythrocyten. Archiv. f. d. ges. Physiol. 262, 23.

Pauling, L., Itano, H.A., Singer, S.J. and Wells, I.C. (1949). Sickle cell anemia, a molecular disease. Science 110, 543.

Pearce, R.M. (1904). The experimental production of liver necrosis by the intravenous injection of hemagglutinins. J. med. Res. 12, 329.

Pearson, H.A. and Vertrees, K.M. (1961). Site of binding of chromium-52 to haemoglobin. Nature (London), 189, 1019.

Ponder, E. "Haemolysis and Related Phenomena". J. & A. Churchill Ltd. London. 1948.

Prankerd, T.A.J. (1955). The metabolism of the human erythrocyte. A review. Brit. J. Haemat. 1, 131.

Prankerd, T.A.J. (1960). Studies on the pathogenesis of haemolysis in hereditary spherocytosis. Quart. J. Med. N.S. 29, 199.

Prankerd, T.A.J. (1963). The spleen and anaemia. Brit. med. J. 2, 517.

Prankerd, T.A.J., Altman, K.I. and Young, L.E. (1955). Abnormalities of carbohydrate metabolism of red cells in hereditary spherocytosis. J. clin. Invest. 34, 1268.

Rappaport, H. and Crosby, W.H. (1957). Auto-immune hemolytic anemia. II. Morphologic observations and clinico-pathological correlations. Amer. J. Path. 33, 429.

Read, R.C. (1954). Studies of red cell volume and turnover using radiochromium. Description of a new "closed" method of red cell volume measurement. New Engl. J. Med. 250, 1021.

Read, R.C., Wilson, G.W. and Gardner, F.H. (1954). The use of radio-active sodium chromate to evaluate the life span of the red blood

cell in health and certain hematological disorders. Amer. J. Med. Sci. 228, 40.

Robinson, M.A., Loder, P.B. and de Gruchy, G.C. (1961). Red cell metabolism in non-spherocytic congenital haemolytic anaemia. Brit. J. Haemat. 7, 327.

Robson, H.N. (1949). Medical aspects of splenectomy. Edin. med. J. 56, 381.

Rose, J.C., Freis, E.D. (1957). Alterations in the systemic vascular volume of the dog in response to hexamethonium and norepinephrine. Amer. J. Physiol. 191, 283.

Rosenthal, M.C., Pisciotta, A.V., Komninos, Z.D., Goldenberg, H. and Dameshek, W. (1955). The autoimmune hemolytic anemia of malignant lymphocytic disease. Blood, 10, 197.

Saint, E.G. and Gardner, H.J. (1952). The successful treatment of severe haemolytic anaemia with adrenocorticotropic hormone (ACTH). Med. J. Aust. 2, 305.

Schloesser, L.L., Korst, D.R., Clatanoff, D.V. and Schilling, R.F. (1957). Radioactivity over the spleen and liver following the transfusion of chromium-51 labeled erythrocytes in hemolytic anemia. J. clin. Invest. 36, 1470.

Schultze, M. (1865). Ein heizbarer Objecttisch und seine Verwendung bei Untersuchungen des Blutes. Arch. f. mikr. Anat. 1, 1.

Schweigger-Seidel, F. (1862). Untersuchungen über die Mils. Virchows Arch. path. Anat. 23, 526.

Selwyn, J.G. and Dacie, J.V. (1954). Autohemolysis and other changes resulting from the incubation in vitro of red cells from patients with congenital hemolytic anemias. Blood 9, 414.

Shahidi, N.T. and Diamond, L.K. (1959). Enzyme deficiency in erythrocytes in congenital non-spherocytic hemolytic anemias. Pediatrics 24, 245.

Shen, S.C., Ham, T.H. and Fleming, E.M. (1943). Studies on the destruction of red blood cells. III. Mechanism and complications of hemoglobinuria in patients with thermal burns: spherocytosis and increased osmotic fragility of red blood cells. New Engl. J. Med. 229, 701.

Shemin, D. and Rittenberg, D. (1946). The life span of the human red blood cell. J. biol. Chem. 166, 627.

Shils, M.E., Sass, M. and Goldwater, L.J. (1952). A micro-hematocrit method and its evaluation. Amer. J. clin. Path. 22, 155.

Silbermann, O. (1890). Untersuchungen über der Krankheitserscheinungen und Urachen des raschen Todes nach schweren Hautuer brennungen. Virchows Arch. f. path. Anat. 119, 488.

Simon, E.R. and Ways, P. (1964). Incubation hemolysis and red cell metabolism in acanthocytosis. J. clin. Invest. 43, 1311.

Singer, K. and Dameshek, W. (1941). Symptomatic hemolytic anemia. Ann. int. Med. 15, 544.

Singer, K., King, J.C. and Robin, S. (1948). The life span of the megalocyte and the hemolytic syndrome of pernicious anemia. J. lab.

clin. Med. 33, 1068.

Sitarz, A.L., Ultmann, J.E. and Wolff, J.A. (1963). Erythrocyte life span and sites of destruction in thalassaemia major. Acta Haemat. 30, 204.

Spain, D.M., Molomut, N. and Haber, A. (1950). Biological studies on cortisone in mice. Science 112, 335.

Spanton, W.D. (1895). On splenectomy, with notes of three cases. Brit. med. J. 2, 1090.

Spiegler, E. (1896) cited by Shen, S.C., Ham, T.H. and Fleming, E.M. (1943) New Engl. J. Med. 229, 701.

Stanley, D. (1895). Splenectomy. Brit. med. J. 2, 1197.

Sterling, K. and Gray, S.J. (1950). Determination of the circulating red cell volume in man by radioactive chromium. J. clin. Invest. 29, 1614.

Strumia, M.M., Sample, A.B. and Hart, E.D. (1954). An improved micro-hematocrit method. Amer. J. clin. Path. 24, 1016.

Strumia, M.M., Taylor, L., Sample, A.B., Colwell, L.S. and Dugan, A. (1955). Use and limitations of survival studies of erythrocytes tagged with Cr^{51}. Blood 10, 429.

Stukely, W. (1722) cited by Prankerd, T.A.J. (1963) Brit. med. J. 2, 517.

Sturgeon, P. and Finch, C.A. (1957), Erythrokinetics in Cooley's anemia. Blood 12, 64.

Sutherland, D.A., McCall, M.S., Groves, M.T. and Muirhead, E.E. (1954). The survival of human erythrocytes estimated by means of cells tagged

with radioactive chromium. A study of the normal state. J. lab. clin. Med. 43, 717.

Swisher, S.N. and Young, L.E. (1954). Studies of the mechanisms of erythrocyte destruction initiated by antibodies. Trans. Assoc. Amer. Phys. 68, 125.

Szeinberg, A., Asher, Y. and Sheba, C. (1958). Studies on the glutathione stability in erythrocytes of cases with past history of favism or sulpha drug-induced hemolysis. Blood 13, 348.

Talbot, T.R. Jr., Sax, M. and Cary, A. (1954). A forty-eight hour procedure for the determination of post-transfusion survival of erythrocytes from preserved blood, employing radioactive chromium-51 (Cr^{51}). J. clin. Invest. 33, 968.

Tietel, P. (1960). Influence of intra erythrocyte K on the shape and the frequency distribution curve of resistance to hypotonicity in an erythrocyte population. Folia Haemat. 77, 209.

Thomas, L. (1953). Cortisone and infection. Ann. N.Y. Acad. Sci. 56, 799.

Thorn, G.W. (1950). Hematologic effects of ACTH and cortisone. Blood 5, 785.

Thorn, G.W., Jenkins, D., Laidlaw, J.C., Goetz, F.C., Dingman, J.F. Arons, W.L., Streeten, D.H.P. and McCracken, B.H. (1953). Pharmacologic aspects of adrenocortical steroids and ACTH in man. New Engl. J. Med. 248, 323.

Todd, C. and White, R.G. (1911). On the fate of red blood corpuscles when injected into the circulation of an animal of the same species; with a new method for the determination of the total volume of the blood. Proc. Roy. Soc. series B 84, 255.

Toghill, P.J. (1964). Red-cell pooling in enlarged spleens. Brit. J. Haemat. 10, 347.

Toghill, P.J. and Prichard, B.N.C. (1964). A study of the action of noradrenaline on the splenic red cell pool. Clin. Sci. 26, 303.

Turner, A.W. and Hodgetts, V.E. (1959). The dynamic red cell storage function of the spleen in sheep. I. Relationship to fluctuations of jugular haematocrit. Aust. exp. Biol. med. Sci. 37, 399.

Turner, A.W. and Hodgetts, V.E. (1960). The dynamic red cell storage function of the spleen in sheep. II. Jugular haematocrit fall after some tranquillizing agents, particularly chlorpromazine. Aust. J. exp. Biol. med. Sci. 38, 79.

Ultmann, J. (1958). The role of the spleen in the hemolytic anemia of cancer patients. Cancer Res. 18, 959.

Unger, L.J. (1951). The effect of ACTH in acquired hemolytic anemia. Amer. J. clin. Path. 21, 456.

Vaughan, J.H., Bayles, T.B. and Favour, C.B. (1950). The effect of ACTH on blood complement, gamma globulins and fibrinogen. J. clin. Invest. 29, 850.

Veall, N. and Vetter, H. Radioisotope Techniques in Clinical Research and Diagnosis. Butterworth and Co. (Publishers) Ltd. London. 1958. (a) p. 115, (b) p. 178.

Vincent, P.C. (1959a). The effects of heavy metal ions on the human erythrocyte. II. The effects of lead and mercury. Aust. J. exp. Biol. med. Sci. 36, 589.

Vincent, P.C. (1959b). The effects of heavy metal ions on the human erythrocyte. III. Inhibition of the effects of lead and mercury. Aust. J. exp. Biol. med. Sci. 37, 83.

Vincent, P.C. and Blackburn, C.R.B. (1958). The effects of heavy metal ions on the human erythrocyte. I. Comparison of the action of several heavy metals. Aust. J. exp. Biol. med. sci. 36, 471.

von Euler, U.S. (1952). Some aspects of clinical physiology of noradrenaline. Scandinav. J. clin. lab. Invest. 4, 254.

von Lesser, L. (1880). Über die Todesursachen nach Verbrennungen. Virchow's Arch. f. Path. Anat. 79, 248.

Wagley, P.F., Shen, S.C., Gardner, F.H. and Castle, W.B. (1948). Studies on the destruction of red blood cells. VI. The spleen as a source of a substance causing agglutination of the red cells of certain patients with acquired hemolytic jaundice by an anti-human serum rabbit serum (Coombs' serum). J. lab. clin. Med. 33, 1197.

Wagner, H.N., McAfee, J.G. and Winkelman, J.W. (1962). Splenic disease diagnosis by radioisotope scanning. Arch. int. Med. 109, 673.

Wagner, H.N., Razzak, M.A., Gaertner, R.A., Caine, W.P. and Feagin, O.T. (1962). Removal of erythrocytes from the circulation. Arch. int. Med. 110, 90.

Watson, C.J. and Paine, J.R. (1942). A study of the splenic venous blood. Trans. Assoc. Amer. Phys. 57, 249.

Wearn, J.T., Warrens, S. and Ames, O. (1922). The length of life of transfused erythrocytes in patients with primary and secondary anemia. Arch. int. Med. 29, 527.

Weed, R., Eber, J. and Rothstein, A. (1961). Effects of primaquine and other related compounds on the red blood cell membrane. I. Na^+ and K^+ permeability in normal human cells. J. clin. Invest. 40, 130.

Weiner, A.S. (1934). Longevity of the erythrocyte. J. Amer. med. Assoc. 102, 1779.

Weinstein, I.M. and Leroy, G.V. (1953). Radioactive sodium chromate for the study of survival of red blood cells. II. The rate of hemolysis in certain hematological disorders. J. lab. clin. Med. 42, 368.

Weinstein, I.M., Spurling, C.L., Klein, H. and Necheles, T.F. (1954). Radioactive sodium chromate for the study of survival of red blood cells. III. The abnormal hemoglobin syndromes. Blood 9, 1155.

Weisman, R. Jr., Hurley, T.H., Harris, J.W. and Ham, T.H. (1953). Studies of the function of the spleen in the hemolysis of red cells in hereditary spherocytosis and sickle cell disorders. J. lab. clin. Med. 42, 965.

Welch, C.S. and Dameshek, W. (1950). Splenectomy in blood dyscrasias. New Engl. J. Med. 242, 601.

Wertheim, G. (1868) cited by Shen, S.C, Ham, T.H. and Fleming, E.M. (1943) New Engl. J. Med. 229, 701.

Widal, F., Abrami, P. and Brule, M. (1907) cited by Loutit, J.F. and Mollison, P.L. (1946) J. Path. Bact. 58, 711/

Wilson, C. (1890). Some cases showing hereditary enlargement of the spleen. Trans. clin. Soc. London 23, 162.

Wilson, C. and Stanley, D.A. (1893). A sequel to some cases showing hereditary enlargement of the spleen. Trans. clin. Soc. London 26, 163.

Winkelman, J.A., Wagner, H.N., McAfee, J.G. and Mozley, J.M. (1960). Visualization of the spleen in man by radioisotope scanning. Radiology, 75, 465.

Wintrobe, M.M., Cartwright, G.E., Palmer, J.G., Kuhns, W.J. and Samuels, L.T. (1951). Effect of corticotrophin and cortisone on the blood in various disorders in man. Arch. int. Med. 88, 310.

Wiseman, B.K. and Doan, C.A. (1942). Primary splenic neutropenia. A newly recognised syndrome closely related to congenital hemolytic icterus and essential thrombocytopenic purpura. Ann. int. Med. 16, 1097.

Wright, C.S., Doan, C.A., Bouroncle, B.A. and Zollinger, R.M. (1951). Direct splenic arterial and venous blood studies in the hypersplenic syndromes before and after epinephrine. Blood 6, 195.

Yoffey, J.M., Metcalf, W.K., Herdan, G. and Nairn, Y. (1951). Effect of ACTH and suprarenal extract on bone marrow. Brit. med. J. 1, 660.

Young, L.E., Izzo, M.J. and Platzer, R.F. (1951). Hereditary spherocytosis. I. Clinical, hematologic and genetic features in 28 cases, with particular reference to the osmotic and mechanical fragility of incubated erythrocytes. Blood 6, 1073.

Young, L.E. and Miller, G. (1953). The long term picture in autoimmune hemolytic disease. Trans. Ass. Amer. Phys. 66, 190.

Young, L.E., Miller, G. and Christian, R.M. (1951). Clinical and laboratory observations on autoimmune hemolytic disease. Ann. int. Med. 35, 507.

Young, L.E., Platzer, R.F., Ervin, D.M. and Izzo, M.J. (1951). Hereditary spherocytosis. II. Observations on the role of the spleen. Blood 6, 1099.

Zinkham, W.H. and Lenhard, R.E. Jr. (1959). Metabolic abnormalities of erythrocytes from patients with congenital non-spherocytic hemolytic anemia. J. pediatrics 55, 319.

PUBLICATION OF PAPERS

Kimber, R.J. and Lander, H. (1964). The effect of heat on human red cell morphology, osmotic fragility and subsequent survival in vivo. J. lab. clin. Med. 64, 922.

Kimber, R.J., Lander, H. and Robson, H.N. (1965). The sequestration of NEM-treated red cells in normal and abnormal subjects: A test of splenic uptake function. J. lab. clin. Med. Accepted for publication 8th February, 1965.

Kimber, R.J. and Lander, H. (1965). Noradrenaline and the splenic red cell pool. To be submitted to Clin. Sci.

Material from this thesis has also been presented at the following scientific meetings.

The Haematology Society of Australia. 1st May, 1964. "The effect of heat on human red cell morphology, osmotic fragility and survival."

The Australian Society for Medical Research. 14th August, 1964. "The fate of human red cells treated with a sulphydryl inhibitor (NEM) in normal and abnormal subjects."

The Haematology Society of Australia. 10th April, 1965. "Studies on the presence of a splenic red cell pool in man."