Nitroblue Tetrazolium - Its Use in the Diagnosis of Infection and in the Study of Leukocytes, Lipoproteins and Liposomes

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by

Anthony Walter Segal, M.B.,Ch.B.,M.Sc.,M.R.C.P.

Northwick Park Hospital and Clinical Research Centre.

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"There is at bottom only one genuinely scientific treatment for all diseases, and that is to stimulate the phagocytes. Stimulate the phagocytes. Drugs are a delusion."

G. B. Shaw (1906)

The Doctor's Dilemma, Act I.

ABSTRACT

A rapid, objective indicator of pyogenic infection would be of great value in the practice of clinical medicine. On the basis of earlier studies it was claimed that the nitroblue tetrazolium (NBT) test might fulfill such a role. In view of the potential value of this test, it was reassessed in order to determine its diagnostic accuracy and clinical value. The results obtained in this study did not conform with those previously published. Elevated NBT scores were not diagnostic of pyogenic infection, there was a wide overlap of the results of tests performed on patients with pyogenic disease, patients with other diseases and normal subjects. In addition, there was a significant observer error in the interpretation of the slide preparations. The extent of this error was reduced with experience, but was still considerable in the hands of experienced observers.

In the NBT test, the dye enters neutrophils by phagocytosis of NBT in particulate form, complexed to heparin and/or fibrinogen. The proportion of neutrophils which phagocytose these complexes seems to be related to the severity of illness of the patient. As serum from these patients is capable of enhancing phagocytosis of complexed dye by normal cells, a humoral factor could be responsible for the increased phagocytosis of complexed NBT indicated by a positive test. Of the compounds tested, in an in vitro model system designed to simulate the NBT test,

α^1-acid glycoprotein, immunoglobulins and endotoxin, in concentrations that occur in vivo, enhanced NBT reduction. Any one of these compounds, singly or in combination, could be responsible for positive NBT tests.

There was a poor correlation between the results of NBT test performed by the method of Park et al (1968) on blood taken into heparin as anticoagulant, and by the method of Gordon et al (1973) with EDTA as anticoagulant. This suggests that phagocytosis of the dye in the two tests might be modified by different factors. In the test on blood with EDTA as anticoagulant there was good evidence for the enhancement of NBT reduction by a humoral factor and the NBT score correlated very well with the serum concentration of α^1-acid glycoprotein which could possibly contribute to the positive results of NBT tests performed by this method. When heparin was used as anticoagulant, the evidence suggesting the enhancement of NBT reduction by a humoral factor was less clear, and the correlation between the NBT score and serum concentration of α^1-acid glycoprotein was less significant. This discrepancy could result from the fact that heparin itself enhances NBT reduction, or that heparin, which is highly acidic, might mask any opsonising effect of α^1-acid glycoprotein which is dependent upon its acidic nature.

The physiological roles of heparin and α^1-acid glycoprotein in man, and fetuin in some animals, are unknown. They might act as non-specific opsonins. Heparin might be

released as a result of a focal insult to act as a local opsonin, whereas the acid glycoproteins are produced predominantly at times of stress and could function as a non-specific circulating opsonising system, for the phagocytosis of inert particles or autologous debris, or of invading microorganisms in the early stages of infection, while a specific immunological counter attack is in preparation.

The dose response of phagocytosis by neutrophils from the blood of normal subjects to endotoxin stimulation can be used as a standard for comparison with the dose response of other neutrophils. In this system it was demonstrated that neutrophils from patients with Crohn's disease, diabetes mellitus, geriatric patients, patients receiving therapy with sodium aurothiomalate and prednisone, and the majority of patients with hypogammaglobulinaemia, phagocytose complexed NBT and reduce the dye normally. It would also appear extremely unlikely that reports that immunoglobulins or complement are obligatory for dye reduction, are correct. However, it was demonstrated that immature bone marrow neutrophils, and neutrophils previously exposed to immune complexes, do not demonstrate the normal increment of NBT reduction after endotoxin stimulation. It was demonstrated in the quantitative NBT test that immature neutrophils have a normal capacity to reduce the dye, so the abnormally low reduction in a stimulated NBT test is almost certainly a result of diminished phagocytosis. False-negative NBT tests in severely ill patients probably result from the replacement of normal

circulating neutrophils by a pool of less mature neutrophils, or much less commonly, as a result of circulating immune complexes.

The NBT test does not seem to have much wide application as a diagnostic test for infection, however, it may prove to be very useful in assessing the maturity of circulating neutrophils and as a screening test for the diagnosis of, and for monitoring the response to therapy of, patients with immune complex disease.

The quantitative NBT test is different to the NBT test. It is performed on leukocytes suspended in a protein free solution rather than in whole blood. It is useful in the diagnosis of chronic granulomatous disease of childhood (CGDC) and in assessing whether the failure of NBT reduction by neutrophils in the NBT test results from the failure of the phagocytosis of complexed NBT or whether the cell has an abnormally low capacity to reduce the dye. Reduction of the dye in this test system is closely related to the release of LDH, a cytoplasmic marker enzyme, from the cells. This suggests that the dye is reduced by compounds released from the cells as a result of the toxicity of NBT, which is enhanced by endotoxin and latex, on the outer membrane of the cell. The actual enzymes and mechanisms responsible for the bactericidal activity of neutrophils, and for reduction of dye, are still unclear. The combined failure of both these processes in neutrophils of patients with CGDC or neutrophils of normal subjects after exposure to very

high concentrations of hydrocortisone, suggests that they might share a common mechanism. Ascorbic acid could play an important role in the transport of electrons from the HMP shunt to superoxides, or other free radicals, which could be responsible for both the final step of dye reduction and microbial death.

CGDC is characterised by chronic infection resultant upon defective leukocyte microbicidal activity. Liposomes might be useful vehicles for the transport of compounds into neutrophils and macrophages. They could be used, initially on an experimental basis in vitro, to establish whether or not entrapped antibiotics, ascorbic acid, various H_2O_2 generating systems, or enzyme systems enhance cellular bactericidal capacity. The tissue distribution of these particles after intravenous injection was traced by using entrapped NBT as a marker, which indicated not only the distribution of these particles, but which also gave some idea of the rate of liposomal degradation. The distribution of these lipid spherules to phagocytic cells of the RES suggests that they might be useful in the in vivo treatment of CGDC with microbicidal systems demonstrated to be effective in vitro. They may also be of use in the treatment of chronic infective, metabolic and neoplastic conditions involving phagocytic cells.

In vitro, the selective binding of reduced NBT to lipoproteins was discovered by investigating the observation that reduced NBT is found both in the cellular elements of blood and

in plasma. This property allows the use of reduced NBT as a prestain in the electrophoretic separation of lipoproteins. It is specific, has a vivid blue colour, does not require an organic solvent and does not seem to change the immuno reactivity of lipoproteins. The electrophoretic separation of lipoproteins, prestained with reduced NBT, on polyacrylamide, was found to be simple, accurate and reproducible. The classical lipoprotein bands were demonstrated and in addition several slow α bands were observed in serum from normal subjects and an inter-β band, possibly the S_f 12-20 fraction of the LDL, was seen in the serum of some normal and hyperlipoproteinaemic subjects. If it is shown that NBT uniformly stains lipoproteins, rather than being selectively concentrated in any particular lipid, and that densitometry of electrophoretic strips accurately reflects the lipoprotein concentration, this technique could be a very useful, simple, cheap and rapid method for lipoprotein quantitation.

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CONTENTS

CHAPTER I

"As often happens, unraveling a pathological situation leads to better comprehension of the normal. The study of chronic granulomatous disease of childhood (CGD), sometimes called 'fatal granulomatous disease', supports this truism. It has certainly spurred work on the whole question of how leukocytes - polymorphonuclear leukocytes in particular - ingest and kill bacteria. However, only wishful thinking could permit the conclusion that the critical questions have indeed been properly answered".

Manfred L. Karnovsky, 1973.

1.1. The introduction of the NBT test into clinical medicine

Janeway and colleagues (Janeway et al, 1954) first observed that certain children had the same susceptibility to infection as patients with agammaglobulinaemia, despite having elevated levels of circulating immunoglobulins. "Fatal granulomatous disease" was defined as a distinct clinical entity by Berendes, Bridges and Good (Berendes et al, 1957, Bridges et al, 1959), and this condition was later found to be associated with a decrease in intraleukocytic microbicidal activity (Quie et al, 1967).

It was known that the phagocytic process of normal leukocytes is associated with a burst of metabolic activity (Beck, 1968, Karnovsky, 1968) in which glucose and oxygen consumption

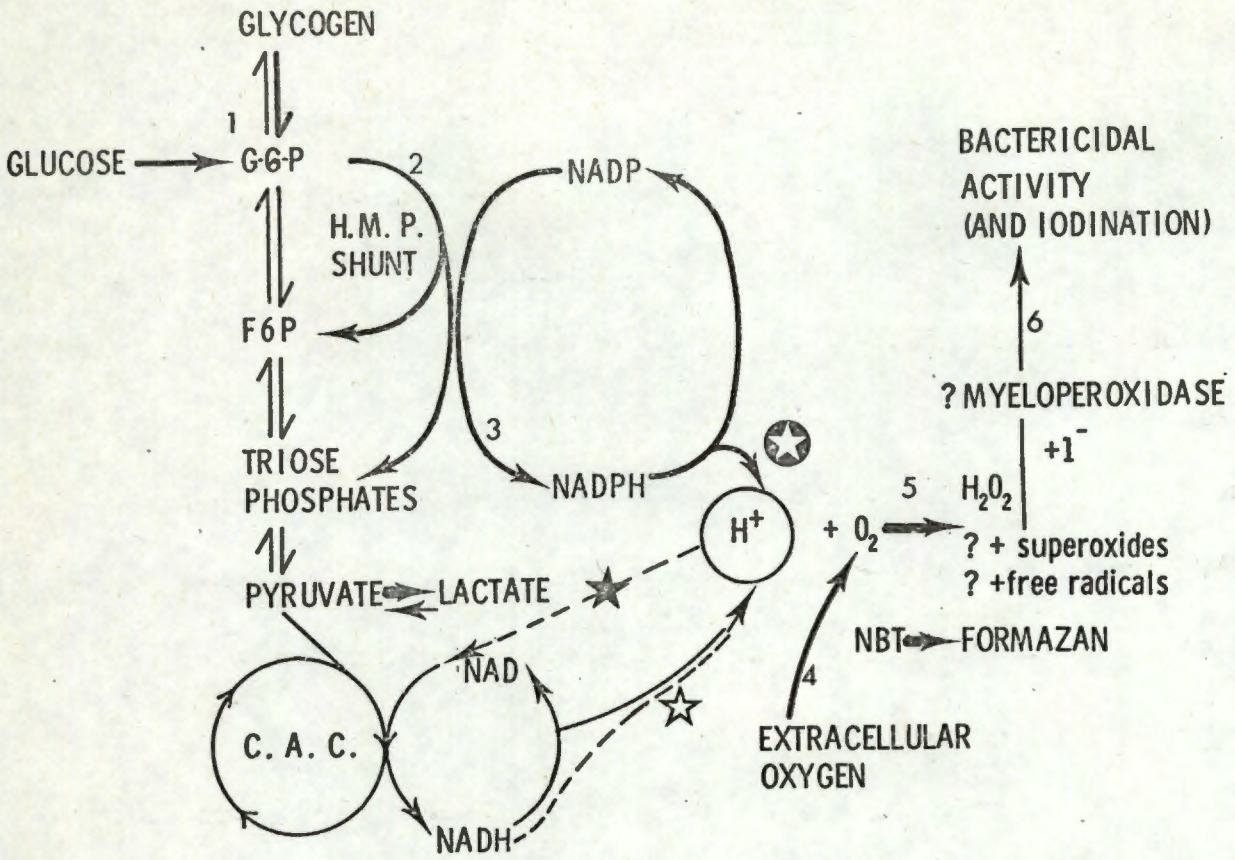


FIGURE 1.1.

Diagrammatic representation of the 'burst of metabolic activity' associated with phagocytosis by neutrophils. Glucose-6-phosphate production 1, H.M.P. shunt activity 2, NADPH production 3, oxygen consumption 4, H_2O_2 production 5, and bacterial iodination 6, are all increased.

The microbicidal activity of these cells appears to be related to the combination of reducing equivalents with oxygen, which may be promoted by NADPH-oxidase ✪ or NADH-oxidase ☆ (in conjuction with a NAD^+-NADPH transhydrogenase ★) enzymes.

(Sbarra and Karnovsky, 1959, Cohn and Morse, 1960) and H_2O_2 (Iyer et al, 1961, Paul and Sbarra, 1968) and lactate production (Sbarra and Karnovsky, 1959, Cohn and Morse, 1960) are increased. A large proportion of glucose catabolism, as measured by $^{14}CO_2$ production from glucose-1-^{14}C, occurs through the hexose monophosphate shunt (HMP shunt) (Evans and Karnovsky, 1962, Zatti et al, 1965). The three key metabolic changes that occur as a result of phagocytosis are the increased oxygen consumption, HMP shunt activity and H_2O_2 production (Fig. 1.1). A discussion of other metabolic changes associated with phagocytosis is included in the review article by Klebanoff (1971).

The phagocytosis induces stimulation of O_2 consumption, glucose-1-C oxidation and formate oxidation as a measure of H_2O_2 formation was found to be greatly depressed in leukocytes of patients with chronic granulomatous disease of childhood (CGDC) (Holmes et al, 1967, Baehner and Nathan, 1967). The bactericidal defect in the neutrophils of these patients was thus linked to defective H_2O_2 production. Cells of these patients are capable of killing organisms such as streptococci, pneumococci (Mandell and Hook, 1969) and lactobacilli (Klebanoff and White, 1969), which themselves generate H_2O_2, but they have an impaired ability to kill bacteria like Staphylococcus aureus and certain low-grade gram-negative pathogens which do not produce H_2O_2. This bactericidal defect can be partially reversed by redox dyes such as methylene blue and phenazine, which "produce H_2O_2" (Lehrer, 1969), or by

the introduction of a H_2O_2 generating system in the form of the enzyme glucose oxidase, into the cell (Baehner et al, 1970a, Johnston and Baehner, 1970). H_2O_2 formed by one organism may be utilised for the destruction of a second non-H_2O_2 generating organism (Klebanoff and Smith, 1970).

The importance of H_2O_2 in the bactericial mechanism was obvious, but the mechanism by which it results in cell death was uncertain. Klebanoff (1968) showed that myeloperoxidase, an enzyme present in high concentrations in the primary granules of the neutrophil, which is released into the phagocytic vacuole during granule lysis, has potent bactericidal activity in the presence of H_2O_2 and an anionic cofactor, particularly a halide (Klebanoff, 1971). Iodination of bacteria occurs both in a cell free system containing myeloperoxidase, H_2O_2 and iodide, and in the intact leukocyte following phagocytosis, and a correlation was found to exist between iodination and bacterial death (Klebanoff, 1967). Iodination by intact cells is greatly depressed or absent in leukocytes devoid of myeloperoxidase or leukocytes of patients with CGDC, and it has been suggested that iodination of cells, catalysed by myeloperoxidase, may be the mechanism by which H_2O_2 exerts its bactericial effect (Klebanoff, 1971). However, only one of five reported patients with an absolute myeloperoxidase deficiency had an increased susceptibility to infection, which suggests that myeloperoxidase does not play an essential role in the bactericidal mechanism.

Deficient production of H_2O_2 is the only clearly documented biochemical derangement that results in decreased bactericidal activity. Therefore, by studying the condition of CGDC in which the production of H_2O_2 is defective, it was hoped that the pathway of electrons from the HMP shunt to oxygen would be established.

Two key facts related to this pathway are that; one of the early metabolic changes in the phagocytosing granulocyte is the oxidation of reduced nicotinamide adenine dinucleotide phosphate (NADPH) (Zatti and Rossi, 1965) and that the phagocytosis-induced respiratory burst is insensitive to inhibition by lmM cyanide (Sbarra and Karnovsky, 1959, Rossi and Zatti, 1964). Iyer and Quastel (1963) found an enzyme in guinea pig leukocyte homogenates which catalysed the oxidation of NADPH, and to a lesser extent reduced nicotinamide adenine dinucleotide (NADH), by O_2 with the formation of H_2O_2, but this enzyme and peroxidases, which can also catalyse the oxidation of NADPH (Akazawa and Conn, 1958) are partially inhibited by lmM cyanide, and the remaining activity is considered to be inadequate to account for the respiratory burst (Baehner et al, 1970b).

Karnovsky and co-workers (Evans and Karnovsky, 1961, Cagan and Kamovsky, 1964, Baehner et al,1970b,Karnovksy, 1973) proposed an alternative enzyme, NADH oxidase, as the primary oxidase. It is distributed equally between lysosomes and the cytoplasm (Stossel et al, 1971), is insensitive to lmM cyanide, and its activity, which is increased by phagocytosis, is considered

adequate to account for the respiratory burst (Baehner et al, 1970b). Baehner and Karnovsky (1968) demonstrated defective activity of this enzyme in patients with CGDC, but this observation has not been supported by other workers (Holmes - Gray and Good, 1971).

If NADH oxidase is the primary oxidase responsible for transport of electrons to oxygen, NADH must be rapidly regenerated as there is no change in the NAD+/NADH ratio with phagocytosis (Zatti and Rossi, 1965, Selvaraj and Sbarra, 1967). A NAD+-NADPH transhydrogenase enzyme has been detected in human leukocytes (Evans and Kaplan, 1966), but guinea pig leukocytes, which have a greater respiratory burst than human leukocytes, had minimal activity (Baehner et al, 1970b). A NADPH-linked lactate dehydrogenase has been suggested as the coupling enzyme (Evans and Karnovsky, 1961). Its activity has been demonstrated in guinea pig, but not in human leukocytes (Baehner et al, 1970b). A dehydroascorbate-NADPH reductase was also detected in leukocyte granules, but its activity under the experiment conditions used, was considered insufficient to account for the observed increase in HMP shunt activity (Baehner et al, 1970b).

It is therefore known that during a burst of metabolic activity, associated with phagocytosis, there is increased activity of the HMP shunt culminating in the production of H_2O_2 which plays an important bactericidal role. This process is deranged in the condition of CGDC. The normal pathway of electrons from NADPH, produced by the HMP shunt, to oxygen, and the basic defect in CGDC are unknown.

NBT-nitroblue tetrazolium

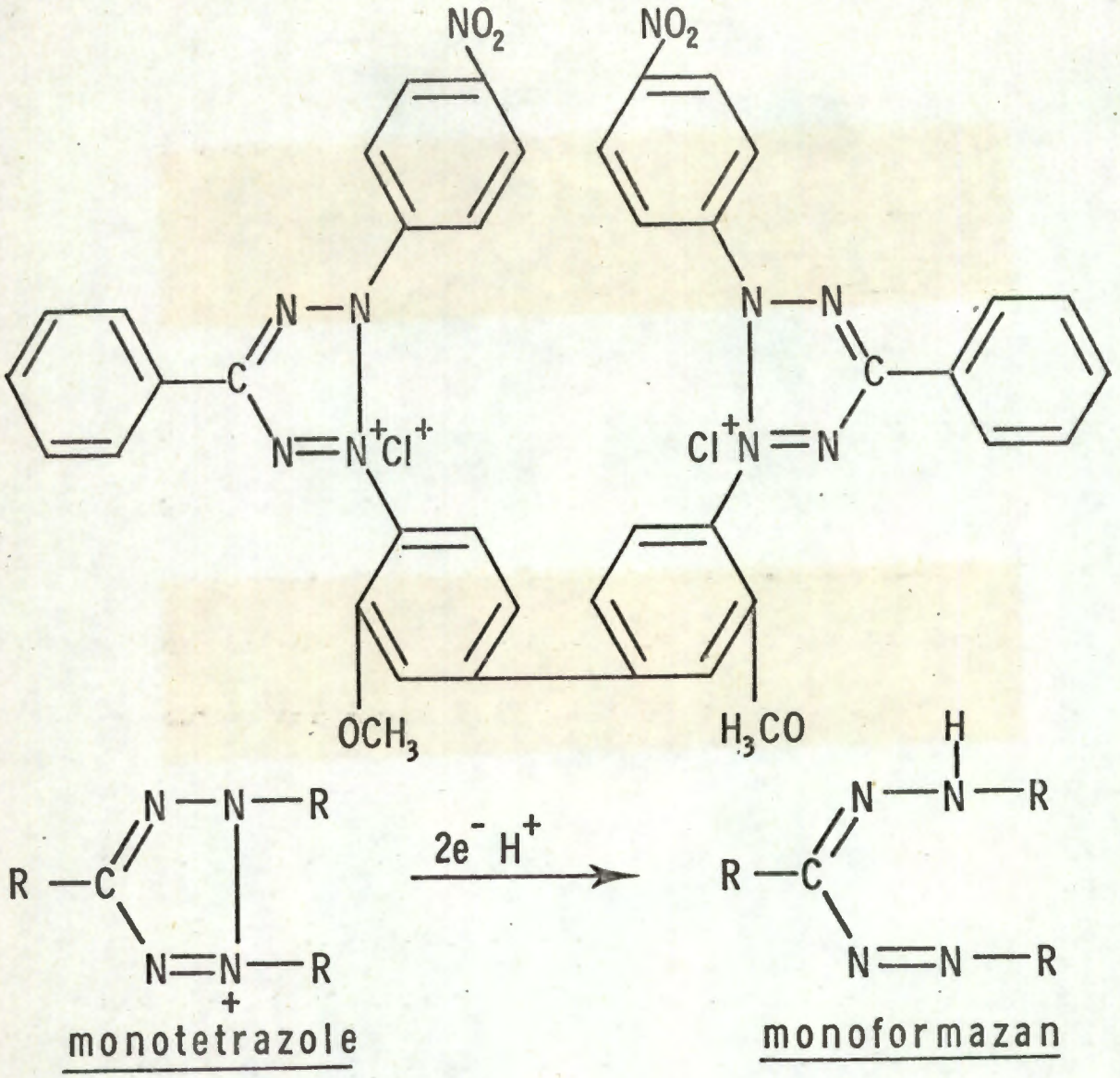


FIGURE 1.2.

Diagrammatic representation of the molecular structure of NBT (above). The site at which reduction occurs is also shown (below).

NBT was first synthesised in 1956 for use as a histochemical indicator of dehydrogenase activity (Nachlas et al, 1957). It is a p-nitrophenyl substituted ditetrazole (Fig. 1.2) which has a strong affinity for electrons. When reduced it changes from a light yellow, water soluble compound to a dark blue, water insoluble, diformazan (formazan).

Baehner and Nathan (1967) observed that NBT was reduced by neutrophils of normal subjects suspended in a balanced salt solution, that reduction was enhanced by phagocytosis and that neutrophils from patients with CGDC reduced the dye at an abnormally slow rate in the presence or absence of a phagocytic stimulus. The cells and NBT were then mixed and incubated under standard conditions in the presence of lmM potassium cyanide, and NBT reduction measured spectrophotometrically after extraction with pyridine. By using this 'quantitative NBT test' a clear distinction could be made between the amount of NBT reduction by neutrophils of normal subjects and those from patients with CGDC. Whereas the former exhibited a fourfold increase in NBT reduction in the presence of latex particles as a phagocytic stimulus, NBT reduction by neutrophils of patients with CGDC was not increased under similar circumstances. (Baehner and Nathan, 1968).

The quantitative NBT test is the standard procedure for the definitive diagnosis of CGDC. It is, however, time consuming and intricate and requires cells from a relatively large volume of blood (25 ml). Gifford and Malawista (1970) developed

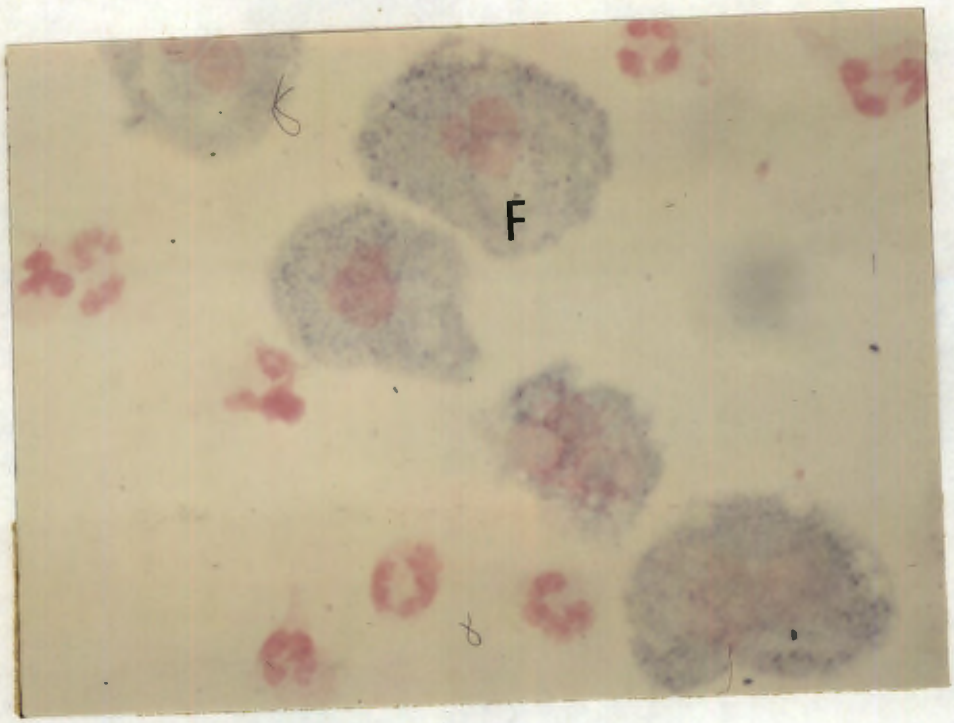


FIGURE 1.3.

Light micrograph of neutrophils after adhesion to glass slides and exposure to NBT by the method of Gifford and Malawista, showing NBT reduction by 'formazan cells' (F), but not by other neutrophils

Safronin x1,200

a simple micromethod of exposing cells to NBT, for use as a screening test for CGDC. In this test, a drop of blood is incubated on a glass slide, the neutrophils are isolated by washing off the adherent blood clot and then covered with a solution of NBT. After further incubation the NBT is washed off and the cells counterstained. Approximately half the adherent neutrophils from normal subjects are blue in colour, amorphous and swollen and appear degenerate with apparent disintegration of their nuclei (Fig. 1.3). These cells have been called 'formazan cells'. These changes are not observed in neutrophils of patients with CGDC.

Enhanced NBT reduction by neutrophils of normal subjects exposed to a phagocytic stimulus, which is known to be associated with a burst of metabolic activity, and failure of enhanced reduction by neutrophils of patients with CGDC in which this burst of metabolic activity does not occur, led Park et al (1968) to postulate that neutrophils of patients with pyogenic infection, exposed to the phagocytic stimulus of the infecting organism, would reduce the dye to a greater extent than normal, unstimulated, neutrophils. In this test, hence referred to as the NBT test, blood, taken into heparin as anticoagulant, is mixed with an equal volume of a 0.1% solution of NBT in phosphate buffered saline. After incubation for 15 minutes at 37°C and a further 15 minutes at room temperature, slide preparations are made, counterstained, and a count made of the percentage of neutrophils

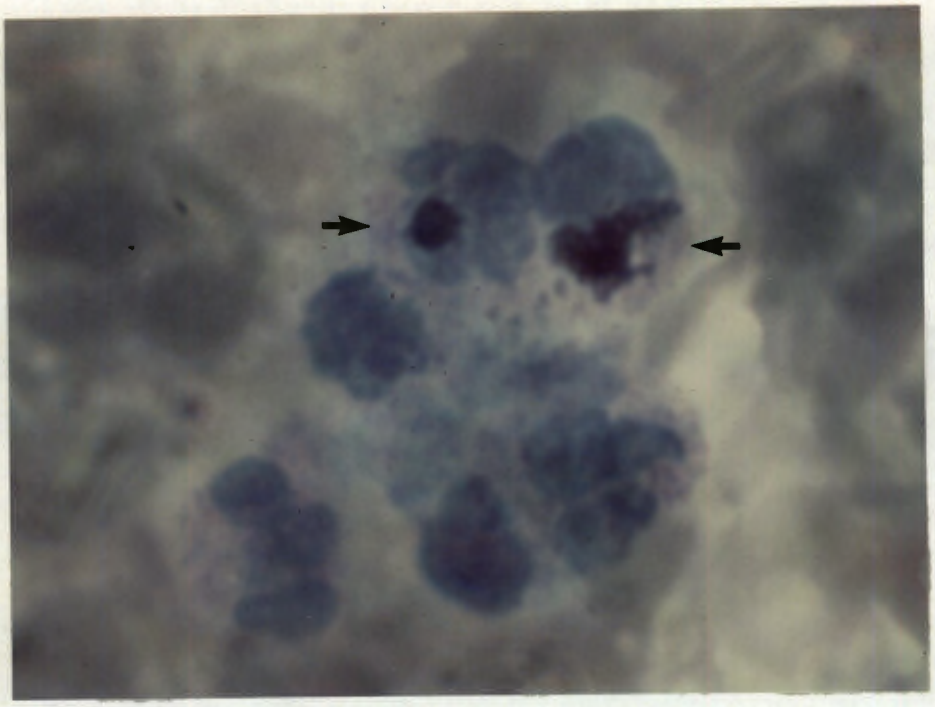


FIGURE 1.4.

Light micrograph of a typical NBT test slide preparation made by the method of Park et al (1968) (heparin as anticoagulant). Neutrophils are aggregated around amorphous material and two of the neutrophils contain dense, blue-black formazan deposits (arrows).

Leishman's x2,000

containing descrete deposits of formazan (Fig. 1.4.). The percentage of neutrophils containing formazan deposits is referred to as the NBT score. By these methods, Park et al (1968) observed a clear distinction between the high NBT score from blood of patients with pyogenic infection, and the low NBT score from the blood of normal subjects and patients with non-pyogenic disease.

The value of the test as a diagnostic aid in clinical medicine was given strong support by further studies on both children and adults (Matula and Paterson, 1971a, Feigin et al, 1971. Humbert et al, 1971, Sobel et al, 1973) and by leading articles in major medical journals (Park, 1971, Feigin, 1971, Lancet, 1971). These encouraging reports, and the simple techniques involved (allowing its application by even the most modestly equipped laboratory), were probably responsible for the intense worldwide interest that subsequently developed in the NBT test. Within a single eighteen month period (1972-mid 1973), twenty one letters relating to the test appeared in the correspondence columns of 'The Lancet' alone.

An important technical difficulty is the tendency of neutrophils to clump after incubation with NBT (Graham, 1973, Neuwirtova and Setkova, 1973). Close contact and superimposition of clumped neutrophils and deposits of formazan complicate the identification and evaluation of individual cells, while a reciprocal decrease in the number of easily identified, unclumped neutrophils

makes counting both laborious and time consuming. One method of facilitating counting is neutrophil concentration by centrifugation, and this has been employed both before (Will and Grauman, 1973, Gordon et al, 1973) and after (Strukelj and Zemva, 1973) incubation with NBT. Clumping can be prevented by the use of ethylenediamine tetra-acetate (EDTA) as anticoagulant instead of heparin, but this decreases the proportion of neutrophils reducing the dye, in infected patients, or after in vitro endotoxin stimulation (Park and Good, 1970, von Anner et al, 1971, Sobel et al, 1973). Gordon et al (1973) showed that the addition of the sucrose polymer 'ficoll' to the incubation mixture prevented both this loss of sensitivity and clumping of cells, while maintaining cellular morphology. It thus appeared that a very powerful tool had been developed for the diagnosis of pyogenic infection and that a major technical difficulty in its application had been circumvented.

A test of this kind would have tremendous clinical application. The body has a fairly non-specific response to disease of diverse aetiology, locally in the form of inflammation, and generally, as evidenced by fever, leukocytosis and changes in humoral factors. Specific therapeutic manoeuvres depend upon accurate diagnosis of the aetiology of the disease. Pyogenic bacteria are generally susceptible to therapy with antibiotics, but conventional methods for differentiating bacterial infection from other acute diseases are unsatisfactory. When the pathological lesion, or products thereof, are directly accessible, microscopy of

specimens may reveal the causal organism; however, under other circumstances, diagnosis depends upon culturing the organism, or the demonstration of a specific antibody response, both of which are time consuming. In general, the initial diagnosis of bacterial infection depends upon the recognition by the clinician of a clinical syndrome, aided by helpful but generally non-specific investigations such as radiology and examination of the peripheral blood, which produce results early in the course of the disease. In severely ill patients, therapy is then usually commenced with antibiotics likely to be effective against the probable causal organism. The diagnosis and treatment of bacterial infection in the early stages of the disease process is very subjective, and its success depends to a large extent upon the acumen of the clinician.

There is great need for a rapid, simple, accurate, objective indicator of bacterial infection. It would have wide application in clinical medicine. If acute pyogenic infection could be accurately distinguished from other diseases, antibiotic therapy could be instituted in acutely ill patients while at the same time protecting patients with non-pyogenic infective disease from side effects of this therapy (Brumfitt, 1970). It would give direction to further investigation, it would simplify the identification of infective complications of non-infective diseases, in which the clinical picture is obscured by symptoms and signs attributable to the primary pathology, or drug therapy thereof, and would allow patients at special risk of infection to be more closely monitored.

Other tests have been proposed for this purpose. Leukocyte alkaline phosphatase activity was found to be elevated in patients with bacterial infection (Wachstein, 1946) but this has since been shown to be a non-specific effect (Valentine and Beck, 1951, Beisel, 1967).

The gelationof lysates of amoebocytes of the horseshoe crab, limulus polyphemus, has been proposed as a highly sensitive and specific bioassay for endotoxin, which is useful in the diagnosis of systemic infections caused by gram-negative bacilli (Levin et al, 1970). However, this test was found to be relatively insensitive and non-specific when reassessed by Stumacher et al (1973).

In view of; the great clinical value of a simple diagnostic test of pyogenic infection; the fact that the NBT test is the only test of this kind that has been found to be of value (Park et al, 1968, Matula and Paterson, 1971a, Feigin et al, 1971., Humbert et al, 1971, Sobel et al, 1973, Gordon et al, 1973); reports of conditions resulting in false-negative and false-positive results (Tables 2.1 and 2.2); the absence of any study in which a direct comparison has been made between the results of tests performed on blood anticoagulated with heparin (Park et al, 1968) or with EDTA (Gordon et al, 1973), the initial purpose of the studies in this thesis was to re-evaluate the clinical application and diagnostic accuracy of the NBT test, and to compare the results of tests performed on the same blood sample anticoagulated either with heparin or with EDTA. Further studies were conducted to determine the mechanisms of entry of the dye into neutrophils and

to examine factors which might be responsible for positive and for false-negative test results. During the course of these studies it was observed that reduced NBT is not confined to the cellular elements of blood, but also occurs in solution in the plasma. As reduced NBT is insoluble in water, and binds to cellular protein (Nachlas et al, 1957), studies were then undertaken to determine whether or not this reduced NBT was maintained in solution by binding to plasma proteins. The discovery that reduced NBT was selectively bound to lipoproteins led to further studies to determine the value of this compound as a lipoprotein stain. Finally, the use of liposomes as a vehicle for the transport of therapeutic agents into cells was proposed as a possible new approach to the treatment of patients with CGDC. The anatomical destination of these particles after intravenous administration is unknown and was studied by using entrapped NBT as a histochemical marker.

CHAPTER 2

A re-evaluation of the value of the NBT test in the diagnosis of bacterial infection

2.1. INTRODUCTION

The Nitroblue Tetrazolium (NBT) test was developed in 1968 by Park et al (1968) as a non-specific in vitro diagnostic test for bacterial infection. In this test, blood anti-coagulated with heparin (75-100 i.u./ml) is incubated with a dilute solution of NBT and a count is made of the percentage of neutrophils containing intracytoplasmic deposits of dark blue formazan, the reduced product of NBT. In patients with bacterial or fungal infections the percentage of neutrophils containing formazan, called NBT-positive cells, was greater than 10%; in healthy controls and patients with non-pyogenic diseases the percentage of NBT-positive cells was 10% or less. Similar findings were recorded in children (Feigin et al, 1971 , Humbert et al, 1971) and adults (Matula and Paterson, 1971a, Freeman and King, 1972a).

Occasional false-negative results were attributed to an inability of neutrophils to respond to the stimulus of infection (Park and Good, 1970). An in vitro stimulation test using Escherichia coli endotoxin, was developed (Park and Good, 1970) to indicate the ability of neutrophils to respond to stimulation, so distinguishing between true and false-negative results.

TABLE 2.1.

Conditions in which inappropriately normal or depressed NBT test results have been obtained in the presence of bacterial infection:

CONDITION	AUTHORS
Antibiotic therapy	Ridgway and Johnson 1973 Rubenstein and Pelet 1973 Freeman et al 1973a Hawkins 1973
Appendicitis	Drysdale 1972
Bacterial meningitis	Esposito and De Lalla 1972
Chronic granulomatous disease of childhood	Park et al 1968 Park 1971
Glucocorticoid therapy	Miller and Kaplan 1970 Matula and Paterson 1971b Ng et al 1972
Glucose-6-phosphate dehydrogenase deficiency	Feigin 1971
Hypogammaglobulinaemia	Park 1971
Lipochrome histiocytosis	Park 1971
Myeloperoxidase deficiency	Feigin 1971
Nephrosis	Park 1971
Phenylbutazone and salicylate therapy	Douwes 1972
Premature infants (infected)	Cocchi et al 1969
Sickle cell disease	Park 1971
Streptococcal pharyngitis	Randall et al 1973
Systemic bacterial infection	Ng et al 1972 Hawkins 1973
Systemic lupus erythematosus	Douwes 1972 Wenger and Bole 1973

TABLE 2.2.

Conditions other than bacterial infection in which positive NBT tests have been reported:

CONDITION	AUTHORS
Chediak- Higashi Syndrome	Grush and Mauer 1969
Fungal infection	Park et al 1968 Matula and Paterson 1971a
Haemophilia	Humbert et al 1971
Hepatitis	Elgefors and Olling 1972 Hellum and Solberg 1973a
Lymphoma	Catovsky 1971 von Anner et al 1971 Soonattrakul and Anderson 1973
Malaria	Matula and Paterson 1971a Anderson 1971 Chretien and Garagusi 1971
Mycoplasma infection	Freeman and King 1972c
Myelofibrosis	von Anner et al 1971 Ng et al 1972
Myocardial infarction	Lauter et al 1973
Neonates	Park et al 1969 Cocchi et al 1971a Humbert et al 1970 Douwes 1972
Neoplasia	Ashburn et al 1973 Hellum and Solberg 1973a
Oral contraceptive therapy	Norden and Reese 1972
Osteogenesis imperfecta	Humbert et al 1971 Douwes 1972
Parasitic disease	Chretien and Garagusi 1971
Polycythaemia vera	Ashburn et al 1973
Pregnancy	Drysdale 1972
Reactional lepromatous leprosy	Goihman-Yahr et al 1973
Toxoplasma infection	von Anner et al 1971
Typhoid vaccination	Grush and Mauer 1969
Viral infection	Kitani et al 1972 Humbert et al 1971

Neutrophil clumping may complicate the identification and counting of individual cells and is a major technical difficulty of the test of Park et al. (1966). Gordon et al. (1973) reported that cell clumping could be inhibited by the use of EDTA instead of heparin as anticoagulant, and that the loss of sensitivity of the test that results from the use of this agent (Park and Good, 1970), was prevented by the addition of the sucrose polymere, ficoll, to the incubation mixture. It thus appeared that the NBT test was a simple, rapid and accurate method of distinguishing pyogenic infection from other acute diseases, and that a major technical difficulty had been eradicated.

The test was re-evaluated in this study because of the important potential clinical application of a test of this kind, and because reports of false-negative and false-positive test results (Tables 2.1 and 2.2) cast doubt upon its specificity and accuracy. Although the methods of Park et al and Gordon et al, which use heparin and EDTA as anticoagulant respectively, were independently reported as being of diagnostic value, and had been assumed to have the same basic mechanism (Gordon et al, 1973), they had not been directly compared.

A prospective study was initiated to: re-evaluate the role of the NBT test as a useful indicator of pyogenic infection using modifications of the methods of both Park et al (1968) and Gordon et al (1973); to compare the results obtained by the two methods; to investigate the individual and interobserver error in

the quantitation of NBT reduction in blood smears, and to compare NBT reduction with the total white blood cell count (WBC), neutrophil count, a shift to the left of the myeloid series of white blood cells, the presence of toxic granulations in the cytoplasm of neutrophils, the blood platelet concentration, the erythrocyte sedimentation rate (ESR), and the serum concentration of C-reactive protein (CRP) and α^1-acid glycoprotein.

2.2. METHODS

Solubilisation of NBT

A 0.2% solution of NBT in 0.15 M NaCl was made by the addition of 100 mg of solid NBT to 50 ml of 0.15 M NaCl in a conical flask which was partially immersed in hot water ($\pm$80°C), the mixture of NBT was mixed with a magnetic stirrer for 30 minutes and then filtered through a Millipore filter (0.22μ). This solution was stored at 4°C and aliquots were diluted to 0.1%, with an equal volume of phosphate buffered saline containing 9 mM phosphate buffer, pH 7.2, and 0.15 M NaCl.

NBT is more soluble in H_2O in which it was rapidly dissolved by mixing solid NBT and warm water ($\pm$50°C) by vigorous manual shaking. The mixture was not filtered. These methods of dissolving NBT were used throughout the studies that follow.

2.2.1. Patients and Controls

Details of the 202 patients and 26 controls are included in the attached appendices. The number, age (mean and

TABLE 2.3. Details of subjects

	Normal Controls	Untreated Pyogenic Infection	Treated Pyogenic Infection	Tuberculosis	Non-Bacterial Infection	Non-Infective Disease	Un-Diagnosed Disease	Total
Group	1	2	3	4	5	6	7	
Number of test subjects	26	40	26	7	21	91	17	228
Number of male subjects	9	20	14	3	5	37	8	96
Mean age	33.3	41.5	37.9	35.7	31.2	48.5	50.8	42.4
Standard deviation of age	15.3	24.9	20.2	22.2	16.6	22.2	24.7	21.3

standard deviation), sex and classification of the subjects is shown in Table 2.3.

Group 1 (Appendix A)

25 healthy hospital staff and 1 normal child.

Groups 2 and 3 (Appendices B and C)

66 patients with pyogenic infections. Patients in Group 2 had not recieved any antimicrobial therapy (Table 2.4) while those in Group 3 (Table 2.5) had received antimicrobial chemotherapy at the time of the test; 4 patients had received therapy for longer than 1 week; the mean period of treatment for the remainder was 2.2 days. The criteria for inclusion of patients in these groups was the clinical diagnosis supported by either bacteriological identification of the organism (in 38 patients), the detection of significant quantities of pus (in 32 patients with abscesses), or the histological picture of acute suppuration in appendicitis (2 patients).

All patients in Groups 2 and 3 were assessed clinically by the author and subdivided, on the basis of the severity of systemic manifestations, into 3 grades. These were absent or minimal in patients classified as grade A, which included patients with small localised abscesses or post-operative abdominal wound abscesses. Grade C patients were severely ill with infections of the order of severity of septicaemia or liver abscess. Grade B patients had illnesses of intermediate severity.

TABLE 2.4. Details of patients with untreated pyogenic infections (Group 2).

DISEASE	NUMBER OF PATIENTS
Abscesses - Site	
Abdominal wound	12
Perianal	3
Liver	2
Appendix	1
Lung	1
Subphrenic	1
Other infections	
Pneumonia	4
Urinary tract infection	4
Appendicitis	2
Endocarditis	1
Erysipelas	1
Gastroenteritis	1
Infected burn	1
Infected uterus	1
Otitis media	1
Otitis media and pneumonia	1
Pharyngitis	1
Portal pyaemia	1
Pyonephrosis	1
TOTAL	40

TABLE 2.5. Details of patients with treated pyogenic infections (Group 3)

DISEASE	NUMBER OF PATIENTS
Abscesses - Site	
Abdominal wound	6
Appendix	2
Liver	1
Pharyngeal	1
Subphrenic	1
Tubo-ovarian	1
Other diseases	
Cellulitis	2
Generalised peritonitis	2
Septicaemia	2
Arthritis (suppurative)	1
Bronchopneumonia	1
Cholecystitis	1
Oesophagitis	1
Otitis media	1
Prostatitis	1
Pyelonephritis	1
Osteitis	1
TOTAL	26

Group 4 (Appendix D)

7 patients with bacteriologically proven active tuberculosis.

Group 5 (Appendix E)

21 patients with non bacterial infections; 1 with hepatic schistosomiasis; 1 with massive Ascaris infestation and 19 with viral disease of which 16 were confirmed serologically; a clinical diagnosis was made in 2 cases of rubella and in 1 case with Herpes zoster infection.

Group 6 (Appendix F)

91 hospital patients with a diverse range of non-infective disease including patients with diabetes, haematological pathology, 'auto immune' disease, neoplasia, inflammatory bowel disease and a number of miscellaneous conditions.

Group 7 (Appendix G)

17 patients in whom bacterial infection was suspected but not proven.

2 patients were included in group 2 (numbers 18 and 23) and group 6 (numbers 72 and 47).

Selection of test subjects

Patients in groups 2-5 and 6 were for the most part patients who had been recently admitted to one of the wards of Northwick Park Hospital, who could be clearly classified into one of the study groups or in whom this seemed likely after further investigation. Most of the patients in group 6 were outpatients at Northwick Park Hospital on whom blood tests were being performed.

13 of the patients in group 5 were part of a clinical trial at the Common Cold Research Centre, Salisbury. There was a slight bias towards selection of patients with haematological disease, in view of reports of false positive tests on these patients, and patients with inflammatory bowel disease because of the author's clinical commitment to a department of gastroenterology. Patients were unselected as far as disease severity was concerned and no patients were removed from the study after initial selection.

2.2.2. N B T Tests

Blood was taken by venepuncture with a disposable plastic syringe and 1.0 ml was transferred to plastic test-tubes containing 0.05 ml of a heparin solution containing 1000 i.u./ml. (50 i.u./ml) and 5.0 ml to glass bottles containing 12.0 mg of disodium E D T A (2.4 mg/ml). All tests were done immediately or within 1 hour of venepuncture after storage of blood samples at 4°C.

For the test with heparinised blood, 0.2 ml was incubated at 37°C for 10 minutes in a plastic test tube. 0.2 ml of a 0.1% solution of N B T in phosphate buffered saline was then added. The mixture was gently shaken, then incubated at 37°C for 15 minutes, then at room temperature (±25°C) for another 15 minutes. The sample was then applied to a glass slide by running a drop of the test mixture down the slide as described by Wollman et al (1972). This method is here referred to as the heparin method.

The E D T A blood was tested as above, except

that the N B T solution contained ficoll. (200 mg/ml); the mixture was incubated at 37°C for 30 minutes and then at room temperature for 15 minutes; and the sample was applied to glass slides by smearing. This method is referred to as the E D T A /ficoll method. Smears were air dried and stained.

2.2.3. Counting

The smears were counted independently by three observers who did not know the patient details; one observer (observer 1) the author, was very experienced (having previously counted 3000 such preparations), one (observer 2) was relatively experienced in that she was a trained light microscopist and had counted 200 slide preparations before commencing the study, and the third had minimal experience of light microscopy and no previous experience with these tests (observer 3). One hundred neutrophils were counted on each slide to obtain the 'N B T score'.

The 45 preparations on which the greatest counting difference between the two more experienced observers was obtained were recounted by both observers to obtain an artificially corrected result for comparison with other data. 20 slides prepared by the heparin method and 20 prepared by the E D T A /ficoll method were randomly selected and recounted to assess the observer error.

The scores obtained by observer 1, modified in the light of observer 2's results by correcting obvious counting errors, were regarded as the test result for intergroup comparison and for relating to the other measurements.

2.2.4. Other Investigations

The white blood cell counts were performed on a Coulter Counter model S.

Neutrophil counts and the assessment of platelet concentration, and the presence or absence of a shift to the left of the myeloid series and toxic granulations in neutrophils were performed by an experienced haematology technician. The platelet concentration was roughly graded into 5 grades of concentration (< 1-4+) purely on a subjective assessment of the peripheral blood smear. The erythrocyte sedimentation rates were determined by the Westergren method (Westergren, 1921) in disposable plastic tubes ('Dispette').

The serum C-reactive protein concentration was measured by the Mancini radial immunodiffusion technique (Mancini, et al, 1965), using commercially available 'Partagen' plates and standard C R P preparations on 18 patients from groups 2 and 3 and 18 patients from group 6. The serum concentration of α'-acid glycoprotein was measured by the same technique on 78 patients and normal subjects. 0.4 ml of specific rabbit anti-human serum, titre 0.4 mg/ml, was mixed with 36.0 ml of 1.0% agarose in a solution containing Barbital buffer, pH 8.6, ionic strength 0.05, and calcium lactate 1 mM at a temperature of 48°C. The mixture was rapidly poured onto a glass slide (10.7 x 10.7 cm, Chance Glass) on a metal level plate, and allowed to gel. 26 evenly spaced wells

were punched in the gel. Serum samples and standard solutions of α^1-acid glycoprotein (50, 100 and 200 mg per ml) were diluted 1 in 11 in 0.15M saline and 3 μl of each specimen was measured into each well by means of a Hamilton syringe. Standard samples were applied in duplicate to each plate in random order. The plates were placed in a damp box for 48 hours at room temperature after which they were washed in 0.15M saline for 24 hours and then in water for 2 hours, covered with filter paper (Whatman Number 1) and air dried. Staining was performed with 0.1% amido black in a mixture of methanol, water and glacial acetic acid in the ratios of 5:5:1 by volume. The plate was destained for 30 minutes in the methanol, water, glacial acetic acid mixture and finally rinsed in methanol. The diameter of the precipitation rings was measured in two directions, and the value of the product, D^2, calculated. The values obtained from the standard solutions were used to plot a calibration curve (D^2 against concentration) from which serum concentrations were calculated.

2.2.5. Statistical Analysis

To equalise the variance, the N B T scores were transformed to angles (Fisher and Yates, 1963) and the E S R, W B C and neutrophil counts to logarithms (to the base 10) before analysis. A small number of analyses were performed with untransformed values.

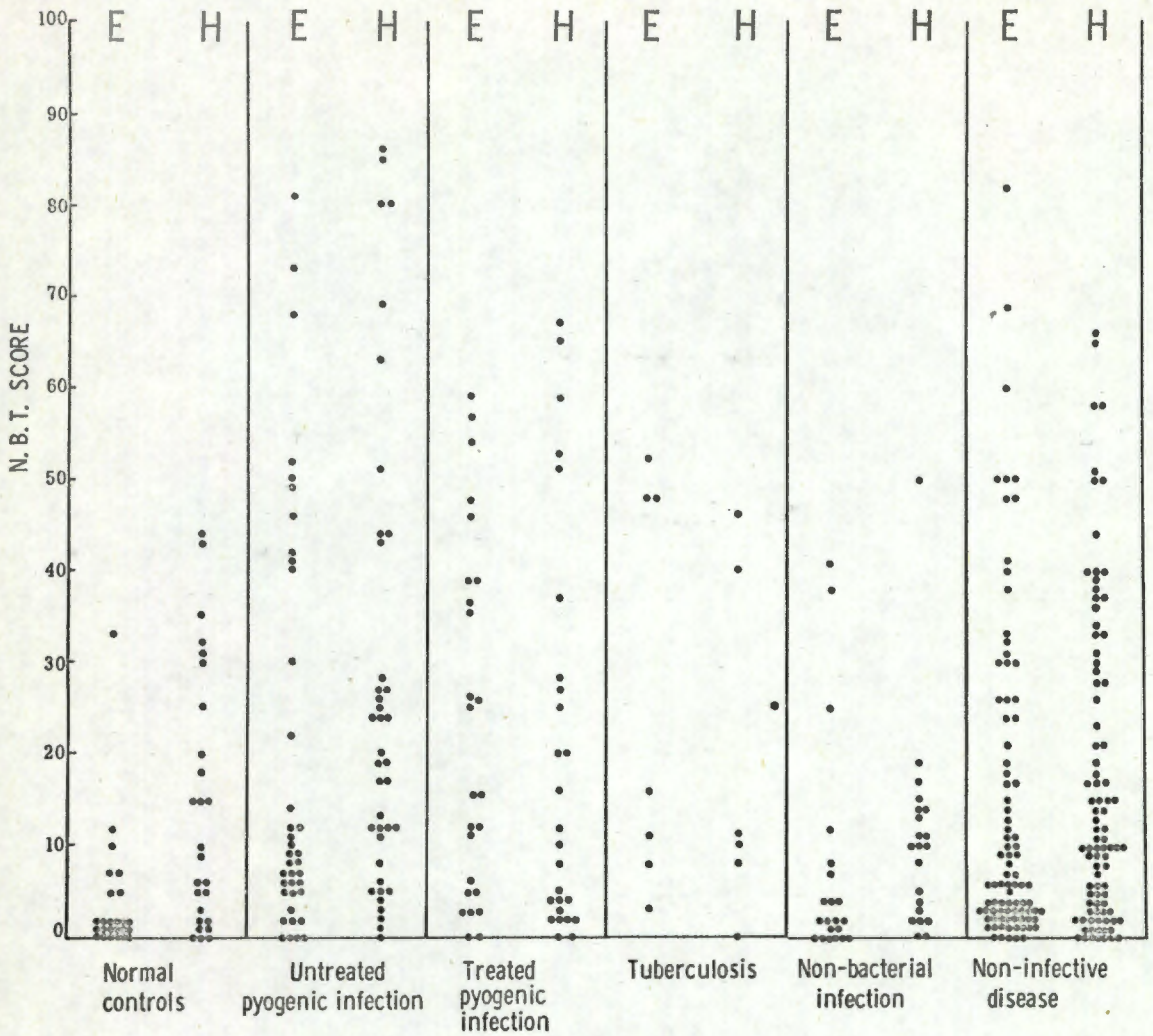


FIGURE 2.1.

NBT scores obtained with the EDTA/ficoll (E) and heparin (H) methods in different subject groups.

2.3 Results

The EDTA/ficoll and heparin NBT scores obtained by Observer 1 in subject groups 1-6 are shown in Fig. 2.1. The controls gave consistently low NBT scores by the EDTA/ficoll method. In all other groups there was considerable overlap of the NBT scores. Consistently high scores were obtained in patients from group 6 with inflammatory bowel disease, erythema nodosum, myelofibrosis and diabetic precoma. (Appendix H).

The results of the agreed EDTA/ficoll and heparin NBT scores, the ESR, WBC and neutrophil count (Mean and S.E.) for the different groups are shown in Table 2.6.

The NBT scores were compared with the ESR, WBC and neutrophil counts in groups 1,2,3,5 and 6, and the correlation coefficients obtained are shown in Table 2.7. There was a highly significant overall correlation between the neutrophil count and the heparin score and the EDTA/ficoll score and the ESR. Significant correlations were obtained between the heparin score and the neutrophil count in groups 1 and 2, and between the EDTA/Ficoll score and the neutrophil count in group 1, the WBC in group 2 and neutrophil count overall.

The mean number of NBT positive neutrophils in each group is shown in Table 2.8.

Feigin et al (1971) constructed nomograms on which are plotted the NBT score and total number of NBT positive neutrophils

TABLE 2.6. The NBT scores, erythrocyte sedimentation rate (ESR), white blood count (WBC) and neutrophil count in the different groups. Statistical analysis has been done on a transformed scale (angles and logs) and the standard errors apply to the transformed means. The absolute (detransformed) values are also given.

INVESTIGATION	GROUP						
	1	2	3	4	5	6	7
EDTA/ficoll score							
Mean (percentage)	2.4	15.0	19.2	23.5	3.9	10.8	10.5
Mean (angles)	9.0	22.8	26.0	39.0	11.4	19.2	18.9
S.E.	2.8	2.3	2.8	5.3	3.1	1.5	3.4
Number	26	39	25	7	21	88	17
Heparin score							
Mean (percentage)	10.2	23.9	14.4	11.1	8.4	14.0	11.5
Mean (angles)	18.6	29.3	22.3	19.5	16.8	22.0	19.8
S.E.	2.8	2.3	2.8	5.4	3.1	1.5	3.5
Number	26	40	26	7	21	91	17
E.S.R.							
Mean (mm in 1 hr.)	6.4	35.2	42.9	35.2	8.2	23.8	23.4
Mean (logs)	0.80	1.55	1.63	1.55	0.91	1.38	1.37
S.E.	0.100	0.082	0.102	0.182	0.108	0.054	0.134
Number	20	30	19	6	17	67	11
W.B.C.							
Mean (x 10^3)	6.5	10.2	9.6	6.7	7.8	8.2	8.4
Mean (logs)	0.81	1.01	0.98	0.83	0.89	0.91	0.12
S.E.	0.037	0.029	0.037	0.070	0.041	0.019	0.045
Number	26	40	26	7	21	91	17
Neutrophil count							
Mean (x 10^3)	3.5	7.1	6.8	4.6	4.4	4.9	5.2
Mean (logs)	0.55	0.85	0.84	0.66	0.65	0.69	0.72
S.E.	0.050	0.040	0.051	0.096	0.055	0.027	0.063
Number	26	40	25	7	21	91	16

TABLE 2.7. The correlation between the NBT score obtained by the EDTA/ficoll (E) and heparin (H) methods and the erythrocyte sedimentation rate (ESR), total white blood count (WBC) and neutrophil count. Results are expressed as the correlation coefficient with number of comparisons shown. The significant level (p value) is indicated where significant correlations were obtained.

GROUP	METHOD	ESR	p	WBC	p	NEUTROPHIL COUNT	p
1.	H	0.17(20)		0.15(26)		0.42(26)	<0.05
	E	0.27(20)		0.27(26)		-0.38(26)	<0.05
2.	H	-0.06(29)		0.27(26)		0.45(39)	<0.01
	E	-0.10 (30)		0.41(40)	<0.01	0.23(38)	
3.	H	-0.07(19)		0.12 (26)		0.15 (25)	
	E	0.19 (19)		-0.09(25)		-0.1 (24)	
5.	H	-0.21 (17)		0.17 (21)		0.14 (21)	
	E	0.47(17)		-0.21 (21)		-0.10 (21)	
6.	H	0.05(67)		-0.02(91)		0.11 (90)	
	E	0.11 (67)		-0.02(88)		0.10 (87)	
TOTAL	H	0.12 (169)		0.18 (228)		0.28 (224)	<0.001
	E	0.29(170)	<0.001	0.17 (223)		0.16 (219)	<0.05

TABLE 2.8. The mean and standard error of the total number of NBT positive neutrophils/c.mm of blood (NBT score x Neutrophil count). Statistical analysis has been done on a transformed scale (logs) and the standard errors apply to the transformed means. The absolute figures and number of tests are also shown.

METHOD		GROUP							TOTAL
		1	2	3	4	5	6	7	
Heparin	Mean (absolute figure)	109.6	1000.0	288.4	33.1	131.8	275.4	257.0	269.0
	Mean (logs)	4.04	5.00	4.46	3.52	4.12	4.44	4.41	4.43
	S.E.	0.31	0.17	0.29	0.92	0.31	0.14	0.32	0.09
	Number	26	39	25	7	21	90	16	224
EDTA/ficoll	Mean (absolute figure)	12.6	281.8	398.1	802.8	12.3	208.9	138.0	128.8
	Mean (logs)	3.10	4.45	4.60	4.91	3.09	4.32	4.14	4.11
	S.E.	0.35	0.27	0.30	0.18	0.45	0.13	0.42	0.10
	Number	26	38	24	7	21	87	16	219

TABLE 2.9. Comparison between the NBT score and the concentration of C reactive protein (CRP), α^{I}-acid glycoprotein and the sum of the two. Results are expressed as the correlation coefficient. The number of comparisons made is shown in brackets and the significance level (p value) is shown where statistically significant.

PROTEIN	EDTA/ficoll		p	HEPARIN		p
α^{I}-Acid glycoprotein	0.39	(78)	< 0.001	0.25	(77)	< 0.05
CRP	0.52	(26)	< 0.01	0.297	(26)	
α^{I}-Acid glycoprotein + CRP	0.38	(12)		0.65	(15)	< 0.01

Fig. 2.2.

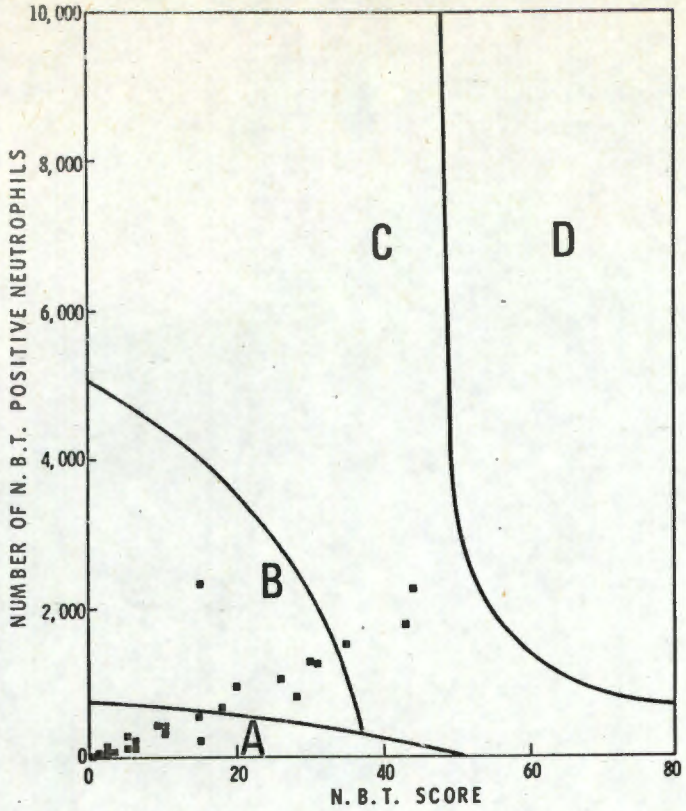
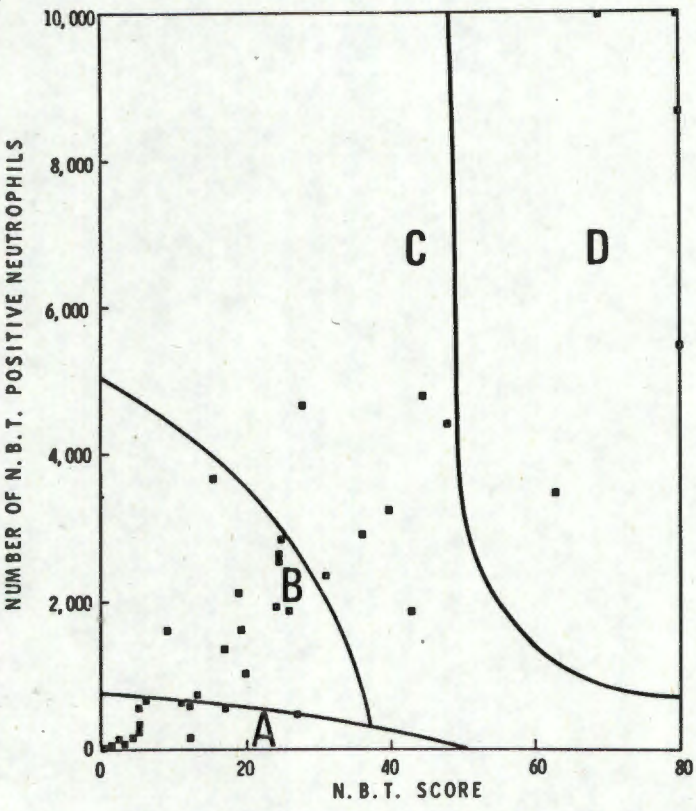


Fig. 2.3.



FIGURES 2.2 and 2.3.

Results of NBT tests on normal subjects (Fig. 2.2) and patients with untreated pyogenic infection (Fig. 2.3) plotted on nomograms constructed by Feigin et al (1971).

to magnify the discrimination between the groups. Use of these nomagrams did not result in greater accuracy in the interpretation of the results of either the EDTA/ficoll or heparin NBT tests (Figs. 2.2 and 2.3).

The NBT scores were compared to the serum concentration of C R P, α'-acid glycoprotein and the sum of these proteins (Table 2.9.) There was a highly significant correlation ($p < 0.001$) between the EDTA/ficoll score and the serum concentration of α'-acid glycoprotein.

A comparison of the clinical assessment of severity of illness of patients in groups 2 and 3 with the NBT scores showed an increased NBT score with increasing severity. This was more striking for the heparin than the EDTA/ficoll method. (Table 2.10.p.58). A shift to the left of the myeloid cell series and the presence of toxic granulations in the cytoplasm of neutrophils was most marked in groups 2 and 4. (Table 2.11)

TABLE 2.11. The percentage of each group of patients showing either a shift to the left of the myeloid series or the presence of toxic granulations in their neutrophils.

GROUP	1	2	3	4	5	6	7
Shift to the left of the myeloid series.	0	22	4	29	5	19	6
Toxic granulations	0	15	8	14	5	10	0

TABLE 2.10. The effect of severity of illness on NBT scores in bacterial infections

Mean scores (angular transformation) with standard error shown.

		SEVERITY OF ILLNESS					
		A Mild		B Moderate		C Severe	
		Numbers of patients	NBT score	Numbers of patients	NBT score	Numbers of patients	NBT score
Untreated pyogenic infection	E.D.T.A./ficoll method	23	19.8(±3.4)	13	25.4(±4.5)	3	34.6(±9.4)
	Heparin method	22	25.1 (±3.3)	15	31.8 (±3.9)	3	47.6(±8.8)
Treated pyogenic infection	E.D.T.A./ficoll method	11	26.0(±4.9)	9	23.3(±5.4)	5	30.7(±7.3)
	Heparin method	11	14.5 (±4.6)	10	21.1 (±4.8)	5	42.2(±6.8)

TABLE 2.12. Comparison between the EDTA/ficoll (E) and heparin (H) NBT scores of patients in groups 2 and 6 in whom a shift to the left of the myeloid series and toxic granulations in the neutrophils were either present or absent.

Results are expressed as the correlation coefficient. The number of comparisons is shown in brackets. The significance level (p value) is shown where statistically significant.

	NBT SCORE			
	Group 2		Group 6	
Investigations	E	H	E	H
Shift to the left: present	23.6±7.2(9)	41.6±7.0(9)	14.1±3.7(17)	24.0±5.1 (16)
		p <0.05		
Shift to the left: absent	18.6±4.1 (30)	23.7±4.3(31)	15.1±2.1 (71)	16.7±1.9 (75)
Toxic granulations: present	27.8±10.3(6)	50.0±11.0(6)	16.9±6.1 (9)	27.2±7.5(9)
		p <0.02		
Toxic granulations: absent	18.3±3.7(33)	23.7±3.7(34)	21.3±5.2(76)	17.0±1.8 (82)

A comparison was made in groups 2 and 6 between the NBT scores in those patients in whom these parameters were positive and those in whom they were negative (Table 2.12). A statistically significant difference was only obtained in patients in group 2 between the NBT scores obtained by the heparin method in the presence and absence of a shift to the left and toxic granulations ($p < 0.05$ and

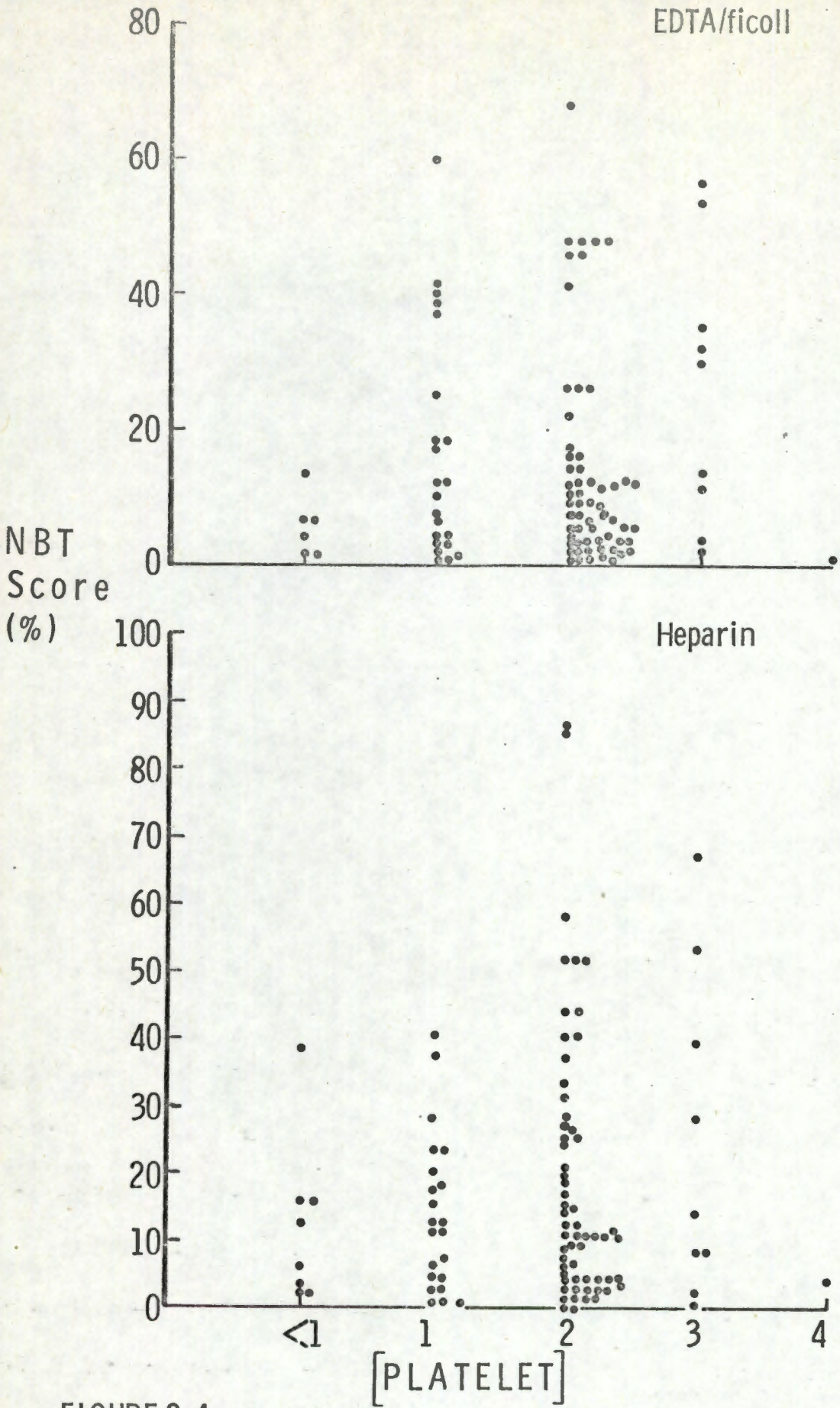


FIGURE 2.4

Relationship between results of NBT tests performed by the EDTA/ficoll and heparin methods and platelet concentration quantified by examination of the peripheral blood smear and expressed as increasing gradiations from <1 to 4.

<0.02 respectively). (Table 2.12).

There was no obvious relationship between the NBT score and platelet concentration (Fig. 2.4).

2.3.1. Counting Error

The discrepancies between duplicate counts tended to be smaller when the counts were themselves small, and to counteract this the counts were transformed to angles (Fisher and Yates, 1963) before analysis (2.2.5.) In these units the error variances were 20.5 and 15.1 and 17.6 and 20.3 for counts performed by observers 1 and 2 on slides prepared by the EDTA/ficoll and heparin methods respectively. These appear to be consistent with no difference between methods or between observers. The average variance is 18.4 giving a standard deviation of $\pm$ 4.28°; this corresponds to $\pm$ 7.5% at a count of 50%, or $\pm$ 5% at a count of 10%. Counting 100 cells in itself gives rise to a variance of 8.2. After allowing for counting errors the correlations between duplicate counts by observers 1 and 2 were very high (Figs. 2.5 and 2.6), estimated at 1.00 and 0.99 for preparations made by the EDTA/ficoll and heparin methods respectively. With the recounts on EDTA/ficoll preparations, observer 1 gave rather different readings on the two occasions counting lower on the second occasion, the mean difference

Fig. 2.5.

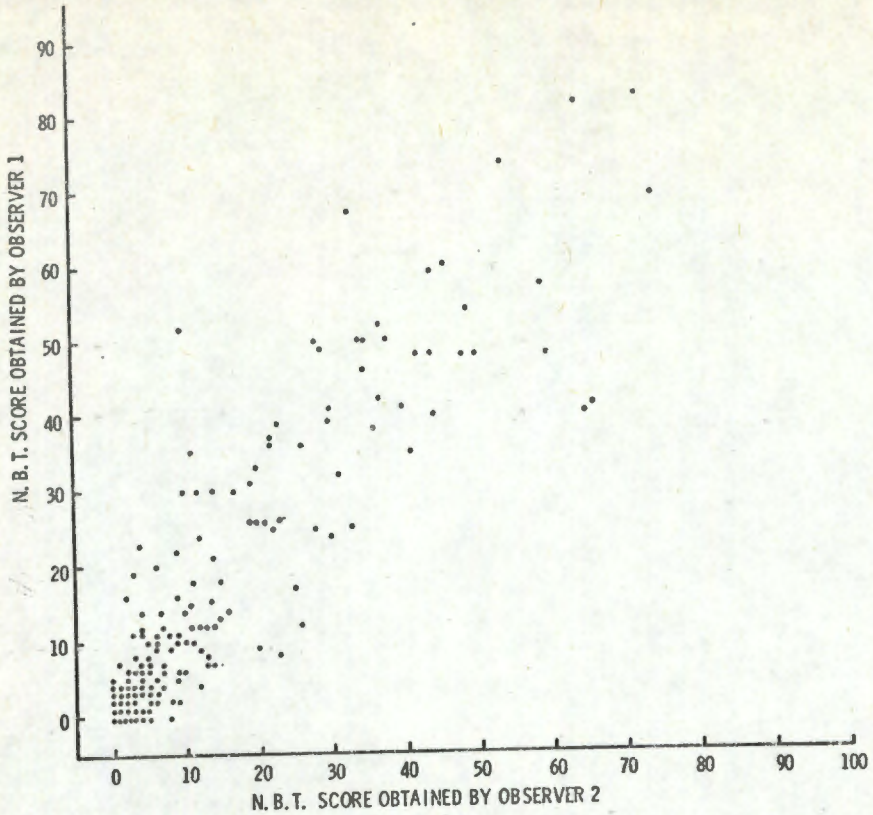
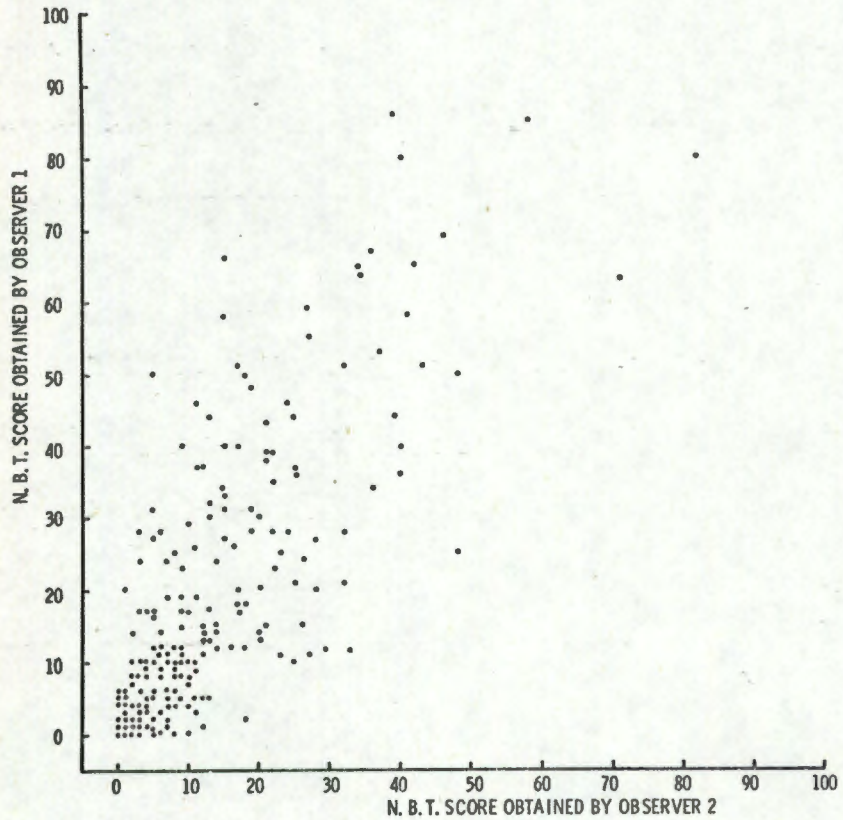


Fig. 2.6.



FIGURES 2.5 and 2.6

The relationship between duplicate counts by experienced observers on slides prepared by the EDTA/ficoll (Fig. 2.5) and heparin (Fig. 2.6) methods.

of which was $4.9^{\circ} \pm 1.43$. Despite these close correlations there was an inter-observer difference in the absolute results obtained, observer 1 consistently counting higher than observer 2 on the preparations from the heparin tests. There was closer agreement of the counts performed on slides made by the EDTA/ficoll method (Figs. 2.5 and 2.6).

Again using angular transformations, comparisons were made between observers 1 and 2, both experienced observers, and between 1 and 3, an inexperienced observer on counts from all the test specimens. The correlations between observers 1 and 2 are high, but those between observers 1 and 3 are considerably lower. Observer 3 seemed to improve with experience with EDTA/ficoll counts but not with heparin counts, the mean of which is consistently lower than that of observer 1 (Table 2.13).

TABLE 2.13. Comparisons between duplicate counts by observers 1 and 2 and 1 and 3 on preparations made by the EDTA/ficoll (E) and heparin methods (H). Results are expressed as the correlation coefficients observed.

OBSERVERS	E.	H.
1 and 2	0.88	0.78
1 and 2 corrected for counting error	0.97	0.87
1 and 3 first 100 readings	0.32	0.58
last 100 readings	0.57	0.53

The EDTA/ficoll and heparin scores obtained by observer 1 were compared and revealed a very low correlation coefficient of 0.34 which was only raised to 0.37 when allowance was

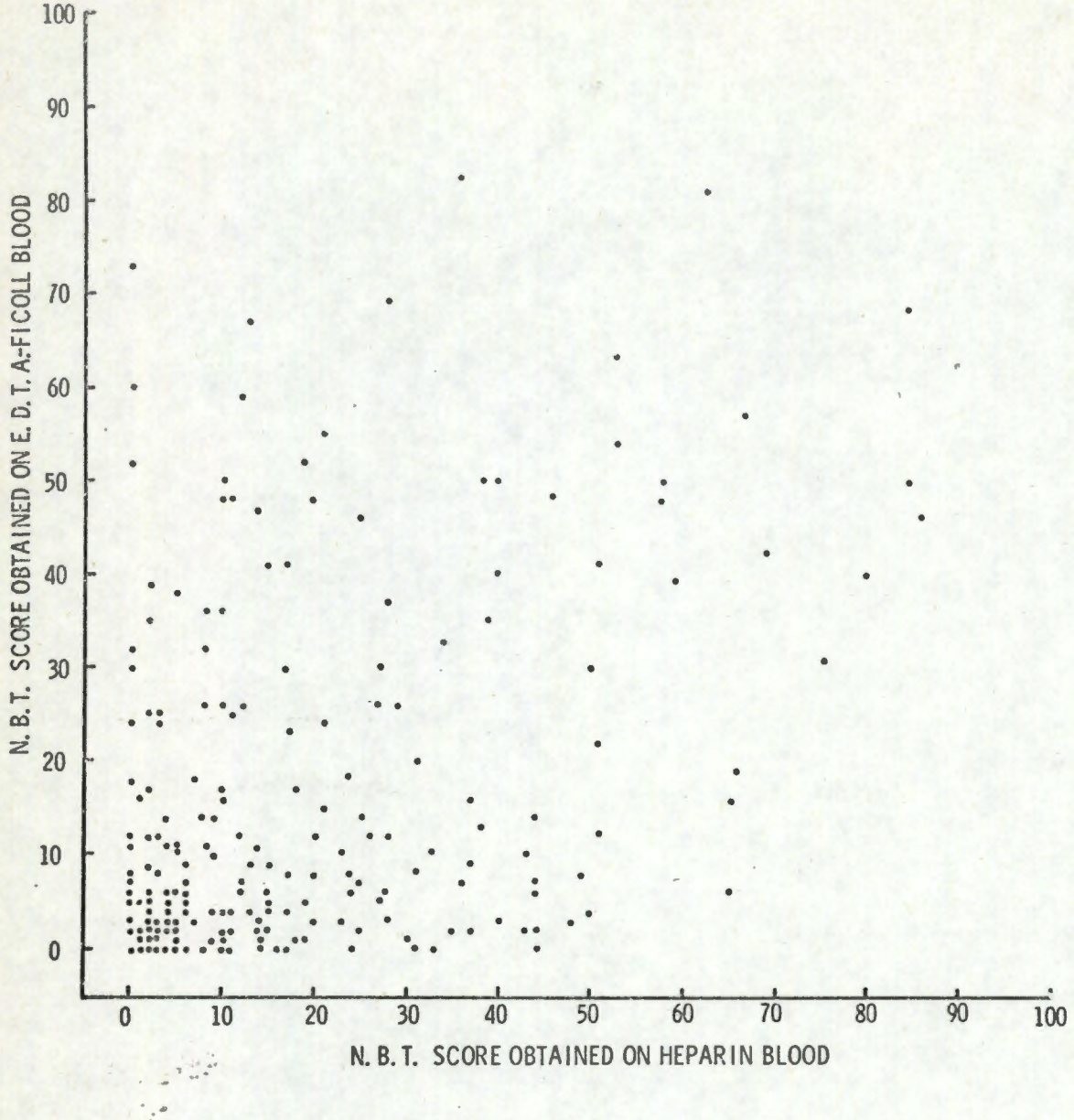


FIGURE 2.7.

Comparison between the NBT scores obtained when the test was performed simultaneously on the same blood sample by the EDTA/ficoll and heparin methods.

made for observer error (Fig. 2.7.).

2.4. Summary of Results

A diagnostic test should clearly distinguish between the subject group that it is designed to define, and other subject groups, if it is to have broad clinical application. This was not found with the N B T test. There was a wide overlap in the results from patients with pyogenic infection - whether or not antimicrobial treatment had commenced - in non-pyogenic infection and in non-infective disease. In addition, a wide range of values was obtained in healthy controls when the test was performed on heparinised blood.

There was a poor correlation between test results when the test was performed simultaneously on the same patients by the methods of Park et al (1968) and Gordon et al (1973), suggesting a basic difference in the mechanism underlying the two tests.

The counting error has not been previously commented upon. In this study a significant counting error, amounting to a standard deviation of $\pm$ 7.5% at an N B T score of 50%, was found when the slide preparations were quantitated by experienced observers. This is not surprising in view of the subjective nature of the counting procedure; an evaluation must be made not only of the amount of formazan within a cell that qualifies the cell as N B T positive, but also whether or not the cell is a neutrophil - large intracellular formazan deposits distort cellular morphology and make destinction between neutrophils and macrophages difficult. An N B T score of 10% had a standard deviation of $\pm$ 5% in the hands of experienced

observers and thus the statement (Freeman and King, 1972a) that N B T scores above 10% are almost universally positive would seem to ignore the existence of observer error, and appears unfounded. The inexperienced observer recorded very different results to the experienced observers, between whom there was very little inter-observer error after corrections were made for the counting error, indicating the importance of experience in accurate interpretation of the test result.

A significant relationship existed between a very crude assessment of the magnitude of systemic manifestations of illness in patients with bacterial infection and the N B T scores. High results were also obtained by both methods of performing the test in severely ill patients with non-infective conditions such as diabetic precoma, acute bronchospasm and paraffin inhalation. Unfortunately, severity of the systemic manifestations of other disease processes was not noted and could not therefore be correlated with the N B T scores. It would seem that the N B T score may be related to the severity of the illness rather than its causation and this matter will be further examined in Chapter 3 together with the relationship between the N B T scores and the neutrophil count, E S R , platelet concentration and the serum concentration of C R P and α'-acid glycoprotein.

A high proportion of patients with tuberculosis, inflammatory bowel disease and myelofibrosis gave false positive results by both methods of performing the test. This has been described in myelofibrosis (Ng et al, 1972) but not in inflammatory bowel disease.

Conflicting reports have been produced on results obtained in tuberculosis. Negative results were described in tuberculosis when the test was performed in heparinised blood (Park et al, 1968, Humbert et al, 1971 , Matula and Paterson, 1971a) but Park (1971) later described positive tests in tuberculous meningitis and miliary tuberculosis. Fikrig et al (1973) have subsequently described negative tests on 5 patients with tuberculous meningitis. In the present study high heparin test scores were obtained in a patient with ileocaecal and another with miliary tuberculosis. Gordon et al (1973) found high scores in 3 of the 6 patients with pulmonary tuberculosis and in a single child with tuberculous meningitis, whereas in this study 6 of the 7 patients with tuberculosis had high E D T A /ficoll scores.

2.5. General discussion on the value of the N B T test as a diagnostic aid

The availability of a rapid indicator of acute pyogenic infection has important clinical application. It gives direction to further lines of investigation and helps safeguard against indiscriminate therapeutic manoeuvres.

Resultant upon the findings of early studies (Park et al, 1968, Matula and Paterson, 1971a, Humbert et al, 1971 and Feigin et al, 1971) the N B T test stimulated world wide interest and was accepted as just such a test in the editorial columns of leading medical journals (Lancet, 1971, Park, 1971, Feigin, 1971). The question arises as to the reason for the difference between the results obtained in the present study in which the usefulness of the test is refuted and

TABLE 2.14. 'Revised' results of major studies in which the NBT test was performed on subjects with and without pyogenic infection. The number of subjects in each group is shown and the results are expressed as the percentage of correct and incorrect test results in each group.

Study Ref.	Author	Year	Subjects Studied					
			With Pyogenic Infection			Without Pyogenic Infection		
			Number	Test Results(%)		Number	Test Results(%)	
				True positive	False negative		True negative	False positive
A	Park et al	1968	29	100	0	95	? A few false positives	
B	Matula & Paterson	1971	65	93.8	6.2	102	99.2	0.8
C	Humbert et al	1971	73	63.0	27.0	197	90.6	9.4
D	Feigin et al	1971	47	66.6	23.4		Not stated	
E	Sobel et al	1973	29	60.7	39.3	109	88.1	11.9
F	Gordon et al	1973	56	66.8	23.2	108	94.4	5.6

the results reported from previous studies which led to the general belief in its value. The major factors which could be responsible for such a conflict of results are methodological disparity, and differences in subject selection and in the interpretation of results obtained. Five major studies have been performed using the heparin method and one, that of Gordon et al (1973) by the E D T A /ficoll method, and reference will be made to these studies by the reference figures listed (Table 2.14).

As far as the technique of performing the test is concerned, there are three major variables; the anticoagulants used, the duration and method of storage of the blood and of incubation of the mixture of blood and N B T , and the criteria by which neutrophils are classified as either N B T positive or negative. Despite assumptions to the contrary (Gordon et al, 1973) the E D T A /ficoll and heparin methods of performing the test are not directly comparable as indicated by the poor correlation obtained by duplicate tests performed in this study on the same patients by both methods. In subsequent analysis of the studies, that of Gordon et al will be dealt with separately.

The concentration of heparin has a profound effect on the N B T score obtained on a blood sample. An increase in the heparin concentration causes a parallel rise in the N B T score until significantly toxic levels are reached. This effect has been reported by other investigators (von Anner et al, 1971, Hicks and Bennett 1971, Bjorksten and Solheim, 1973 and Hellum and Solberg 1973a) and will be discussed more fully in Chapter 3. The concentrations of heparin

advocated by Park et al (1968) induce NBT reduction in a major proportion of normal neutrophils. With regard to the low NBT scores obtained in control subjects in studies A, B, C, D and E it is possible that Park et al (1968) did not adhere to the heparin concentration described and that investigators in studies B, C, D and E, in which the methods of Park et al (1968) were followed, did not rigidly adhere to the method with regard to the concentration of heparin used.

It is possible that the effect of heparin on NBT reduction varies with the preparation used although the widespread reports of the effect of heparin on NBT reduction referred to above would argue against this. One possibility is that the heparin concentration was regarded as insignificant and that blood was taken into commercially available heparinised containers of the type used by Wollman et al (1972) which contain economically small quantities of heparin. Similar containers in England contain 15 i.u. heparin/ml (Greyward Laboratories, Bedford).

The concentration of heparin used in the present study was standardised at 50 i.u. per ml because at the time that the study was initiated the relevance of the concentration of heparin was not realised. In retrospect it was too high and could account for the high NBT scores obtained in some of the control subjects. This does not invalidate the study as the heparin concentration was standard and one of the most significant findings was the high proportion of patients with pyogenic infection in whom the test gave a false-negative result.

The time lapse between venesection and commencement of

the test is only stated in studies C and E, in which it was less than 30 minutes. In the present study a period of one hour was allowed because there was no significant decrease of N.B.T. reduction over this time period when blood was stored at 4°C. Blood samples were preincubated at 37°C for 10 minutes to ensure that the temperature was constant when the N B T was added. Test tubes incubated in a water bath result in more consistent incubation conditions than the concave microslides used in the method of Park et al (Hellum and Solberg 1973b).

Incubation of the mixture of blood and the N B T solution for 15 minutes at 37°C followed by 15 minutes at room temperature is optimal and the protracted incubation at 37°C employed in studies B and E would be expected to increase the overlap of the N B T scores between normal and abnormal subject groups (Hellum and Solberg, 1973b). The reason for the period of incubation at room temperature is unknown and has not been stated (Park et al, 1968), but was probably found to be technically convenient during the development of the method. For the sake of conformity, the incubation times employed by Park et al (1968) and Gordon et al (1973) for heparin and E D T A blood specimens respectively were blindly followed in this study.

The criteria for the classification of a cell as N B T positive varied from study to study. In studies B and E any formazan deposit, in C, localised deposits larger than neutrophil granules, and in A and D, a 'large' formazan deposit, were all taken as the distinction between a negative and positive cell. In the present study

a positive cell was standardised as one in which the formazan deposits were at least the size of a lobe of the nucleus. The criteria adopted should not greatly effect the intergroup results of individual studies if standardised within each study and the more rigid criteria adopted in this study should tend to result in comparatively low NBT scores.

The E D T A /ficoll method employed here is identical to that employed by Gordon et al (1973) with the exception that centrifugation prior to incubation with NBT was dispensed with. This should not greatly effect the results, and the increase in time taken in evaluating the slides made from whole blood must be weighed against that employed in preparative centrifugation if the relative merits of the two methods are to be compared. The discrepancy in the results of the present study and those reported from other studies is unlikely to be due to methodological differences, although high concentrations of heparin could account for the high NBT scores obtained on some of the uninfected subjects in this study, and thus other possible factors must be investigated.

Subject selection varied enormously from study to study. Park et al (1968) and Humbert et al (1971) compared a group of normal adults and children with a group of diseased children, Matula and Paterson (1971a), normal adults with a group of diseased adults, and Gordon et al (1973), normal adults with an unspecified group of diseased subjects. No details of the criteria used for the selection of subjects were included in these studies. Sobel et al (1973) compared

afebrile diseased and normal adults and children with febrile adults and children and Feigin et al (1971), preoperative afebrile children with febrile children. Diseased children have not been directly compared with normal children which probably reflects the technical or ethical difficulty of obtaining blood from otherwise healthy children. In the groups of subjects comprising both adults and children, the relative proportion of each have not been detailed.

The NBT test discriminates clearly between patients with pyogenic infection and other subjects according to the results obtained in the two earliest studies (Park et al, 1968 and Matula and Paterson, 1971a). The results of subsequent studies refute the role of this test as being of any practical value as a reliable indication of pyogenic infection in a broad clinical context. The misconception as to its value is due both to the incorrect evaluation of the results obtained in some of the studies and due to widespread acceptance of the test despite this. 'Revised' results of the major studies are listed in Table 2.14. They are expressed as the percentage of true and false positive and negative results obtained in patients with and without pyogenic infection obtained by comparing the test result with the upper limit of normality of each individual study. All test subjects are included in this table, Humbert et al (1971) having excluded 25% of their subjects with non-infective disease from their final analysis after finding false positive test results in a large proportion of patients with haemophilia, and in a large proportion of patients with osteogenesis imperfecta and their immediate relatives. The percentage of false

negative results varies from 0 to 39.3 with a mean of $\pm$ 20% and that of false positive results (where stated) from 0.8 to 11.9 with a mean of $\pm$ 7%.

The results of the studies of Park et al (1968) and Matula and Paterson (1971a)differ significantly from those obtained in other studies with regard to the low incidence of fallacious results obtained by these authors. This difference is most likely to result from different methods of patient selection. Matula and Paterson (1971a) compared untreated patients with pyogenic bacterial infection with other diseases in the majority of which definitive bacteriological cultures were obtained but in whom the offending organism was adjudged non pathogenic. Park et al (1968) do not indicate whether or not their patients with acute bacterial or fungal infection had received treatment and compared these patients to 65 patients with other diseases of whom only 14% did not have viral diseases or tuberculosis and 6% had systemic lupus erythematosus, a disease in which false negative results are common (Douwes,1972, Wenger and Bole, 1973). Most acute pyogenic infections are emminently treatable with antibiotics. It is very unlikely that treatment was withheld from these patients, and the NBT tests at least in the study of Matula and Paterson (1971a) would have been performed in a very acute stage of the disease. The weakness of these two studies lies in the poor documentation of not only the disease processes included in the groups of patients with non-pyogenic disease, but also the severity of such disease and time during the course of the disease at which the test was performed.

It is not helpful to compare acutely ill patients with pyogenic infection with relatively asymptomatic patients with viral disease, or with patients in an inactive or convalescent phase of the disease, and to detect a difference between the groups. This in no way mimics the clinical dilemma so often posed by an acutely ill patient in whom pyogenic infection or non-pyogenic disease could be equally responsible, and underlines the weakness of any trial other than a true prospective one in assessing a test of this kind.

Hints that 'all was not well with the NBT test' came from reports from Park and Good, (1970) of false negative tests, which provoked the development of the "NBT test stimulated." This measures the capacity of neutrophils to reduce NBT after an endotoxin stimulus, and when compared to the true NBT score, can indicate a true negative test result, where a high NBT score is obtained after stimulation but not by the NBT test proper. False negative tests in patients with bacterial infection cannot be excluded if the NBT test result is low before and after endotoxin stimulation. Matula and Paterson, (1971b) described a severely ill patient with gross abdominal sepsis in whom the spontaneous NBT test was normal but who had a normal response of the NBT test to endotoxin. This disparity was attributed to the massive doses of prednisone administered to the patient, despite the observation by the same authors that patients receiving large doses of steroids may have spontaneously high test scores.

True prospective trials were performed by Feigin et al

(1971) and Sobel et al (1973) where febrile patients were compared, thus ensuring some uniformity of the severity of disease and more accurately reflecting the problem that the NBT test was designed to solve. In a high percentage of these patients the NBT results did not discriminate between the two disease groups. They also found much lower scores in treated than in untreated patients with acute pyogenic infection, a point which will be dealt with in depth in the next chapter (3.3.). Feigin et al (1971) recorded false negative results in 23% of their patients with pyogenic infection and although the false positive tests are not individually detailed there is an obvious overlap between the groups of patients with and without pyogenic infection. Despite a considerable proportion of incorrect test results, the authors of these major studies still supported the value of the NBT test in the diagnosis of infection. Gordon et al state that "the NBT test is shown to possess a useful capacity to distinguish patients with active bacterial infection from those with non-bacterial inflammatory disease in both adults and childhood populations", despite the facts that viral infection was the only non-bacterial inflammatory disease studied, and that no note was made of the ages of the subjects studied. Sobel et al concluded that "while the NBT dye test may be of value to the clinician, results require cautious interpretation with attention being given to the therapy received as well as to the haematological and immune status of the patient", broad cloaks for non conformist results, and added that "a decreasing NBT value even in the absence of clinical change may reassure the physician

of early clinical improvement", which is an unsubstantiated, erroneous and very dangerous assumption. Feigin et al (1971) concluded that "the NBT dye test is a useful aid in differentiating patients with febrile disorders. It is apparent, however, that classification of every patient on the basis of the NBT dye test without regard to the patients' history, physical findings, or ancillary laboratory data cannot be accomplished with perfect reliability." Humbert et al (1971) felt that the test should be further evaluated because of its potential importance in the diagnosis of acute bacterial infections but that "because of the number of 'false-positive' and 'false-negative' results, it should not replace standard microbiological techniques in such clinical situations."

The bulk of the blame for the widespread interest in, and acceptance of, the NBT test as a diagnostic test for pyogenic infection in a broad clinical context rests with editorials in major medical journals (Feigin, 1971, Park, 1971, Lancet, 1971). These communications are compiled by authorities who as such have a responsibility to report clearly and accurately to the body of the medical profession, who rely upon such reports for guidance. All these articles note the conditions in which false negative and false positive test results occur but it is the emphasis given and the general concept transmitted which has given such strong support to the NBT test. Feigin described his earlier study and states that "discriminant analysis was used to prepare a normogram permitting easy replacement of patients into one or another group". The

replacement may have been easy, but it certainly was not accurate (Table 2.15).

TABLE 2.15. 'Results of retrospective classification of 197 individuals' Feigin et al,1971 (Retrospective classification according to normograms compiled by discriminant analysis).

Group A - Control subjects.

Group B - Viral infection, partially treated bacterial infection and non-infectious febrile illnesses.

Group C - Untreated bacterial infections.

Group D - Ineffectively treated bacterial infections.

GROUP (Final diagnosis)	NUMBER OF SUBJECTS	A	B	C	D
		Classification of nomogram			
A	20	14	6	0	0
B	130	29	70	28	3
C	36	1	9	19	7
D	11	0	1	4	6

He states that "it is imperative to realise that the NBT dye test is not infallible. Correctly used it is a valuable adjunct to history, physical examination and other laboratory data to support the clinical diagnosis of bacterial or systemic fungal infection; as an aid to differentiating patients with bacterial infection receiving ineffective antibiotic therapy and as a prospective aid in monitoring patients at high risk of infection." The test is of use when clinical and other

evidence is equivocal. If reliance was placed on a combination of the NBT test results obtained by Feigin , classified in a retrospective manner using his normograms, 28% of the patients with acute bacterial infection would have been denied antibiotic therapy and in 5 of the 11 patients receiving ineffective antibiotic therapy, this fact would not have been appreciated. As an aid in the monitoring of patients at risk of infection the NBT test may have a role and this will be discussed later. Park quotes the work of Feigin et al (1971) as evidence to support his original study and then lists a large number of subject groups on whom further studies have been performed. He states that "the results disclosed that the original hypothesis is correct with a few exceptions", the few is not quantitated nor are the exceptions detailed. He also listed a large number of conditions in which "normal or nearly normal" results were obtained. No further reference is made to what is meant by nearly normal. His conclusions were similar to those of Feigin (1971). The leading article in 'the Lancet' was a broad, uncritical literature survey which concluded that the introduction of the NBT test "into the repertoire of the routine hospital laboratory seems justified."

The present study and a critical reappraisal of the literature in no way supports the concept that the NBT test is of practical use in the specific diagnosis of pyogenic infection in a broad clinical context. The next question to be answered is to whether or not it has any part to play in specific clinical situations or in monitoring patients at serious risk of infection.

Wollman et al,(1972) found the test was useful in distinguishing between infection and rejection in patients after renal transplantation. The number of patients in each group are very small and the criteria for the diagnosis of rejection are not detailed. The creatinine clearance, a reduction of which is an early sign of rejection (Calne, 1967), is equal in the infected group and the group in which rejection was said to be present. It is of interest that two of the eight patients had very high NBT scores preoperatively at a time when they were presumably uninfected and one of the six infected uraemic patients also studied had a normal NBT score despite a pneumococcal septicaemia, which further emphasises the unreliability of the test in the diagnosis of infection per se. This study is of interest and the NBT test could be a valuable tool in transplantation where the problem of distinguishing infection from rejection is a difficult one (Cullum et al, 1972) and in which the successful therapy depends upon prompt action. Before any such action is taken, a much larger, carefully controlled trial is essential to delineate the usefulness of the test in distinguishing rejection from infection.

Fikrig et al, (1973) studied the descriminatory value of the NBT test in children with meningitis. They found that 34 of 36 patients with bacterial meningitis only 3 of 67 with viral meningitis and no patients of 5 with tuberculous meningitis had positive NBT tests. The test may be of value as an additional diagnostic parameter, but it is of interest that in two of the patients with pyogenic meningitis the test was initially negative, only reverting to a positive result at a later

stage in the disease.

Positive NBT tests were observed in patients with myocardial infarction by Lauter et al (1973). On serial testing the NBT score fell to normal after the first 7 days except in the two patients with a complicated clinical course. This emphasises the fact that positive test results can occur in non-infective conditions. The test may be of use in the diagnosis of complications after infarction, but some advantage over present day methods would have to be shown to warrant further involvement in what has been shown to be a rather non-specific inaccurate test.

Patients with cystic fibrosis of the pancreas are liable to recurrent bacterial pulmonary infections (Addington et al, 1971). Sullivan et al (1973) correlated NBT test results with the clinical and radiological assessment of the severity of illness of the patients and found that they were relatively closely related. They also compared the effect of treatment of infected patients on the clinical response to the treatment and on serial measurement of the NBT score and showed that in those patients who responded clinically there was a fall in the NBT score and that this did not occur in patients remaining clinically unwell. It was concluded that "the NBT test is a valuable parameter, along with a history, physical examination and the chest roentgenogram in the surveillance and management of patients with cystic fibrosis". This is not so. The NBT test was not shown to have any advantage over a clinical assessment of the patient's condition either in the

diagnosis of infection or its response to therapy; in fact 11% of the NBT scores correlated poorly with the clinical assessment assumed as the standard. No evidence of the NBT test being "a valuable adjunct in the surveillance of patients with cystic fibrosis" is given. It may be argued that the clinical assessment of a patient is subjective whereas the NBT test is objective, but the present study has shown that there is also a strong subjective component in the interpretation of the NBT test preparations.

Freeman and King (1972a,b,c and d, Freeman, et al, 1973a, Freeman et al, 1973b) are strong proponents of the test and have staunchly defended its reputation against reports of false negative results (Freeman and King, 1972a) and methodological difficulties in the presence of neutropenia (Freeman and King, 1972b). They found the test useful as an indication of infective complications in patients with intravenous catheters (Freeman and King, 1972d) and after open heart surgery (Freeman et al, 1973b) and in the management of patients with infections (Freeman et al, 1973a).

Studies on patients with intravenous catheters revealed that all 9 patients with a positive NBT test had positive cultures from the catheter tip, in some of whom the test result reverted to normal after catheter removal, whereas the 6 patients with sterile catheter tips all had negative NBT tests. It is very surprising that none of the patients with infected catheter tips gave positive blood cultures. In a large study positive blood cultures were obtained on

all patients in whom thrombophlebitis occurred in association with intravenous cannulas (Altemeier et al, 1971). These results suggested to Mogensen (1972) that the catheters were probably contaminated during removal through the skin. In reply Freeman and King (1972e) denied this and stated that patients' pyrexia and NBT score fell to normal upon removal of the "infected" catheter. It was implied that this improvement occurred in all patients in whom the infected catheter was removed, which is in conflict with previous statements. It is possible that both the fever and high NBT scores resulted from phlebitis, a common complication of prolonged vascular cannulation (Medical Research Council Subcommittee Report, 1957), and that this resolved, with a concommitant fall in the fever and NBT score, upon removal of the catheter.

As a means of monitoring patients after open heart surgery for infection, the NBT test gave 25% false-positive results but no false-negative results. The occurrence of a positive test after myocardial infarction and its complications suggest that the test is unlikely to distinguish infective and non-infective complications of open heart surgery, which would be its most valuable role.

Freeman et al (1973a) described the use of serial NBT tests as a guide to the clinical management of two infected patients. Unsubstantiated assumptions were made linking false-negative results to steroid therapy, and clinical, and bacteriological evidence clearly indicated infection when a positive NBT result was obtained. No conclusions as to the diagnostic value of the test can

be drawn from this study.

2.6. Summary

The role of the NBT test as a specific diagnostic marker of pyogenic infection was re-evaluated. The test was performed simultaneously by two methods on blood from the same subjects which included normal controls and patients with infective and non-infective diseases.

Poor correlation was obtained when the results obtained from the two methods were compared. Although higher NBT scores and total numbers of NBT positive neutrophils were found in the subjects with pyogenic infection than in normal subjects, similar high results were obtained in subjects with a wide variety of diseases. The overlap of results between subject groups and the previously unrecognised error in interpretation of the test samples suggest that the test has no useful discriminatory value in the specific diagnosis of pyogenic infective disease.

A careful survey of the literature on the NBT test has shown the prime factors responsible for its acceptance as a specific diagnostic index of pyogenic infection to be misinterpretation of the results of poorly designed and documented studies and the amplification of such misconceptions by the editorial columns of leading medical journals.

The NBT test does not appear to have any role as a non-specific index of pyogenic infection. The available evidence of its value in specific clinical situations, including the surveillance of patients particularly at risk of infection is scanty and inconclusive.

The NBT test has not lived up to its promise as a specific index of pyogenic infection. Further claims as to its clinical value merit close scrutiny.

CHAPTER 3

The mechanisms involved in the reduction of NBT by neutrophils

Although a considerable literature has accumulated about the spontaneous NBT test in bacterial infection and conditions resulting in false negative and false positive results (Chapter 2), little attention has been paid to the basic mechanisms involved. In the first section of this chapter an attempt will be made to explain how the NBT enters neutrophils. The following two sections will examine the conditions governing its entry. In the final section an investigation will be made of the effect of NBT on the cells themselves and the possible metabolic pathways involved in dye reduction.

3.1.1. SECTION 1: The entry of NBT into neutrophils in the NBT test

The hypothesis that the NBT test would be a useful test for infection was based upon the assumption that in vivo phagocytosis of bacteria would induce metabolic changes in a neutrophil, sufficient to cause increased spontaneous in vitro reduction of NBT as is observed in the quantitative NBT test after the phagocytosis of latex (Park et al, 1968). Both the mechanism of this reduction and the site at which it occurs, whether extracellularly followed by phagocytosis of the formazan, or intracellularly, were unknown. Baehner (1972) was of the opinion that the dye does not penetrate intact cell membranes because of its negative

charge (it is in fact cationic), but that an alteration or disruption of the outer membrane of the leukocyte would allow access of reducing substances to the NBT. Similar views were expressed by Park (1971). Neither of these theories satisfactorily explained the observed morphological chatacteristics of the formazan deposits (Fig. 1.4) which vary greatly in size and shape, differing totally from that of any intracellular organelle. If NBT reduction was resultant upon an increase in cellular metabolism and membrane permeability it would be expected to be a more diffuse process than that giving rise to the focal density staining deposits that are observed.

In this section, it will be shown that for microscopic visualisation in the NBT test, the dye must enter the cell by phagocytosis. For this to occur two processes are obligatory; the cell must be stimulated to phagocytic activity and the NBT must be presented to the cell in a form suitable for phagocytosis. Cells suspended in serum and exposed to endotoxin are unable to reduce significant quantities of the dye, whereas cells in plasma do. The addition of fibrinogen or heparin to serum corrects this deficiency. NBT precipitates and complexes with fibrinogen and heparin and is phagocytosed by neutrophils as part of this complex.

3.1.2. METHODS

3.1.2.a Preparation of cells

Blood samples were collected from healthy volunteers between the ages of 18 and 30 years. Blood samples from different

individuals were used for each experiment.

A portion (25 ml) of heparinised blood (50 i.u./ml) was centrifuged at 450 G for 10 min at 15°C. The buffy coat (approximately 5 ml) was aspirated with a Pasteur pipette and washed three times in 50 ml of Hanks balanced salt solution at 4°C. After each wash, the suspension was centrifuged at 200 G for 10 min at 15°C. After the final wash, most of the supernatant was discarded and the washed cells were mixed by gentle shaking. A portion (0.4 ml) of the washed cell preparation was mixed with 0.6 ml of the suspending media.

3.1.2b Suspending media

The washed cells from four individuals were suspended in each of the following solutions: (i) autologous serum; (ii) autologous serum containing disodium EDTA (4.0 mg/ml); (iii) autologous serum containing heparin (70 i.u./ml); (iv) Hanks solution; (v) Hanks solution containing heparin (10 i.u./ml); (vi) Hanks solution containing disodium EDTA (4.0 mg/ml). Fibrinogen in sodium chloride solution (0.15 M) was added to aliquots of each of suspensions (ii)-(vi) to give a final concentration of 5 mg/ml. Additional studies were done with solutions (i) and (ii).

3.1.2.c Neutrophil stimulation

To cause stimulation, 10 μg of Escherichia coli endotoxin (type 0127 : B8) in 0.05 ml of phosphate (0.090 M) saline (0.15 M) mixture, (pH 7.2) was added to a 1.0 ml sample of each of the above suspensions in a disposable plastic test tube and the tubes

were incubated for 10 min at 37°C. This amount of endotoxin was used because in a dose-response study it was the smallest concentration necessary to give a maximal response (3.2). Similar amounts of suspension were incubated without endotoxin.

3.1.2.d Incubation with NBT

To 0.5 ml of the stimulated and unstimulated cell suspensions was added 0.5 ml of a 0.1% solution of NBT in phosphate buffered saline. The mixture was gently shaken and incubated at 37°C for 30 min.

Staining and counting of neutrophils

Staining and counting was performed as in 2.2. Twenty slides were re-examined and the standard deviation of a single count (percentage of positive cells) was estimated to be 2.2.

3.1.2.e Precipitation of fibrinogen by NBT

To 0.6 ml samples of fibrinogen (5 mg/ml) in Hanks solution with and without EDTA (4.0 mg/ml) and to plasma from blood anticoagulated with EDTA (2.4 mg/ml) was added 1.0 ml of 0.1% solution of NBT in phosphate-buffered saline. These mixtures were gently shaken and incubated in a water bath at 37°C for 30 min. After centrifugation at 500 G for 10 min, the deposit was washed three times with distilled water. The washed deposit was dissolved in Hanks solution by incubation in a water bath at 37°C for 30 min. The solution was then centrifuged at 1000 G for 10 min. Thrombin was

added to the supernatant (50 i.u./ml) and the mixture observed for the presence of a clot. Precipitates produced by the addition of NBT to plasma anticoagulated with EDTA were redissolved in a similar manner. Electrophoresis of these redissolved precipitates and a standard solution of fibrinogen in saline (5.0 mg/ml) against whole human antiserum was performed by a modified Laurell technique (Minchin Clarke and Freeman, 1968).

3.1.2f Precipitation of heparin by NBT

To 0.6 ml of a solution of heparin (70 i.u./ml) in Hanks solution and 0.6 ml of plasma from blood anticoagulated with heparin (50 i.u./ml) was added to 1.0 ml of a 0.1% solution of NBT in phosphate-buffered saline. The solutions were examined microscopically, using ordinary and polarized light, for precipitated particles, as were control solutions of heparin in Hanks solution and plasma, and the 0.1% solution of NBT. Reduction of the solutions was performed by the addition of phenazine methosulphate (300 μM) and NADH (10 mM). To investigate whether or not the precipitates obtained above contained NBT, they were dialysed against water for 72 hours using a cellulose membrane and then reduced by the addition of phenazine methosulphate (300 μM) and NADH (10 mM) and examined for blue coloration.

3.1.2g. Measurement of the relationship between the concentration of NBT, the precipitation of fibrinogen and heparin, and the percentage of positive neutrophils

A portion (1.0 ml) of solutions of NBT in phosphate-buffered saline in concentrations of 0.1-0.0032% were added to 1.0 ml of

whole blood anticoagulated with EDTA (2.4 mg/ml) or heparin (50 i.u./ml). Endotoxin stimulation, incubation, staining and counting were carried out as described above. Portions (1.0 ml) of solutions of NBT in phosphate-buffered saline in concentrations of 0.2-0.0032% were added to 0.6 ml of plasma from blood anti-coagulated with EDTA (2.4 mg/ml), to which was added 0.03 μCi of ^{125}I-labelled fibrinogen and to 0.6 ml of plasma from heparinised blood (50 i.u./ml) containing 0.03 μCi of ^{125}I-labelled fibrinogen plus 0.28 μCi of heparin (^{35}S) sulphate (corresponding to 60 μg). After incubation for 30 min at 37°C the mixtures were centrifuged at 4000 G for 30 min. The gamma activity in the supernatant was counted using a well-type sodium iodide detector. The samples containing no NBT gave about 14,000 c.p.m. and those with the highest concentration of NBT gave about 1200 c.p.m., the background being 20 c.p.m. The ^{35}S activity was counted in a liquid-scintillation system, the corresponding count rates being about 25,000 c.p.m. to about 1000 c.p.m. with a background count of 25 c.p.m. Each sample was counted for 100 seconds with a cut-off at 10,000 counts. The observed beta counts were corrected for the contribution due to ^{125}I which amounted to 13.6% of the observed gamma counts.

The concentration of fibrinogen was measured in the supernates of plasma containing EDTA, after incubation with NBT, by a tanned red cell haemagglutination inhibition immunoassay (Merskey et al, 1969).

3.1.2.h Effect of centrifugation of the NBT solution on the reduction of NBT by neutrophils

A solution of 0.1% NBT in phosphate-buffered saline was centrifuged at 30,000 G for 16 hours. A portion (1.0ml) of the suspension solution was added to 1.0ml of blood anticoagulated with either EDTA (2.4 mg/ml) or heparin (50 i.u./ml) with and without prior stimulation with endotoxin as described above. Incubation, staining and counting were performed as above. After centrifugation the solution of NBT was examined microscopically, using ordinary and polarized light, for the presence of particles.

3.1.2.i Substitution of NBT precipitates of heparin and plasma proteins for NBT in solution

To 1.0 ml of a solution of heparin (1000 i.u./ml) and to 0.6 ml of plasma anticoagulated with EDTA (2.4 mg/ml) was added 1.0 ml of a 0.2% solution of NBT in saline. The mixtures were gently shaken and incubated in a waterbath at 37°C for 60 minutes. After centrifugation at 1000G for 10 minutes, the supernates were discarded and the pellets (approximately 0.1 ml) resuspended in 1.0 ml of phosphate-buffered saline by vigorous shaking on a mechanical mixer. The suspension of precipitated heparin was added to 1.0 ml of heparinised blood (50 i.u./ml), and the plasma precipitate to 1.0 ml of blood anticoagulated with EDTA (2.4 mg/ml). To each was added 100 μl of endotoxin (200 μg/ml) in phosphate-buffered saline to give a final concentration of 10 μg/ml. The

mixtures were gently shaken, incubated for 20 minutes at 37°C, smeared and stained.

3.1.2.j. The effect of ficoll on the phagocytosis of latex particles and on the reduction of NBT by neutrophils

Blood from 2 normal adult males (ages 28 and 29) was anticoagulated with EDTA (2.4 mg/ml). To 2.7 ml of blood was added 0.3 ml of undiluted, dialysed latex particles. The mixture was gently shaken and to 1.0 ml of this mixture was added 1.0 ml of 0.09% NBT in phosphate-buffered saline to which there had been no additions or to which had been added either endotoxin or ficoll in final concentration of 20 μg and 200 mg/ml respectively. The mixtures were gently shaken, incubated at 37°C for 30 min, specimens were smeared on slides and stained. 100 neutrophils were counted and the percentage of cells with and without associated latex particles, formazan reduction in association with these latex particles or as large clumped deposits, and the relationship between these deposits and the presence of latex particles in cells was observed.

3.1.2.k. Electron microscopic studies of leucocytes after exposure to NBT

A portion (1.0 ml) of 0.1% NBT was added to 1.0 ml of whole blood, using EDTA (2.4 mg/ml) and heparin (50 i.u./ml) as anticoagulant after prior incubation of the blood with endotoxin (10 μg/ml) at 37°C for 10 min. The mixture was incubated in a water bath at 37°C for 15 min, centrifuged at 400 G for 60 min and the buffy coat aspirated with a Pasteur pipette, fixed by the method of Hirsch

and Fedorko (1968) and embedded in Epon. Thin sections were cut and examined unstained and after staining with uranyl acetate and lead citrate (Renolds, 1963) in an A.E.I. EM6B electron microscope. Control samples were prepared by excluding the NBT or endotoxin from the above mixtures.

3.1.3. RESULTS

3.1.3.a Reduction of NBT by stimulated neutrophils suspended in various media

A small percentage of neutrophils were found to reduce NBT when suspended in serum or Hanks solution with or without the addition of disodium EDTA (Table 3.1). Prior stimulation with endotoxin did not affect these results. The addition of fibrinogen resulted in a small increase in the percentage of positive cells in the absence of stimulation but in a marked increase with stimulated cells. In the presence of heparin, a large percentage of the neutrophils suspended in serum or Hanks solution became positive regardless of whether or not fibrinogen was added. The discrepancy between the percentage of positive cells found when stimulated cells were suspended in serum plus heparin (70 i.u./ml) or Hanks solution plus heparin (10 i.u./ml) can be attributed to the different concentrations of heparin added to the two solutions, a lower concentration being added to Hanks solution to prevent the clumping of cells caused by higher concentrations.

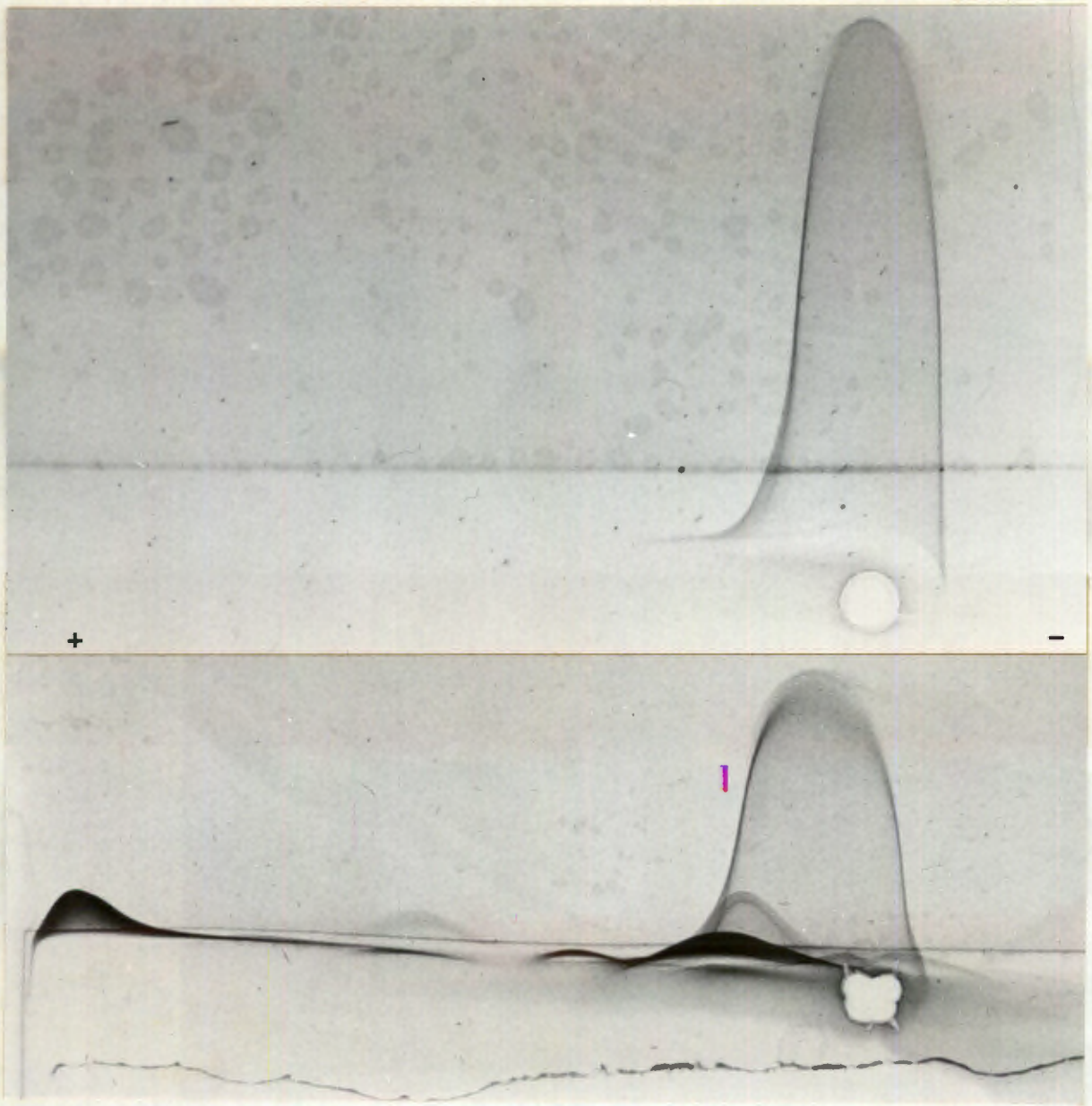


FIGURE 3.1.

Immunoelectrophoresis of proteins precipitated from plasma by NBT and redissolved in Hank's solution (below), and pure fibrinogen (above), showing that fibrinogen constitutes the major component of these precipitates.

TABLE 3.1. Effects of the addition of endotoxin and/or fibrinogen to cells suspended in autologous sera and various media. Results are expressed as the percentage (mean and range) of NBT-positivive cells

ADDITIONS		SUSPENDING MEDIUM					
Endo-toxin	Fibri-nogen	Serum	Serum + EDTA	Serum + heparin	Hanks Soln.	Hanks Soln.+ EDTA	Hanks Soln.+ heparin
-	-	0(0-3)	0(0-1)	10(0-37)	0(0)	1(0-1)	1(0-4)
+	-	1(0-3)	0(0-2)	38(9-73)	1(0-2)	0(0-1)	12(0-32)
-	+	-	2(0-2)	8(2-12)	3(0-7)	5(0-10)	7(0-11)
+	+	-	14(7-36)	30(18-39)	16(10-25)	28(18-45)	50(39-58)
No. of experiments		10	6	4	4	4	4

3.13b Precipitation of fibrinogen by NBT

Solutions of fibrinogen in Hanks solution with or without added EDTA, or plasma anticoagulated with EDTA all yielded a precipitate after incubation with NBT. The precipitates were dissolved and subsequently formed clots upon the addition of thrombin, indicating the presence of fibrinogen. Immunoelectrophoresis of the redissolved precipitate from plasma showed that fibrinogen, identified by its electrophoretic mobility, formed the major protein component (Fig. 3.1). A number of minor protein peaks were also seen.

3.13c Precipitation of heparin by NBT

Numerous small precipitated particles could be seen on microscopy with both ordinary and polarized light after the addition of NBT to the solution of heparin. These particles developed a dark blue colour upon reduction suggesting that NBT was incorporated in these particles.

Fig. 3.2.

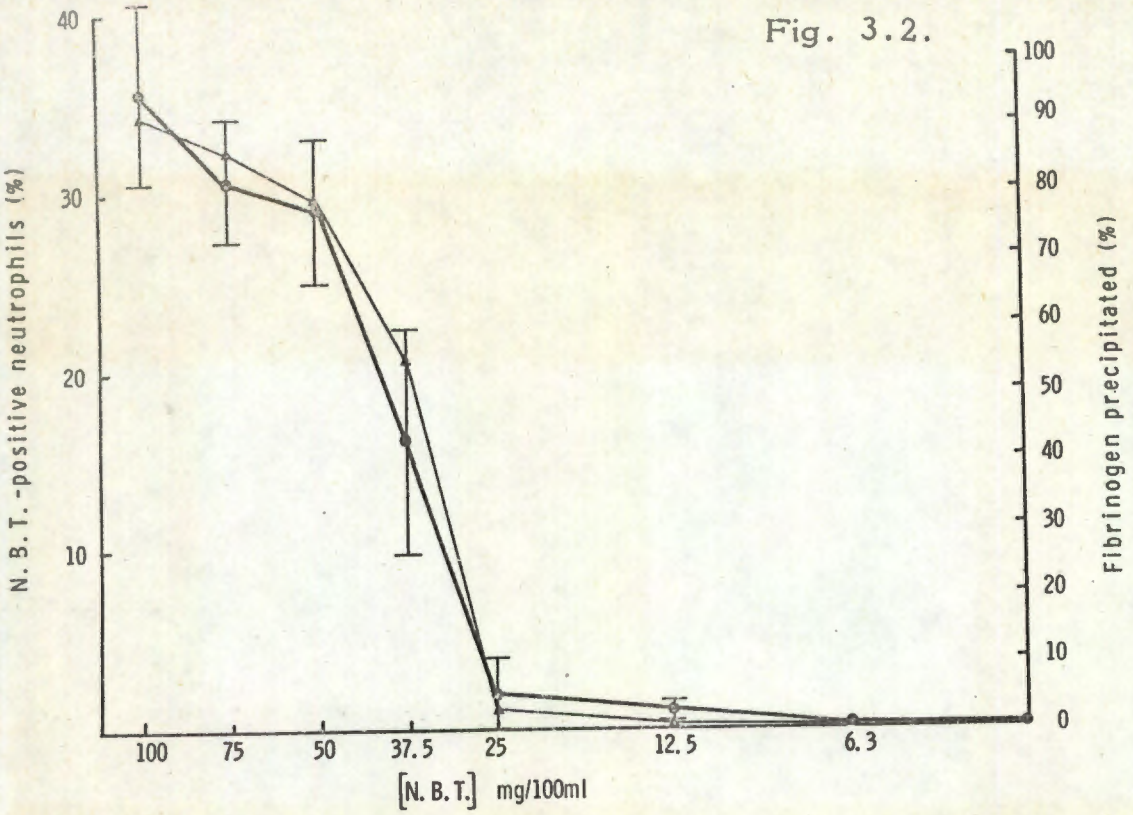


Fig. 3.3.

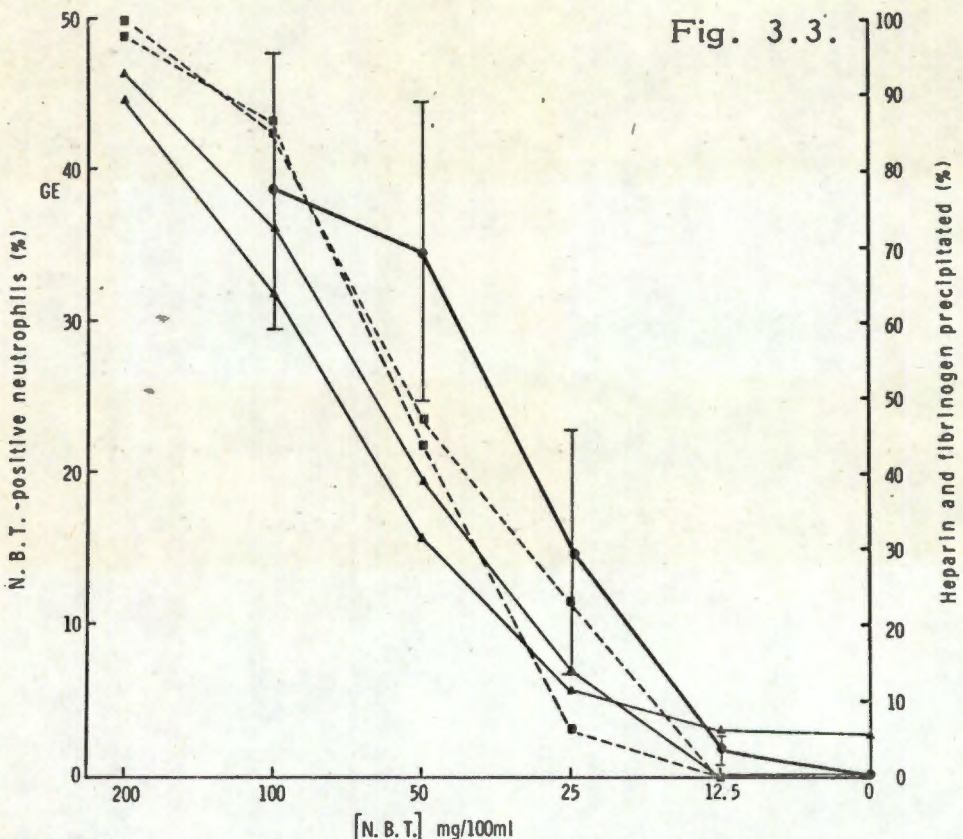


FIGURE 3.2.

Relationship between the percentage of NBT-positive neutrophils in blood anticoagulated with EDTA and stimulated with endotoxin (●, mean of five studies ±1 SEM) and the percentage of ^{125}I-labelled fibrinogen precipitated from EDTA-treated plasma upon the addition of varying concentrations of dye (▲, mean of duplicate values shown).

FIGURE 3.3.

Relationship between the percentage of NBT-positive neutrophils from heparinised blood after endotoxin stimulation (●, mean of five studies ±1 SEM) and the precipitation of ^{125}I-labelled fibrinogen (▲) and ^{35}S-labelled heparin (■) from heparinised plasma (individual experiments) upon the addition of varying concentrations of NBT.

3.1.3.d Demonstration of NBT in the precipitates of fibrinogen and heparin

Unreduced NBT was found to be freely dialysable across a cellulose membrane. After dialysis against water for 72 hours the precipitates of fibrinogen and heparin were incubated with a reducing solution and the development of a deep-blue coloration due to the presence of formazan indicated that NBT was not dialysed off the the precipitates.

3.1.3.e Effect of decreasing the concentration of NBT on the precipitation of fibrinogen and heparin and on the reduction of NBT by stimulated neutrophils (Appendix M).

In EDTA anticoagulated blood and plasma, concentrations of NBT greater than 0.05% caused almost total precipitation of fibrinogen and resulted in a significant percentage of stimulated neutrophils becoming NBT-positive. Concentrations of NBT of 0.025% or less resulted in little fibrinogen precipitation or NBT reduction by neutrophils. The critical concentration of NBT necessary for reduction by a significant percentage of neutrophils and for marked fibrinogen precipitation was similar (Fig. 3.2). . In heparinised plasma, NBT caused the precipitation of both fibrinogen and heparin. The precipitation of fibrinogen and heparin and the percentage of positive cells were decreased in parallel by dilution of the dye (Fig.3.3)

3.1.3.f Effects of centrifugation of the NBT solution

No particulate matter could be detected on microscopic

examination of the solution of NBT after centrifugation at 30,000 G for 16 hours. Use of this NBT solution did not decrease the percentage of NBT-positive neutrophils in stimulated blood when compared with solutions of NBT prepared in the standard manner.

3.1.3.g Substitution of NBT precipitates of heparin and plasma proteins for NBT in solution

Approximately half the neutrophils from heparinised blood and 5% (absolute values 48% and 5%) of those in EDTA anticoagulated blood contained formazan deposits. These deposits were morphologically identical to those obtained when NBT in solution is mixed with blood.

3.1.3.h The effect of ficoll on the phagocytosis of latex particles and the reduction of NBT by neutrophils

In comparison with the blood to which only latex was added, endotoxin and ficoll caused a marked, roughly parallel, increase in the percentage of neutrophils associated with latex particles and those in which formazan deposits were observed (Table 3.2). Most of the formazan deposits were in the form of large clumps.

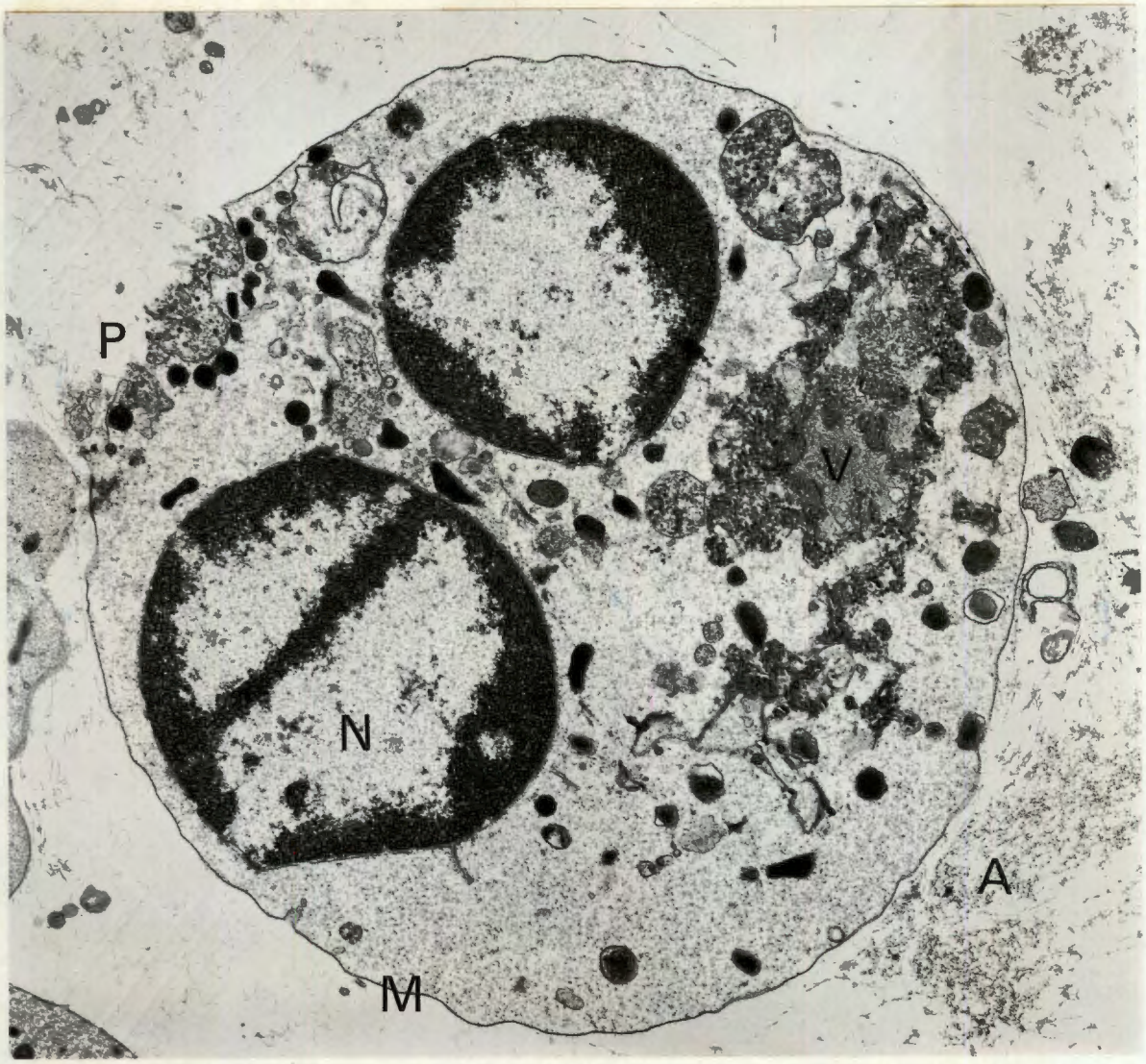


FIGURE 3.4.

Stained electron micrograph of a neutrophil after exposure to NBT and endotoxin, showing membrane-lined vacuoles containing amorphous material (V), amorphous material outside the cell (A) and undergoing phagocytosis (P), nucleus (N) and outer membrane (M).

Uranyl acetate and lead citrate x15,000.

TABLE 3.2. The effect of endotoxin and ficoll on the reduction of NBT by, and association of latex particles with neutrophils, in blood exposed to a mixture of latex and NBT. Results of the two studies are expressed as percentages.

Addition to Blood	Morphological characteristics of neutrophils				
	No Latex	Latex + formazan around latex particles	Latex + large formazan clump	Latex No formazan	Formazan No latex
NBT	72,72	9, 4	12, 4	5,22	2, 0
NBT + Endotoxin	17,41	6, 8	72,44	4, 6	0, 1
NBT + ficoll	20,39	18, 6	51,45	11, 10	0, 0

3.13i Electron-microscopic studies of leucocytes after exposure to NBT

Membrane-bound vacuoles, containing densely staining amorphous material, were found in the cytoplasm of a large proportion of the monocytes and neutrophils from blood exposed to both NBT and endotoxin. Similar amorphous less densely staining material was also seen both extracellularly and undergoing phagocytosis (Fig.3.4) The neutrophils appeared swollen and their outer membranes were partially ruptured. The amorphous material was seen purely extracellularly in blood exposed to NBT alone but was not seen after exposure to endotoxin in the absence of NBT. Neither cellular

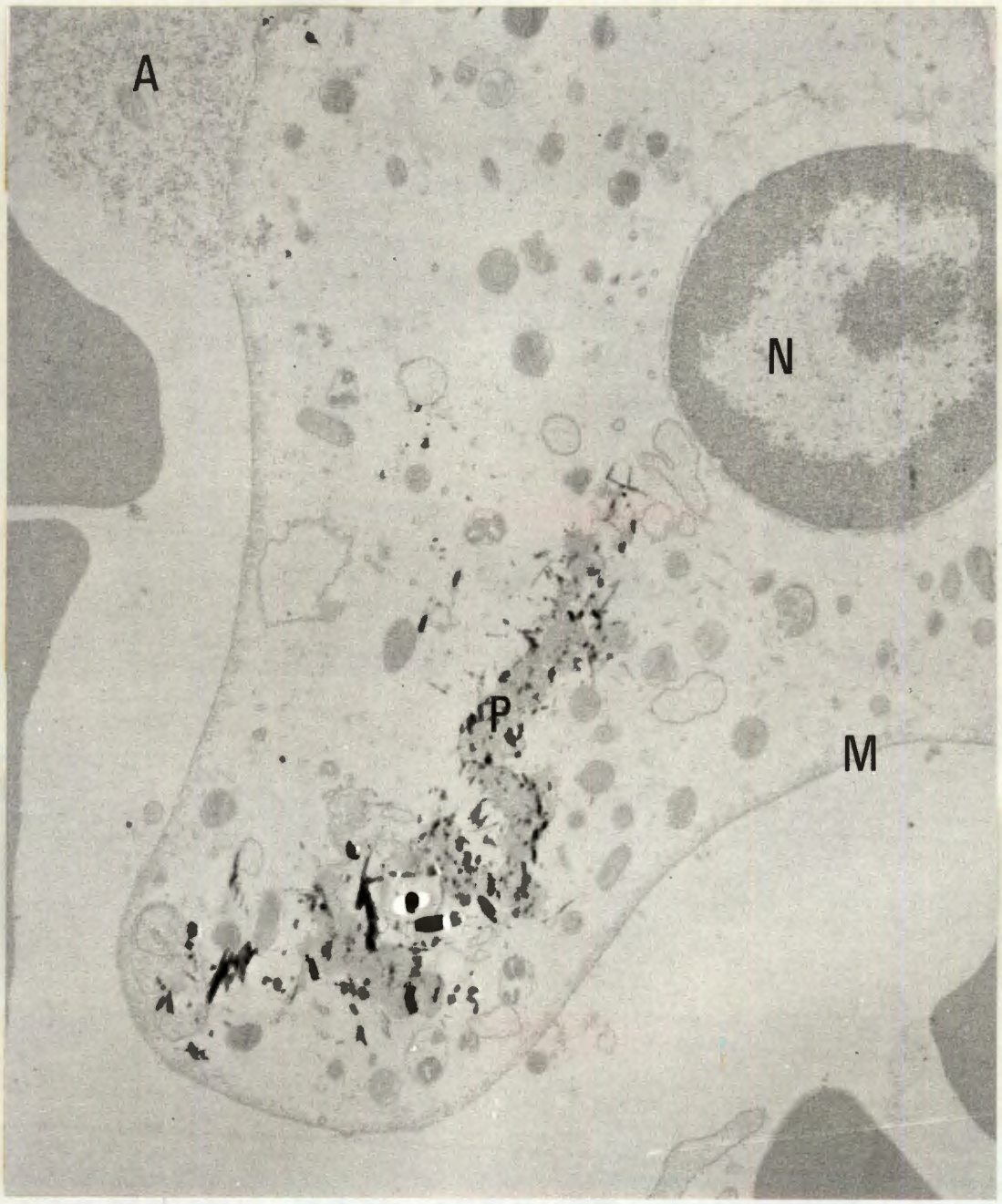


FIGURE 3.5.

Unstained electron micrograph of a neutrophil after exposure to NBT and endotoxin showing amorphous material outside the cell (A), amophous material containing electron-dense particles within the cell (P), nucleus (N) and outer membrane (M). x15000.

swelling nor abnormalities in the outer membrane of neutrophils were found in cells that were not exposed to a combination of both endotoxin and NBT. Unstained sections showed the amorphous material to contain electron-dense, elongated, somewhat needle-shaped particles approximately 150-500 nm in length. These particles were most numerous when the amorphous material was within phagocytic vesicles and could represent formazan deposits (Fig. 3.5).

3.14 DISCUSSION

The studies in this section were consequent upon a small pilot study in which an attempt was made to increase NBT reduction by normal neutrophils suspended in the serum of patients with pyogenic infection. Very little reduction was observed. Endotoxin, which had at that time been shown to result in a fairly standard increase in the reduction of NBT by neutrophils in whole blood (3.2) failed to enhance NBT reduction by cells suspended in autologous serum. It thus became obvious that a factor, present in plasma but absent from serum, was having a permissive effect on the stimulation of NBT reduction by endotoxin. The most apparent difference between plasma and serum is the presence of fibrinogen, clotting factors and anticoagulant in the former and thus the experiments on cells suspended in serum were repeated in the presence and absence of fibrinogen and EDTA (the addition of fibrinogen alone causes serum to clot).

It was found that the addition of fibrinogen re-established

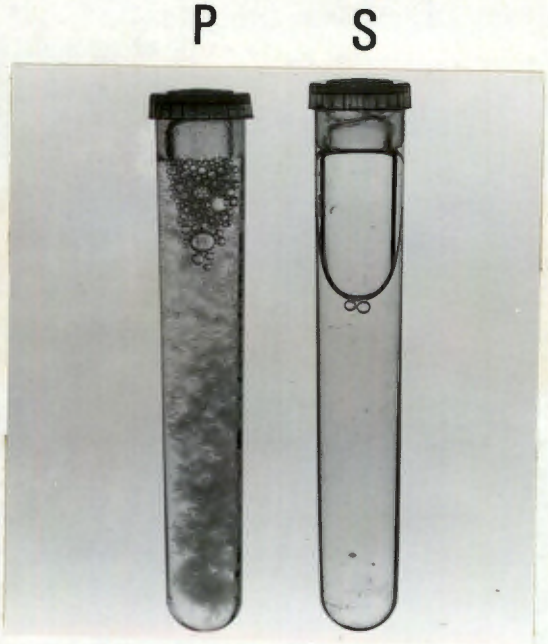


FIGURE 3.6.

A flocculant precipitate is produced by the addition of NBT to plasma (P) but not to serum (S).

the ability of endotoxin to increase NBT reduction by neutrophils and that fibrinogen added to cells suspended in a balanced salt solution retained this property in the absence of other serum factors.

Interest was then aroused as to the mechanism of augmentation of NBT reduction by fibrinogen. It was noticed in other experiments that if NBT and blood were mixed in the standard concentration and the cellular components rapidly separated from the liquid phase by centrifugation, the clear supernate became opalescent and developed a flocculant precipitate within a short time. A mixture of EDTA plasma and NBT in the same concentrations and under the same conditions as are employed in the NBT test formed an identical precipitate (Fig. 3.6). The precipitates were redissolved and subsequently formed a firm clot upon the addition of thrombin, indicating the presence of functional fibrinogen. Immunoelectrophoresis of the redissolved precipitates showed that fibrinogen, identified by its immunoelectrophoretic mobility, formed the major protein component. (Fig. 3.1)

Significant NBT reduction occurs in cell suspensions containing endotoxin and heparin in the absence of fibrinogen. Heparin is also precipitated by solutions of NBT in the same concentrations as employed in the in vitro NBT test. The cationic precipitation or heparin (Scott, 1968), and of fibrinogen (Surgenor, 1952) have been described, and although the precipitation of these substances by NBT was not further investigated, this would seem a

likely mechanism.

The light microscopic appearance of formazan deposits is that of irregular amorphous craggy lumps, of varying size, which appear to have an intracellular location. The evidence for an intracellular location is strengthened by observations by Nathan et al, (1969) that the reduction of NBT, in this case transported into the cell on zymosan particles, occurs almost exclusively within the phagocytic vacuole. It had been found that there is an absolute requirement for fibrinogen and/or heparin to allow significant reduction of NBT in the model system and that NBT caused the precipitation of these substances. It therefore seemed likely that the entry of NBT into neutrophils in visible amounts was by phagocytosis of the dye in association with precipitated fibrinogen and heparin. This theory was strongly supported by the finding that the percentage of neutrophils reducing the dye and the precipitation of fibrinogen and heparin depend upon the concentration of NBT, and that a close, parallel relationship exists between these processes upon exposure to the same serial dilutions of the dye (Fig. 3.3). Electron microscopy of stimulated neutrophils in the presence of plasma and NBT revealed the presence of amorphous material outside the cell, in the process of endocytosis and within membrane-bounded cytoplasmic vacuoles. The needle-shaped crystals seen in unstained preparations are almost certainly formazan deposits. Humbert et al (1973) reported almost identical electron microscopic findings but drew

conflicting conclusions from them. They reported that "the intracellular formazan was amorphous (noncrystalline) and was located mainly along smooth endoplasmic membranes adjacent to Golgi structures, and in smooth membrane-bound cisterns. It was occasionally observed within vacuoles. Occasional small deposits of formazan were also seen scattered in the cytoplasm. When aggregates of neutrophils were seen, extracellular formazan was often localised at the cell interfaces." They deduced that "some reduced NBT was occasionally seen on the outer membrane of neutrophils, particularly when two cells were in intimate contact. This finding raises the possibility that part of the formazan observed within the cells could have been formed outside and then carried into the cell by way of pinocytosis or phagocytosis. However, if such was the case, one would expect most of the reduced NBT to be found within vacuoles. Our observations revealed that most vacuoles were devoid of formazan; furthermore, formazan was mostly seen within a membranous system, analogous to smooth endoplasmic reticulum (SER) which had no connections with vacuoles."

It would seem that they equated amorphous material with formazan when in fact it was more likely to have been a macromolecular complex of NBT and precipitated fibrinogen and heparin, in which case their assumption that the material outside the cell was formazan rather than NBT is unfounded. Large membranous cisterns associated with the SER were absent from their electron micrograph of a neutrophil from a control subject. Phagocytic

vacuole formation is related to phagocytosis and the finding of empty vacuoles suggests that they initially contained material lost during tissue processing. The analogy between the membranous systems and smooth endoplasmic reticulum was probably drawn because of the irregular contour of both membranous systems; most electron microscopic data on phagocytic vacuoles records them as having a regular contour because of the regular structures of ingested materials studied. The phagocytic vacuole would be expected to conform in shape to accommodate its contents, which if irregular in outline would result in an irregular vacuole (Simson and Spicer, 1973). These authors have attempted to link NBT reduction by the hexose monophosphate shunt (HMPS) - linked oxidative pathway and the location of these enzymes on the SER in neutrophils by their interpretation of electron microscopic findings. They fail to explain why formazan deposition is localised to "SER cisterns" rather than the smooth endoplasmic reticulum as a whole. They conclude that "further ultrastructure and ultracentrifugal studies will be needed to infirm or confirm that speculation." The study described above would seem to 'infirm' the conclusions they have drawn from their observations, the hypothesis as to the location of the HMPS - linked oxidative pathway in neutrophils on the SER membranes remains still very much an hypothesis.

So far it has been demonstrated that NBT is phagocytosed by neutrophils in association with particulate matter. Prolonged

dialysis of NBT precipitated heparin and plasma proteins led to partial redissolution of the plasma proteins but not of heparin. Exposure to a reducing solution caused blue colouration of the residual precipitates indicating that NBT was not dialysed off the precipitate and that phagocytosis of these precipitates leads to concommitant phagocytosis of the associated dye.

As a prelude to engulfment, particles must attach to the outer membrane of the neutrophil, and this attachment may result from electrophilic bridging (Penny et al, 1966) by divalent cations between terminal carboxyl groups on the neutrophil membrane (Bangham, 1964) and negatively charged groups on the object of phagocytosis.

When the NBT test is performed on the blood of patients with bacterial infection, a smaller percentage of the neutrophils become NBT-positive if EDTA is used as anticoagulant, this has been interpreted as resulting from the inactivation of complement by EDTA (Park and Good, 1970), a point which will be further discussed, (3.3). EDTA in concentrations less than half those used in the present study (10 mM) causes a marked reduction of phagocytosis of bacteria (Allison and Lancaster, 1965) and latex particles (Kvarstein, 1969) by human neutrophils. Although the mechanism of this effect is unknown, it is likely to be due to the chelation of divalent cations (Kvarstein, 1969). For phagocytosis of macromolecular complexes, as demonstrated above, to occur in the presence of EDTA some binding mechanism must be envisaged.

NBT may displace calcium ions from chelation by EDTA, in which event the blood would be expected to clot, a process which occurs in the presence of NBT after the addition of Ca^{++} to blood anti-coagulated with EDTA. It is more probable that NBT, itself a divalent cation, can replace calcium as a linking ion in the binding of the fibrinogen complexes to the cell surface. Stossel has shown (1973) that the phagocytosis of aggregated albumin by human neutrophils does not occur in the absence of divalent cations and that phagocytosis is restored by the replacement not only of Ca^{++} or Mg^{++}, but of Mn^{++} or Co^{++} as well. The permissive effect of divalent cations on phagocytosis seems to be related to the non-specific divalent character of the ion rather than its specific atomic structure. The absence of free divalent cations could account for the relatively low NBT score obtained when preformed NBT-fibrinogen complexes instead of NBT in solution were added to blood anticoagulated with EDTA (3.1.3.g).

Gordon et al (1973) found that the addition of the sucrose polymer, ficoll, to the incubation mixture restored the level of the NBT scores obtained from the blood of patients with pyogenic infection from the low levels obtained by testing blood anticoagulated with EDTA in the absence of ficoll (Park and Good, 1970, Freeman and King, 1972b) to those obtained on blood anticoagulated with heparin. The experiment described above revealed that both endotoxin and ficoll stimulated the association (the term associated rather than phagocytosis has been used because of the difficulty in distinguishing between

adherent and phagocytosed particles) of latex particles with, and the reduction of NBT by, neutrophils. The bulk of formazan deposits are not around phagocytosed latex particles, but as discrete irregular lumps as seen with NBT reduction in the absence of latex. Only a small percentage of neutrophils from normal subjects reduce NBT when exposed to ficoll in the absence of endotoxin or latex (2.3.) It would thus appear that both endotoxin and ficoll enhance latex association with and NBT reduction by neutrophils, the latter largely as a result of increased phagocytosis of macromolecular complexes of NBT with fibrinogen. The mechanism by which ficoll enhances phagocytosis is unknown, and has not been further investigated in this study. Evidence to support the finding that ficoll enhances phagocytosis comes from a report by Cohn and Parks (1968) in which similar concentrations of ficoll were found to induce pinocytosis by macrophages.

NBT tends to crystallise out of solution in the concentrations in which it is used in the test of Park et al (1968). Phagocytosis of isolated NBT crystals is not a significant mode of entry of the dye into neutrophils because significant dye reduction does not occur in the control tests in the absence of heparin or fibrinogen and removal of these residual crystals by high-speed centrifugation does not decrease the number of neutrophils becoming NBT positive.

The phagocytosis of NBT as a component of a macromolecular complex with heparin and/or fibrinogen explains how NBT enters neutrophils in quantities visible as formazan by light microscopy,

and why the formazan deposits from such bizarre formations within the cytoplasm. It also explains the conformation attained by clumps of neutrophils around previously unnoted amorphous material (Fig. 3.7) which is almost certainly the same macromolecular complex surrounded by neutrophils in attempted phagocytosis.

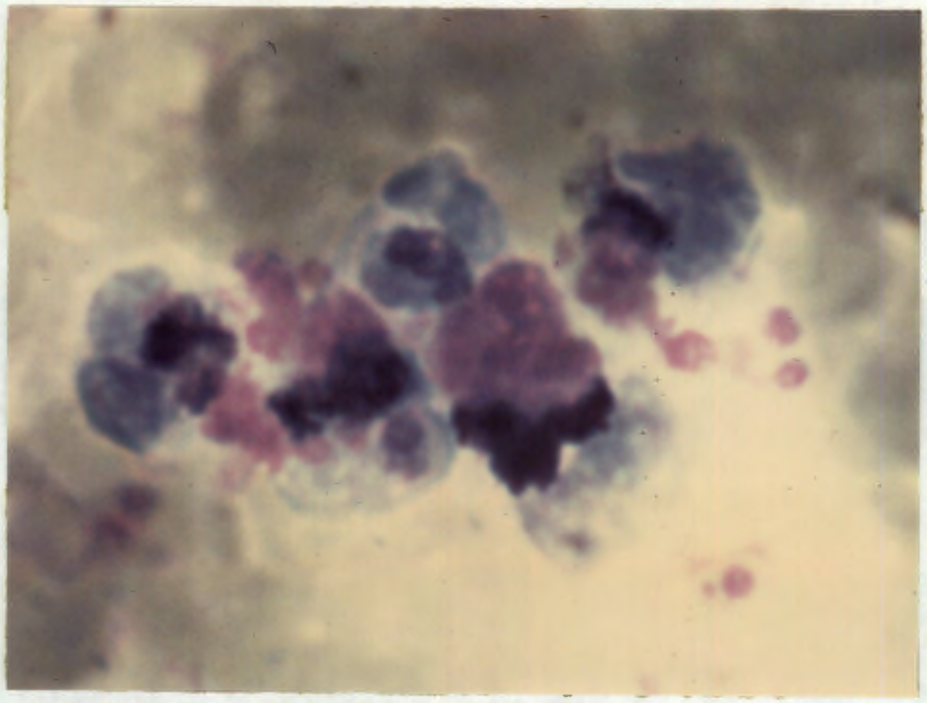


FIGURE 3.7.

Light micrograph of NBT test preparation (heparin as anticoagulant) showing neutrophils clumped around an amorphous precipitate, and formazan deposits at sites of mutual contact.

Leishman's x2,000

3.1.5. SUMMARY OF SECTION I

In this section a model system was constructed to investigate the mechanism of entry of NBT into neutrophils in the NBT test. The model system is basically similar to the standard NBT test, but differs in that endotoxin was used as an artificial stimulus of neutrophil activity.

It was found that NBT enters neutrophils in quantities visible as formazan by light microscopy as a result of phagocytosis. The dye is not phagocytosed when presented to the cell in solution but must be transported into the cell as a component of a macromolecular complex. The nature of this complex is dependent upon the anticoagulant used; in the presence of EDTA, fibrinogen, and with heparin, both fibrinogen and the heparin itself are precipitated from solution by, and complex with, NBT. These complexes are then phagocytosed in significant quantities only by neutrophils stimulated to phagocytic activity by endotoxin.

The synthetic sucrose polymer, ficoll, enhances NBT reduction by promoting phagocytosis.

3.2. Studies on some of the factors which may enhance NBT reduction by neutrophils in the NBT test

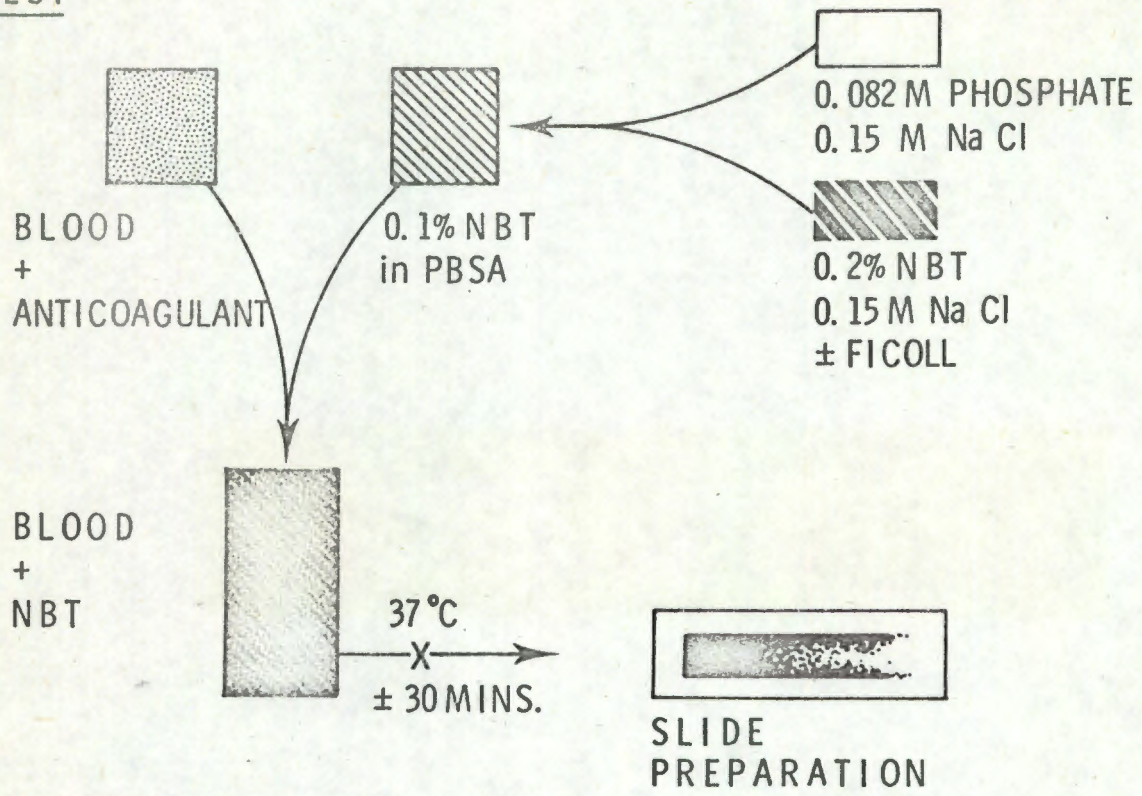
3.2.1. INTRODUCTION

In the last section it was shown that in the NBT test, the dye enters neutrophils by the phagocytosis of the NBT complexed to precipitates of heparin and/or fibrinogen and that the NBT itself is responsible for the formation of the complexes. These macromolecular complexes are present in all blood specimens to which NBT is added, but are phagocytosed to a varying degree, governed to some extent by the clinical condition of the blood donor and the conditions under which the test is performed (Chapter 2). There is thus a variable phagocytic response by neutrophils exposed to a fairly uniform suspension of particles. In this section a study will be made of some of the factors which may be responsible for enhanced phagocytosis.

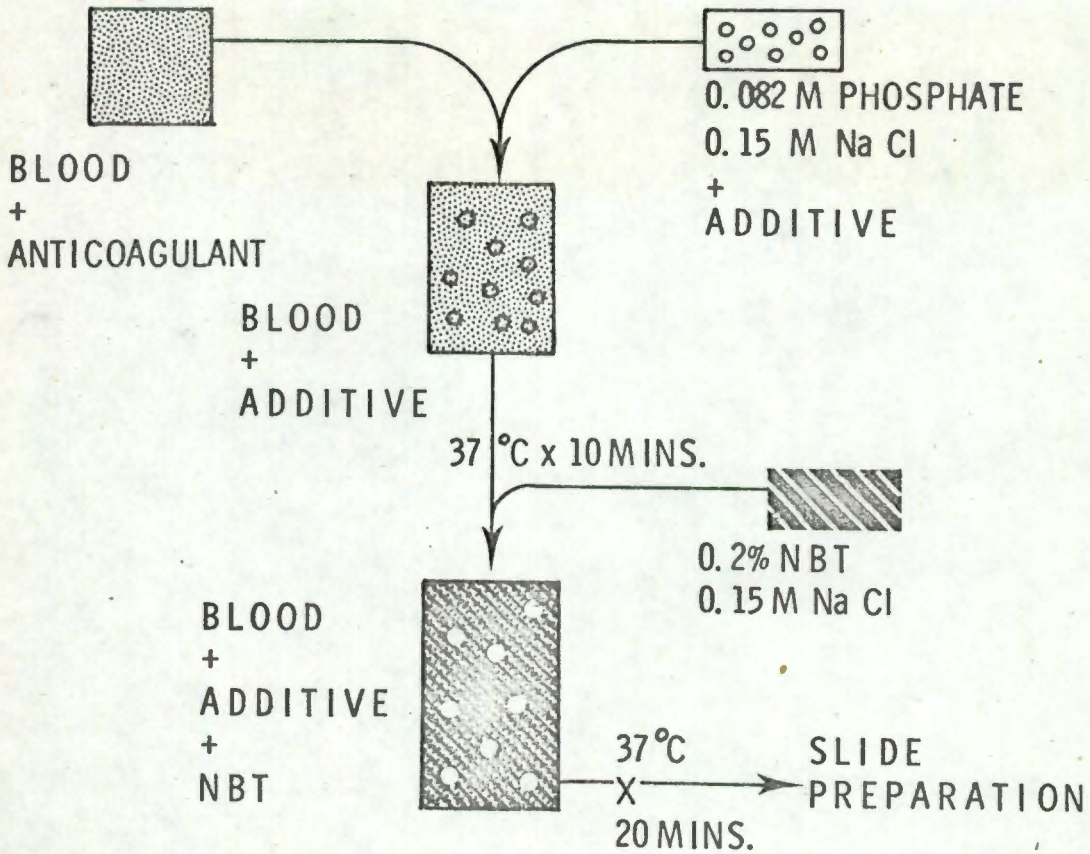
A model system was constructed in order to test the in vitro effects of various substances on NBT reduction. Substances tested included endotoxin, heparin and a variety of other organic and inorganic chemical compounds and mixtures.

It will be shown that endotoxin and heparin result in a fairly standard enhancement of NBT reduction, and that this increase in NBT reduction is dependent upon the concentration of these agents. The effect of endotoxin on NBT reduction by the neutrophils of patients

NBT TEST



MODEL SYSTEM



with Crohn's disease, diabetes mellitus, immune deficiency states, geriatric patients and patients receiving therapy with sodium aurothiomalate and prednisone is examined and compared to the dose response pattern in normal subjects.

3.2.2. METHODS

The model was designed to result in the same final concentrations of component mixtures as those used in the standard NBT test (Fig. 3.8).

One volume (usually 0.25 ml) of the additive, or dilutions thereof, in phosphate buffered saline was mixed with two volumes (usually 0.5 ml) of blood anticoagulated with EDTA or heparin. After incubation at 37°C for 10 minutes, one volume (usually 0.25 ml) of 0.2% NBT in saline was added to this mixture which was gently shaken, incubated at 37°C for a further 20 minutes, and slide preparations made and counted in the usual way. Control experiments using phosphate buffered saline with or without endotoxin (30 μg/ml) were also performed.

3.2.2.a The effect of endotoxin on NBT reduction by the neutrophils of normal subjects and patients with a varity of conditions

Subjects. Details of the normal subjects (38) and patients with Crohn's disease (6), diabetes (5), immune deficiency (26), geriatric

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FIGURE 3.8. (Opposite)

Outline of the model system used to study the effect of the addition of various substances to blood on the reduction of NBT by neutrophils.

patients (6), patients receiving therapy with sodium aurothiomalate (8) and prednisone (6), the single patients with the Chediak-Higashi syndrome and another with CGDC are shown in Appendices I, J and K.

Method. In this study blood was anticoagulated with EDTA (2.4 mg/ml) and one or more dilutions of endotoxin added to give final concentrations of from 100 μg to 1 pg/ml. Blood with heparin as anticoagulant (50 i.u./ml) from the patient with the Chediak-Higashi syndrome was treated by identical methods.

Immunoglobulin concentrations in the serum of patients with immune deficiency were measured by the Mancini radial immunodiffusion technique (Mancini et al, 1965) at the Immune Deficiency Referal Laboratory for South East England.

3.2.2.b The effect of varying concentrations of heparin on NBT reduction by neutrophils

Blood was taken from 5 normal healthy adults and 0.5 ml immediately added to each of 7 plastic test tubes containing a mixture of 0.25 ml of phosphate buffered saline and sodium heparin (preservative free, 162 i.u./mg, Evans Medical) to give final concentrations of 0.75, 6.6, 33, 66, 120, 175 and 330 i.u./ml. The standard procedure was then followed.

3.2.2.c The effect of various compounds on NBT reduction by neutrophils

Screening tests were done with a large number of compounds, in varying concentrations, on the blood of normal adults

anticoagulated with either EDTA (2.4 mg/ml), or heparin (2-50 i.u./ml) (Appendix L. Table 3.8). Those experiments in which obvious changes in the amount of NBT reduction were observed were generally repeated a number of times together with controls (Appendix L, Tables 3.9, 3.10). The effects of extracts of pus and thrombus were also tested in the model system. The preparation of these extracts and modifications of the model system now follow.

Exposure of blood to pus

1.0 ml of sterile pus from an abdominal wound abscess was thoroughly mixed with 9.0 ml of phosphate buffered saline. 5.0 ml of this mixture was homogenised in a Potter's homogeniser. The homogenate and residual cell suspension were then centrifuged at 2000 G at 20°C for 10 minutes. The supernates, and dilutions thereof, in phosphate buffered saline were substituted for phosphate buffered saline in the model system.

Exposure of blood to thrombus supernate and lysate

10.0 ml of blood from a normal subject was defibrinated by continuous stirring with a wooden orange stick. The adherent clot was divided in two, one half was incubated in 0.5 ml phosphate buffered saline at 37°C for 30 minutes. The other half was homogenised in 0.5 ml phosphate buffered saline in a Potter's homogeniser. Both preparations were centrifuged at 2000 G at 20°C for 10 minutes. The supernates were substituted for phosphate buffered saline in the model system.

Modifications of the model system

i. Exposure of blood to neuraminidase prior to incubation with NBT

0.2 ml of blood anticoagulated with heparin (20 i.u./ml) was mixed with 50 μl of neuraminidase (Vibrio cholerae, 500 i.u./ml) or 50 μl of heat inactivated neuraminidase (100°C for 1 hour) and incubated at 37°C for 1 hour. 0.25 ml of 0.1% NBT in phosphate buffered saline was added to and mixed with the blood and the standard procedure was then followed.

ii. Exposure of blood to abnormally high and low oxygen concentrations

0.5 ml blood samples in plastic test tubes were gassed for 1 minute with either 100% 0_2 or N_2. The blood was gently shaken, gaseous equilibration allowed for 5 minutes, the tubes were regassed for a minute and incubated at 37°C for 30 minutes. The standard procedure was then followed.

iii. Freezing and thawing and sonication of blood samples

Heparinised blood samples were frozen and thawed once or sonicated for 15 seconds at an interpeak amplitude of 10 microns on a 150 watt MSE ultrasonic disintegrator with a 3.0 mm titanium probe emersed in the blood. The standard procedure was then followed.

iv. Exposure of blood to phosphate buffers of varying pH

Aqueous solutions of 0.15M NaH_2, PO4 and 0.15 M Na_2H PO4 were mixed to give solutions with a pH range of 5.5 to 7.5. These solutions were then substituted for phosphate buffered saline (pH 7.2) and the standard procedure was followed. The pH of the blood phosphate mixtures was not measured.

v. The addition of Cytochalasin B to blood

Cytochalasin B was dissolved in dimethylsulphoxide (DMSO, 1.0 mg/ml) which was diluted to a final concentration of 33 μg/ml in phosphate buffered saline immediately before use, and the standard procedure was then followed. Control studies were performed with DMSO alone.

vi. Exposure of blood to retinol

Retinol was dissolved in ethanol (25 mg/ml and 1.0 mg/ml) immediately before use. Volumes of 1-10 μl were dispensed into plastic test tubes with a Hamilton syringe. 1.0 ml of blood was added to the test tubes during the course of continuous mixing on a mechanical mixer. The mixtures of blood and retinol were incubated at 37°C for 10 mins, 1.0 ml of 0.1% NBT was added and the standard procedure was then followed. Control experiments were performed using ethanol alone. All procedures involving retinol were performed in a darkened room.

3.2.3. RESULTS

With EDTA as anticoagulant in the model system normal blood gave very low levels of NBT reduction. Higher levels produced by the addition of compounds to the test system were therefore readily detected. The observation that the use of EDTA as anticoagulant reduced the sensitivity of the NBT test (Park and Good, 1970) and the possibility that chelation of divalent cations such as Ca^{++} and Mg^{++}, might modify the effects of additives led to the use of heparin as anti-coagulant in some experiments. Before the realisation that heparin enhanced NBT reduction, varying concentrations were used, resulting

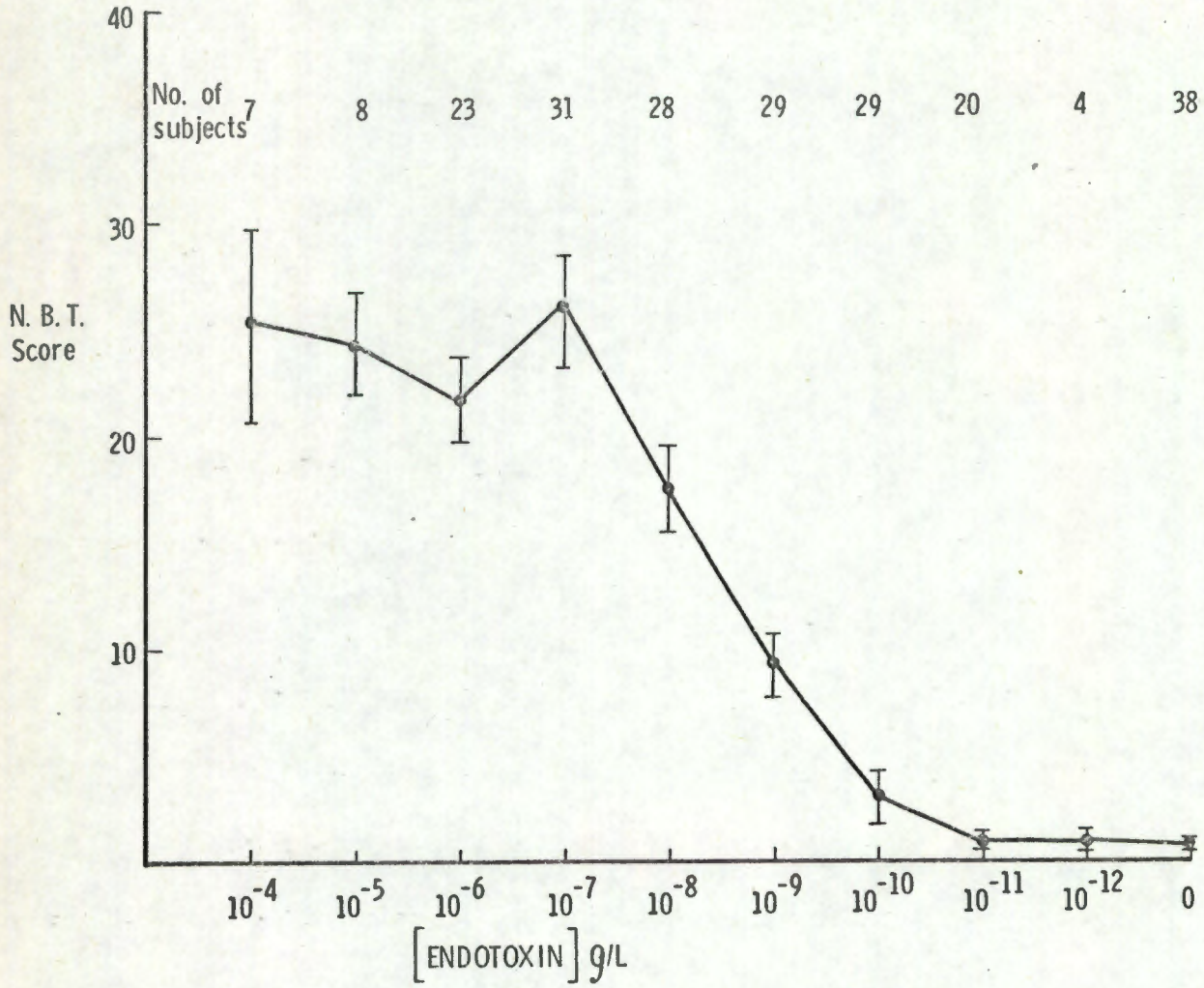


FIGURE 3.9.

Effect of varying concentrations of endotoxin on NBT reduction by neutrophils of normal subjects (EDTA as anticoagulant).

(• Mean + 1 S.E.).

in different amounts of basal NBT reduction by neutrophils in the absence of any additives. Even when the same amount of heparin was used there was marked intersubject variation of NBT reduction. Analyses of the results that follow must therefore be made with knowledge of the anticoagulant used. The effect of an additive on NBT reduction by neutrophils in blood anticoagulated with heparin is best interpreted by comparing the effects of different concentrations of the same compound, or the effects of different compounds, processed simultaneously on the same blood sample. These results can be compared with baseline levels of reduction in the absence of any additives, and with levels of reduction obtained after stimulation with supramaximal concentrations of endotoxin.

3.2.3.a <u>The effects of endotoxin on NBT reduction by neutrophils of normal subjects and patients with various diseases</u>

The results of exposing blood of normal subjects, anticoagulated with EDTA, to varying concentrations of endotoxin are shown in Table 3.3 and Fig. 3.9. There was a linear relationship between the NBT score and endotoxin concentration at concentrations of between 100 pg/ml and 100 ng/ml. At concentrations below 100 pg/ml there was no significant increase of NBT reduction above control levels and at concentrations above 100 ng/ml the NBT scores plateaued at approximately 25%.

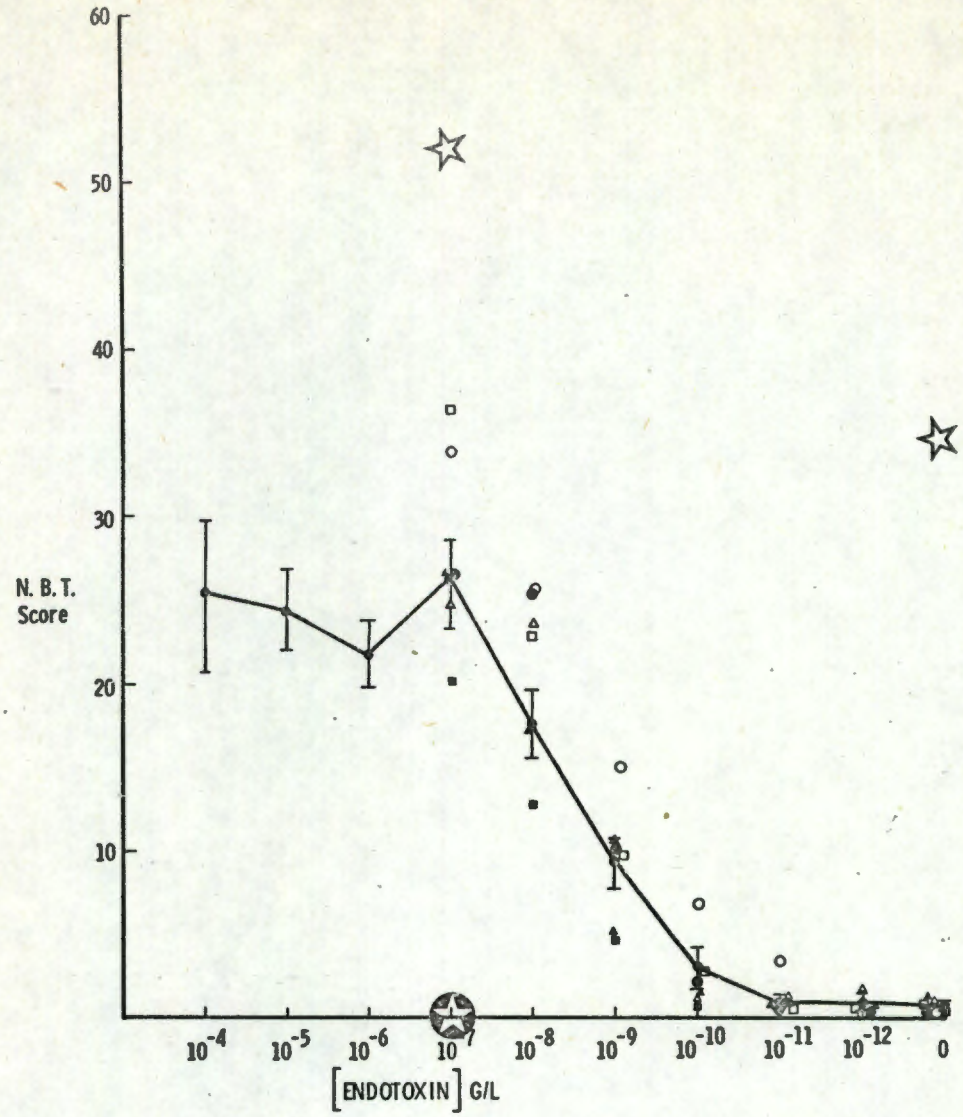


FIGURE 3.10.

Effect of varying concentrations of endotoxin on NBT reduction by neutrophils from the blood of patients with diabetes mellitus △, Crohn's disease ●, hypogammaglobulinaemia ■, geriatric patients, □ patients receiving therapy with sodium aurothiomalate ▲ and prednisone ○, and individual patients with the Chediak-Higashi syndrome ☆ and CGDC ✪ compared to the response in normal subjects ●—●.

TABLE 3.3. The effect of varying concentrations of endotoxin on NBT reduction by neutrophils from blood of normal subjects (with EDTA as anticoagulant). Results are expressed as the mean and S.E. NBT score.

[Endotoxin] g/ml	$1x10^{-4}$	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}	10^{-11}	10^{-12}	0
No. of studies	7	8	23	31	28	29	29	20	4	38
NBT score Mean	25.3	24.4	22.8	26.2	17.5	9.2	3.0	1.1	1.0	0.7
S.E.	4.7	2.2	2.0	2.5	2.1	1.4	1.1	0.4	0.5	0.3

There was no significant deviation from the above pattern of NBT reduction in patients with Crohn's disease, diabetes mellitus, geriatric patients and patients receiving therapy with prednisone or sodium aurothiomalate. Blood, whether anticoagulated with heparin or EDTA from the patient with the Chediak-Higashi syndrome gave a high level of NBT reduction in both the presence and absence of endotoxin, whereas no reduction occurred in the blood of the patient with CGDC (Table 3.4, Fig. 3.10).

TABLE 3.4. The effect of endotoxin concentration on NBT reduction by neutrophils from EDTA anticoagulated blood of patients with various conditions. Results are expressed as the mean and S.E. NBT score. The results of studies on individual patients with the Chediak-Higashi syndrome and CGDC are also shown.

PATIENTS			[ENDOTOXIN]					
Condition	No.	Age (years)	1×10^{-7}	10^{-8}	10^{-9}	10^{-10}	10^{-11}	(g/ml) 0
Diabetes mellitus	5	53.0 (7.1)	24.4 (4.8)	23.2 (2.7)	10.2 (4.0)	0.8 (0.3)	1.0 (0.6)	0.6 (0.2)
Crohn's disease	6	48.0 (6.2)	26.2 (4.6)	25.3 (2.9)	10.2 (2.9)	2.3 (0.6)	0.6 (0.5)	0.3 (0.3)
Gold therapy	8	56.8 (2.3)	26.6 (4.7)	17.5 (4.0)	5.1 (2.1)	2.0 (0.7)	1.3 (0.4)	1.6 (0.5)
Prednisone therapy	6	44.5 (9.1)	33.5 (10.0)	25.5 (6.5)	7.8 (3.0)	7.0 (3.5)	3.8 (1.4)	0.3 (0.2)
Geriatric subjects	6	83.3 (2.9)	36.3 (4.9)	22.7 (5.0)	9.5 (3.2)	2.0 (0.5)	0.8 (0.6)	0.3 (0.2)
Hypogammaglobulinaemia	24	24.5 (3.3)	19.7 (2.7)	12.6 (2.1)	4.6 (1.0)	0.8 (0.2)	1.0 (0.7)	0.8 (0.5)
Chediak-Higashi	1	3	43,60	-	-	-	-	32,36
CGDC	1	4	0	-	-	-	-	0

The majority of patients with hypogammaglobulinaemia showed the same pattern of response to endotoxin stimulation. One patient (Appendix K, No.7), a young man with childhood onset hypogammaglobulinaemia, was initially tested when he had a septic arthritis of the knee, retested when convalescent and again when fully recovered. The NBT scores showed a progressive rise with improvement in his clinical condition. The neutrophils of a 12 year old boy with congenital sex linked hypogammaglobulinaemia (Appendix K,

No. 3) and a persistent viral meningoencephalitis showed an almost complete inability to reduce NBT after exposure to endotoxin. A young man (Appendix K, No. 15) with adolescent onset hypogammaglobulinaemia failed to show normal NBT reduction upon endotoxin stimulation. He was subsequently found to have a bacterial hepatitis caused by one of the Borrelia species of bacteria. During the course of, and after, intensive prolonged intravenous penicillin therapy, NBT reduction by his neutrophils gradually increased as his clinical condition improved. Low NBT scores after endotoxin stimulation were also found in three other clinically well patients (Appendix K, No.'s 1,4 and 5), all of whom had congenital or sex linked hypogammaglobulinaemia. Brothers with sex linked disease both reduced NBT poorly (Appendix K, No.'s 3 and 4) while an affected male cousin (Appendix K, No. 9) and the maternal sibs (Appendix I, No.'s 33 and 35) reduced the dye normally.

Washed cells of the three of those patients whose neutrophils responded poorly when suspended in normal ABO blood group AB, plasma showed a slightly increased, but still highly abnormal NBT reduction in response to endotoxin stimulation. Washed normal ABO blood group O cells showed normal NBT reduction after suspension, together with endotoxin, in the plasma of these patients. This suggests that the defective NBT reduction by these patients has a cellular rather than humoral basis (Tables 3.5 and 3.6).

TABLE 3.5. NBT scores of washed cells from two sibs with sex linked hypogammaglobulinaemia (Appendix K, No.'s 3 and 4) and their parents exposed to endotoxin after suspension in autologous or heterologous plasma from blood with EDTA as anticoagulant. Duplicate studies on cells from the same blood samples are shown.

	Hypogamma. Sibs.		Parents (Normal)	
	Number 3	Number 4	Mother	Father
Autologous Plasma	0, 0	0, 5	10, 16	28, 14
Heterologous Plasma (ABO group AB)	5, 6	4, 15	15, 17	23, 18

TABLE 3.6. NBT scores of washed cells from a hypogammaglobulinaemic patient (Appendix K, No.15) and a normal subject suspended in autologous plasma or heterologous, ABO blood group AB, plasma. The normal cells were also suspended in the hypogamma. plasma. NBT scores from separate studies on cells from the same blood sample are shown. All plasma had EDTA as anticoagulant.

		CELLS	
		Patient	Normal Subject
PLASMA	Hypogamma. Patient	2, 6, 5	7, 8, 14
	Normal Subject		17, 16, 13
	Heterologous Normal (ABO Group AB)	6, 0, 9	18, 11, 22

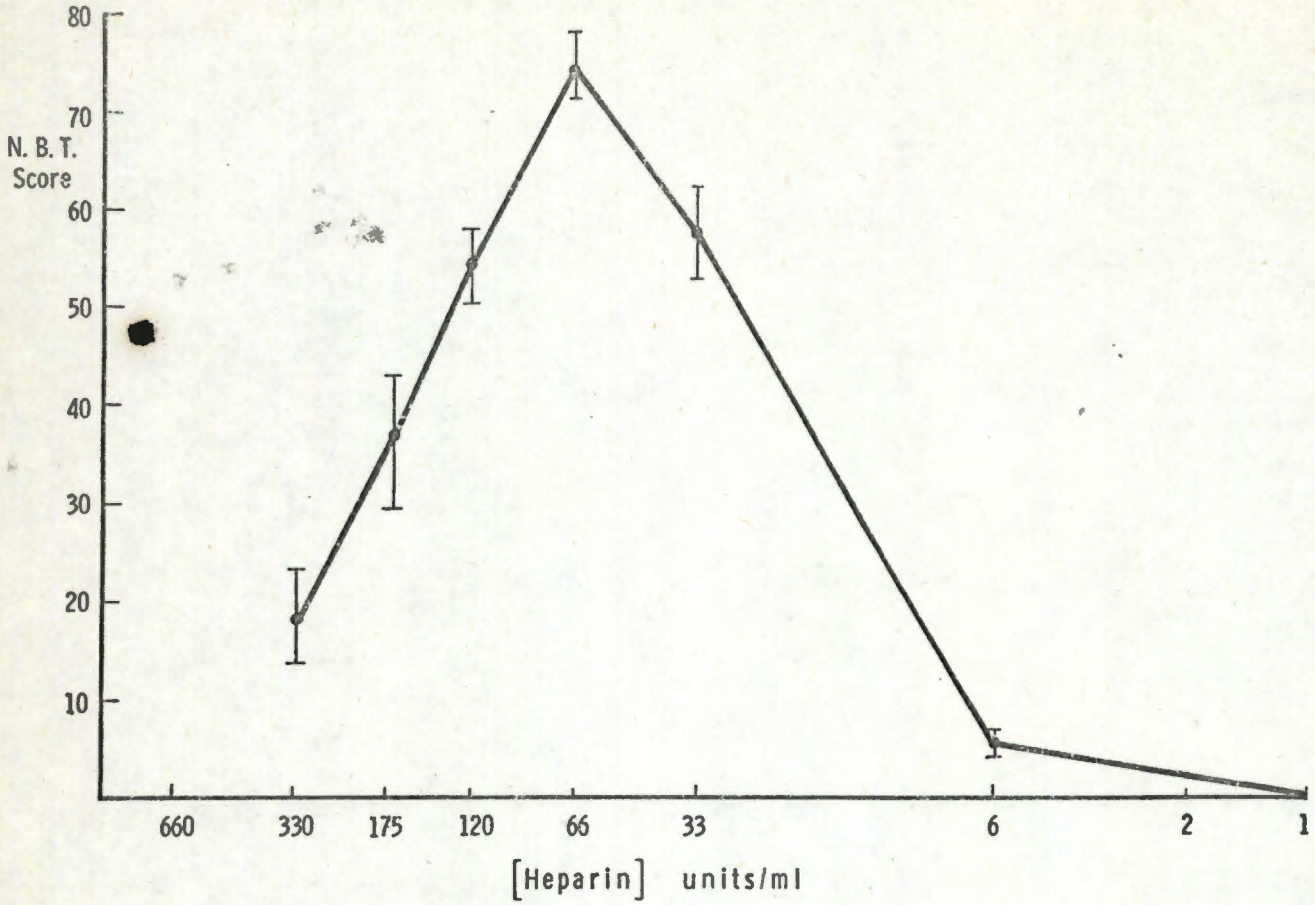


FIGURE 3.11.

Effect of varying concentrations of heparin on NBT reduction by neutrophils of normal subjects. (• Mean ± 1 S.E.).

There was no relationship between the serum concentration of the various classes of immunoglobulins and NBT reduction after endotoxin stimulation, which was normal even in the absence of measurable immunoglobulin. The general pattern of reduction by the group as a whole was normal (Fig. 3.10).

3.2.3.b The effect of varying concentrations of heparin on NBT reduction by neutrophils

Increasing concentrations of heparin from 6.75 to 66 i.u./ml resulted in a marked increase in the NBT score (Table 3.7, Fig. 3.11). With higher concentrations of heparin there was a progressive diminution of NBT reduction accompanied by increasingly marked morphological changes in the blood smears; the neutrophils became clumped and stained poorly with Leishman's stain. The cell clumps contained only very small amounts of reduced NBT.

TABLE 3.7. The effect of heparin concentration on NBT reduction by neutrophils in the blood of normal subjects. Results are expressed as the NBT score. The mean and S.E. NBT score are also shown.

Subject	[Heparin] i.u./ml						
	330	175	120	66	33	6	0.75
1	-	41	52	82	39	1	0
2	13	15	50	75	64	5	0
3	8	28	53	68	57	4	3
4	24	57	70	81	72	7	0
5	30	44	59	65	57	9	1
Number of studies	4	5	5	5	5	5	5
Mean	18.8	37.0	56.8	74.2	57.8	5.2	0.5
S.E.	(4.4)	(6.4)	(3.2)	(3.0)	(4.9)	(1.2)	(0.5)

TABLE 3.8 Screening tests of the effects of various substances on NBT reduction by neutrophils in blood containing EDTA or heparin as anticoagulant.

ADDITIVE	Final concentration (± range)/ml	BLOOD + EDTA	BLOOD + Heparin	Minimal effective concentration per ml
α^1-acid glycoprotein	3.3 mg	↑	↑	1.7mg
ADP	1.0mg-1.0 ng	-	-	
Adrenaline	10^{-3}-10^{-9}M		↑	10^{-3}M
Corticosteroids				
Cortisone acetate	10^{-3}-10^{-9}M		↓	10^{-3}M
Hydrocortisone hemisuccinate	10^{-1}-10^{-11} M		↓	10^{-1} M
Cyanide (potassium)	10^{-3}M		↑	
Cyclic AMP	1.75 µg-175pg		-	
Cytochalasin B	10µg		↑	
Endotoxin	100 µg-1pg	↑	↑	1 ng
Fetuin	10mg-16µg	↑	↑	400µg
Immunoglobulins	3.3 mg		↑	
Hexoestrol propionate	10^{-4}-10^{-14}M	↓	↑	10^{-4}M
Isoprenaline	10^{-3}-10^{-14}M	-	-	
Lactate	10^{-1}-10^{-5} M	-	-	
Neuraminidase				
Active	100 units		↑	
Heat denatured	"		↑	
Noradrenaline	10^{-3}-10^{-9}M		↓	10^{-3}M
Phosphate buffers	pH 5.5-7.5	-	-	
Prostaglandins E_1	1µg-100ng		↓	1 g-100ng
A_1	"		↓	"
F_2	"		-	"
Pus		-		
Retinol	250 µg-0.5µg	-	-	
Sodium fluoride	10^{-2}M		↓	
Triton X 100	0.05-0.01%	-	-	
Anoxia and Hyperoxia			-	
Thrombus		-	-	
Freeze thaw	x 1		Diffuse	
Lysolecithin	100-1 µg		stippling	1µg
Phospholipase C	10 µg-100ng		of	100ng
Sonication	15 secs x 10µ		neutrophils	

KEY: Enhanced reduction ↑

Diminished reduction ↓

No effect -

3.2.3.c The effect of some other substances on NBT reduction by neutrophils

Screening tests were performed with a number of substances on blood containing EDTA or heparin as anticoagulant (Table 3.8 opposite).

In more complete studies, fetuin, immunoglobulins, α^1-acid glycoprotein (Table 3.9), neuraminidase, active and heat inactivated, Cytochalasin B and KCN (Table 3.10) enhanced NBT reduction. All these substances also promoted neutrophils clumping, which was very marked after the addition of Cytochalasin B and neuraminidase. Formazan deposits observed in the preparations containing Cytochalasin B did not appear to have the normal intracellular location, but seemed rather to be adherent to the outer neutrophil membrane. DMSO, the solvent in which Cytochalasin B was solubilised, did not promoted NBT reduction or cell clumping.

TABLE 3.9. The effect of fetuin, α^1-acid glycoprotein and immunoglobulins compared to phosphate buffered saline (PBSA) ± endotoxin on NBT reduction by neutrophils in the blood from five normal subjects (A-E) containing heparin (20 i.u./ml) as anticoagulant. Results are expressed as the NBT score. The mean and S.E. NBT scores are also shown.

ADDITIVE Concentration		SUBJECTS A	B	C	D	E	MEAN NBT Score (S.E.)
Fetuin	(3.3mg/ml)	30	16	48	12	3	21.8 (7.0)
α^1-acid glycoprotein	"	62	27	71	35	13	41.6 (9.7)
Immunoglobulin	"	21	30	36	21	8	23.2 (4.2)
PBSA	-	9	5	33	2	0	9.8 (5.4)
PBSA + Endotoxin	(10μg/ml)	35	35	61	38	14	36.6 (6.7)

TABLE 3.10. The effect of active and heat inactivated neuraminidase, Cytochalasin B, KCN, NaF, anoxia and hyperoxia, compared to phosphate buffered saline ± endotoxin, an NBT reduction by neutrophils in the blood from five normal subjects containing heparin (20 i.u./ml) as anticoagulant. The results are expressed as the NBT score.

ADDITIVE	Concentration	SUBJECTS 1	2	3	4	5
Neuraminidase						
Active	100i.u./ml	36	53	50	39	-
Inactivated	"	-	78	70	42	-
Cytochalasin B	10 μg/ml	45	83	92	86	-
KCN	10^{-3}M	51	-	-	-	50
NaF	10^{-2}M	4	-	-	-	-
Anoxia		11	-	-	-	-
Hyperoxia		17	-	-	-	-
PBSA		19	56	34	20	20
PBSA + Endotoxin	10 μg/ml	56	81	70	73	-

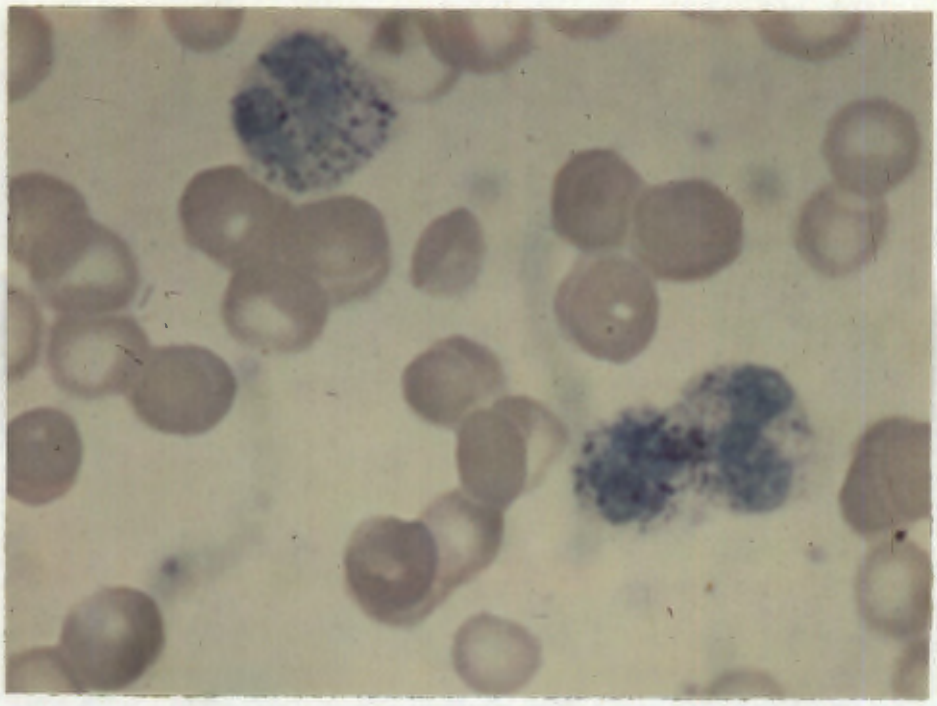


FIGURE 3.12.

Light micrograph of a slide preparation made from a blood sample which was sonicated before incubation with NBT. The neutrophils show diffuse punctate formazan deposits.

Leishman's x 2,000

Freezing and thawing, sonication, lysolecithin and phospholipase C, all resulted in NBT reduction by most of the neutrophils. This reduction did not have the usual appearance but consisted of diffuse, punctate, stippled formazan deposits which appeared to be confined to the outer membrane of the cell (Fig. 3.12).

Cortisone acetate and hydrocortisone hemisuccinate, hexoestrol propionate and noradrenaline all inhibted NBT reduction, but only at very high, totally unphysiological levels (Table 3.8). At these levels the corticosteroids inhibited reduction not only at a microscopic level but also in the supernatant plasma, where the customary blue colour failed to developed even after 18 hours of incubation. Adrenaline stimulated NBT reduction, but only at completely unphysiological levels. Isoprenaline reduced NBT directly, but did not enhance NBT reduction by neutrophils.

Prostaglandins E_1 and A_1 caused partial diminution and NaF a marked decrease of NBT reduction. NBT reduction was partially inhibited by the higher concentration of retinol, but this was probably as a result of the high concentration of ethanol (1.0%) which had a similar effect in the absence of retinol.

ADP, Cyclic AMP, Isoprenaline, lactate, phosphate buffers, prostaglandin $F_2\alpha$, sterile pus, Triton X 100, anoxia and hyperoxia, and products of coagulation did not effect NBT reduction by neutrophils, in the test conditions employed.

3.2.4. DISCUSSION

In the previous section it was shown that NBT enters neutrophils by phagocytosis of a macromoledular complex incorporating

the dye. In normal neutrophils, reduction of NBT appears to be related to the "burst of metabolic activity" that accompanies phagocytosis (Park et al, 1968, Klebanoff, 1971). Thus the NBT test is a measure of the phagocytic activity of neutrophils. In exceptional circumstances, such as in CGDC, phagocytosis proceeds normally (Stossel et al, 1972) but reduction of the dye is absent or delayed (Nathan et al, 1969).

In this section there are three groups of studies. The first study investigated the effect of various compounds, including endotoxin and heparin, on NBT reduction by the neutrophils of normal subjects. In the second study, a standardised endotoxin stimulation test was used to investigate neutrophil function in patients with a variety of diseases. Thirdly, the model was used to investigate the effect of NBT reduction of factors, the serum concentrations of which are elevated in disease, and which could be directly responsible for the enhanced reduction of the dye observed in a positive NBT test.

3.2.4.a <u>The effect of endotoxin, heparin and other compounds on NBT reduction by neutrophils</u>

i. <u>The effect of endotoxin on NBT reduction</u>

Enhanced NBT reduction has been demonstrated after the addition to blood of endotoxin (Park and Good, 1970), cell free bacterial culture filtrates (Matula and Paterson, 1971a) and bacteria (Cocchi et al, 1971b). The model system described here differs from that of Park and Good (1970) in that they used blood with heparin as anticoagulant and a single very high concentration (20 μg/ml) of endotoxin. EDTA was used as anticoagulant in this study as it gives

more reproducible baseline results than heparin, which itself enhances NBT reduction. The single high concentration of endotoxin used by Park and Good provided a supramaximal stimulus, and they did not investigate the dose response of NBT reduction by neutrophils from normal or abnormal subjects.

In the model system employed in this study, in normal subjects, an increase in the concentration of endotoxin from 100 pg/ml to 100 ng/ml resulted in a linear increase in the proportion of neutrophils that reduced NBT. Endotoxin concentrations below 100 pg/ml did not stimulate, and concentrations greater than 100 ng/ml did not further enhance NBT reduction.

This test was also performed on patients with CGDC and with hypogammaglobulinaemia because of reports of false-negative NBT tests in these subjects (Table 2.1), and on the patient with the Chediak-Higashi syndrome in which false-positive NBT tests have been observed (Table 2.2). Diabetic patients were investigated because they are liable to infection (Seymour and Phear, 1963), and patients with Crohn's disease because it is a granulomatous disease, the aetiology of which is unknown and which could be related to defective neutrophil function. The effect of old age and therapy with sodium aurothiomalate was also investigated.

It was found that all the groups of patients responded with a similar pattern to the normal subjects (Fig. 3.10, Table 3.4). These findings indicate that the parameters of neutrophil function measured by a stimulated NBT test, the enhancement of phagocytosis by endotoxin,

and reduction of the dye, are normal in the majority of individuals in the various groups of patients studied.

The finding of normal NBT reduction by patients receiving therapy with glucocorticoid drugs is at variance with the decreased reduction observed in these patients by Chretien and Garagusi (1973) which may be a reflection of the different stimuli used to enhance NBT reduction, or the short duration of steroid therapy in three of their five patients. A few patients with hypogammaglobulinaemia showed diminished, and the patients with CGDC complete absence of NBT reduction. The patient with the Chediak-Higashi syndrome revealed greatly enhanced NBT reduction in both the presence and absence of endotoxin, which correlates well with the observation (Stossel et al, 1972) that phagocytosis of relatively inert particles is increased in this condition.

ii. <u>The effect of heparin on NBT reduction</u>

Heparin resulted in a concentration dependent enhancement of NBT reduction in initial concentrations of up to 66 i.u./ml (final concentration 50 i.u./ml). At higher concentrations of heparin the neutrophils appeared damaged and NBT reduction was decreased (Table 3.7, Fig. 3.11). These results indicate that heparin itself enhances phagocytosis of complexed NBT and that the use of high concentrations of this agent as anticoagulant would result in false-positive results in the NBT test of Park et al (1968) (2.5). A discrepancy exists between the uniform enhancement of NBT reduction by heparin in this study, and NBT reduction by neutrophils in blood

of normal subjects in which a standard (initial and final concentrations of 50 and 25 i.u./ml respectively) concentration of heparin was used as anticoagulant in the re-evaluation of the NBT test (2.3). In the latter, a wide range of NBT scores was obtained. Possible explanations of this discrepancy are that in the present study a different preparation of heparin was used (preservative free), blood was added to heparin in phosphate buffered saline rather than heparin alone, and blood from all five subjects was studied simultaneously, under the same conditions on the same day.

Further studies on the NBT test performed on blood anti-coagulated with heparin should utilise a standard low concentration of heparin to prevent enhancement of NBT reduction by heparin itself.

iii. The effect of other compounds on NBT reduction

Cytochalasin B was studied because it is known to partially inhibit phagocytosis (Davies et al, 1973a), and therefore might be expected to diminish NBT reduction. In view of this it was surprising to find that it enhanced NBT reduction (Table 3.10). The marked cell clumping induced by this agent, as observed in this study, and the inhibition of glucose transport into cells (Zigmond and Hirsch, 1972) suggests an effect on the cell membrane. Increased NBT reduction may result from increased adhesion of complexed NBT to the cell surface, a situation in which it may be susceptible to NBT reduction by the same mechanisms as are responsible for dye reduction within the phagocytic vacuole. This concept will be elaborated upon in section 4 (3.4.4.).

The effect of neuraminidase was investigated as it has been shown to enhance phagocytosis by macrophages (Weiss et al, 1966). It enhanced NBT reduction by neutrophils (Table 3.10) and also caused marked cell clumping. These effects are discussed in the next section (3.3.5.).

Screening tests on the effects of other compounds on NBT reduction were also performed. The results of these were not pursued further and are only included in the discussion for the sake of completeness.

In accordance with the findings of Park et al (1969), NaF inhibited NBT reduction, suggesting that glycolysis is important for NBT reduction. KCN enhanced NBT reduction (Table 3.10), and this finding, together with the observation which was not reproduced in the single experiment in this study, that high concentrations of oxygen inhibit NBT reduction (Cocchi et al, 1971b), suggest that anaerobic respiration may enhance phagocytosis.

Increased lactate production accompanies phagocytosis (Klebanoff, 1971). Neither lactate itself nor acidic phosphate buffers enhanced NBT reduction.

Surprisingly, extracts and homogenates of sterile pus did not enhance NBT reduction in the single experiment in which these were tested.

Exposure of cells to phospholipase C, lysolecithin, sonication and freezing and thawing, procedures which result in damage to the cell membrane, all enhanced NBT reduction. The

reduced NBT did not have the characteristic appearance of focal, discrete lumps of formazan, but took the form of minute punctate deposits. These may result from the reduction of NBT at breaks in the neutrophil membrane, as will be discussed in section 4 (3.4.5.).

3.2.4.b In vivo factors which might be responsible for enhanced NBT reduction in the NBT test

The original hypothesis as to the mechanism of induction of NBT reduction in neutrophils (Park et al, 1968) was that it occurred as a result of a change in the neutrophil membrane resultant upon the burst of metabolic activity induced by the phagocytosis of microorganisms. These workers found that a mean of 5825 neutrophils/c.mm in infected patients contained formazan deposits, which, taking into account the turnover rate of peripheral blood neutrophils (3.3.6.), would suggest that vast numbers of bacteria, in the order of magnitude of $1 \times 10^9 - 1 \times 10^{10}$ were exposed to neutrophils every few hours for the duration that the test remains positive, which may be a matter of weeks (Matula and Paterson, 1971a). Such a situation could be contemplated for a limited period of time in a patient with an overwhelming septicaemia but is hardly likely to be responsible for the positive tests reported in patients with streptococcal pharyngitis (Randall et al, 1973) or parasitic disease (Chretien and Garagusi, 1971) or to be responsible for the diverse conditions resulting in a false positive test. (Table 2.2).

NBT reduction has been ehanced in vitro by the addition to blood of bacterial endotoxin (Park and Good, 1970). In this study endotoxin in concentrations of greater than 1ng/ml enhanced NBT reduction and could be one of the factors responsible for a positive NBT test in infected patients in whom endotoxaemia of the same order of magnitude has been recorded (Levin et al, 1970, Caridis et al, 1972, McGill et al, 1970).

Endotoxaemia is not limited to gram negative bacterial infection, but also occurs in non-infective disease, possibly as a result of a failure of the hepatic clearance of bacteria, or products thereof, originating in the gut (Caridis et al, 1972). False positive NBT tests could result from a secondary endotoxaemia, but the severity of the non-infective illnesses resulting in such an endotoxaemia (Caridis et al, 1972) would suggest such a mechanism to be a rare one, at least in the patients included in the re-evaluation of the test performed in this study (2.1.).

As a result of the observation (Chapter 2, Table 2.10) that the NBT score was related to the degree of constitutional symptoms in infected patients and the fact that myocardial infarction results in positive NBT tests (Lauter et al, 1973), factors known to be increased in the serum of acutely ill patients were investigated for their ability to enhance NBT reduction by normal neutrophils.

Serum concentrations of glucocorticoid hormones are increased in stressful situations (Smith and Hoijer, 1962), and there has been a divergence of opinion as to the effect of therapy with

exogenous glucocorticoids on results obtained from the NBT test. Ng et al (1972) and Miller and Kaplan (1970) felt that prednisone therapy decreased NBT reduction in infected patients, while Matula and Paterson (1971b) attributed false positive results to this drug. Wollman et al (1973) concluded that even massive doses of glucocorticoids do not have a prolonged effect upon NBT test results but that intravenous administration of a bolus of methyl prednisone caused a sharp drop in the NBT score soon after injection. In the model system, neutrophils of patients undergoing treatment with prednisone reduced NBT normally after endotoxin stimulation. Cortisone acetate and hydrocortisone hemisuccinate inhibited NBT reduction, but only at the highly unphysiological levels of 1×10^{-3} and 1×10^{-1}M respectively. Concentrations of 1mM, about 50 times greater than that achieved in the blood of an adult after the intravenous administration of 500mg of hydrocortisone (Peterson et al, 1955) had no effect. It is unlikely that endogenous glucocorticoid hormones promote NBT reduction in sick patients, or that the results of NBT tests are grossly modified by therapy with these agents.

Physiological concentrations of adrenaline and noradrenaline, serum levels of which are acutely raised by stress (Smith and Hoijer, 1962) did not influence NBT reduction. Similarly, isoprenaline, a synthetic β adrenergic receptor stimulant (Harrison et al, 1967) was ineffective. Adrenaline and isoprenaline are thought to mediate their effect through the "second messenger", cyclic adenosine 3', 5'-monophosphate (Robinson et al, 1971). Although levels of this

compound are elevated in phagocytosing neutrophils (Park et al, 1971) it did not itself stimulate NBT reduction. Raised neutrophil levels of cyclic AMP may be a sequel to, rather than a promotor of, phagocytosis.

Prostaglandin E_1 increases cyclic AMP levels in various tissues (Horton, 1969) including leukocytes (Scott, 1970) and stimulates leukocyte margination (Kaley and Weiner, 1971). The prostaglandins E_1 and A_1 have been shown to promote an acute inflammatory response (Kaley and Weiner, 1971). These substances in similar concentrations partially inhibited NBT reduction in the model system, whereas $F_2\alpha$ was without effect.

Thrombocytosis may accompany acute tissue necrosis following trauma or inflammation, and may also occur in tuberculosis and Hodgkin's disease (Shaw and Oliver, 1958, Ozer et al, 1960, Fountain and Losowsky, 1962). Positive NBT tests may occur under the same circumstances. Although the platelet concentration in the blood smears of some of these patients correlated poorly with the NBT score (2.3.), the possibility existed that some humoral factor, released from platelets, might enhance NBT reduction. ADP is a natural platelet aggregant and is released from platelets after adhesion (Hovig, 1963). NBT reduction was not enhanced by ADP itself or by the supernatant fluid in which thrombus had been incubated.

The 'acute phase reactants' constitute a poorly defined group of plasma proteins including fibrinogen, haptoglobins, α^1-acid glycoprotein, α^1-antitrypsin, CRP, and antichymotrypsin. The

concentration of these proteins is elevated as a non-specific sequel to inflammatory disease (Boltax and Fischel, 1956), myocardial infarction (Johansson et al, 1972) or surgical trauma (Aronsen et al, 1972). The patients with myocardial infarction with various complications sustained high levels of α^1-antitrypsin, α^1-acid glycoprotein, CRP and antichymotrypsin. NBT tests are positive after myocardial infarction and remain positive in patients with complications (Lauter et al, 1973).

The function of some of the "classical" acute phase reactants is uncertain. Fibrinogen has an important role in the coagulation system (Seegers, 1967) and α^1-antitrypsin and antichymotrypsin may function as inhibitors of the proteolytic lysosomal enzymes released from damaged tissues (Janoff et al, 1962). CRP has been shown to enhance phagocytosis and it has been suggested that it may function as an opsonin (Hokama et al, 1962) but the role of α^1-acid glycoprotein remains an enigma. Fetuin has a number of features in common with α^1-acid glycoprotein. It is also an acidic glycoprotein (pK 3.5) with the electrophoretic mobility of an α^1-globulin (Pederson, 1945). It occurs in the serum of those foetal and neonatal animals in whom the placenta is impermeable to maternal immunoglobulins (Jameson et al, 1942, Hansen and Philips, 1947, Barboriak et al, 1958, Lecce et al, 1961). The serum concentrations of fetuin falls to very low levels within three to four days of birth, when serum immunoglobulins are acquired from maternal colostrum (Smith, 1946, Hansen and Philips, 1947).

Human syncitial and foetal membranes become permeable to IgG but not to IgM after the first trimester of pregnancy. Human foetal plasma comprises at least one protein species not normally demonstrable in the adult, which displays certain features similar to those of fetuin but which disappear early in foetal life. There is also a possibility that human neonatal serum has an α^1-globulin of a similar nature, but the evidence is conflicting and awaits experimental clarification (Schultze and Heremans, 1966). CRP levels are frequently elevated in the first weeks of life (Hanson and Nilsson, 1962) possibly related to stress induced by adaptation to extrauterine life (Sandor, 1966a).

Fetuin and α^1-acid glycoprotein both enhance NBT reduction (Table 3.9) in physiological concentrations and serum concentrations of α^1-acid glycoprotein correlate well with NBT scores on the same blood (Table 2.9).

Myeloma (aspecific) immunoglobulins IgG and IgM, but not IgA, function as opsonins (van Oss and Stinson, 1970, Stinson and van Oss, 1971). A non-specific rise in the γ globulins occurs in a vast number of diseases of diverse aetiology (Sandor, 1966b). Immunoglobulins enhanced NBT reduction in the model system (Table 3.9). Elevated concentrations of these proteins could be responsible for false positive NBT tests in a large proportion of the conditions in which these have been reported (Table 2.2).

In this study, many factors enhanced the phagocytosis of complexed NBT by neutrophils. Serum concentrations of a number of compounds are commonly elevated in disease. Of those tested, only α^1-acid glycoprotein, immunoglobulins and endotoxin promoted NBT

reduction in vitro. It is tentatively suggested that any one of these substances, singly or in varying combinations, or other as yet unidentified substances, may be responsible for the increased phagocytosis of NBT indicated by a positive NBT test.

3.2.4.c The physiological role of heparin, α^1-acid glycoprotein and fetuin

The preceding studies demonstrated that heparin, α^1-acid glycoprotein and fetuin promote the phagocytosis of complexes of fibrinogen and NBT. The physiological roleof these compounds is obscure. It is suggested that they function as a non-specific opsonising system to enhance the phagocytosis of endogenous debris as well as exogenous particulate matter. Current knowledge of non-specific opsonins and the role of heparin will be briefly discussed, after which this hypothesis will be expanded upon.

i. Opsonins

When Metchnikoff defined the process of phagocytosis in 1884 (Metchnikoff, 1884), he placed little importance on the role of serum. Wright and Douglas (1903, 1904) established that serum facilitated the phagocytosis of pathogenic bacteria, that its permissive effect was achieved by modification of the micro-organism rather than the neutrophil, and that immunity to bacterial infection developed as a result of changes in the serum rather than in the neutrophils themselves. They called this "an 'opsonic' effect (opsono - I cater for; I prepare victuals for)" and suggested the term "'opsonins' to designate the elements in the blood fluids which produce this effect".

Subsequent investigation has established that specific antibody in the absence and in the presence of complement (Winkelstein, 1973), aspecific (myeloma) IgG and IgM (van Oss and Stinson, 1970, Stinson and van Oss, 1971), and a peptide product of the proteolysis of γ globulin, "tufsin" (Najjar and Nishioka, 1970), all facilitate phagocytosis by neutrophils.

Proteins other than immunoglobulins have also been implicated as opsonins. Surgenor (1952) and Tullis and Surgenor (1956) demonstrated the presence of two heat labile fractions with the electrophoretic mobility of an α^1-globulin and β globulin respectively, in normal human plasma. These fractions promoted the phagocytosis of inert particles by leukocytes and were termed the phagocytosis-promotion factors (PPF). The identity of these proteins is unknown. When the third component of complement (C3), a β globulin, is activated by the classical pathway, or the alternate pathway, which requires properdin, it acts as an opsonin (Winkelstein, 1973). Tullis and Surgenor (1956) observed that both heat inactivation of complement and inhibition of the alternate pathway by adsorption of properdin with zymosan reduced the activity of PPF by approximately 35%. C3 could be a component of the β globulin fraction of the PPF.

C reactive protein (CRP) is a β globulin (McCarty, 1947). It also causes pneumococcal capsular swelling and complexes with a number of micro-organisms in the presence of calcium ions (Löfstrom, 1944). Approximately 25% of the PPF activity was removed by the addition of powdered barium sulphate which adsorbs CRP (Ganrot and Kindmark, 1969). CRP may be another

component of the β globulin fraction of the PPF (Ganrot and Kindmark, 1969) although it is only present in trace amounts in the serum of healthy adults (Laurell, 1972). α^1-acid glycoprotein is one of the three major α^1-globulins (Laurell, 1972). Its function is unknown. It could be a component of the α^1 fraction of the PPF.

The non-specific opsonising system and PPF system are both inactivated by heating serum to 56°C for 20 minutes (Smith and Wood, 1969, Tullis and Surgenor, 1956). The effect of heat on opsonisation by CRP and α^1-acid glycoprotein has not yet been investigated. This procedure should help to clarify whether or not these proteins are constituents of the non-specific opsonising systems so far described.

Opsonisation is also important for phagocytosis by the reticuloendothelial system (RES). RES blockade, in which an initial intravenous (i.v.) injection of particulate matter results in reduced clearance from the circulation of a second i.v. dose of the same material, was thought to result from physical saturation of the system (Biozzi et al, 1953, Benacerraf et al, 1954). Blockade is related to the surface properties of the colloid (Murray, 1963a and b), preopsonisation of particles overcomes it (Jenkings and Rowley, 1961) and it has been proposed that reticuloendothelial blockade might result from depletion of opsonin (Saba and Di Luzio, 1965). In the isolated perfused liver, a heat labile, 'exhaustible' plasma factor, independent of haemolytic complement, has been shown to opsonise particulate carbon (Filkins and Smith, 1965, Filkins et al, 1965).

The nature of these opsonins is unknown, although a barium sulphate adsorbable α_2 globulin enhances the phagocytosis of colloidal particles by Kupffer cells (Saba et al, 1966) and α and β globulins have been found to bind to colloidal gold (Simon, 1954). The non-specific systems that enhance phagocytosis by neutrophils and the RES appear to have a number of features in common.

Tests of neutrophil phagocytosis and its modification by various substances, have largely involved measurement of the uptake of microorganisms and inorganic particles. Such systems are crude as the particles are generally amenable to endocytosis and a gross phagocytic defect is required to incur an abnormal result. No quantitative studies have been performed on the phagocytosis of fibrin or cell debris and as a result very little is known of the circumstances necessary for endocytosis. There are a number of disease states in which tissue specific antibodies are associated with pathology, for which they may be casual (Holborow, 1968). It is possible that the phagocytosis of autologous material requires opsonisation by a system which is both non-specific and non-cytopathic. This probably precludes specific immunoglobulins which although capable of opsonic activity are unlikely to distinguish autologous debris from normal constituents of the body on which they would probably exert a pathological effect.

ii. Heparin

Heparin occurs almost exclusively within the mast cell (Ehrlich and Stivala, 1973) in which it is contained within granules, bound to histamine and 5-hydroxy tryptamine by a basic granule

protein (Uvnäs et al,1970, Bergendorff and Uvnas, 1972). Degranulation of the mast cell is readily induced by many relatively minor physical and chemical changes in the environment (Selye, 1965a) and heparin is released from the basic granule proteins in the presence of cations (Uvnas et al, 1970, Bergendorff and Uvnas, 1972).

The number of visible mast cells are acutely reduced by trauma (Wichman, 1955) or acute inflammation (McGovern, 1957), suggesting release of their contents, but are increased in areas surrounding chronic inflammation and neoplasia (Selye, 1965b).

The physiological function of heparin is unknown although various roles have been attributed to it. It is known to have a lipolytic action (Robinson and French, 1953) and the anatomical situation of mast cells predominantly around blood vessels (Selye, 1965c) has led to postulations that the focal release of heparin inhibits thromboembolic disease (Petterson and Hjelmman, 1964) and atherosclerosis (Pollak, 1957, Wexler, 1964).

In addition to its known anticoagulant action (Ehrlich and Stivala, 1973), heparin has a thrombolytic effect (Fitzgerald et al, 1967, Fitzgerald, 1969), the mechanism of which is not understood. Neutrophils are important in the resolution of fibrin in inflammatory exudate and in intravascular thrombus (Barnhart, 1966). The efficacy of low dose heparin therapy in the prophylaxis of deep vein thrombosis is unexplained, as the quantities administered do not reach anticoagulant levels although concentrations of coagulation factor 10a are reduced (Kakkar et al, 1971).

iii. An hypothesis as to the role of α^1-acid glycoprotein, fetuin, heparin and other compounds as promotors of phagocytosis

It is known that (Winkelstein, 1973) there are two systems of opsonins. The specific immunoglobulins are directed against micro-organisms and may be directed against autologous tissue in pathological situations. There is also a system of non-specific opsonins, including C3 and possibly CRP, α^1-acid glycoprotein, fetuin and heparin, produced in preparation for, or at time of, stress which augment phagocytosis of foreign material and possibly also of endogenous particulate waste.

Serum levels of the "acute phase reactants", CRP and α^1-acid glycoprotein are elevated by stress. Fetuin is present in the neonatal animal when it is first exposed to invasion by micro-organisms and deficient in specific opsonins. Serum levels of fetuin decline at the same time that specific antimicrobial opsonins are acquired from maternal colostrum. Heparin is released at the site of inflammation where opsonisation of the aggressive organism and the resolution of damaged tissues and products of inflammation are most needed. It is possible that the release of heparin is a focal phenomenon, as a result of the decreased survival value that would be afforded by a substance with both opsonic and anticoagulant properties in a stressful situation, when haemostasis may be vital.

These substances together with immunoglobulins and ficoll were all shown to promote phagocytosis of

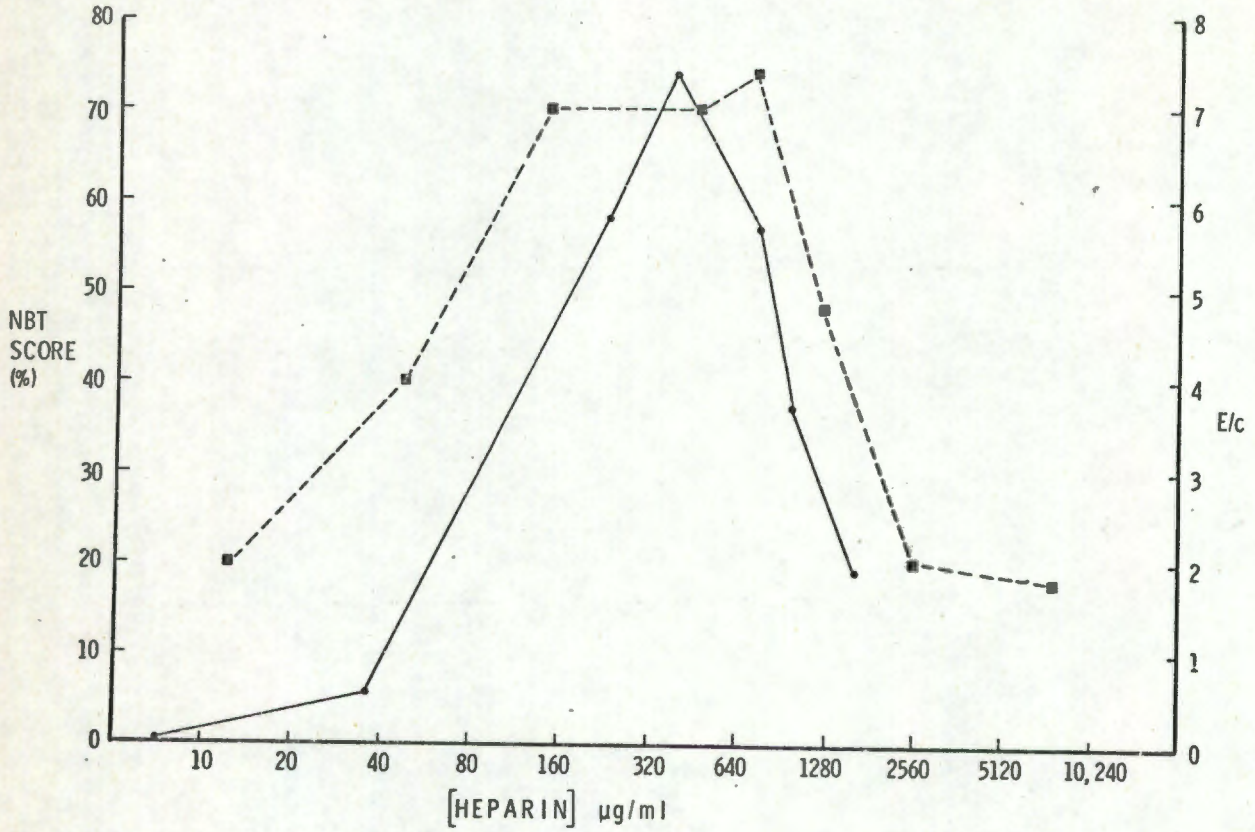


FIGURE 3.13

The effect of varying concentration of heparin on the reduction of NBT by neutrophils (this study) and on pinocytosis by mouse macrophages in an in vitro culture system (Cohn and Parks, 1967). The pinocyte index, E/C, is an expression of the ratio between the number of pinosomes observed in cells exposed to heparin (E) and in control cells (C).

complexed NBT. In studies (Conn and Parks, 1967) on factors inducing microscopically visible pinocytosis in a mouse macrophage culture system, the same compounds were observed to promote, and heparin in high concentrations to decrease, endocytosis and the concentrations necessary to exert these effects were similar in both studies (Table 3.11, Fig. 3.13).

TABLE 3.11. The concentration (± range) of compounds shown to enhance phagocytosis of complexed NBT (this study) or pinocytosis by mouse macrophages (Conn and Parks, 1968).

Additive	Neutrophil Phagocytosis	Mouse Macrophage Pinocytosis	Units
Fetuin	0.4 - 10.0	0.5 - 20,0	mg/ml
γ globulin	3.3	2.0 - 20.0	"
Ficoll	200.0	10.0 - 50.0	"
Heparin	5.0 - 400.0	13.0 - 700.0	μg/ml

CRP, raised serum levels of which are found after stress and in some neonates, enhances phagocytosis of bacteria and other particles by neutrophils (Hokama et al, 1962, Ganrot and Kindmark, 1969). In the latter study α^1-acid glycoprotein was found to be without effect. Its effect may have been present but masked by heparin present in the test system, as both compounds are very acidic. Heparin has been shown to facilitate thrombolysis which may have resulted from phagocytosis of the thrombus (Fitzgerald, 1969) and its effect in the prophylaxis of venous

thrombosis in subanticoagulant doses may be mediated by the enhanced removal of microthrombi.

The concentration of heparin necessary to enhance NBT reduction cannot be directly compared with the blood levels of heparin reached with low dose heparin therapy (0.2 i.u./ml - Yin et al, 1973), because NBT precipitates heparin, and it has not been established whether free or complexed NBT is responsible for opsonisation. Heparin in a concentration of 1.0 i.u./ml (the lowest concentration studied) markedly enhanced the phagocytosis of colloidal gold by Kupffer cells (Filkins and Di Luzio, 1966), demonstrating opsonisation at relatively low concentrations.

CRP, α^1-acid glycoprotein, fetuin and heparin are all acidic. Cohn and Parks observed the promotion of endocytosis almost exclusively by acidic compounds. Fetuin, α^1-acid glycoprotein and immunoglobulins are rich in carbohydrate, and heparin and ficoll are carbohydrate polymers. This may be relevant to the binding of these compounds to the cellular membrane as it is known that the carbohydrate component of immunoglobulins resides in the hinge section of the Fc component of the heavy chain (Edelman, 1971) which is responsible for binding the molecule to specific receptors on the neutrophil membrane (Messner and Jelinek, 1970).

3.2.5. SUMMARY

Using a model system it was found that in normal subjects, patient with Crohn's disease, diabetes mellitus, geriatric

subjects and patients receiving therapy with sodium aurothiomalate and prednisone, there was a standard response of NBT reduction to varying concentrations of endotoxin. Abnormal results were obtained in some hypogammaglobulinaemic patients and in a patient with CGDC and another with the Chediak-Higashi syndrome. The model system evolved may be of value in the in vitro assessment of neutrophil function.

The concentration of endotoxin necessary to induce NBT reduction in vitro were of the same order of magnitude as are found in endotoxaemia in vivo. Endotoxin may be one of the humoral factors responsible for a positive NBT test.

The serum concentration of many substances is increased under stressful circumstances. Some of these were investigated for their ability to enhance NBT reduction in vitro. This was observed with the "acute phase reactant", α-acid glycoprotein, and with fetuin, heparin and immunoglobulins.

An opsonic function as part of a non-specific opsonising system was proposed for some of the "acute phase reactants" as well as for fetuin and heparin.

It was proposed that a number of humoral factors could be responsible for the increased phagocytosis of complexed NBT indicated by a positive NBT test, and that they may act singly, or in combination, in an individual test subject.

3.3. SECTION 3 Possible causes of false negative NBT tests and studies on the effect of heterologous serum on NBT reduction by normal neutrophils.

3.3.1. INTRODUCTION

A significant proportion of neutrophils fail to reduce NBT despite exposure to infection in vivo (Park et al, 1968, Matula and Paterson, 1971a, Feigin et al, 1971, Fikrig et al, 1973, Gordon et al, 1973) or bacterial products (Matula and Paterson, 1971a, Koch, 1973), bacteria (Cocchi et al, 1971a) or endotoxin and heparin (this study) in vitro.

It thus appeared that a population of neutrophils exists that does not respond to infection in vivo, or stimulation in vitro, by the phagocytosis and reduction of NBT. For reasons that will be delineated later (3.3.5.) it was felt that this absence of NBT reduction might be a reflection of cellular immaturity. In this section the reduction of NBT by immature, bone marrow, neutrophils is examined and will be shown to be decreased in comparison with circulating neutrophils. It will also be shown that preincubation with immune complexes diminishes NBT reduction by neutrophils. In the light of these findings, the effect of sera from infected and uninfected patients on whom NBT tests were performed (Chapter 2), upon NBT reduction by normal neutrophils will be examined.

3.3.2. METHODS

3.3.2.a Exposure of autologous blood and bone marrow to various compounds

Sternal bone marrow and autologous peripheral blood samples were obtained simultaneously from subjects with a variety of non-malignant disease. These specimens were exposed to various compounds by the methods outlined in 3.2.2. and to latex particles by the method described in 3.1.3.1.

i. Exposure of autologous blood and bone marrow, with heparin as anticoagulant, to endotoxin, Cytochalasin B, neuraminidase and latex particles.

Peripheral blood and bone marrow samples (2.0 ml) from 6 subjects, of whom 5 had macrocytosis in the absence of megaloblastosis and the sixth, cyclical neutropenia, were taken into plastic tubes containing heparin (50 i.u./ml) and exposed to either phosphate buffered saline alone or phosphate buffered saline containing endotoxin (10 μg/ml), neuraminidase (100 i.u./ml) or latex particles, or to Cytochalasin B (10 μg/ml) in DMSO.

ii. Exposure of autologous blood and bone marrow to endotoxin in the presence of EDTA as anticoagulant

Autologous blood and bone marrow specimens (2.0 ml) with EDTA as anticoagulant (2.4 mg/ml), from 5 patients were exposed to endotoxin (10 μg/ml).

iii. Exposure of autologous blood and bone marrow to various concentrations of heparin

Autologous blood and bone marrow samples from two

male subjects aged 62 and 59 years with macrocytosis in the absence of megaloblastosis were exposed to heparin in varying concentrations.

3.3.2.b The effect of immune complexes on NBT reduction by human neutrophils

i. Manufacture of rabbit ovalbumin-antiovalbumin immune complexes

Serum from two rabbits (Himalayan), hyperimmunised against ovalbumin, was a gift from Dr. David Brown, Department of Immunology, The Hammersmith Hospital, London. The animals had been immunised by deep intramuscular injection of ovalbumin (20mg) in Freund's complete adjuvant, followed by several booster doses of antigen (20mg in Freund's complete adjuvant) at monthy intervals. The antiovalbumin antibodies consisted almost entirely of the IgG class of immunoglobulins.

0.5 ml of serum was incubated in 12 plastic test tubes with 0.5 ml of phosphate buffered saline containing serial doubling dilutions of ovalbumin (20mg-10μg/ml) at 37^{o}C for 30 minutes. The point of equivalence was found to be 1.25 mg/ml of ovalbumin as measured by quantitative precipitation (Kabat, 1961). Tubes number 1 and 2 (10 and 5 mg antigen/ml respectively) had immune complexes in gross antigen excess, 11 and 12 (9.8 and 4.9 μg antigen/ml respectively) complexes in gross antibody excess, tubes number 4 and 5 (2.5 and 1.25 mg antigen/ml respectively) as having complexes in slight antigen excess and at equivalence, and tubes 8 and 9 (78 and 39 μg antigen/ml respectively) as having complexes in slight antibody excess.

ii. The effect of antiserum containing varying antigen concentrations on NBT reduction by neutrophils

Blood from a normal subject was anticoagulated with heparin (20 i.u./ml). 2 x 100μl samples of this blood were each mixed in plastic test tubes with 50 μl of serum/antigen mixtures from tubes 1, 2, 4, 5, 8, 9, 11 and 12 and incubated at 37°C for 15 minutes. To one series of the mixtures was added 20 μl of endotoxin in phosphate buffered saline (300 μg/ml, final concentration of $\pm$ 25 μg/ml) and to the other tubes, 20 μl of phosphate buffered saline. The mixture was gently shaken, then incubated at 37°C for 10 minutes. 50 μl of 0.2% NBT in saline was added, the mixture was gently shaken and the standard procedure was then followed.

Further studies were done on blood anticoagulated with heparin (50 i.u./ml) using various dilutions of the serum from both animals, which contained complexes in antibody excess (39 μg/ml of antigen).

iii. The effect on NBT reduction of exposing neutrophils in whole blood to complexes in antibody excess, endotoxin and NBT in varying combinations and sequence

Blood anticoagulated with heparin (20 i.u./ml) was exposed to complexes in antibody excess (39 μg antigen/ml) in the undiluted serum from one of the rabbits as above (ii) with variations in the sequence of addition of the components of the mixtures. In the mixtures from which the complexes or the complexes and endotoxin were excluded, the 50 μl of 0.2% NBT in saline was replaced by

100 μl of 0.1% NBT in phosphate buffered saline, and the 20 μl of phosphate buffered saline containing endotoxin was replaced by 20 μl of phosphate buffered saline without endotoxin (Table 3.12).

TABLE 3.12 Exposure of neutrophils in whole blood to immune complexes, endotoxin and NBT in varying combinations and sequence. The figures (except for NBT scores) refer to the time in minutes after the beginning of the experiment that the component mixtures were added to heparinised blood (single study)

Summary of sequence of addition of components to reaction mixture	Endotoxin	Immune complexes	NBT	Slide preparation made	NBT score (%)
Endotoxin then complexes and NBT	0	10	10	30	73
Endotoxin then complexes then NBT	0	10	25	45	12
Endotoxin and complexes then NBT	0	0	15	35	22
Complexes then endotoxin then NBT	5	0	20	40	12
Complexes then endotoxin then NBT	15	0	25	45	11
Endotoxin no complexes	0	-	10	30	74
No endotoxin or complexes	-	-	10	30	26

3.3.2.c Animal experiments

i. The effect of vitamin C deficiency in NBT reduction

Guinea pigs were reared on autoclaved proprietary rabbit food containing little or no ascorbic acid. Control animals received green vegetables in addition. After 3 weeks the guinea pigs on the scorbutic diet showed evidence of vitamin C deficiency as manifest by weight loss, loss of subcutaneous fat and diarrhoea. Blood from 2 scorbutic and 2 control animals was exposed to endotoxin and then to NBT by the standard method.

ii. The effect of congenital deficiencies of the complement system on NBT reduction

Blood from 2 adult rabbits (Himalayan) with a congenital deficiency of the sixth component of complement (C6), and 2 guinea pigs with a congenital deficiency of the fourth component of complement (C4) was exposed to endotoxin and then to NBT by the standard method.

iii. Effect of immune complexes in vivo on NBT reduction by neutrophils

Ovalbumin was injected intravenously into a hyperimmunised rabbit (Himalayan) in a concentration calculated to result in the formation of immune complexes in antibody excess after in vitro titration of dilutions of antigen against serum (40 μg/Kg). Blood was taken from a marginal ear vein 30 minutes after antigenic challenge and exposed to endotoxin and NBT by the standard method.

Source of animals. Complement deficient animals were bred, and the rabbit immunised, in the laboratory of Dr. Peter Lachman, The Hammersmith Hospital, London.

3.3.2.d The effect of the suspension of normal neutrophils in the sera of patients with a variety of disease processes on whom NBT tests had been performed.

i. Resuspended cells exposed to EDTA plasma

Washed buffy coat preparations of a normal, ABO blood group O positive, subject were prepared as described in 3.1. To each of 38, 100 μl aliquots of this preparation in plastic test tubes was added 100 μl of serum from different subjects, the mixture was gently shaken and incubated at 37°C for 15 minutes. 100 μl of plasma from the blood of the cell donor (EDTA as anticoagulant, 2.4 mg/ml) and 100 μl of 0.2% NBT in saline containing ficoll (400 mg/ml) were added, the tubes were capped, mixed by gentle shaking, incubated at 37°C for 30 minutes and then at room temperature for 15 minutes and slide preparations made and counted in the usual manner. This treatment of the resuspended cells is analogous to the EDTA/ficoll NBT test (2.2.2.)

ii. Resuspended cells exposed to a solution of fibrinogen and heparin

To each of 29, 100 μl aliquots of the washed cell preparation was added 100 μl of sera from different patients. After mixing and incubation at 37°C for 30 minutes, to each tube was added 100 μl of a solution of fibrinogen (10 mg/ml) and heparin (50 i.u./ml) in phosphate buffered saline and 100 μl of 0.2% NBT in saline. The suspension was mixed by gentle shaking, incubated at 37°C for 15 minutes, at room temperature for a further 15 minutes and the samples smeared, stained and counted. This treatment of the re-

suspended cells is analogous to the heparin NBT test (2.2.2.)

3.3.3. RESULTS

3.3.3.a Exposure of autologous blood and bone marrow to various compounds

i. Exposure of autologous blood and bone marrow to endotoxin, Cytochalasin B, neuraminidase and latex particles

A much higher NBT score was obtained, under all circumstances examined, with peripheral blood neutrophils than with bone marrow neutrophils (Table 3.13). NBT reduction was enhanced in peripheral blood by endotoxin. It was also enhanced by Cytochalasin B and neuraminidase, but cell clumping in the presence of these substances was very extensive and the clumps contained a lot of reduced NBT. Counting of unclumped cells containing reduced NBT would seem to underestimate the percentage of cells containing this compound.

The reduction of NBT by bone marrow neutrophils was increased by endotoxin, latex, neuraminidase and Cytochalasin B. Cell clumping was also observed with Cytochalasin B and neuraminidase, but it was less extensive than in peripheral blood. Examination of the peripheral blood neutrophils in the single patient with cyclical neutropenia was impossible because of the profound neutropenia (200 neutrophils/cmm), but the marrow neutrophils revealed low levels of NBT reduction which was enhanced by Cytochalasin B and latex particles.

TABLE 3.13. The NBT scores obtained upon exposure of autologous blood (B) and bone marrow (M) to NBT after incubation with phosphate buffered saline (PBSA), endotoxin (ENDO), Cytochalasin B (CB), latex particles (latex) and neuraminidase (N). The mean and S.E. of the NBT scores are also shown.

SUBJECTS			NBT SCORE									
			PBSA		ENDO		CB		LATEX		N	
Age	Sex	Condition	B	M	B	M	B	M	B	M	B	M
80	M	Macrocytosis	26	4	57	11	29	15	31	4	-	-
78	M	"	43	13	50	49	69	80	39	25	44	15
81	M	"	83	0	74	16	91	34	70	12	38	11
62	M	"	9	3	47	13	7	2	22	16	-	-
81	M	"	39	0	87	30	14	3	46	13	68	22
68	F	Neutropenia	-	0	-	0	-	8	-	28	-	0
		Mean	40.0	4.0	63.0	23.8	42.0	26.8	41.6	14.0	50.0	12.0
		S.E.	11.0	2.1	6.8	6.4	14.6	13.0	7.3	3.0	7.5	4.0

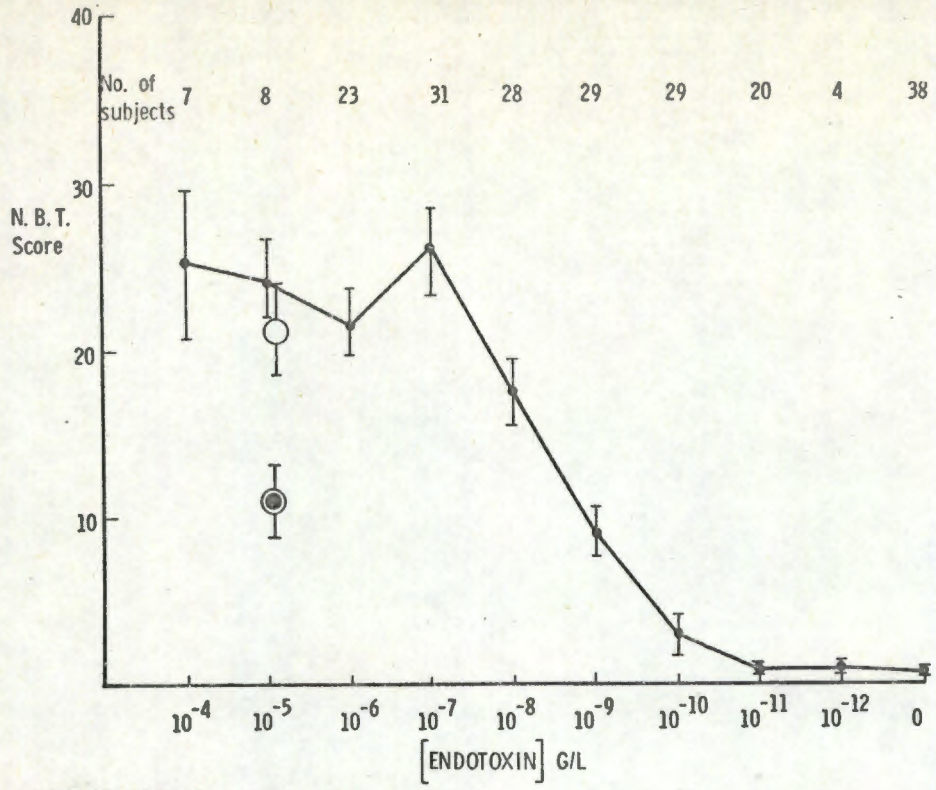


FIGURE 3.14.

Effect of endotoxin on NBT reduction by neutrophils from peripheral blood O and bone marrow ◉ . The dose response of neutrophils from the peripheral blood of normal subjects is also shown • (Mean ± 1 S.E. shown).

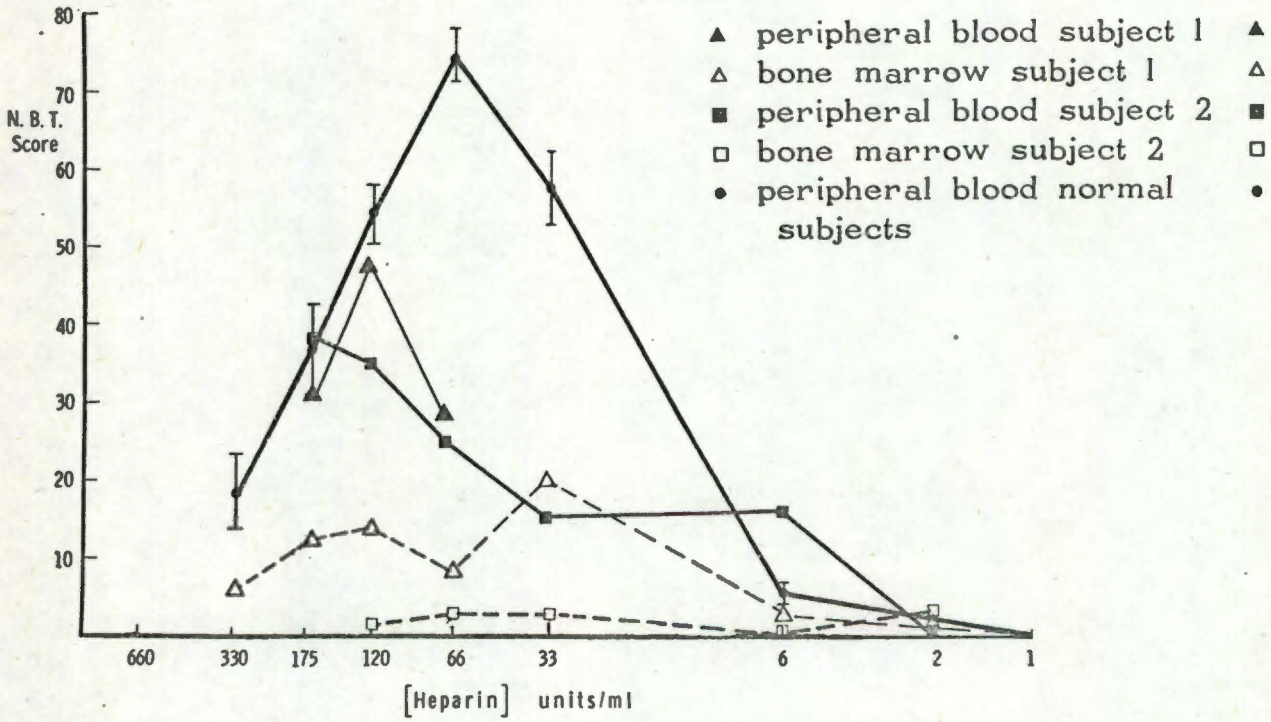


FIGURE 3.15

Effect of varying concentrations of heparin on NBT reduction by neutrophils from the peripheral blood and bone marrow of two subjects. The effect of varying concentrations of heparin on NBT reduction by neutrophils of normal subjects is also shown •

ii. NBT reduction by autologous peripheral blood and bone marrow neutrophils exposed to endotoxin in the presence of EDTA as anticoagulant

NBT reduction by peripheral blood neutrophils was normal. In comparison, dye was reduced by a much lower proportion of marrow neutrophils (Table 3.14, Fig. 3.14).

TABLE 3.14 NBT reduction by autologous peripheral blood and bone marrow neutrophils of 5 patients (EDTA as anticoagulant) exposed to endotoxin. Results are expressed as the NBT scores obtained. The individual results and the mean of three tests on a single blood and bone marrow sample from each subject, and the overall mean and standard error are shown.

SUBJECTS			NBT SCORE			
			BLOOD		BONE MARROW	
Age	Sex	Condition	Individual Results	Mean	Individual Results	Mean
45	F	Normal	12, 8, 5	8.3	3, 4, 2	3.0
79	M	Macrocytosis	48, 33, 23	34.7	28, 22, 25	25.0
26	F	Epilepsy	10, 12, 17	13.0	3, 1, 3	2.3
80	M	Macrocytosis	29, 27, 19	26.0	18, 11, 17	15.3
68	M	Macrocytosis	27, 27, 22	25.3	3, 3, 13	9.7
MEAN (S.E.)				21.3 (2.8)		11.0 (2.3)

iii. The reduction of NBT by autologous peripheral blood and bone marrow neutrophils exposed to varying concentrations of heparin

TABLE 3.15. The effect of various concentrations of heparin (i.u./ml) on NBT reduction by autologous peripheral blood (B) and bonemarrow (M) neutrophils of two patients. Results are expressed as the NBT scores obtained.

(HEPARIN) i.u./ml	NBT SCORE			
	PATIENT			
	1		2	
	B	M	B	M
2	0	3	--	0
33	16	0	-	20
66	15	3	29	8
120	25	3	48	14
175	35	1	30	12

Increasing concentrations of heparin resulted in increased NBT reduction by peripheral blood neutrophils. This was lower than that observed in normal neutrophils (3.2.3.b). This discrepancy may result from some abnormality of the neutrophils of these subjects or from experimental variation.

A much lower proportion of bone marrow neutrophils reduced the dye under similar circumstances (Table 3.15, Fig. 3.15).

3.3.3.b The effect of rabbit ovalbumin-antiovalbumin immune complexes on NBT reduction by human neutrophils

i. The effect of varying antigen concentration

Immune complexes at equivalence, and in slight antibody excess from both rabbits, caused markedly diminished NBT reduction by neutrophils in comparison with NBT reduction by neutrophils of animals exposed to complexes in gross antigen or antibody excess and neutrophils of control animals (Table 3.16).

TABLE 3.16 The effect of immune complexes formed by different antigen/antibody combinations on NBT reduction by neutrophils in blood anticoagulated with heparin (20 i.u./ml) in the presence and absence of endotoxin. Results are expressed as the NBT score.

COMPLEXES	ANTIGEN μg/ml	NBT SCORE (%)			
		SERUM DONOR 1		SERUM DONOR 2	
		Endotoxin	No Endotoxin	Endotoxin	No Endotoxin
Gross antigen excess	1.0×10^4	20	19		
	5.0×10^3	10	15	21	20
At equivalence	1.25×10^3	8	2		
Slight antibody excess	6.3×10^2	3	15	11	6
	7.8×10^1	0	2	4	4
	3.9×10^1	7	4		
Gross antibody excess	9.8	30	2		
	4.9	29	14	20	3
No antigen	Control 1 (serum + PBSA	29	17	-	-
No antigen or antibody	Control 2 (PBSA)	23	21	-	-

ii. The effect of varying concentrations of immune complexes on the reduction of NBT by neutrophils

Complexes in antibody excess were most inhibitory when undiluted but significant inhibition was still apparent when diluted 1 in 25 in phosphate buffered saline (final antigen concentration of 1.5 μg/ml, Table 3.17).

TABLE 3.17 The effect of dilutions of immune complexes on the inhibition of NBT reduction by neutrophils in blood anticoagulated with heparin (50 i.u./ml). Results are expressed as the NBT score.

DILUTIONS OF COMPLEXES Dilution	ANTIGEN μg/ml	NBT SCORE (%)	
		SERUM DONOR 1	SERUM DONOR 2
0	3.9×10^{1}	2	8
1 in 5	7.5	15	24
1 in 25	1.5	41	33
1 in 125	3.0×10^{-1}	70	68
1 in 625	6.0×10^{-2}	57	42
No complexes or serum	0	72	66
No complexes, serum or endotoxin	0	41	55

The enhanced reduction of NBT when compared with the previous experiment can be related to the higher heparin concentrations used in this experiment.

iii. The effect of the sequence of addition of the component mixtures on NBT reduction by neutrophils.

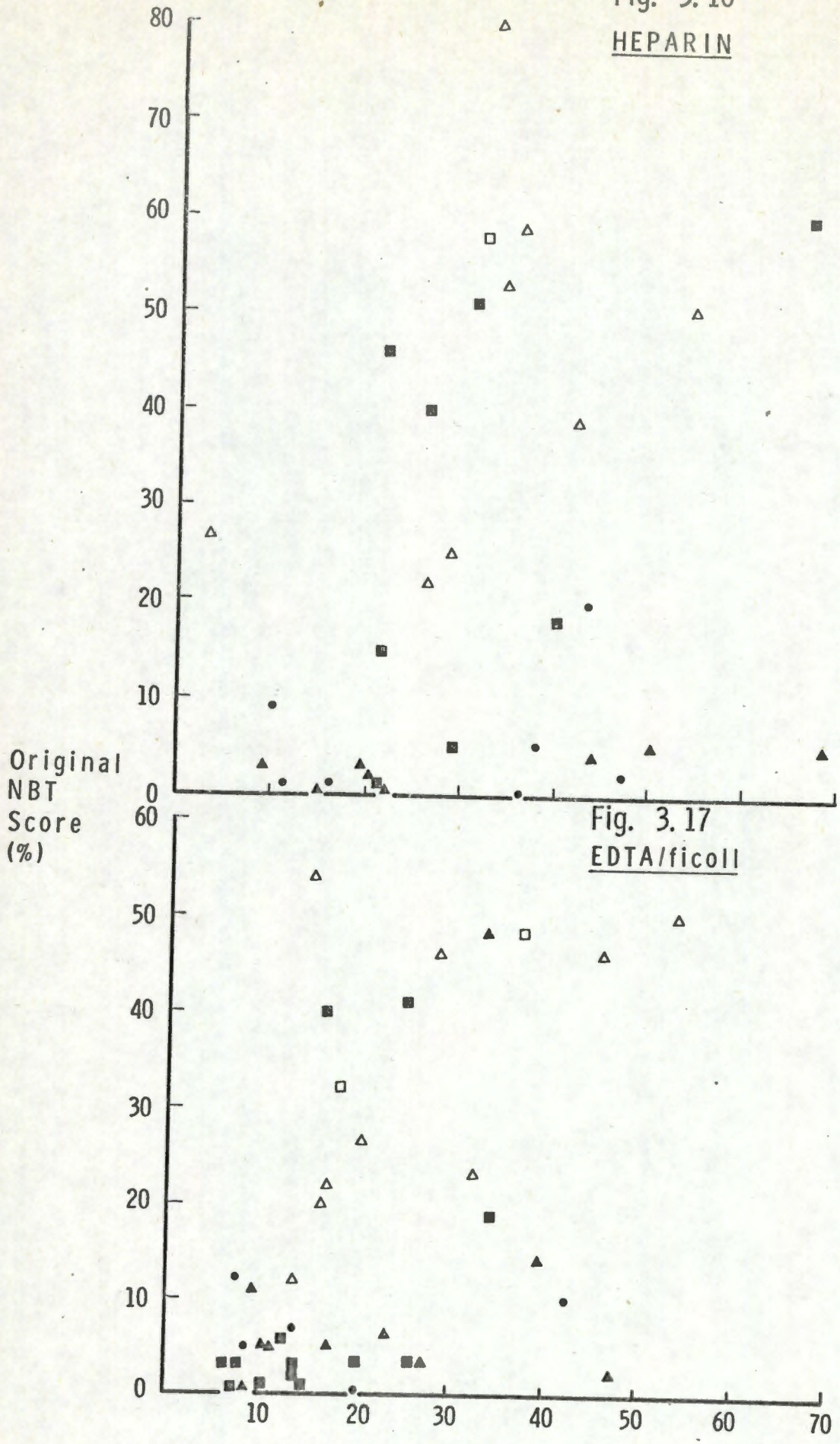
The addition of endotoxin to the test system before, after, or simultaneously with the complexes did not grossly alter the inhibitory effect of the complexes on NBT reduction. However, if the complexes and NBT were added simultaneously, reduction was not diminished (Table 3.12).

3.3.3.c Animal experiments

In limited studies complement deficient animals appeared to reduce NBT normally (Table 3.18).

TABLE 3.18. NBT reduction by neutrophils of normal animals, rabbits deficient in the sixth component of complement (C6), guinea pigs deficient in the fourth component of complement (C4), a hyperimmune rabbit after the intravenous injection of antigen and scorbutic guinea pigs, in the presence and absence of endotoxin. Quantified results are expressed as the NBT score.

Test Animal	Anticoagulant	NBT SCORE Endotoxin (10 μg/ml)	NBT SCORE PBSA Control
Normal rabbit 1	EDTA	33	2
C6 deficient rabbit 2		16	5
Normal rabbit 1	Heparin(20i.u./ml)	46	24
Normal rabbit 2		22	8
C6 deficient rabbit 1		37	11
C6 deficient rabbit 2		24	14
Hyperimme rabbit after I.V. ovalbumin	"	5	0
Normal guinea pig	Heparin(20i.u./ml)	20	4
C4 deficient guinea pig		22	3
Normal guinea pig	"	Normal appearance of smear with cell clumping and NBT reduction.	
Scorbutic guinea pig		Neutrophils abnormal. Badly damaged by endotoxin and NBT. Less so by NBT alone. Poor uptake of stain with very little NBT reduction	



FIGURES 3.16 and 3.17

Relationship between the results of EDTA/ficoll and heparin NBT tests performed on normal subjects •, patients with acute △ and chronic ▲ pyogenic infection and other acute □ and chronic ■ diseases, and NBT reduction by normal neutrophils suspended in serum from the same subjects.

In comparison with normal rabbits, there was diminished reduction of NBT by the neutrophils of a hyperimmune rabbit after antigenic challenge (Table 3.18). Lack of knowledge of the ability of neutrophils from this rabbit to reduce NBT before antigenic challenge detracts from the value attributable to these results.

3.3.4.d The effect of heterologous sera on NBT reduction by normal neutrophils

TABLE 3.19 Comparison between the NBT scores of normal neutrophils exposed to NBT, by the methods analogous to the EDTA/ficoll or heparin NBT test, after resuspension in heterologous sera, and the NBT scores on blood of the serum donors, serum concentration of CRP and serum concentration of α^1-acid glycoprotein. Results are expressed as the correlation coefficients obtained. The significance level (p value) is shown where significant correlation was obtained.

	Method	Original NBT score	CRP	α^1-acid glycoprotein
Resuspended NBT score of normal cells in heterologous sera	EDTA/ ficoll	0.43 (38) $p < .01$	0.01 (12)	0.01 (33)
	Heparin	0.18 (29)	0.09 (9)	0.27 (24)

The same normal neutrophils, suspended in different heterologous sera, reduced NBT in varying proportions (Figs. 3.16 and 3.17). The NBT scores of the resuspended cells compared best with the original NBT test results of patients with acute disease. A large proportion of patients with chronic disease had a low NBT test result, while a higher NBT score was obtained from normal cells

suspended in serum from these patients.

There was a significant correlation ($p < 0.01$) between the NBT scores of normal cells suspended in heterologous sera, and exposed to NBT by a method analogous to the EDTA/ficoll NBT test, and the NBT scores obtained on the blood of the serum donors (Table 3.19).

There was poor correlation between the NBT scores obtained on blood when the NBT test was performed by the heparin method and the NBT scores of normal neutrophils suspended in serum from the same subjects and exposed to NBT by an analogous method.

There was poor correlation between the NBT scores of normal neutrophils resuspended in heterologous sera and the serum concentrations of CRP and α^1-acid glycoprotein.

3.3.5. DISCUSSION

Circulating cells comprise but a small fraction of the body's total complement of neutrophils. The bone marrow contains approximately 1.8×10^{10} neutrophils and neutrophil precursors per kg of body weight in normal humans. Approximately half of these cells constitute a marrow storage pool of band and segmented neutrophils totalling a minimum thirteen times the number in blood (7×10^8 cells/kg) (Boggs, 1967). Blood neutrophils are distributed in approximately equal number between a circulating pool and a marginal blood pool (Athens et al, 1961). The latter comprises a pool of neutrophils marginated along venule and capillary walls, from which they can be

induced to re-enter the circulating pool by exercise or the administration of adrenaline.

Segmented neutrophils develop from last dividing myelocytes in approximately 2-3 days and subsequently exit from the marrow after a further 4-6 days, totalling a transit time through the non-dividing neutrophil compartment of 6-9 days. Infection apparently shortens neutrophil marrow residence time by about 2 days. The appearance of band neutrophils in the circulation preceeds segmented forms by 1 day, but no sequential relationship has been found to exist between the time of appearance of neutrophils and the number of nuclear lobes, suggesting the degree of segmentation should not be used as a measure of neutrophil age (Fliedner et al, 1964, Athen et al, 1965, Galbraith et al, 1965).

The neutrophils have a half life in the circulating pool of about 6.6 hours and egress occurs in a random fashion, irrespective of age (Cartwright et al, 1964). The fate of the neutrophil after it leaves the circulation and marginates is unknown. There is some evidence that they can survive in the circulation for at least 50 hours (Rosse and Gurney, 1959). Entry into the tissues is random and is unrelated to the time after release from the bone marrow (Fliedner et al, 1964).

The relationship between neutrophil age and functional capacity is unknown. This probably reflects the great difficulty in establishing the relative age of segmented neutrophils and of separating neutrophils into groups of different chronological age for study

purposes. The surface properties of granulocytes vary with cellular development. As cells mature the negative surface change density is reduced, they become more deformable, adhere more readily to glass or plastic on which they spread more prominently, migrate more rapidly, become more actively phagocytic and form pseudopodia more actively (Lichtman and Weed, 1972).

Neutrophil bactericidal activity is diminished in infected subjects (Alexander and Meakins, 1972), an effect which is most marked in the presence of a shift to the left of the myeloid series of leukocytes (Solberg and Hellum, 1972). This bactericidal defect may be related to the premature release of neutrophils from the marrow pool and a younger circulating neutrophil population as a result of increased neutrophil turnover observed in infected subjects (Fleidner et al, 1964, Athens et al, 1965, Galbraith et al, 1965).

Bone marrow samples obtained by aspiration must be contaminated to a variable degree by peripheral blood. Despite this limitation, it is a simple method of obtaining immature neutrophils. In this study NBT reduction was observed in a much smaller proportion of marrow neutrophils than in autologous circulating neutrophils in the presence of endotoxin, heparin and various other compounds, and in the single patient with severe neutropenia it was almost completely absent. Marrow neutrophils have a normal capacity to reduce NBT in a quantitative NBT test (3.4) and thus diminished reduction by these cells probably reflects their inability to phagocytose complexed NBT.

It is postulated that the finding of negative NBT tests in patients in whom the result should otherwise be positive usually indicates replacement of the normal blood neutrophil pool by a population of less mature neutrophils. There is no method of accurately measuring the age of circulating neutrophils and thus there is no means of comparing cellular maturity with spontaneous or stimulated NBT reduction by neutrophils. Reliance must be placed on circumstantial evidence linking the occurrence of a high proportion of young neutrophils in the circulation with false positive NBT test results.

An abnormally high proportion of neutrophils reduce NBT in uninfected neonates (Park et al, 1969). Neutrophil precursors and immature neutrophils comprise a significant percentage of the total circulating neutrophil pool in neonates with major infections (Xanthou, 1972). In such patients the NBT test result is greatly diminished in the absence (Cocchi et al, 1969) and presence (Cocchi et al, 1971) of an in vitro phagocytic stimulus.

The conversion of positive to negative results in infected patients after treatment with antibiotics and lower initial results in treated than untreated patients has been attributed to the antibiotics themselves (Matula and Paterson, 1971a , Sobel et al, 1973, Feigin et al, 1971). The decrease in NBT reduction may be purely a function of time. Present day antibiotic therapy is commenced very soon after the diagnosis of pyogenic infection. Thus studies on infected subjects in which comparisons are drawn between a group of untreated patients and a group of patients after the initiation of chemotherapy are unlikely

to be comparing groups of subjects at the same temporal stage of the disease. Observations that reductions in the NBT score of patients receiving antibiotic therapy may occur before clinical improvement (Matula and Paterson, 1971a, Hawkins, 1973, Ridgway and Johnson, 1973) suggest that the inhibitory effect is probably unrelated to eradication of the disease. In addition, Rubenstein et al. (1973) have shown that a number of commonly used antibiotics, in therapeutic concentrations, have no effect on the reduction of NBT by neutrophils in vitro.

The likelihood exists that in the face of systemic infection, demargination of neutrophils moves large numbers of mature neutrophils into the circulating neutrophil pool, and that these cells may be provoked to phagocytose complexed NBT leading to a positive NBT test result. As the disease process continues, the more mature neutrophils migrate to the site of pathology and are replaced by prematurely released neutrophils from the marrow pool. Because egress from the circulating pool is random, considerable time may elapse before the newly released neutrophils, themselves ageing, but possibly not yet mature enough to reduce NBT, constitute the majority of circulating neutrophils which may be reflected by a false negative NBT test. In support of this Esposito and De Lalla (1972) studied 12 patients with advanced untreated bacterial meningitis; NBT tests performed on peripheral blood samples were universally negative, whereas in all 7 cases in which tests were performed on the cerebrospinal fluid from the same patients, a very high percentage of the neutrophils reduced the dye.

Failure of enhanced NBT reduction in infected subjects is not an optimal indicator of the inability of neutrophils to reduce NBT because of the variability of disease processes. The exposure of blood to endotoxin in vitro produces a more uniform stimulus to the cells. Under these circumstances failure of NBT reduction was found in a patient following a 25 pint blood transfusion, when most of the patient's blood and circulating leukocytes would have been replaced by transfused blood, and in the same patient after a major episode of infection (Freeman et al, 1973a). Suppressed NBT reduction was found in those patients with burns whose lesions became infected (Curruri et al, 1973), a complication which has been shown to reduce the bactericidal capacity of neutrophils (Alexander and Meakins, 1972), and in infected diabetic patients (Pujol-Moix, 1973). The development of the 'NBT test stimulated' which reveals the inability of neutrophils to reduce NBT after an endotoxin challenge (Park and Good, 1970) is in itself an indication that infection can predispose to a diminution of NBT reduction, as no such depression has been found in normal subjects (3.2.3.a).

Suppressed NBT reduction after endotoxin stimulation was found in 9 of 93 subjects studied (3.2.3.a). Six of these patients had hypogammaglobulinaemia, of whom 2 had major chronic infection, a third had acute pyogenic arthritis and the other three had childhood onset hypogammaglobulinaemia in the absence of serious infection.

In the two patients with major infection that responded to antibiotic therapy, there was an increased ability of their neutrophils to reduce NBT after endotoxin stimulation, while the third, a boy with

viral meningoencephalitis, did not respond to therapy and there was no change in the amount of NBT reduction by his neutrophils. These findings are consistent with the hypothesis that NBT reduction is diminished by prolonged infection. The reason for poor reduction by the neutrophils of the 3 patients with hypogammaglobulinaemia in the absence of obvious infection is unknown. One possible explanation is that a rapid turnover of both the myeloid and lymphocytic cell series is responsible for immaturity of both neutrophils and lymphocytes resulting in a diminished ability to reduce NBT and deficient immunoglobulin synthesis respectively.

A final indication that neutrophils from the marrow pool are unable to reduce NBT comes from the study of Wollman et al (1972). Massive systemic glucocorticoid therapy did not reduce the NBT score in infected subjects unless the test was performed soon after the intravenous administration of the drug as a bolus. Glucocorticoids promote rapid release of neutrophils from the marrow into the blood pool (Bishop et al, 1968) so a reduction in the NBT score after such therapy may reflect a sudden release of marrow neutrophils into the blood.

Seg mented marrow neutrophils have a higher negative surface charge density than circulating neutrophils. This is due primarily to negatively charged carboxyl groups of N-acetylneuraminic acid (Lichtman and Weed, 1972). Reduction of the surface charge density of monocytes by the action of neuraminidase increased cellular deformability and phagocytosis (Weiss et al, 1966). The

enhancement of NBT reduction in peripheral blood (3.2.3.d) and bone marrow neutrophils (3.3.4.a) in this study by treatment of the cells with neuraminidase, suggests that a smaller proportion of immature neutrophils reduce the dye as a result of the greater surface charge density on these cells. This negative surface charge may prevent NBT reduction by repulsion of the complexed NBT (attempts to measure the nett charge of these complexes failed) or because of a general reduction of the phagocytic process. The decreased surface charge density after treatment of the cells with neuraminidase is also probably responsible for the marked cell clumping, as such charges are important for repulsion between cell membranes (Pethica, 1961). It is difficult to control experiments in which neuraminidase is used, because heat inactivation caused precipitation of the enzyme. The enhanced reduction of NBT observed in the presence of heat inactivated neuraminidase (3.2.3.d) probably results from enhancement of phagocytosis by this precipitate.

The evidence presented is strongly suggestive that relatively immature neutrophils have a diminished capacity to reduce NBT. In order to prove this a number of experiments have been devised, but not as yet implemented. Neutropenia could be induced in animals by the administration of antineutrophilic serum (Lawrence, et al, 1967) or antimetabolic drugs (Shadduck and Nagabhushanam, 1971) and the NBT score after endotoxin stimulation related to the age of the emerging neutrophils. This system would have two major drawbacks; any remnants of the agent employed to induce neutropenia may effect the emerging cells, and implementation of a stimulated NBT test is

very difficult in the presence of neutropenia because of the scarcity of visible cells. Another method would be to deplete the subject of neutrophils by leukopheresis (Craddock et al, 1955), and measure NBT reduction, after endotoxin stimulation, before and after the procedure. One such experiment has been performed and the removal of 4×10^{10} neutrophils from a normal subject resulted in a 50% reduction of the stimulated NBT score after the procedure, This would have to be repeated numerous times under carefully controlled conditions before the results could be regarded as significant.

The correlations between the NBT scores and neutrophil count, total white blood count (Table 2.7) and the presence of a shift to the left of the myeloid series (Table 2.12) probably reflect the relationship between the NBT scores and the severity of the disease process (Table 2.10). Extrapolation cannot be made from these indices as to the relative proportions of cells of differing maturity.

Another possible cause of false negative test results is immune complex disease. Nydegger et al (1973) demonstrated that immune complexes enhanced NBT reduction by neutrophils. In their test system, cells were suspended in serum and simultaneously exposed to NBT and immune complexes. The suspension of cells in serum alone would account for the low level of NBT reduction in the absence of complexes, a particulate phagocytosable substance being obligatory for significant visible NBT reduction (3!.1). Immune

complexes are voraciously engulfed by neutrophils (Hawkins and Peeters, 1971) and probably constituted the particulate object of phagocytosis alongside which NBT passed into the cell. In the present study it was demonstrated (3.3.3.b) that NBT reduction is greatly diminished by prior incubation of blood with immune complexes at equivalence or in slight antibody excess, or by antigenic challenge of a previously immunised animal (3.2.3.c). These conditions closely mimic in vivo immune complex disease, where cells would have prolonged exposure to the complexes before encountering NBT in vitro. Simultaneous exposure of neutrophils to NBT and complexes was also shown to enhance NBT reduction in this study. The mechanism of inhibition of NBT reduction by immune complexes is unknown, but as the neutrophils did not appear morphologically changed, and as there was no evidence of cellular swelling and engorgement, they may have coated the neutrophil membrane, so preventing adherence of complexed NBT to the neutrophil, or may have inhibited phagocytosis as a result of "Kamikaze" cell damage, a process which receives its name as a result of the lack of regranulation and eventual death of neutrophils after phagocytosis (Spicer and Hardin, 1969).

Immune complexes occur in systemic lupus erythematosus (Koffler et al, 1971) and meningococcaemia (Greenwood et al, 1973) and could account for false negative NBT tests in these subjects (Table 2.1). Wollman et al (1972) found the NBT test to be useful in patients after renal transplantation, uniformly low results being found

in patients with rejection, whereas infected patients gave high results. It is possible that the low results obtained in the patients with the rejection phenomena were the more abnormal, resulting from a secondary abnormality of neutrophil function, induced by immune complexes (Milgrom et al, 1971), which inhibited the high NBT test result usually found in acutely ill patients.

If the hypothesis that diminished NBT reduction after endotoxin stimulation reflects a decreased phagocytic capacity of the neutrophils, either as a result of relative cellular immaturity or the "Kamikaze" effect of autodestruction after phagocytosis, is correct, it would have important clinical application. The circulating concentration of 'functional' neutrophils, which may be a more relevant index of host defence than the current measurement of the total neutrophil concentration, could be assessed and might indicate the necessity for "normal granulocyte transfusion therapy" (Graw et al, 1972). A screening test could be devised to detect and monitor the response to therapy of patients with immune complex disease.

Complement inactivation was postulated as the reason for the lower amplitude of NBT reduction when EDTA rather than heparin is used as anticoagulant (Park and Good, 1970). EDTA has not been shown to be anticomplementary in the presence of excessive divalent NBT cations which may replace the essential role of calcium in complement activation. Heparin is anticomplementary (Walton et al, 1957), NBT is reduced by cells suspended in balanced salt solution in the absence of complement (3.1.) and NBT reduction is the same in normal and complement deficient animals (3.3.3.c.). In the light of these

facts it seems very unlikely that complement is important for NBT reduction by neutrophils.

The assumption that immunoglobulins are necessary for NBT reduction (Freeman and King, 1972b) was based solely on the report of Park and Good (1970) that false negative tests had been obtained in hypogammaglobulinaemic patients. The present study (3.2.3.a) indicates that negative stimulation tests are exceptional in these patients and are unrelated to the pattern of immunoglobulin deficiency.

Having investigated possible causes of false negative NBT tests, the effect of humoral factors in serum upon NBT reduction by normal cells can be investigated with the knowledge that factors which may enhance NBT reduction by normal cells may have been unable to exert their effect on autologous cells because of an inate or induced inability of these cells to reduce NBT.

Varying degrees of NBT reduction was obtained by the same cells suspended in different sera. There was good correlation between the initial and resuspended NBT scores when the cells were exposed to NBT in the presence of ficoll and EDTA (Table 3.19 Fig. 3.17) but not in the presence of heparin (Table 3.16, Fig. 3.16). In the majority of cases where high NBT scores were obtained in both the initial test and resuspended cells the patients had acute disease. The majority of patients with chronic disease had low initial NBT scores and high resuspended scores, perhaps indicative of an inability of their cells to reduce NBT. Unfortunately this cannot be established as NBT

stimulated tests were not performed on these patients. There was poor correlation between the resuspended NBT scores and the serum concentrations of CRP and α^1-acid glycoprotein although the number of studies was too small to allow definite conclusions to be drawn (Table 3.19).

The close correlation between the initial and resuspended NBT scores of cells exposed to ficoll and EDTA suggests that there is a humoral factor responsible for promoting the phagocytosis of the complexed NBT. The initial close correlation between the EDTA/ficoll ($p < 0.001$) and heparin ($p < 0.05$) NBT test scores and the serum concentration of α^1-acid glycoprotein (Table 2.9) indicates that this substance may be one of these humoral factors.

However, the NBT scores are increased with increasing severity of disease (Table 2.10) and the acute phase protein levels and NBT scores may both be independently resultant upon disease severity, rather than causally related.

The poor correlation between the NBT score of resuspended cells and the heparin NBT test results, and between cells resuspended by both methods and serum concentrations of α^1-acid glycoprotein and CRP possibly reflect the low number of studies performed, which magnify the errors of the methods and counting techniques employed. It is possible that heparin, which is very acidic, masks the opsonising action of α^1-acid glycoprotein, and that this, together with false positive tests induced by heparin, accounts for the discrepancy between results of NBT tests performed on the same blood

sample by the EDTA/ficoll and heparin methods (2.3.1.)

It is difficult to establish that an increase in the NBT test result reflects opsonisation of complexed NBT by some or all of the acute phase proteins. Enhanced NBT reduction on the addition of purified proteins to blood in vitro may result from modification of the protein by the purification procedures, whereas manouvres designed to increase serum levels of these proteins in laboratory animals will have a concomitant effect on the neutrophil population. A possible method of demonstrating binding of complexed NBT or other particles to the cell surface would be to inhibit phagocytosis, an energy dependent process (Rabinovich, 1967, Sbarra and Karnovsky, 1959) with metabolic inhibitors, or with Cytochalasin B, and to demonstrate the association of the cell and particle in the presence of, but not in the absence of, the opsonin. Such binding could be established by microscopy or by the association of radiolabelled cells, opsonin and particles after separation of unbound particles from cells by manouvres such as cell washing and density centrifugation. Such a system would separate opsonisation per se from increased phagocytosis as a result of enhanced cellular phagocytic activity.

3.3.6. SUMMARY

The relationship between neutrophil maturity and NBT reduction was investigated by comparing NBT reduction by autologous bone marrow and peripheral blood neutrophils. Spontaneous NBT reduction by bone marrow neutrophils was minimal, significantly different from that of circulating neutrophils, but was enhanced by exposure to latex and neuraminidase. This suggests that immature neutrophils are less capable than mature neutrophils of reducing NBT and that false-negative NBT tests might result in conditions in which there is an increased turnover rate of neutrophils leading to a predominance of younger cells in the circulation. The bactericidal capacity of neutrophils may be diminished in younger cells. If the relationship between cellular immaturity and deficient NBT reduction is substantiated, a stimulated NBT test may be a simple method of measuring the proportion of circulating neutrophils comprised by mature cells capable of effective bactericidal activity. Immune complexes inhibit NBT reduction by neutrophils, a stimulated NBT test may also be useful as an indicator of circulating immune complexes.

Normal cells suspended in heterologous sera from normal and diseased subjects in the presence of fibrinogen reduced NBT to a variable extent. There was a close correlation between the NBT scores obtained in the blood of the serum donors, and the NBT scores of normal cells resuspended in serum and exposed to NBT by a method analogous to the EDTA/ficoll NBT test - suggesting a humoral promotor of NBT reduction. There was poor correlation between

the initial and resuspended NBT scores in the presence of heparin. Chronic disease resulted in a low initial but higher resuspended NBT score suggesting the presence of a humoral promotor of NBT reduction, the effect of which was masked in the initial NBT test by an incapacity of the cells to reduce NBT.

3.4. SECTION 4. Mechanisms involved in the reduction of NBT by neutrophils

3.4.1. INTRODUCTION

In the past three sections an examination has been made of the method of entry of NBT into neutrophils in the NBT test, and of factors influencing this entry. This section will be devoted to an investigation of the mechanisms involved in the reduction of the dye by isolated neutrophils; in suspension, in the quantitative NBT test, and adherent to glass slides in the test of Gifford and Malawista (1970). It will be shown that NBT is toxic to neutrophils and that it is probably reduced as a consequence of damage to the outer membrane of the cell allowing free contact between the dye and intracellular reducing substances.

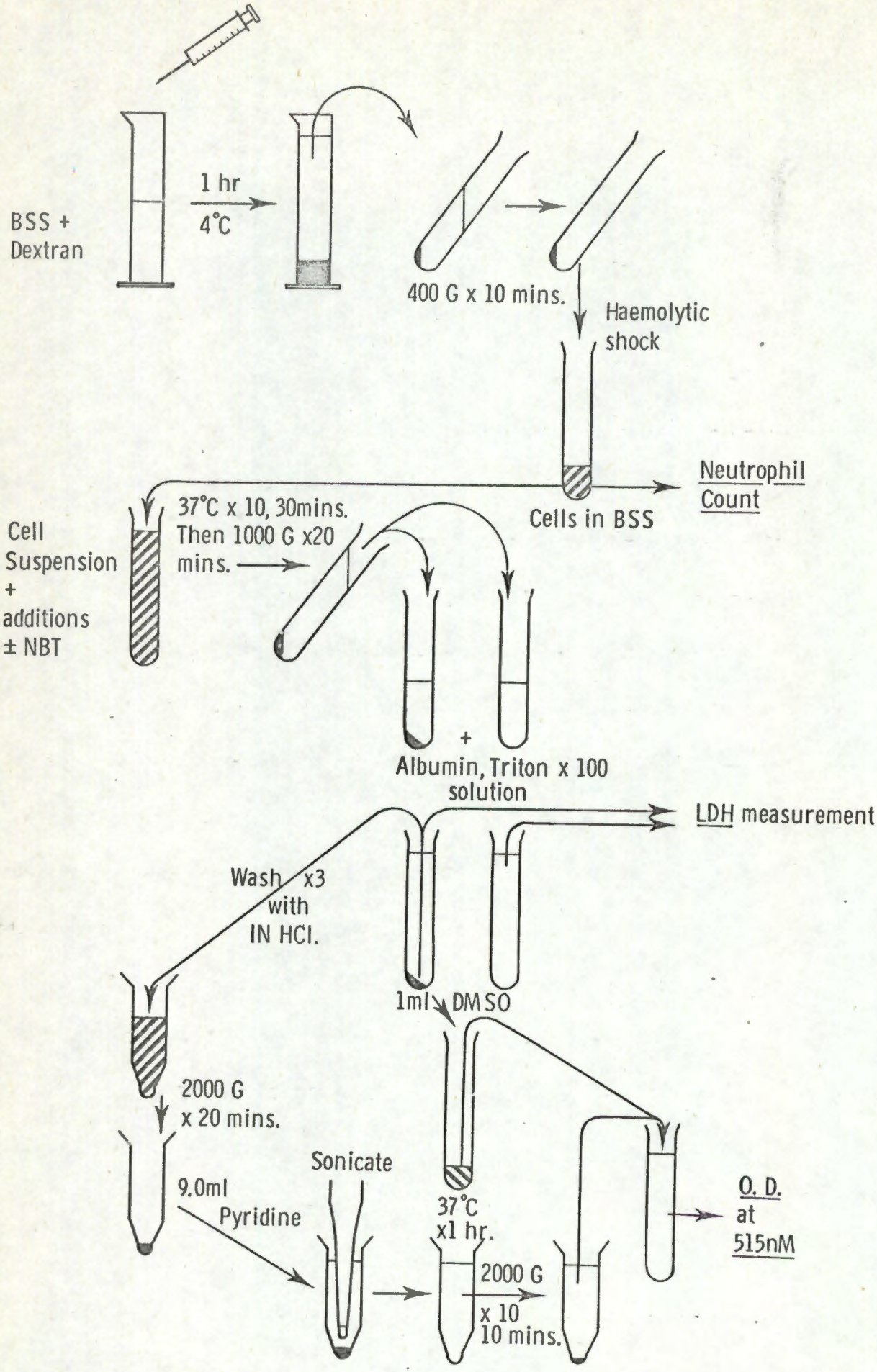
Damage to the neutrophil membrane was assessed by the release of the cytoplasmic enzyme lactate dehydrogenase (LDH). Dye reduction was quantitated spectrophotometrically after extraction from the cells with pyridine.

3.4.2. METHODS

3.4.2.a The effect of saline, endotoxin, latex and heparin on lactate dehydrogenase release in the presence and absence of NBT, and on the quantitative reduction of NBT by neutrophils.

i. Separation of leukocytes

Venous blood (15-100ml) from 8 normal subjects, taken into heparin (4 i.u./ml) was immediately mixed with an equal volume of cold (4°C) separating solution, containing dextran in a balanced salt solution (Blackburn et al, 1973), in a plastic measuring cylinder.


BSS +
Dextran
1 hr
4°C
400 G x 10 mins.
Haemolytic
shock
Neutrophil
Count
Cells in BSS
Cell
Suspension
+
additions
± NBT
37°C x 10, 30mins.
Then 1000 G x20
mins.
+
Albumin, Triton x 100
solution
LDH measurement
Wash x3
with
IN HCl.
1ml DMSO
2000 G
x 20 mins.
9.0ml
Pyridine
Sonicate
37°C
x1 hr.
2000 G
x 10
10 mins.
O. D.
at
515nM

After sedimentation at 4°C for 1 hour the erythrocyte poor supernate was aspirated and centrifuged at 400 G at 4°C for 10 minutes in a Sorval centrifuge. The supernate was discarded and erythrocytes destroyed with a haemolytic shock by the addition of 7.5 ml of cold (4°C) sterile water to the cell pellet, which was resuspended by gentle pipetting up and down approximately 10 times. After 60 seconds 2.5 ml of cold (4°C) saline (0.54M) was added to and mixed with the cell suspension to restore isotonicity. 20 ml of cold (4°C) balanced salt solution (BSS) was added to and gently mixed with the cell suspension which was then centrifuged as above.

ii. Incubation of cells with NBT and other materials

The supernate was discarded and the cell pellet resuspended in BSS (2-10 ml depending upon the final volume required) by gentle pipetting up and down. A small sample of the cell suspension was removed to determine the neutrophil concentration (which ranged from 2-16 x 10^6/cmm). To each of 2, 1.0 ml samples of the cell suspension in disposable plastic test tubes was added 50 μl of either saline, endotoxin (300 μg/ml) in saline, dialysed latex particles or heparin (2000 and 200 i.u./ml, final concentration of 100 and 10 i.u./ml). The tubes were capped, mixed by inversion and incubated at 37°C for 10 minutes. A further 50 μl of the same additive was added to each tube. To one series of tubes was added 0.9 ml of phosphate buffered saline and to

FIGURE 3.18

Diagrammatic representation of the methods used for cell separation, incubation of leukocytes with various additives in the presence and absence of NBT and the measurement of LDH release from and NBT reduction by these cells.

the other 0.9 ml of 0.1% NBT in phosphate buffered saline. The suspensions were mixed by gentle shaking and after incubation at 37°C for 30 minutes they were centrifuged at 1000 G at 4°C for 20 minutes. 1.0 ml of the supernate from each tube was added to 1.0 ml of an aqueous solution containing Triton X 100 (0.1%, a detergent) and bovine albumin (0.1%, to stabilise the LDH). This solution is henceforth referred to as the supernate fraction.

iii. Cell lysis

1.0 ml of the aqueous solution of Triton X 100 and albumin was added to the remaining supernate and cell pellet, mixed by vigorous manual shaking and centrifuged at 2000 G at 4°C for 20 minutes. 0.2 ml of the supernate, henceforth referred to as the subnate lysate was transferred to fresh plastic tubes and stored at 4°C.

iv. Extraction of formazan

The tubes to which NBT had been added, containing the remainder of the subnate lysate and cell debris or cell debris and formazan, were washed 3 times with 2.0 ml of IN HCl, the washings from each tube were pooled in 15 ml glass centrifuge tubes and centrifuged at 2000 G at room temperature for 20 minutes. The supernate was discarded, 9.0 ml of pyridine was added to each tube which was then; sonicated in a MSE 150 Watt ultrasonic disintegrator at an inter peak amplitude of 10 microns for 15 seconds, with the 3.0 mm titanium probe tip positioned immediately above the cell button; incubated at 37°C for 1 hour and then centrifuged at 2000 G at room temperature for 10 minutes. Residual formazan was extracted from the plastic tubes with 1.0 ml of dimethyl sulphoxide (DMSO) which was incubated in the

tube at 37°C for 1 hour and then added to and mixed with the corresponding extract of formazan in pyridine. The optical density was read at 515 nm on a Gifford spectrophotometer against a blank containing a mixture of 9.0 ml of pyridine and 1.0 ml of DMSO.

v. Measurement of LDH

Lactate dehydrogenase (LDH) was assayed in the supernate fraction and subnate lysate by determining the rate of oxidation of NADH at 340 nm following exactly the instructions issued with Biochemica Test Combination LDH UV-test No. 15948 TLAC. Assays were always completed within a few hours of obtaining the samples. The release of LDH into the supernate fraction was expressed as a percentage of the total LDH concentration assayed in the subnate lysate.

3.4.2.b The effect of NBT, in the presence and absence of latex, on LDH release and NBT reduction by peripheral blood neutrophils of two patients with CGDC and a patient with hypogammaglobulinaemia, and by bone marrow neutrophils from two normal subjects

Duplicate studies were performed by the same methods on neutrophils from two patients with CGDC and a patient with hypogammaglobulinaemia who reduced NBT poorly after endotoxin stimulation in the stimulated NBT test (Appendix K No.15). The patients with CGDC were both patients under the care of Professor J.F. Soothill (patients number 3 and 9 in the study of Thompson and Soothill, 1970).

Studies were also performed on neutrophils from the bone marrow of two normal subjects. 2.0 ml of sternal marrow aspirate was mixed with 10.0 ml of autologous plasma anticoagulated

with heparin (4 i.u./ml) and processed as in 3.4.2.a. Differential cell counts were performed on the isolated marrow cells.

3.4.2.c. The effect of endotoxin and of polyvinyl pyrrolidone (PVP) on NBT reduction by cells in the slide test of Gifford and Malawista (1970)

1.0 ml of venous blood was added to 0.1 ml of 0.15M saline or saline containing endotoxin (100 μg/ml) in a plastic test tube, rapidly mixed by shaking, and 1 drop of the mixture was placed on a clean glass slide and incubated at 37°C in a moist chamber. The superficial blood clot was washed off with 0.15 M saline, the excess fluid drained off by tilting the slide, and the adherent leukocytes covered with a mixture containing pooled human serum and 0.2% NBT in 0.15 M saline in the ratios of 5:9 by volume, with and without PVP (2.0 mM), and incubated in a moist chamber at 37°C for 20 minutes. The serum NBT mixture was washed off with 0.15 M saline, rapidly air dried, fixed by immersion in methanol for 1 minute, washed with water, dried and counter-stained with 0.7% aqueous safronin for 5 minutes. 100 cells were counted in 4 different areas on the slide and the mean percentage of swollen, blue "formazan cells" recorded. Studies were performed on the neutrophils from 25 normal subjects in the presence and absence of endotoxin, on a further 16 subjects in the absence of endotoxin and on 2 subjects in the presence and absence of PVP.

3.4.3. RESULTS

3.4.3.a The effect of endotoxin, latex and heparin on the release of LDH by cells in the presence and absence of NBT and on the quantitative reduction of NBT by neutrophils.

LDH release from cells was increased when NBT was present in the incubation mixture. Latex and endotoxin markedly enhanced LDH release in the presence but not in the absence of NBT (Tables 3.20and 3.21).

TABLE 3.20. The effect of endotoxin and latex, in the presence and absence of NBT, on the release of LDH from cells. LDH release (as a percentage of the total LDH released after cell lysis) is expressed as a percentage of LDH release from control cells (exposed to saline only). The mean, S.E. and number of studies are shown.

Additive to cell suspension	LDH activity in supernate compared with control (saline only)	Number of studies
Saline	100%	8
Endotoxin	103.2 (8.2)	3
Latex	86.3 (5.7)	3
NBT	132.2 (11.2)	8
NBT + Endotoxin	222.8 (25.7)	8
NBT + Latex	268.9 (40.6)	8

TABLE 3.21 LDH release (LDH) and NBT reduction by neutrophils from 8 normal subjects exposed to saline, endotoxin, latex particles and NBT in various combinations. The LDH release is expressed as a percentage of total LDH release after cell lysis and NBT reduction is expressed as optical density units x 10^{-3} per 1 x 10^{6} cells. The mean and standard error of the measurements is also shown. The formazan/LDH ratio is a measure of the NBT reduction in O.D. units x 10^{-6} per unit of LDH released (3.4.3.a).

Additive to cell suspension	Change measured	Subject								
		1	2	3	4	5	6	7	8	Mean (S.E.)
Saline	LDH	2.4.	2.2	3.10	3.46	1.30	1.66	6.9	3.59	3.08 (0.88)
Endotoxin	LDH	2.0	2.5	3.50	-	-	-	-	-	2.67 (0.36)
Latex	LDH	1.96	1.7	3.10	-	-	-	-	-	2.25 (0.35)
NBT	LDH	3.10	2.92	4.90	3.87	1.45	2.12	6.03	7.15	3.94 (0.65)
	NBT reduction	-	-	1.08	1.92	1.57	1.71	6.26	8.45	3.50 (1.15)
NBT + Endotoxin	LDH	3.46	6.13	7.20	4.86	4.75	3.46	11.02	9.20	6.26 (0.90)
	NBT reduction	-	-	8.21	6.54	6.02	4.68	7.67	10.52	7.27 (0.75)
NBT + Latex	LDH	10.85	4.84	11.3	3.94	4.38	2.81	10.00	12.55	7.58 (1.30)
	NBT reduction	-	-	17.8	6.41	8.07	3.42	11.17	17.33	10.70 (2.19)
	Formazan/LDH ratio	-	-	2.51	20.68	2.46	9.79	2.96	3.40	6.97 (2.71)

Fig. 3.19.

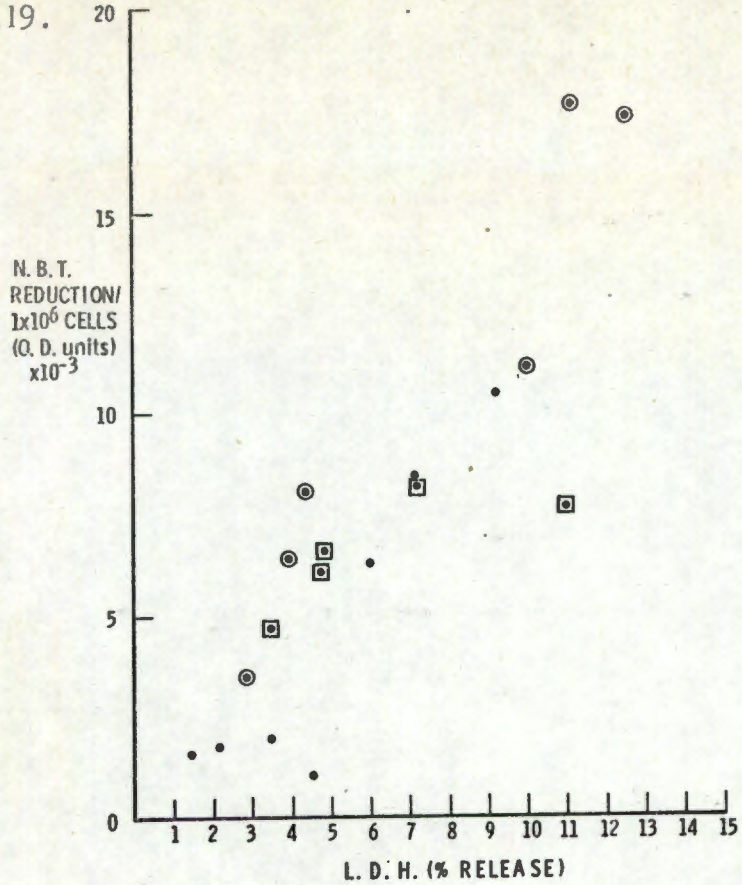
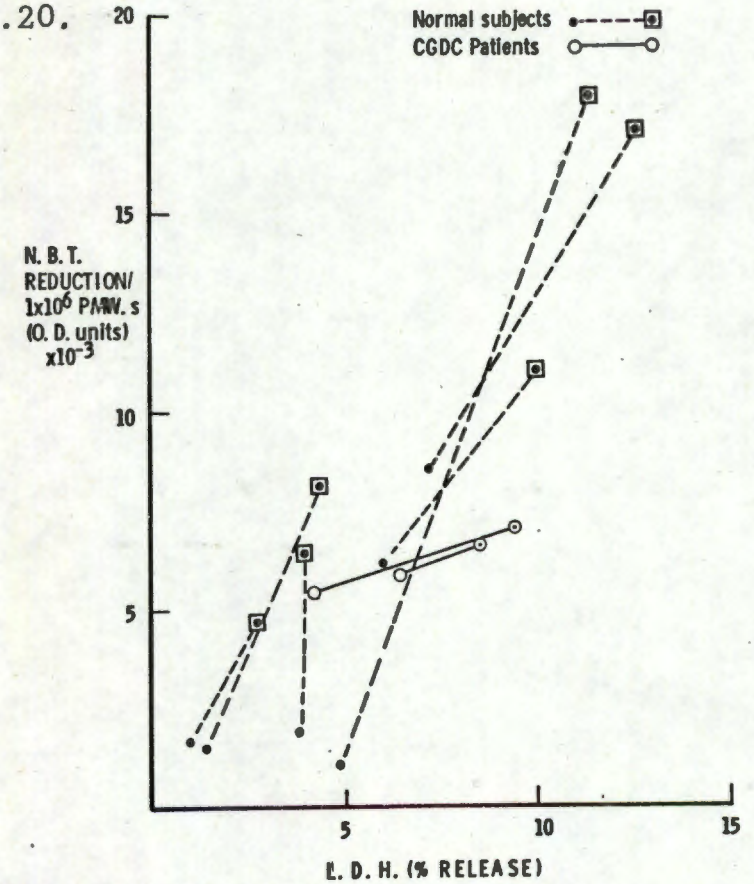


Fig. 3.20.



FIGURES 3.19 and 3.20.

The relationship between the release of LDH from, and NBT reduction by; neutrophils of 6 normal subjects exposed to saline • , endotoxin ◙ or latex particles ◉ in a modified Quantitative NBT test (Fig. 3.19); neutrophils of 6 normal subjects and two patients with CGDC, before •, o, and after ◙, ⊙, the addition of latex particles to the same test system (Fig. 3.20).

Heparin promoted the release of LDH in the presence and absence of NBT (Table 3.22) and in a concentration of 100 i.u./ml the cell suspension became very viscid, suggesting the release of nuclei acids as a result of cell rupture.

TABLE 3.22. The effect of heparin in concentrations of 10 and 100 i.u./ml on LDH release from neutrophils of 4 normal subjects. The results are expressed as a percentage of total LDH release after cell lysis.

Additive to cell suspension	Subject			
	1	2	3	4
Saline	2.4	2.2	3.1	1.9
Saline and Heparin	12.5	21.7	3.9	3.0
NBT	3.1	2.9	4.9	6.8
NBT + Heparin	41.9	12.5	8.4	8.3
Heparin concentration (i.u./ml)	100	100	10	10

There was a close correlation between NBT reduction and LDH release by cells exposed to NBT alone or in the presence of endotoxin or latex particles (Fig. 3.19). Three such comparisons between LDH release and NBT reduction in six normal subjects resulted in a correlation coefficient of 0.89 which is highly significant ($p < 0.001$). This relationship can be expressed as the increase in optical density (in OD units x 10^{-6}) at 515 nm per additional unit of LDH released

Fig. 3.21.

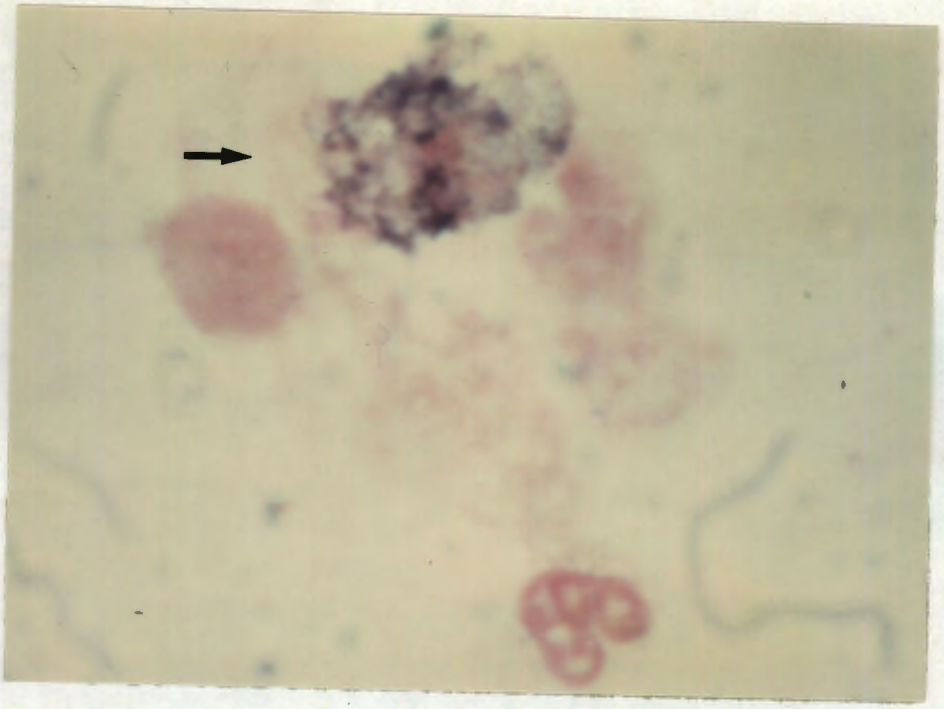
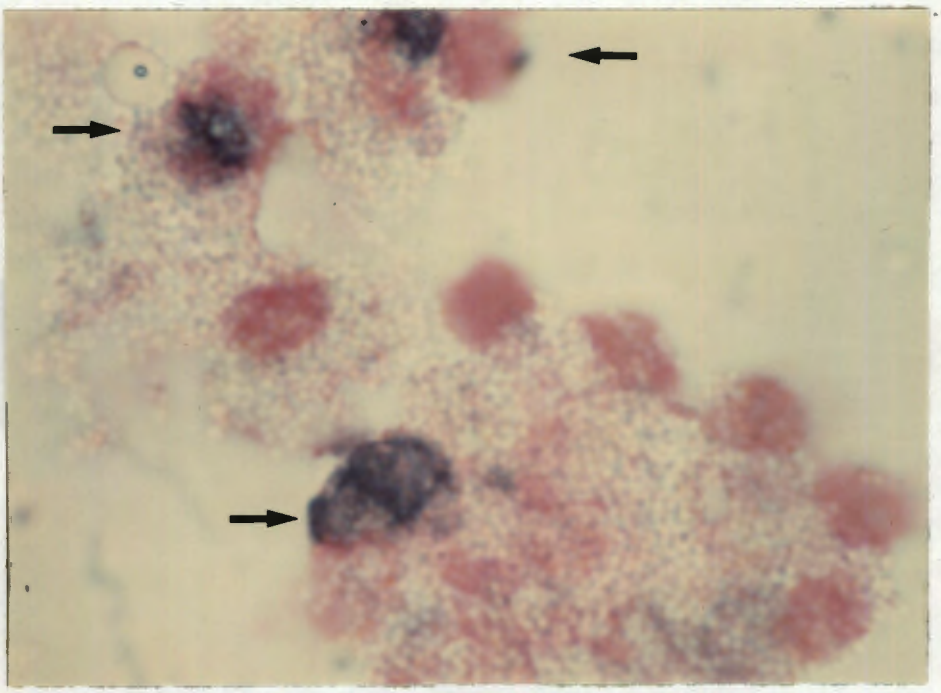


Fig. 3.22.



FIGURES 3.21 and 3.22

Photomicrographs of neutrophils exposed to NBT in a modified Quantitative NBT test in the absence (Fig. 3.21) and presence (Fig. 3.22) of latex particles. Only some of the neutrophils (arrows) demonstrate NBT reduction.

Safronin x2,000.

induced by the presence of latex particles in the cell suspension. In six normal subjects these units, termed "formazan/LDH units" had a mean value of 6.97 with a standard error of 2.71.

Only some of the cells exposed to NBT, or to NBT and latex particles, reduced the dye (Figs. 3.21 and 3.22). Those cells that did reduce the dye closely resembled the formazan cells of Gifford and Malawista (1970). They were swollen and amorphous and exhibited dye reduction both as faint blue cytoplasmic staining and as denser deposits which appeared to be associated with the outer membrane of the neutrophil.

3.4.3.b NBT reduction and LDH release by peripheral blood neutrophils of two patients with CGDC and a patient with hypogammaglobulinaemia, and by bone marrow neutrophils of two normal subjects.

A greater than normal percentage of LDH was released by neutrophils of the patients with CGDC upon the addition of saline alone. NBT reduction by unstimulated cells was normal, however, the characteristic increase of NBT reduction after the addition of latex particles to the cell suspension was greatly diminished (Table 3.23 Fig. 3.20). Expressed in absolute figures, the enhanced NBT reduction amounted to 0.062 and 0.434 formazan/LDH units, which is significantly different from NBT reduction (expressed in the same units) by neutrophils of normal subjects ($p < 0.01$. The absolute values were transformed to logarithms and compared by Students t test).

TABLE 3.23. Details of LDH release and quantitative NBT reduction by neutrophils from the bone marrow of 2 normal subjects and from the blood of patients with chronic granulomatous disease of childhood (CGDC) and a patient with hypogammaglobulinaemia in whom NBT reduction was diminished in the NBT stimulation test (Appendix K, No.15). The LDH release is expressed as a percentage of total LDH release after cell lysis and NBT reduction is expressed as optical density units x 10^{-3} per 1 x 10^{6} cells. The formazan LDH ratio is a measure of NBT reduction in O.D. units x 10^{-6} per unit of LDH released.

Additive to cell suspension	Change measured	Hypogamma. patient	CGDC Patient 1	CGDC Patient 2	Bone Marrow 1	Bone Marrow 2
Saline	LDH	1.9	7.2	5.8	-	1.8
Saline + Latex	LDH	2.1	4.8	4.1	6.1	2.50
NBT	LDH	7.0	6.3	4.2	12.2	6.90
	NBT reduction	4.6	5.9	5.4	11.0	10.0
NBT + Latex	LDH	13.7	8.5	9.4	18.0	10.6
	NBT reduction	25.8	6.5	7.1	48.0	19.2
	Formazan/ LDH ratio	3.5	0.062	0.43	3.13	1.21

Normal results were obtained from a hypogammaglobulinaemic patient (Appendix K, No.15) whose neutrophils reduced NBT poorly when whole blood was incubated with NBT in the presence of endotoxin (Table 3.23).

The differential counts in the marrow samples are shown in Table 3.24.

TABLE 3.24. The differential cell count on marrow cell suspensions. Results are expressed as a percentage.

CELL TYPE	MARROW SAMPLES	
	1	2
Segmented neutrophils	10	11
Stab cells	26	38
Metamyelocytes	22	13
Myelocytes	20	8
Lymphocytes	3	3
Erythrocytes	0	1
Other cells	19	26

LDH release by bone marrow neutrophils was slightly greater than normal as was NBT reduction. When expressed in formazan/LDH units, values of 3.13 and 1.21 indicate that the relationship between LDH release and NBT reduction is similar to that found with circulating neutrophils.

3.4.3.c The effect of endotoxin and PVP on NBT reduction in the slide test of Gifford and Malawista

TABLE 3.25. The effect of endotoxin (10 μg/ml) and PVP (2.0 mM) on NBT reduction by neutrophils adherent to glass slides. Results are expressed as the mean and standard error of the percentage of neutrophils in which dye reduction was observed. The number of studies is shown. The absolute figures of the two studies performed in the presence and absence of PVP are also shown.

Additive	Formazan Cells	Number of Studies
Nil	42.6 $\pm$ 3.0	41
Endotoxin	90.5 $\pm$ 1.6	25
PVP	0 0	2
No PVP	60 28	2

In the absence of endotoxin approximately half the neutrophils were converted to formazan cells whereas endotoxin stimulation resulted in NBT reduction by almost all the cells. In the two studies performed, PVP completely inhibited NBT reduction by, and structural disorganisation of, the cells (Table 3.25.).

Attempts to damage the cells by the action of phospholipase C, lysolecithin and sonication failed because exposure to these agents resulted in almost complete cell loss from the slide after washing.

3.4.4. DISCUSSION

NBT is toxic to neutrophils. Lactate dehydrogenase (LDH) is a cytoplasmic enzyme and its release from cells is a measure of the integrity of the outer membrane of the cell (Davies et al, 1973a).

The addition of NBT to neutrophil suspensions increased LDH released from the cells, and this was further increased in the presence of endotoxin or latex particles. The increase in cell damage is not due to the endotoxin or latex per se, as they do not promote LDH release in the absence of NBT (Tables 3.20 and 3.21). The mechanism of this toxic effect is unknown, but it is unlikely to be due to processes involved in the transfer of electrons to the tetrazolium salt, as in the two patients with CGDC, latex particles markedly increased LDH release from cells in the absence of significant formazan production.

There was a very close correlation ($p < 0.001$) between NBT reduction and LDH release from neutrophils of normal subjects (Fig. 3.19). Damage to the neutrophil membrane sufficient to allow the efflux of LDH, the molecular weight of which is approximately 150,000 (Boyer et al, 1963) would allow the efflux of other cytoplasmic constituents, including dehydrogenase enzyme systems, their substrates and cofactors, and the influx of NBT (molecular weight $\pm$ 750 (Altman, 1972)). This contact would allow the reduction of NBT.

The enhancement of NBT reduction in the presence of latex particles may result from either increased membrane damage, allowing greater contact between the NBT and reducing substances, or because of the increased generation of reducing equivalents in the extramitochondrial cytoplasm induced by endotoxin or latex. Patients with CGDC exhibit normal glycolysis but an absence of the normal burst of HMP shunt activity after the phagocytosis of latex. In two

such patients NBT reduction was minimally increased, despite a marked increase of LDH release in the presence of latex (Fig. 3.22) suggesting that in normal neutrophils the enhancement of NBT reduction by latex phagocytosis results from a concomitant stimulation of NADPH production by the HMP rather than through further damage to the cell membrane.

These results indicate that for the accurate assessment of NBT reduction by neutrophils in the quantitative NBT test of Baehner and Nathan (1968) it is essential not only to quantitate NBT reduction but also to compare this with the amount of LDH release, thereby deriving a standardised value for NBT reduction.

In these studies cyanide was excluded from the cell suspension so as to avoid toxic effects of cyanide on the studies of LDH release by cells exposed to NBT. The classical observation in CGDC is a deficiency of cyanide insensitive NBT reduction. The absence of cyanide from the test system did not hamper the distinction between normal subjects and the patients with CGDC but may have been responsible for higher than normal levels of NBT reduction by unstimulated cells.

In the stimulated NBT test, in which NBT was added to blood or bone marrow in the presence of endotoxin, dye reduction occurred in an abnormally low proportion of bone marrow neutrophils, and the neutrophils of some patients with hypogammaglobulinaemia (3.3.4.a) (3.2.3.a). Quantitative NBT reduction by bone marrow neutrophils and the neutrophils of one of the hypogammaglobulinaemic patients was normal. This indicates that the defect in these cells

in the stimulated NBT test is one of inefficient phagocytosis of the NBT complexes rather than an inability of the cells to reduce the dye. The theory of Cocchi et al (1969) that the failure of NBT reduction by neutrophils of infected premature infants and patients with CGDC shares a common pathogenesis seems unlikely.

In the NBT test, the dye enter neutrophils complexed to heparin or heparin and fibrinogen (3.1). NBT and heparin in solution (Tables 3.21 and 3.22) damage the neutrophil membrane resulting in the extracellular release of cytoplasmic constituents. Cell damage was also observed by electron microscopy in a model system simulating the NBT test (3.1.3.i). Although not quantitated, NBT appears to be concentrated on the precipitates of heparin and/or fibrinogen. It is suggested that when these complexes are engulfed by cells, close contact with the complex is toxic to the membrane of the phagocytic vacuole allowing cytoplasmic dehydrogenase systems to pour over and reduce the NBT. Although the influx of hydrogen ions from increased HMP shunt activity (to be discussed below) is probably the major source of reducing equivalents in normal neutrophils the reduction of NBT by cytoplasmic dehydrogenase systems may be responsible for the slow reduction observed in the phagocytic vacuoles of patients with CGDC (Nathan et al, 1969).

Cytochalasin B partially inhibits phagocytosis, yet it enhanced NBT reduction by neutrophils in whole blood. These effects appear incompatible as NBT reduction under these circumstances is dependent upon the phagocytosis of complexed NBT. However, Cytochalasin B does not reduce the adhesion of micro-organisms to neutrophils (Davies et al, 1973a), and the gross cell clumping observed in the

presence of this agent suggests that it may increase membrane stickiness . The mechanism of NBT reduction in the presence of Cytochalasin B may be envisaged as the adhesion of complexed NBT to the neutrophil membrane, followed by reduction as a result of the release of reducing compounds from the damaged membrane. Reduction is unlikely to be entirely dependent upon the selective exocytosis of lysosomal enzymes induced by this compound (Davies et al, 1973a), as retinol which has a similar effect (Davies et al,1973b) did not enhance NBT reduction.

The mechanisms underlying the NBT test and the quantitative NBT test are basically different. The former is a measure of phagocytosis of NBT complexes whereas the latter is a measure of the toxicity of NBT and the metabolic activity of the cell. This explains the observation of Ashburn et al (1973) that despite widely varying results of the NBT test in normal subjects and patients with infection, chronic granulocytic leukaemia, neoplasia and polycythaemia vera, the results of quantitative NBT tests did not differ significantly between the groups. Indicating that although the phagocytic activity of neutrophils differed in these conditions, the toxic effects of NBT and the burst of metabolic activity associated with phagocytosis did not. A report (De La Vega et al, 1973) of negative NBT tests in an infected diabetic patient, and decreased NBT reduction in the quantitative NBT test when plasma from this patient was added to the incubation mixture, was interpreted as indicating the presence of a plasma factor that inhibited NBT

reduction. The negative NBT tests in this patient probably resulted from a relatively immature neutrophil population (a shift to the left of the myeloid series of leucocytes was evident) and the addition of plasma to the incubation mixture in the quantitative NBT test would cause binding of NBT to fibrinogen, thus reducing the concentration of free NBT and possibly its toxic effects on the neutrophil membrane. Control experiments examining the effect of the addition of normal plasma to the test system were not performed.

Exposure of the neutrophil suspension to NBT under similar conditions to the quantitative NBT test, both in the presence and absence of latex particles, led to reduction by some but not all of the cells (Figs. 3.21 and 3.22). The reasons for the selectivity of this reduction have not been examined. The cells that reduced the dye were swollen and amorphous and dye reduction was predominantly in a granular pattern which appeared to be mainly on the outer membrane of the cell. There was also a light blue diffuse staining which probably represents cytoplasmic staining. These cells closely resemble the "formazan" cells described by Gifford and Malawista (1970) after exposure of neutrophils adherent to glass slides to a solution containing NBT. In this test approximately half the neutrophils reduce the dye in the absence of endotoxin and almost all the neutrophils in the presence of endotoxin (3.4.3.c). These cells look damaged.They are swollen, amorphous and their nuclei are disintegrating. The cytoplasm is diffusely blue and dense granular formazan deposits appear localised to the outer membrane of the cell

(Fig. 1.3). Polyvinyl pyrrolidone (PVP) protects cells from freezing injury (Leibo et al, 1970). It is suggested that NBT alone is toxic to an undefined population of the neutrophils which are adherent to glass, that this toxicity is enhanced by endotoxin, as was shown with cells in suspension, and it is possible that PVP prevents this toxicity, NBT reduction probably occurs predominantly at the site of membrane rupture, but also to a lesser extent within the cytoplasm. The reasons for the variability of NBT reduction by cells, all of which are adherent to glass, is unknown. It may be a function of age, but is difficult to investigate because of the unequal adhesive properties of neutrophils of different age (Lightman and Weed, 1972) which would result in selective detachment of younger cells when the adherent cells are washed in the experimental procedure.

In the NBT test the vast majority of cells which reduce NBT contain deposits of formazan, Occasional cells have a different appearance in which the reduced NBT takes the form of multiple minute dots which appear to be in the outer membrane of the cell (Matula and Paterson, 1971a) (Fig. 3.12). Exposure of neutrophils to lysolecithin, phospholipase C, freezing and thawing and sonication, procedures which result in focal perforation of membranes (Bangham and Horne, 1964, Benedetti and Emmelot, 1966, Leibo et al, 1970, Hughes and Nyborg, 1962) also result in this stippled appearance (3.2.3.d). It is probable that any procedure which causes focal disruption of the outer neutrophil membrane will allow

focal contact between NBT and reducing substances at the aperture, resulting in focal formazan deposition.

The extensive interest in the extremely rare condition of CGDC results from the fact that neutrophils from these patients are unable to kill certain bacteria normally and that specific biochemical abnormalities which could account for this defect have been demonstrated in the cells of these patients. The characterisation of the mechanism underlying these defects may allow specific treatment of these patients, but of more general importance in view of the extreme rarity of this condition, it may help to promote an understanding of the mechanisms underlying the bacteriocidal activity of normal neutrophils.

One of the prime bacteriocidal mechanisms seems to be the halogenation of the bacterial membrane, employing H_2O_2 as an oxidising agent (Klebanoff, 1971). The production of peroxide requires hydrogen ions, the initial source of which is thought to be NADPH produced by the HMP shunt, and a means of combining these with oxygen to form H_2O_2. The production of reducing equivalents can be measured by the activity of the HMP shunt, and their consumption can be measured by cyanide insensitive, therefore non-mitochondrial, oxygen utilisation, or by the reduction of some other hydrogen receptor such as NBT. Neutrophils of patients with CGDC do not exhibit the increased activity of the HMP shunt, oxygen consumption, H_2O_2 production, bacterial iodination or NBT reduction normally promoted by phagocytosis.

The normal increase in the turnover of reducing equivalents is therefore absent. This may result from an inability of the cell to produce the reducing equivalents, or because of their defective transfer from NADPH to oxygen. Although inadequate production has been reported in a single family with chronic granulomatous disease, haemolytic anaemia and complete absence of glucose-6-phosphate dehydrogenase (Gray et al, 1973) the usual finding that methylene blue stimulates HMP shunt activity and NBT reduction (Baehner and Nathan, 1967, Humbert et al, 1973) indicates that the defect in these patients lies in the transport of the reducing equivalents in the face of a normal ability to enhance their production. The kinetics of NBT reduction by neutrophils conform to those of an enzyme mediated reaction with regard to pH and temperature (Baehner and Nathan, 1968). A search was then made for cyanide insensitive enzyme systems capable of oxidising NADPH and/or NADH.

These investigations ignored a vital concept, that of the localisation of the H_2O_2 production. H_2O_2 is toxic to cells (Massie et al, 1972) and must therefore be produced within the phagocytic vacuole itself. Biological membranes are relatively impermeable to the pyridine nucleotides and some shuttle system, as occurs across the mitochondrial membrane (Chappell, 1968) must be envisaged to transport reducing equivalents into the phagocytic vacuole.

The following hypothesis as to the mechanisms of H_2O_2 production and stimulation of the HMP shunt in neutrophils as a result

of phagocytosis is proposed, Ascorbic acid enters the phagocytic vacuole. Initial degranulation of the specific granules which contain lactoferrin and basic protein cause an abrupt increase of the intravacuolar pH (Bainton, 1973). As a result of this pH change, or through catalysis by lactoferrin bound iron, ascorbic acid undergoes non-enzymic convertion to dehydroascorbate with the release of reducing equivalents which in the presence of molecular oxygen form super oxides, or free hydroxyl radicals and subsequently hydrogen peroxide (Slater, 1972a).

The dehydroascorbate enters the cytoplasm where non-enzymic reduction by reduced glutathione (GSH) reforms ascorbate. Reduced glutathione and ascorbate also protect the cells by acting as free-radical scavengers (Slater, 1972b) through enzymic (Bracci et al, 1970) or non-enzymic (Baehner, et al, 1970) reduction of H_2O_2 diffusing into the cell from the phagocytic vacuole. Glutathione (GSSG) is reduced by NADPH-dependent GSSG reductase and the resultant decrease in the NADPH/NADP ratio stimulates the HMP shunt. Superoxides and other free radicals produced from ascorbate are primarily responsible for damage to the bacterial cell wall. When degranulation of the specific granules is followed by degranulation of the azurophilic granules (Bainton, 1973), the intravacuolar pH drops to the optimal pH for myeloperoxidase which then induces halogenation with any residual peroxide. Such a system would be cyanide insensitive and although the final pathways may be non-enzymic, the kinetics of the overall system would conform to those of an enzyme system,

because of dependence upon the enzyme controlled HMP shunt for the supply of reducing equivalents.

The participation of ascorbic acid and glutathione as hydrogen carriers in a mammalian electron transport system involving substrates oxidasable by NADP has been a recurring speculation (Borsook et al, 1936, Kinoshita, 1964, Pirie, 1965) and has been demonstrated in corneal epithelium (Anderson and Spector, 1971). Ascorbate is concentrated in neutrophils (Loh and Wilson, 1970) but its concentration in phagocytic vacuoles is unknown. Although enzymic oxidation of ascorbic acid does not yield detectable H_2O_2, non-enzymic oxidation does (Steinman and Dawson, 1942). H_2O_2 has much greater bactericidal activity in the presence of ascorbate (Miller, 1969). Ascorbate produces free radicals in the presence of oxygen and a metal complex (Slater, 1972a), which damage biological membranes (Slater, 1972c). Ascorbate reduces NBT under alkaline conditions (Nathan et al, 1969), a process which is largely mediated through superoxides when phenazine methosulphate transports reducing equivalents to NBT under aerobic conditions (Nishikimi et al, 1972). Superoxides are produced by neutrophils, a process which is enhanced by phagocytosis (Babior et al, 1973). Superoxides are oxygen molecules containing an additional electron which can be donated to other molecules with resultant ionisation and increased reactivity. It is possible that ascorbate produces superoxides directly, or indirectly after catalysis of H_2O_2 by myeloperoxidase and that these superoxides

are responsible for damage to bacterial cell walls.

Dehydroascorbate crosses plasma membrane by active transport (Loh and Wilson, 1970) and may be reduced by GSH spontaneously (Anderson and Spector, 1971) or enzymatically by dehydroascorbic acid reductase which has been demonstrated in erythrocytes (Christine et al, 1956) and guinea pig tissues (Grimble and Hughes, 1967). The glutathione could be reduced by the glutathione reductase present in human leukocytes (Baehner et al, 1970b). Ascorbic acid has a redox potential very close to that of methylene blue (Burns and Ashwell, 1960) which stimulates HMP shunt activity in patients with CGDC. Dehydroascorbic acid reduces bacterial killing when added to a neutrophil suspension (McCall et al, 1971) an effect which may be explained by competition with oxygen for reducing equivalents, resulting in decreased H_2O_2 production.

It thus seems that ascorbic acid has the potential to transport reducing equivalents from reduced glutathione to oxygen, and to damage bacterial cell walls. Whether its ionic nature (pKa 4.17, Burns and Ashwell, 1960) will allow rapid enough movement of the molecule across plasma membrane has yet to be investigated.

Two experimental systems have been designed to investigate the importance of ascorbate in bacterial killing. Neutrophils form scorbutic and normal guinea pigs will be compared with regard to bacterial killing and the effect of phagocytosis on HMP shunt activity, oxygen consumption, iodination and H_2O_2 production. It is of interest that NBT reduction by neutrophils of scorbutic guinea pigs is defective.

(3.2.) Specific granules will be isolated from normal human neutrophils and oxygen consumption and H_2O_2 production by intact and disrupted granules measured in the presence and absence of ascorbate.

Ascorbic acid may play an important role in the bacteri cidal function of neutrophils. Damage to bacterial cell walls in the absence of myeloperoxidase, could explain why myeloperoxidase deficiency is not always accompanied by liability to bacterial infection (Klebanoff, 1971).

The underlying defect in CGDC may be in the transport of reducing equivalents into the phagocytic vacuole or if the hypothesis as to the release of reducing equivalents from ascorbate by a sudden rise in the intravacuolar pH or the presence of iron bound to lactoferrin is correct, in the sequence of degranulation, or the absence of lactoferrin, as other aspects of degranulation appear normal (Stossel 1973). It would seem appropriate to measure the ascorbic acid concentration in neutrophils of patients with CGDC and to treat these patients with ascorbic acid if a deficiency is observed. Another approach to the treatment of these patients would be to direct an artificially constructed bacteriocidal system into their cells. Liposomes could function as a vehicle for the transport of antibiotics or other bacteriocidal mechanisms (Gregoriadis and Ryman, 1972). Hydrogen peroxide and a halogen could be coentrapped in liposomes and administered to these patients. In patients lacking myeloperoxidase, a separate liposomal preparation containing this enzyme or some other

catalytic system could be administered simultaneously in the hope of union within a phagocytic vacuole harbouring infecting microorganisms. Before contemplating such therapeutic manoeuvres an exact knowledge of the fate of intravenously administered liposomes is essential. An attempt has been made to gain this knowledge as will be fully discussed in Chapter 5.

3.4.5. SUMMARY

NBT is toxic to cells and this toxicity is enhanced in the presence of endotoxin or latex particles. There is a close relationship between LDH release, a marker of cellular integrity, and NBT reduction by cells. This relationship is absent in the cells from patients with CGDC which indicates that the toxicity of NBT is unlikely to be related to the removal of reducing equivalents from the cell, and that the degree of membrane damage alone does not govern NBT reduction. Measurement of LDH release should be made in parallel with NBT reduction in the quantitative NBT test to more accurately predict the amount of NBT reduction that should be expected by correcting for the variation of NBT toxicity on different cell preparations.

It was postulated that ascorbic acid may link the production of reducing equivalents by the HMP shunt to intravacuolar oxygen and that glutathione might act both as a step in this pathway and as a mechanism for the prevention of the toxic effects of H_2O_2 on extravacublar cellular components.

The use of liposome entrapped therapeutic agents was proposed as a possible new approach to the treatment of CGDC.

3.5. SUMMARY

There are basically two different NBT tests. They have different mechanisms of dye reduction and different clinical application.

The first type of test, developed by Park et al (1968) was modified by Gordon et al (1973) by substituting EDTA for heparin as anticoagulant. These have been used as a non-specific diagnostic test of bacterial infection. In this test, the dye enters neutrophils by phagocytosis after which it may be seen by light microscopy as discrete, dense blue black deposits. Before the dye can be phagocytosed it must assume a particulate conformation, which it does by precipitating and complexing with heparin and/or fibrinogen. These precipitated complexes are present in all blood samples after the addition of NBT, but only a very small proportion of the neutrophils from the blood of normal subjects phagocytose the complexes and reduce the dye, whereas a variable proportion of neutrophils from the blood of patients with diverse diseases do so.

Serum from patients with various diseases enhanced phagocytosis and reduction of NBT by neutrophils from normal subjects. There was a close correlation ($p < 0.01$) between the proportion of the patient's neutrophils that reduced the dye in the initial NBT test and the proportion of normal neutrophils that reduced the dye after being suspended in serum from these patients, when both tests were performed in the presence of EDTA as anticoagulant.

This suggests that the cells of both the patient and the normal subject were exposed to similar modification, and as the serum was the only link between the two cell suspensions, that this modification was mediated by humoral factors.

Normal cells suspended in heterologous serum and exposed to NBT in the presence of heparin also showed varying degrees of enhanced phagocytosis but NBT reduction correlated poorly with the initial NBT test result. Possible explanations are that heparin itself may stimulate phagocytosis and produce a false positive test, that heparin may inhibit the action of some of the humoral factors, or it may reflect the small number of studies performed. The effects of heparin may explain the different results of NBT tests performed by the two different methods on the same blood sample.

A number of factors were tested in an in vitro model system for their effect on the phagocytosis of complexed NBT. Heparin, α^1-acid glycoprotein, immunoglobulins, fetuin and endotoxin all enhanced phagocytosis, whereas a number of other factors were without effect. The physiological roles of heparin, fetuin and α^1-acid glycoprotein are unclear - they are all acidic and contain high concentrations of carbohydrate. It was suggested that they may form, together with other acute phase proteins such as CRP, part of a non-specific opsonising system for the resolution of autologous debris or exogenous material, either locally in the case of heparin,

or within the circulation. Low dose heparin therapy may have a prophylactic effect in the prevention of deep venous thrombosis by enhancing the phagocytosis of microthrombi. In a large group of studies the close correlation between the serum concentration of α^1-acid glycoprotein and the results of NBT tests performed by the EDTA/ficoll and heparin methods ($p < 0.001$ and < 0.05 respectively) suggests that this protein may be one of the factors responsible for positive NBT tests, though both may be independently related to disease severity. Complement and immunoglobulins are not obligatory for the phagocytosis and reduction of complexed NBT.

Complexed NBT is not readily phagocytosed by normal neutrophils in the absence of enhancing factors. The fact that NBT is responsible for the formation of these complexes together with the fact that the precipitates contain a 'built in' indicator of phagocytosis, readily identifiable by light and electron microscopy, make this agent a valuable tool in the study of phagocytosis and factors effecting it.

Endotoxin enhanced NBT reduction by neutrophils and resulted in a fairly reproducible dose response in normal subjects. This does response was assumed as a standard for comparison with various groups of patients in whom neutrophil function could have been abnormal. Most patients with hypogammaglobulinaemia, Crohn's disease, diabetes mellitus, geriatric patients and patients receiving therapy with prednisone and sodium autothiomalate showed a similar dose response to normal. This suggests that the

parameters of neutrophil function measured by this test, the ability of neutrophils to be stimulated by endotoxin, to phagocytose complexed NBT and to reduce the dye, are normal in the majority of these patients.

Immature bone marrow neutrophils are unable to phagocytose complexed NBT as well as mature circulating neutrophils. The replacement of the mature circulating pool of neutrophils by a less mature population in infected subjects could account for both the bacteriocidal defects and diminished NBT reduction observed in these patients. If the maturity of circulating neutrophils is indeed shown to be related to NBT reduction, this might be a very simple test system for the measurement of the proportion of immature circulating neutrophils, with a decreased bacteriocidal capacity, and may indicate the necessity for transfusion with normal granulocytes.

Immune complexes also diminish NBT reduction, an effect which could be used to test for circulating immune complexes and the response of the causal disease process to therapy.

The second type of NBT test is the quantitative NBT test which is of value in the diagnosis of CGDC. This test differs from the NBT test used for the diagnosis of bacterial infection because the cells in this test are suspended in a protein free balanced salt solution. The mechanism of dye reduction in this test is also different from that of the test performed on whole blood. In the quantitative NBT test, NBT reduction is related to toxicity of the dye on the cell membrane and the production of reducing equivalents by the cell.

Cell damage, assessed by LDH release, correlates closely with NBT reduction in normal subjects ($p<0.001$). The measurement of LDH release, and its use as a standard against which the observed NBT reduction can be compared, should enhance the accuracy of the quantitative NBT test.

The mechanisms involved in the transfer of reducing equivalents from the HMP shunt to oxygen in the phagocytic vacuole are unknown. It was postulated that ascorbate may be involved in this pathway and that the production of superoxides, other free radicals or H_2O_2 within the phagocytic vacuole by this compound may be an important component of the bactericidal armamentarium of the cell.

CHAPTER 4

The association of reduced NBT with serum lipoproteins and the use of NBT as a lipoprotein stain.

4.1. INTRODUCTION

Incubation of NBT with whole blood results in reduction of the dye by a variable proportion of leukocytes (Chapter 3). If the mixture is left for some hours a blue discolouration of the supernatant plasma develops, indicating that reduced NBT is not confined to the cellular components of blood. This reduced dye appeared to be in solution in the plasma, as it passed through a Millipore filter with a pore size of 0.22 microns and remained in the supernatant plasma after centrifugation at 10,000 G for 30 minutes. As formazan is insoluble in water (Nachlas et al, 1957), some plasma constituent must be responsible for maintaining it in solution.

In this chapter it will be shown that formazan selectively binds to serum lipoproteins. This property together with its vivid blue colour make it a useful prestain for the identification of lipoproteins after separation by electrophoresis.

Its use as a prestain in the separation of serum lipoproteins by acrylamide disc electrophoresis results in the appearance of several bands not normally identified by this separation technique. The identity of these bands and their presence in the serum of normal and hyperlipidaemic subjects is investigated and discussed.

4.2. METHODS

4.2.1. The demonstration of the binding of formazan to serum lipoproteins

Blood samples were allowed to clot at room temperature and electrophoresis of the separated serum was performed within 6 hours. To 1.0 ml of each serum was added 0.5 ml of an aqueous solution of NBT in concentrations from 0.05% - 0.80% and 0.5 ml of an aqueous solution of NADH (10 mM). After gentle mixing, the addition of approximately 100μg of solid phenazine methosulphate resulted in very rapid reduction of the dye and the mixture developed a deep blue colour. This will be referred to as a 'prestained' sample. Unadulterated sera were used as control samples except in immunoelectrophoretic systems where the serum was diluted with an equal volume of 0.15 M saline.

4.2.1.a. Immunoelectrophoresis of NBT prestained and unstained serum

Samples consisted of 5 prestained and control sera and 5 prestained and control sera to which had been added an equivalent volume of both anti-α_1 and anti-β lipoprotein serum. Laurell immuno-electrophoresis of the samples against anti-whole human serum in an agarose/barbitone mixture was performed by the method of Minchin Clarke and Freeman (1968). 'Mono rocket' immunoelectrophoresis was performed on 5 prestained and control samples by the method of Laurell (1966) using 0.2 ml of anti-whole human serum in 18.0 ml of agarose. After completion of the procedures, the lipoproteins in the control samples were stained with Sudan black (saturated solution in 60% ethanol), Oil Red O dye (0.4 g/l in a mixture of equal volumes of acetone and water) or Amido black (2.2.4.).

Antigen-antibody crossed electrophoresis was also performed on prestained plasma from the supernate of a mixture of blood and an equal volume of 0.1% NBT which had been incubated at 37°C for 12 hours.

4.2.1.b Polyacrylamide disc electrophoresis

Samples consisted of sera from 3 normal subjects prestained with either NBT (0.3%) or Sudan black, and serum from 1 subject which was depleted of lipoproteins by repeated ultracentrifugation at a background density of 1.21, retaining the bottom fraction (Hatch and Lees, 1968 - This serum was a gift from Dr. B. Lewis, The Hammersmith Hospital, London) and then prestained with NBT (0.3%).

Electrophoresis was performed by the method of Fring's et al (1971), which is a method of discontinuous acrylamide electrophoresis with concentrating and separating gels containing acrylamide and bisacrylamide in concentrations of 2.50 and 0.63%, and 3.75 and 0.10% in TRIS/HCl buffers of pH 6.7 and 8.6 respectively.

This method was followed exactly for the sera prestained with Sudan black. The sample gel was not used for NBT prestained serum and delipidated serum, which was mixed with a half volume of a saturated solution of sucrose in the TRIS/glycine reservoir buffer (pH 8.3) diluted to 20% of the initial concentration with buffer. 75 μl (equivalent to 25 μl of serum) of this mixture was gently layered on the surface of the concentrating gel immediately before electrophoresis.

4.2.1.c Cellulose acetate and agarose electrophoresis

Ten NBT prestained and control sera were electrophoresed on agarose by the Millipore Agaroslide system following exactly the methods described in the Millipore Application Manual AM 305, and on cellulose acetate following exactly the methods described by De Baets and Lezy (1971).

4.2.2. Further studies with polyacrylamide disc electrophoresis

4.2.2.a Methods of prestaining and sample application

i. The optimal concentration of NBT as a prestain

Normal serum was prestained as described above with aqueous NBT in concentrations of 1.0, 0.8, 0.6, 0.3, 0.2, 0.1 and 0.05%. Electrophoresis was performed as described above (4.2.1.b).

ii. The optimal concentration of sucrose

Serum prestained with NBT (0.3%) was layered on the surface of the concentrating gel after mixing with solid sucrose or with a half volume of a saturated solution of sucrose in TRIS/glycine reservoir buffer, undiluted, or diluted to 80, 60, 40 and 20% of the initial concentration with buffer. The standard procedure was then followed.

4.2.2.b. The lipoprotein pattern obtained in the serum of normal and hyperlipidaemic patients after prestaining with NBT

Serum samples from 50 normal, non-fasting hospital staff (25 males, mean age 33.0, S.D. 12.1 years and 25 females,

mean age 32.3, S.D. 11.7 years) and from 9 hyperlipoproteinaemic patients (2 females) were prestained with NBT (0.3%), mixed with a half volume of a 20% saturated solution of sucrose and electrophoresed by the standard method. (Sera of patients with hyperlipoproteinaemia were characterised by preparative ultra-centrifugation and were a gift from Dr. B. Lewis, The Hammersmith Hospital, London).

All serum samples were tested on the day of collection. Samples from 2 normal subjects were stored at 4°C and re-investigated after 2, 7 and 28 days.

4.2.2.c Characterisation of the lipoprotein bands obtained after separation by acrylamide disc electrophoresis

i. Immunoelectrophoresis

Two dimentional crossed immunoelectrophoresis was performed on samples of NBT prestained serum, and control serum, from a normal subject. A simultaneous primary separation by electrophoresis on polyacrylamide was followed by electrophoresis into agarose containing antiserum. The duration of the polyacrylamide electrophoresis depended upon the lipoprotein under investigation in this part of the study; for the α lipoproteins it was 35 minutes, but 1-2 hours was necessary for adequate separation of the β lipoproteins. The gels were bisected longitudinally and laid cut surface down on a 5 cm x 5 cm glass cover slide (Kodak), parallel to and 1 cm from one of the edges. The cover slide was then covered with 6.0 ml

FIGURE 4.1.

Laurell electrophoresis of serum prestained with reduced NBT showing the localisation of stain in the positions normally occupied by the α and β lipoproteins.

of a solution containing 1.0% agarose and 0.1 ml of anti-β lipoprotein serum, or 0.4 ml of anti-α_1-lipoprotein serum in 0.03M barbitone buffer, pH 8.6, preheated to 48°C. After solidification of the gel the plate was positioned so that the bisected acrylamide strip lay transversely across its cathodal end. Electrophoresis was performed in barbitone buffer for 18 hours at a voltage of 2 volts/cm between wicks.

ii. Electrophoresis of fractionated lipoproteins

Serum fractions from 2 normal subjects were separated into very low density (VLDL, d <1.006 g/ml), low density (LDL, d 1.006-1.063 g/ml) and high density (HDL, d 1.063-1.21 g/ml) lipoprotein fractions by preparative ultracentrifugation. (Hatch and Lees, 1968) (The fractions were a gift from Dr. B. Lewis, The Hammersmith Hospital, London.) The lipoprotein fractions were prestained with NBT and electrophoresed by the standard procedure. The VLDL fraction was precipitated by NBT and to prevent this it was mixed with de-lipoproteinised serum (4.2.1.b) before the addition of NBT.

4.3. RESULTS

4.3.1. Identification of the serum proteins that bind formazan

4.3.1.a Immunoelectrophoresis

i. Antigen-antibody crossed electrophoresis

Blue colouration was found only in the precipitates previously identified as α and β lipoproteins (Minchin Clarke and Freeman, 1968) (Fig. 4.1). The size, shape and position of the lipoprotein arcs did not differ appreciably from those produced when

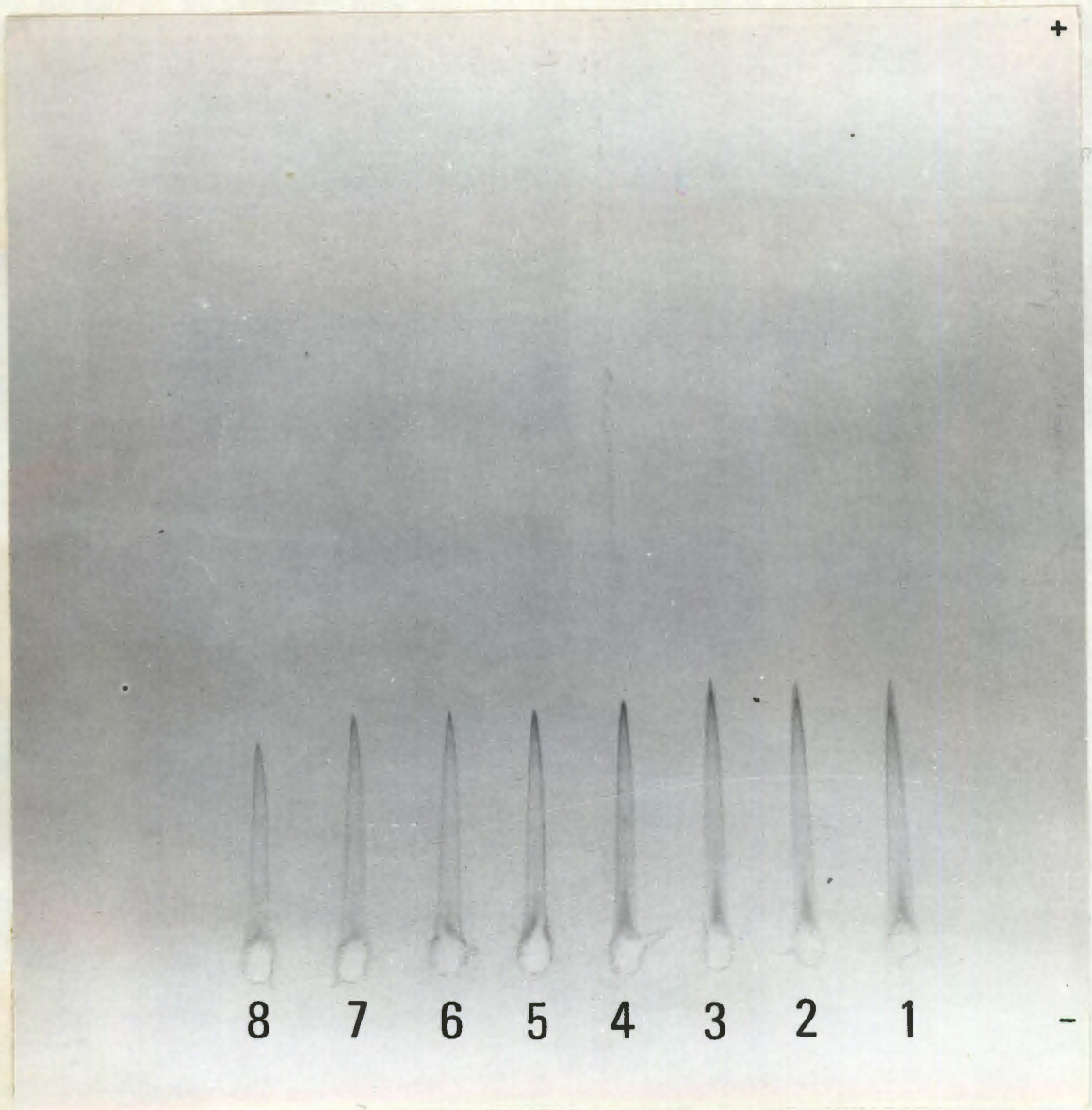


FIGURE 4.2.

'Mono rocket' electrophoresis of serum. Rockets of similar height resulted from the precipitation of the β lipoproteins after electrophoresis of the same serum sample, prestained with NBT (numbers 1-4), or unstained, and then post stained with Sudan black (numbers 5-8).

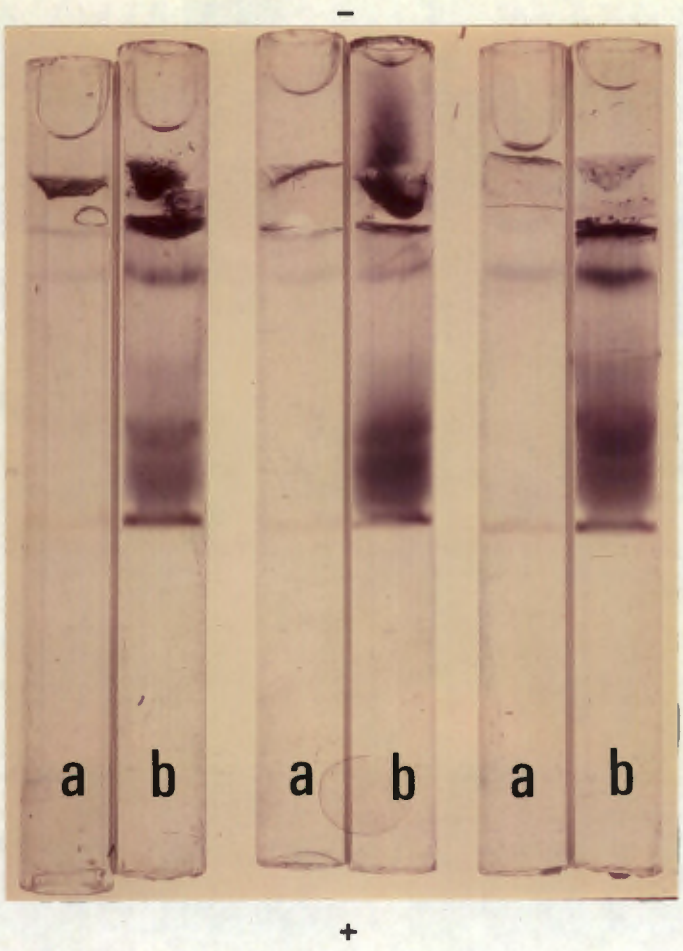


FIGURE 4.3

Polyacrylamide disc electrophoresis of sera, from three normal subjects, prestained with (a) Sudan black and (b) nitroblue tetrazolium.

the control serum was run simultaneously and stained afterwards with Amido black (a protein stain), Sudan black or Oil Red O (lipid stains). Mixing with specific antilipoprotein sera resulted in disappearance of the lipoprotein precipitates from both the NBT prestained and the control sera.

ii. 'Mono rocket' electrophoresis

Repeated single volume samples, prestained with NBT in initial concentrations of less than 0.15% resulted in rockets of fairly constant height which matched the rockets obtained from equal volumes of the control serum and post stained with Sudan black (Fig. 4.2). Increasing concentrations of NBT greater than 0.15% resulted in a progressive reduction in the height of the rockets.

4.3.1.b. Acrylamide disc electrophoresis

Compared with Sudan black as a prestain, NBT gave a greater intensity of colour. The lipoprotein bands were comparable in electrophoretic mobility, but additional bands were demonstrated in the α region (Fig. 4.3.)

When the procedure was performed on the serum fraction of density > 1.21, no staining was seen in the separating gel; all the stain was found as a precipitate on the surface of the condensing gel, suggesting that proteins other than the lipoproteins, which were removed by density centrifugation, do not bind formazan.

4.3.1.c Cellulose acetate and agarose gel electrophoresis

The pattern of lipoprotein separation was similar whether sera were prestained with NBT, at initial concentrations of 0.15% or

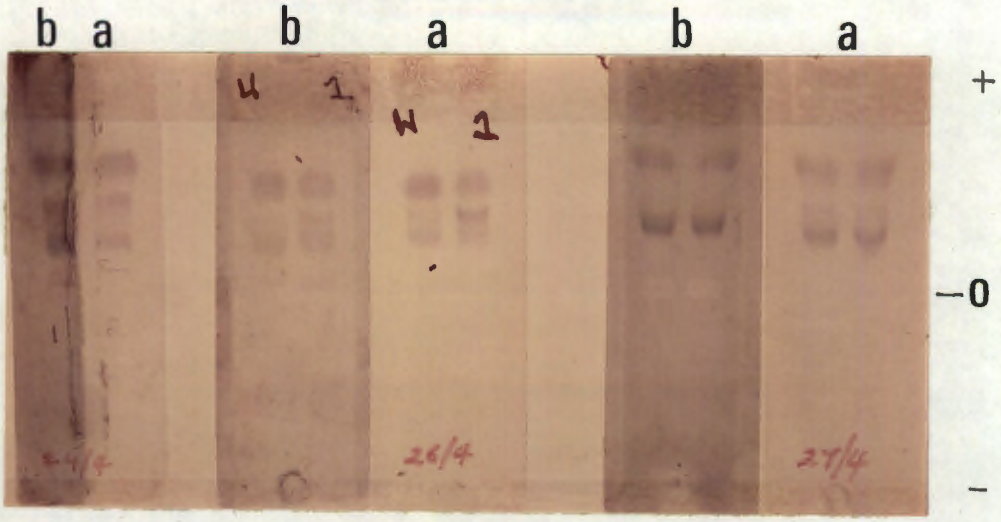


FIGURE 4.4.

Electrophoresis of sera from three normal subjects, prestained with NBT (a) or stained after electrophoresis with Sudan black (b), on agarose by the 'Agaroslide' method.

less, or post stained with Sudan black (Fig. 4.4). Higher concentrations of NBT were only tested on agarose, and resulted in progressively decreased mobility of the pre-β lipoprotein band which fused with the β band when NBT was used in initial concentrations greater than 0.4%.

4.3.2. Polyacrylamide disc electrophoresis

4.3.2.a Identification of the optimal prestaining conditions

i. The optimal concentration of NBT

The use of NBT in initial concentrations of 1.0 and 0.8% caused overstaining and collapse of the concentrating gel layer. Concentrations of less than 0.3% resulted in inadequate staining of the α lipoproteins. Clear delineation of the β lipoproteins together with adequate staining of the α lipoproteins was obtained with initial concentrations of 0.6, 0.4 and 0.3%.

NBT in an initial concentration of 0.3% was used in further studies. Changes in the concentration of NBT resulted in changes in the intensity of staining of the α bands, but not in their electrophoretic mobility.

ii. The optimal concentration of sucrose

The addition of 1 volume of a concentrated sucrose solution diluted in 1 in 5 in buffer, to 2 volumes of prestained serum was found to be optimal. More concentrated solutions, and solid sucrose, caused the concentrating gel to collapse, while the absence of sucrose resulted in inefficient application of the sample, which diffused from the surface of the concentrating gel.

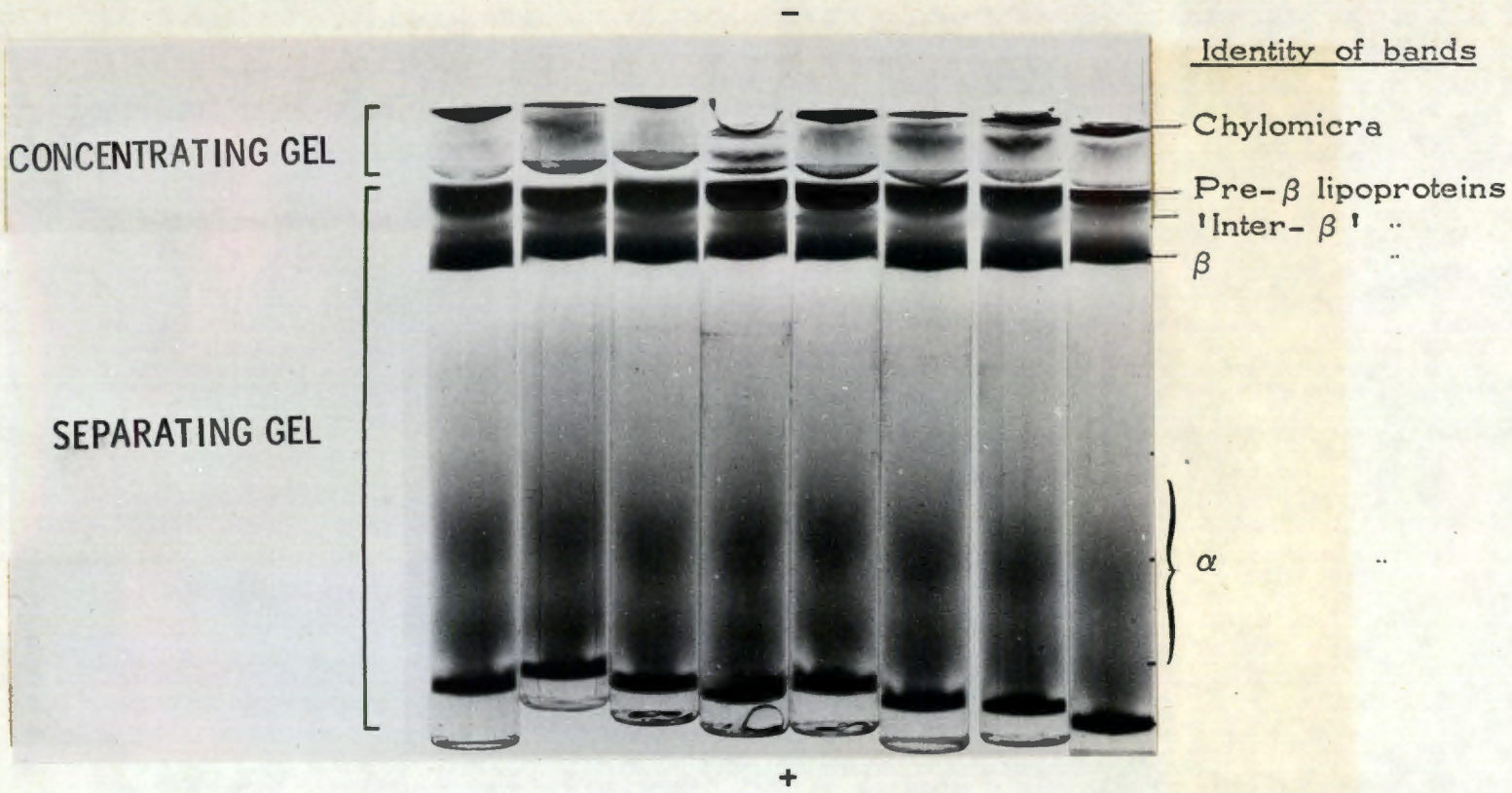


FIGURE 4.5.

Lipoprotein patterns obtained from the serum of a single normal subject separated by simultaneous electrophoresis on 8 separate polyacrylamide discs after prestaining with reduced NBT. The inter-β and minor α bands are clearly visible.

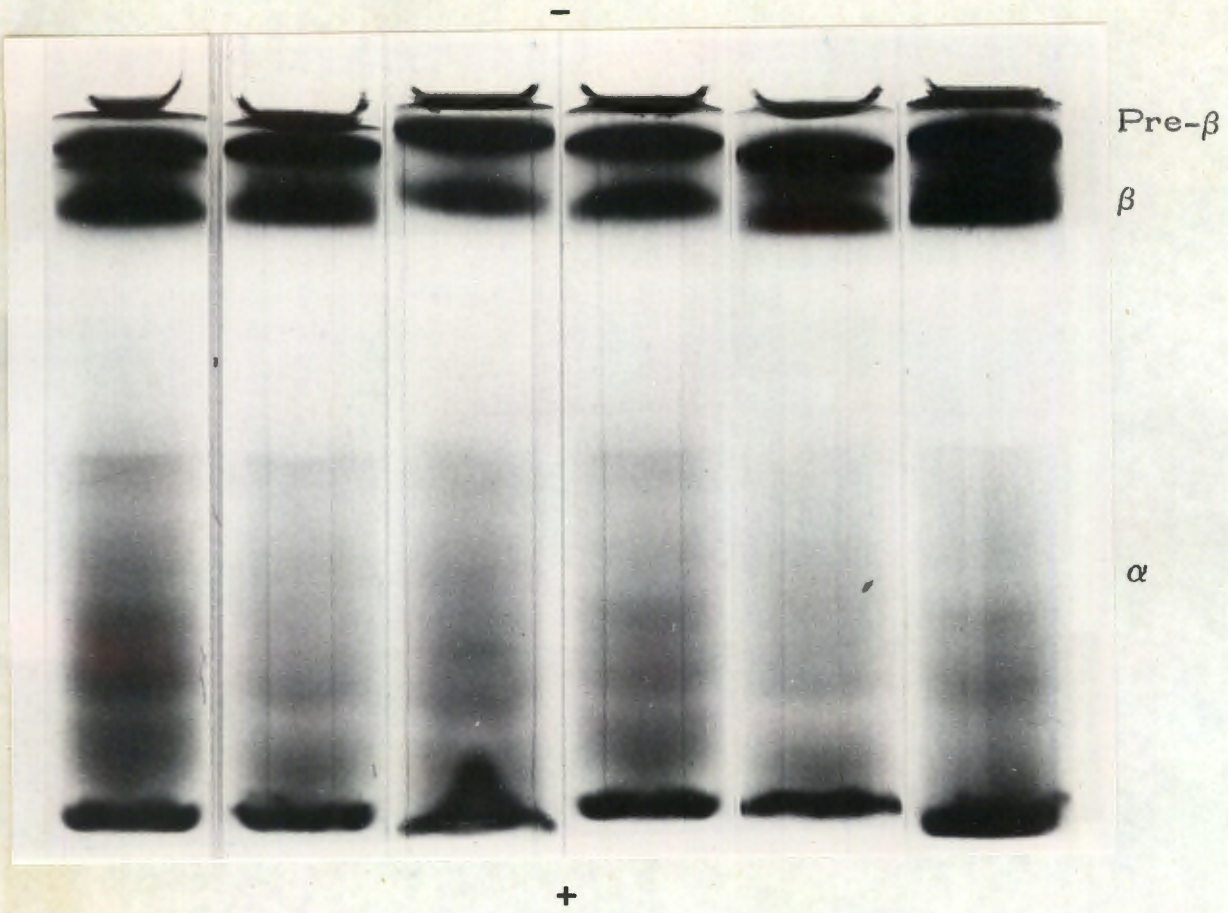


FIGURE 4.6.

NBT prestained sera from 6 normal subjects, after separation by polyacrylamide disc electrophoresis, demonstrating multiple α lipoprotein bands.

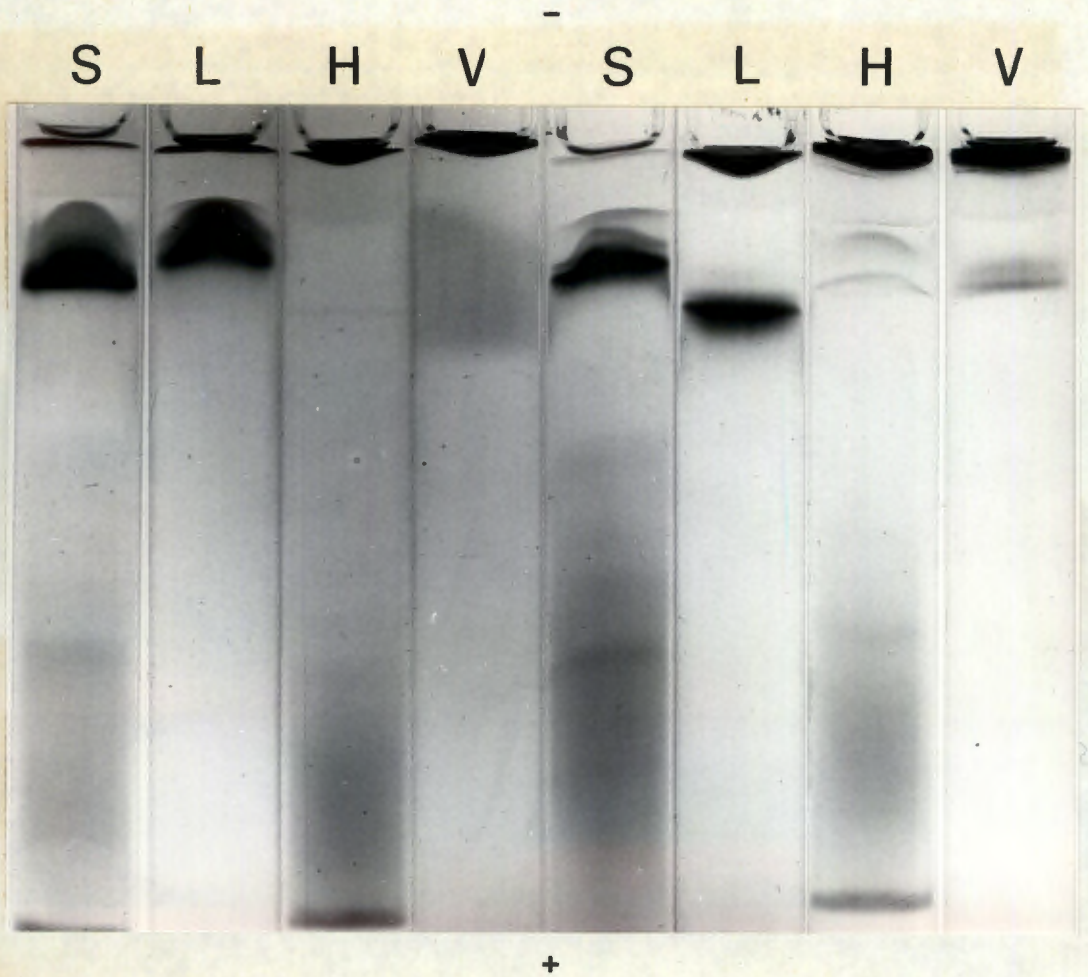


FIGURE 4.7.

Polyacrylamide disc electrophoresis of NBT prestained samples from two normal subjects of serum (S), and serum separated by preparative ultracentrifugation into fractions containing predominantly low density (L), high density (H) and very low density (V) lipoproteins.

4.3.2.b The lipoprotein pattern of normal and hyperlipidaemic sera after separation by polyacrylamide disc electrophoresis

A very reproducible picture was obtained on normal sera (Fig. 4.5). Chylomicra remained localised to the surface of the concentrating gel. The β and pre-β bands occupied the position described by Frings et al (1971). An additional band, referred to as the 'inter β' band in this study, was present between the β and pre-β bands in the serum of 14 males and 6 females. This band remained constant in the sera of 5 individuals restudied over a period of 6 months. Two of the men, both with densely staining β bands, had another faintly staining band anodal to the β band.

The α lipoproteins showed a discrete densely staining band in the position described by Frings et al (1971). An additional 4-5 broad ill-defined 'slow α' bands, present in all normal sera, were found cathodal to the conventional α band (Fig. 4.6). These bands were most clearly defined early in the electrophoretic procedure, before clear separation of the α bands occurred. Prolonged electrophoresis caused the α bands to become broader and less well defined.

The characterisation of these bands was confirmed using ultracentrifugally-isolated VLDL, LDL and HDL serum fractions which migrated to the regions occupied by the pre-β, β and α lipoproteins respectively (Fig. 4.7).

Laurell crossed immunoelectrophoresis was used to further identify the bands. The position of the lipoprotein bands of unstained serum on the acrylamide discs was obtained by comparison

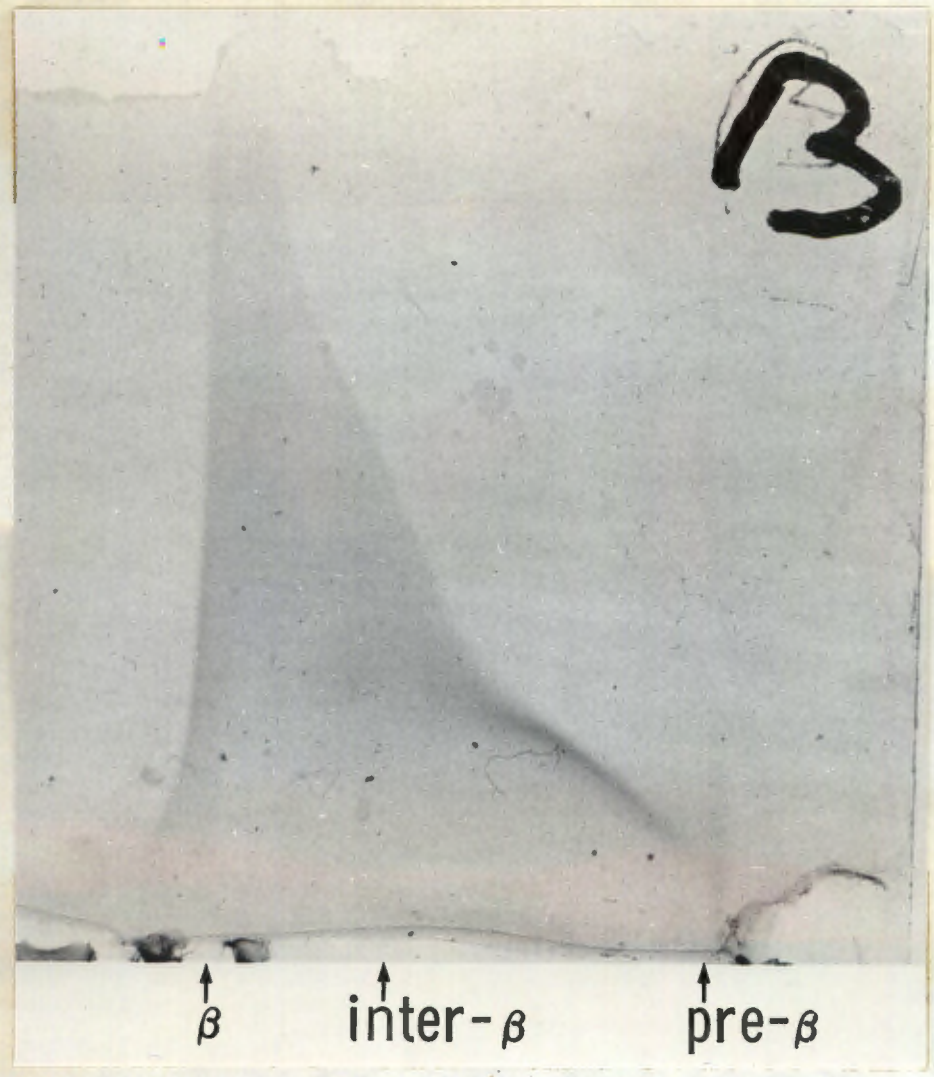


FIGURE 4.8.

Immunoelectrophoresis of normal, unstained serum into agarose containing anti-β lipoprotein serum after preliminary separation on polyacrylamide. The positions occupied by the pre-β, inter-β and β lipoprotein bands of serum, prestained with NBT and electrophoresed simultaneously, are indicated. The position of the inter-β band corresponds with a shoulder on the cathodal margin of the β arc.

Amido black.

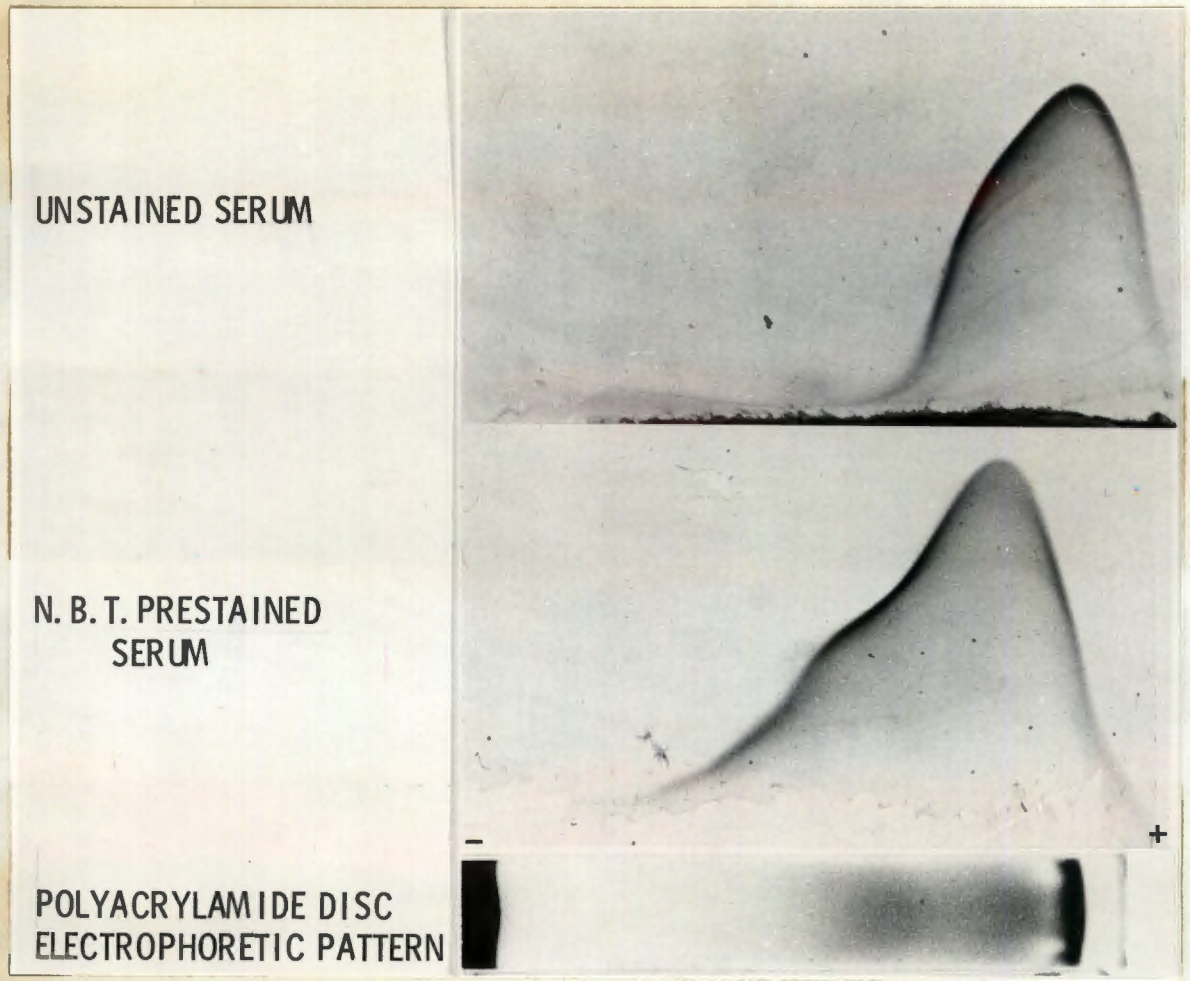
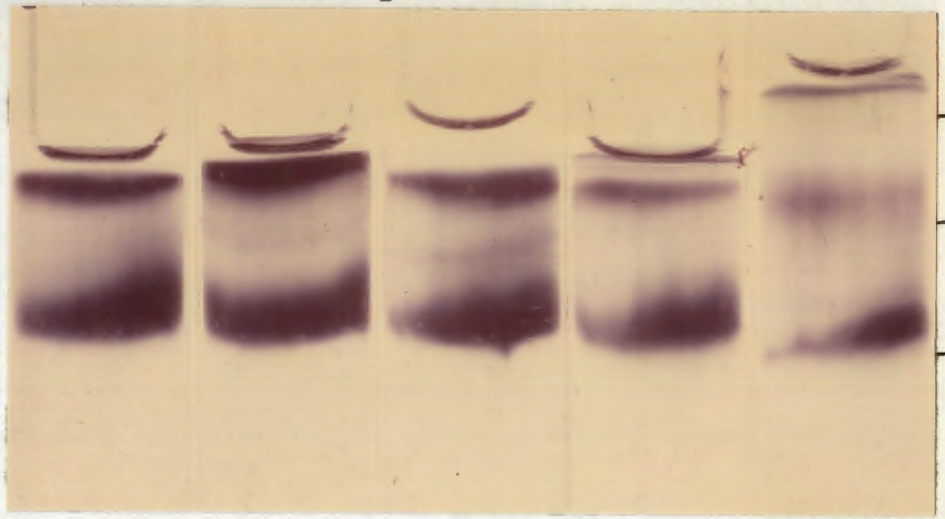


FIGURE 4.9.

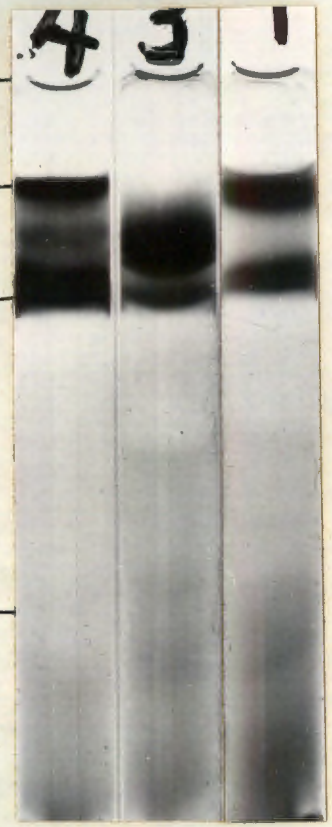
Immunoelectrophoresis of unstained (stained after electrophoresis with Amido black) and NBT prestained serum, from a normal subject, into agarose containing anti-α_1, lipoprotein serum, after preliminary separation on polyacrylamide. The immunoprecipitation arcs are shown, as is the lipoprotein pattern after separation of the same serum, prestained with NBT, on a polyacrylamide disc.

Fig. 4.10



	B	C	C	A	A
Chol. (mg%)	408	296	377	210	190
Trig. "	104	489	222	116	80

Fig. 4.11



	C	D	A
Chol. (mg%)	349	280	190
Trig. "	203	247	80

FIGURES 4.10 and 4.11

Polyacrylamide disc electrophoresis of NBT prestained samples of sera from normal subjects (A), patients with predominantly β (B) and β with pre-β hyperlipoproteinaemia (C), and a single patients with the 'broad β' lipoprotein (D). The concentrations of cholesterol (Chol.) and triglyceride (Trig.) in the plasma of these subjects are shown.

with the position of the same band after simultaneously electrophoresis of NBT prestained serum. All the bands except the pre-β band moved freely from the acrylamide disc into the surrounding agarose gel. In samples of both prestained and control serum, the β bands gave a distinct line of precipitation with anti-β but not with anti-α_1, lipoprotein serum while the 'interβ' band gave a shoulder on the cathodal side of the β arc. (Fig. 4.8). The α lipoproteins gave lines of precipitation with anti-α_1, but not with anti-β lipoprotein serum. Unstained serum revealed a fairly narrow precipitation arc with a distinct shoulder on the cathodal margin. The same serum, prestained with NBT, and electrophoresed simultaneously under the same conditions gave a precipitation line with anti-α_1, lipoprotein serum coinciding with the major band and with a contour skewed towards the cathodal side, showing at least 1 shoulder corresponding to the slow-migrating α lipoprotein components (Fig. 4.9.).

Poor staining of the α bands was observed in all the hyperlipoproteinaemic patients. A general increase in the intensity of staining of the β lipoprotein bands was also observed in these patients. This was predominantly in the pre-β and β regions in patients with pre-β and β hyperlipoproteinaemia respectively. A dense inter-β band was present in sera of patient with hyperβ lipoproteinaemia. An intensely staining inter β band was present in a serum sample containing the 'broad β' lipoprotein in which there was virtually no pre-β band and only a small β band (Figs. 4.10 and 4.11).

Storage of serum samples for up to one month at 4°C

did not result in gross changes in the lipoprotein pattern described above. However, after storage for a few days there was non-specific staining in the β region of the gel between the lipoprotein bands.

Quantitation of the intensity of staining of the individual bands was attempted using a densitometer (Joyce Loebel, Chromoscan); prolonged electrophoresis was necessary before the β bands could be adequately separated for scanning.

4.4. DISCUSSION

Reduced NBT is insoluble in aqueous and lipid media and binds to cellular protein (Nachlas et al, 1957). When reduced in plasma, either spontaneously or with artificial reducing agents, NBT remains in solution. In this study the binding of this compound to serum proteins was investigated.

Reduced NBT was found to bind exclusively to lipoproteins. This was demonstrated by:-

i. The conformity between the localisation of reduced NBT and the localisation of serum lipoproteins, identified by classical lipid stains, after separation by electrophoresis on cellulose acetate, agarose and polyacrylamide or by immunoelectrophoresis on agarose;

ii. The absence of these stained bands from serum depleted of lipoproteins by density centrifugation, and their presence in serum preparations containing the various lipoprotein fractions;

iii. The absence of these bands after the addition to prestained serum of specific antisera;

iv. Precipitation of prestained bands with specific antisera after separation on polyacrylamide.

The mechanism of the binding of reduced NBT to lipoproteins is unknown and has not been investigated in detail.. An aqueous solution of NBT was reduced in the presence of solid cholesterol and phosphatidic acid and liquid triolein. It did not colour these substances, but precipitated in the aqueous phase except in the case of triolein, where a very fine non-particulate layer developed at the interface between lipid and water. Binding studies with delipidated apoproteins were not performed, though this would indicate whether reduced NBT binds to these proteins in the absence of lipid. Formazan might form a monolayer between triglyceride, bound to the lipoproteins, and water as it consists of both hydrophilic and hydrophobic components.

Hyperlipidaemia is associated with a number of pathological conditions, foremost of which is vascular disease (Fredrickson et al, 1967). The disease processes and their treatment (Levy et al, 1972) is dependent to some extent upon the type of hyperlipidaemia. The accepted classification of the hyperlipidaemias has been based upon lipoprotein analyses by electrophoresis and ultracentrifugation (Beaumont et al, 1970).

Analytical centrifugation is the most accurate method of lipoprotein analysis (Hatch and Lees, 1968). However, it is expensive, intricate and time consuming, factors which limit its general application.

The commonly used methods for the investigation of hyperlipidaemia employ inspection of plasma from fasting patients for opalescence, and chemical quantitation of the serum cholesterol and triglyceride concentrations, to indicate the presence or absence of hyperlipidaemia. If present, the pattern of hyperlipidaemia is further investigated. Plasma fractions containing the various classes of lipoproteins are separated by preparative ultracentrifugation and the lipid concentrations in these fractions measured. Electrophoresis of these fractions should also be performed to identify lipoproteins with anomalous centrifugal and electrophoretic characteristics such as the "sinking pre-β " or " floating β " lipoproteins. Preparative ultracentrifugation is expensive and time consuming and is usually reserved for patients on whom the simpler techniques of quantitative measurement of cholesterol and triglyceride and qualitative assessment of the serum lipoprotein pattern by electrophoresis are insufficient for clear classification of the hyperlipidaemia (Beaumont et al, 1970).

Electrophoresis is clearly an important technique in the investigation of abnormalities of the serum lipoproteins. Paper electrophoresis has been extensively used (Lees and Hatch, 1963) but this is a slow process and has been largely superceded by the use of cellulose acetate (Kohn, 1957), agarose (Ressler et al, 1961), starch gel (Lewis, 1961) and finally polyacrylamide (Narayan, 1965).

Electrophoresis involves two processes, separation and identification. The standard electrophoretic methods separate the

lipoproteins into three classical groups, the pre-β lipoproteins, equivalent to the VLDL ultracentrifugation fraction with a density of <1.006 g/ml, the β lipoproteins, equivalent to the LDL fraction with a density of 1.006 - 1.063 g/ml and α lipoproteins, which are equivalent to the HDL with a density of 1.063-1.121 g/ml. Identification of the separated lipoproteins involves staining with standard lipid stains such as Sudan black and Oil Red O. Staining can be performed before or after electrophoresis. Staining after electrophoresis has several disadvantages, it prolongs the duration of the procedure as time must be allowed for both staining of the lipoprotein bands and destaining of the surrounding medium to highlight the regions with specific affinity for the stain. Destaining is often incomplete, or excessive, resulting in destaining of the lipoprotein bands. Prestaining of samples before electrophoresis is the ideal method as it allows observation of the progress of the electrophoretic migration, immediate interpretation of the separation, and eliminates background staining.

The conventional lipid stains have one major disadvantage, they are solubilised in organic solvents. When used as prestains they alter the electrophoretic mobility of the lipoproteins (Hatch and Lees, 1968) and post staining tends to elute the lipoproteins and lipids (Naito and Lewis, 1973). The observation that reduced NBT is an intense lipoprotein stain which results in staining of lipoproteins in the absence of an organic solvent suggested that it might serve as an

efficient prestain for identification of lipoproteins separated by electrophoresis.

Reduced NBT was found to be an efficient prestain for lipoprotein separation by Laurell 'mono rocket' and two dimensional crossed immunoelectrophoresis, and cellulose acetate, agarose and polyacrylamide electrophoresis. In initial concentrations of less than 0.15% it did not appear to alter the electrophoretic separation (except on polyacrylamide), or the immune reactivity of the lipoproteins. The advantages of using NBT as a prestain are clearly shown in the Millipore 'Agaroslide' system. The electrophoresis time can be varied by direct observation of the migration of the lipoproteins and the lipoprotein pattern can be directly recorded by obtaining an immediate tracing from a densitometer. The entire process takes 30 minutes compared with 150 minutes necessary to obtain a result by the prescribed method.

Discontinuous polyacrylamide disc electrophoresis is a rapid process resulting in high resolution of the separated lipoprotein bands. Prestaining is essential, as destaining of acrylamide discs is inefficient, but Sudan black results in relatively poor staining of the lipoprotein bands (Fig. 4.3). The combination of an efficient system for the separation of lipoproteins and a clear indicator of the separated bands should provide the optimal system for the electrophoretic separation of lipoproteins. Further studies were therefore performed with this system to established the normal lipoprotein pattern.

Prestaining of serum with NBT followed by polyacrylamide disc electrophoresis is an efficient method for the separation and identification of serum lipoproteins. The method described has the advantages of being able to dispense with a sample gel, so reducing operating time, while at the same time maintaining reproducibility (Fig. 4.5).

The positions occupied by the β , pre-β and α lipoproteins in the acrylamide discs were established using specific lipoprotein fractions and crossed immunoelectrophoresis. They conformed, in the main, to those described by Frings et al (1971).

Additional bands between the β and pre-β bands have been previously reported (Frings et al, 1971; Grajnert et al, 1972) in the serum from hyperlipidaemic patients. In this study a similar band, here referred to as the 'interβ ' band, has been demonstrated in 56% and 24% of normal male and female subjects respectively. The presence or absence of this band in any one individual was constant over a period of 6 months. The finding of similar bands when Sudan black was used as prestain, and the presence of a shoulder on the immunoprecipitation arc of normal serum corresponding with the inter β band of prestained serum, suggest that its identification in such a large proportion of normal subjects is probably a reflection of the intensity of staining of the lipoproteins by NBT rather than the formation of an artefactual band by the methods used.

Separation by acrylamide electrophoresis is dependent upon both electrical charge and molecular size (Frings et al, 1971).

Electrophoretic mobility is inversely related to molecular size, the large chylomicra remaining on the surface of the concentrating gel and the small α lipoproteins migrating furthest. This accounts for the difference between the migration of the β and pre-β lipoproteins on acrylamide and paper electrophoresis. In the latter, electrophoretic mobility is related almost entirely to charge and the pre-β band migrates more rapidly than the β band, which accounts for its name.

It is though that the large (Sf 20-400) VLDL molecule is catabolised to the smaller (Sf 0-20)LDL molecule, a process accompanied by loss of the VLDL and HDL apoproteins (Levy et al, 1971). The LDL are heterogenous having two major components in the analytical centrifuge (Lewis, 1971). The minor of these two components (Sf 12-20) is possibly an intermediate in the catabolism of VLDL to the smaller LDL molecule (Sf 0-12). This intermediate lipoprotein (Sf 12-20) may be identical to the 'broad β ' lipoprotein which might accumulate as a result of delayed catabolism to the Sf 0-12 fraction (Levy et al, 1971).

The size of the lipoprotein components of the inter band is likely to be intermediate between that of the pre-β and β lipoproteins. It is possible that this band consists of the LDL component with a Sf value of 12-20. This would be compatible with its precipitation by anti-β lipoprotein but not anti-α lipoprotein serum and the presence of a very dense band in this region in serum containing the 'broad β' lipoprotein in which a disproportionate

increase of this subclass of the LDL is characteristic (Beaumont et al, 1970).

If the diagnosis of 'broad β' hyperlipidaemia by the described method of electrophoresis is sufficiently reliable it could have important clinical application as "there is urgent need for a simple diagnostic method" of this condition (Rifkind, 1971).

The HDL are known to be heterogenous. Petek et al (1972) described 2 α lipoprotein bands on agarose gel electrophoresis and Scanu (1965) has shown three bands by immunoprecipitation, only one of which was detected by a lipid stain. The HDL may be composed of a number of subunits, each with its own complement of phospholipid, neutral lipid and the major constituent proteins (Gotto, 1969). It is therefore of interest that a number of α lipoprotein bands have been detected with the above methods. Prestaining with NBT reduces the electrophoretic mobility of some of the α lipoproteins, as shown by a broadening of the immunoprecipitation arc with anti-α_1 lipoprotein serum (Fig.4.9). However, the identical pattern of banding present in almost all normal sera (Fig. 4.6) suggest that this reduction is related to specific subgroups of the α lipoproteins which are heterogenous on the basis of composition or subgroup configuration. The asymmetry of the precipitation arc between unstained serum and anti-α_1 lipoprotein serum is confirmatory of the heterogenous nature of the α lipoproteins. It is therefore suggested that the α lipoproteins are composed of a

number of fractions of like electrophoretic mobility which are modified in a non-uniform manner by the use of NBT as a prestain, resulting in the observation of discrete bands.

Densitometric scanning of the discs was attempted and found to be feasible, but was not further pursued. Because of the variable uptake of standard lipid stains by different lipids (Hatch and Lees, 1968), densitometry gives results which are related to the concentration of the various bound lipids, rather than the concentrations of the lipoproteins themselves. NBT would be of greatest value as a lipoprotein stain if it were shown that the intensity of staining of the various lipoproteins was directly related to the protein, rather than the lipid, concentration. Experiments are at present being performed to compare lipoprotein analyses by analytical ultracentrifugation of unstained sera with densitometer scans on the same sera, prestained with NBT. If the lipoprotein profiles determined by these two methods were found to correspond, the quantitation of serum lipoproteins might be greatly simplified by the combination of electrophoresis and densitometry of NBT prestained serum samples.

Labelling of lipoproteins with reduced NBT may be a useful adjunct to techniques of lipoprotein separation other than electrophoresis, as the lipoproteins are visible and could be quantitated spectrophotometrically.

4.5. SUMMARY

Reduction of NBT is the presence of serum resulted in binding of the dye specifically to lipoproteins. This property together with the intense blue colouration of reduced NBT allow its use as a prestain for the demonstration of lipoproteins separated by electrophoresis. In the correct concentrations, reduced NBT does not alter the electrophoretic mobility of lipoproteins in agarose or cellulose acetate, and does not alter its immune reactivity. Its use as a prestain in polyacrylamide results in the appearance of several new bands. A band, commonly present between the β and pre- β lipoprotein bands, may represent a minor fraction of the LDL. The clear demonstration of this band in the single patient with a 'broad β' lipoprotein, suggests that NBT might be useful in the diagnosis of this condition by electrophoretsis. A number of minor α lipoprotein bands were observed and their significance discussed.

CHAPTER 5

The localisation of intravenously administered liposomes

5.1. INTRODUCTION

In conventional parenteral drug therapy the therapeutic agent is not specifically directed to any particular target or cell. Most cells in the body are exposed to the drug, the therapeutic action of which is dependent upon a qualitative or quantitative metabolic peculiarity of the target cell or organism. Disadvantages of this form of therapy are that normal cells, as well as the target cell or organism, are exposed to the possible deleterious effects of the therapeutic agent, and that this agent is itself vulnerable to metabolic and immunological modification before reaching its target.

Structural and metabolic peculiarities of micro organisms usually allow efficient antimicrobial therapy with drug concentrations well below those toxic to the host. However, when the target is an autonomous quasi-normal cell as in a neoplasia, or when infecting agents are sequestered within cells, normal cells are susceptible to the toxic effects of therapeutically effective concentrations of the drug, the beneficial effects of which must be weighed against its toxic effects on normal tissues.

Inherited enzyme deficiencies in man could possibly be corrected by gene replacement or treated by enzyme replacement (Strauss, 1971), parental administration of enzymes or nucleic acids exposes these substances to immunological or chemical modification within the circulation, without in any way regulating the site of action or eventual destination of such agents.

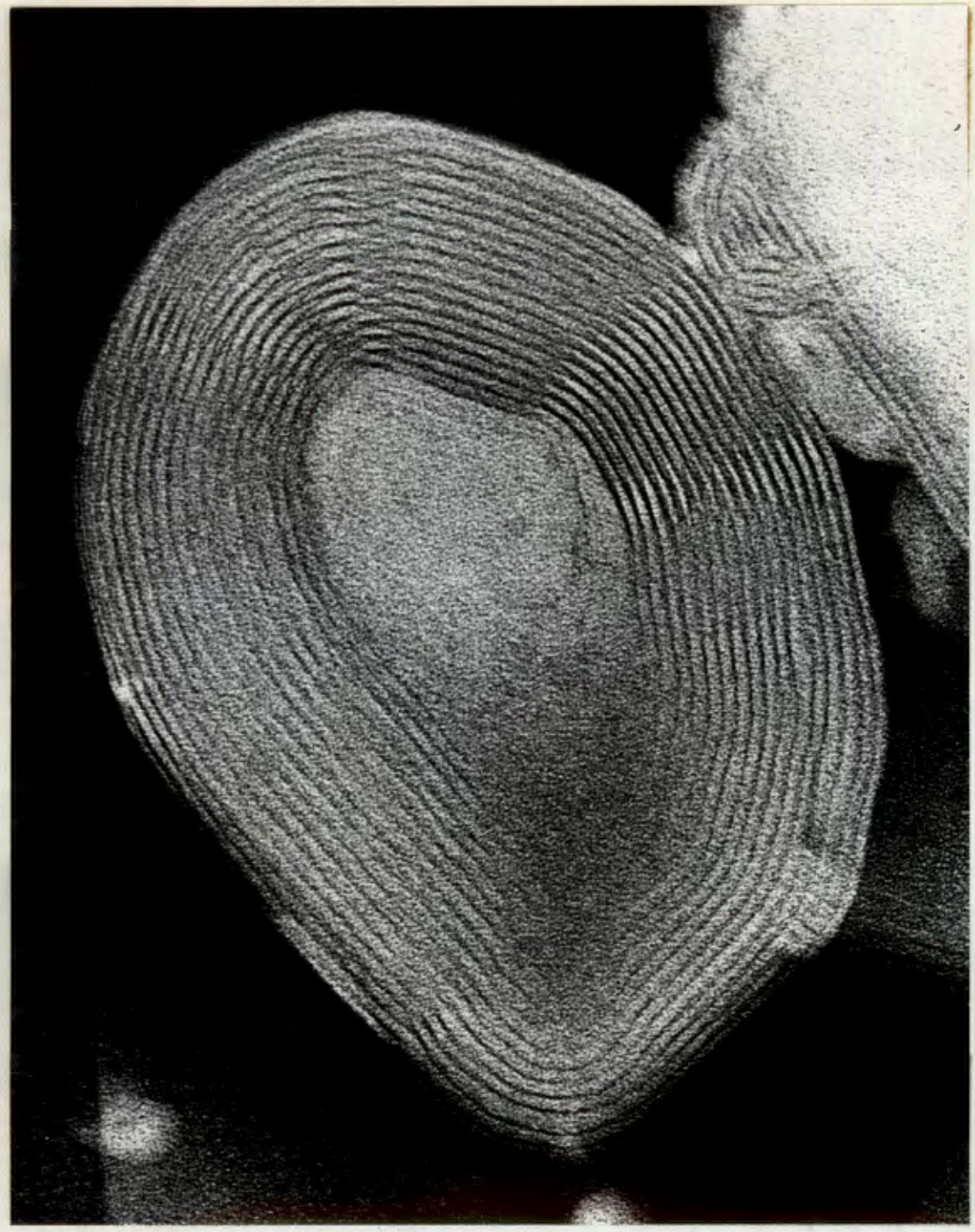


FIGURE 5.1.

Electron micrograph of a liposome, showing it to consist of alternating concentric lamellae of lipid separated by aqueous compartments.

Sodium silico tungstate x360,000.

A system capable of directing therapeutic agents to specific target cells or tissues should be a major therapeutic advance.

Trouet et al (1972) attempted to localise the site of action of a drug to neoplastic tissue by complexing daunorubicin, which inhibits DNA replication, to heat denatured DNA, a carrier designed to direct the drug to cells capable of pinocytic activity. In animal experiments, the toxicity of the drug was reduced, and its therapeutic effect enhanced, and encouraging results were obtained in the treatment of neoplasms in humans. However, they felt that DNA might prove undesirable as a carrier, owing to its informational content, antigenicity, ability to induce plasma DNAase and the fact that its use is limited to drugs capable of combining with it, and suggested that a search should be made for other carriers.

Liposomes (Bangham et al, 1965) are lipid spherules of varying shapes and sizes (approximate diameter: $1 \times 10^2 - 10^4$ A°, Bangham, 1968), composed of a closed system of concentric lipid bilayers approximately 40A° wide in the dried state, separated by aqueous compartments (Fig. 5.1) approximately 55A° wide (Sessa and Weissmann, 1968). Sessa and Weissmann (1970) entrapped lysozyme in the aqueous compartment of liposomes in an attempt to construct an experimental model of lysosomes. Gregoriadis et al (1971) proposed the use of liposome-entrapped

enzymes in the treatment of enzyme deficiency diseases, a procedure which was later shown to be effective in an in vitro model system (Gregoriadis and Buckland, 1973).

The use of liposomes as a carrier system would allow the transport of substances specially tailored to the therapeutic requirements at the target site while at the same time both protecting them from modification within the circulation and preventing non-specific action on normal cells.

It was proposed in Chapter 3 that liposome-entrapped enzymes or therapeutic agents might be useful in the treatment of CGDC, a condition in which the neutrophils are unable to kill certain bacteria, which remain within the cell, protected from the lethal effects of serum and drugs.

The use of so highly selective a form of treatment must hinge upon the exact cellular and subcellular localisation of the transported material after intravenous administration as a liposome-entrapped molecule. Although there is evidence that liposome-entrapped substances localise mainly in the lysosomes of the liver and spleen of animals (Gregoriadis and Ryman, 1972), their precise cellular destination within these organs and the fate of the carrier liposomes within these cells is unknown.

In an attempt to answer these questions, the fate of intravenously administered liposomes was monitored by light and electron microscopy. NBT was chosen as the liposomal

marker because; reduction, either spontaneously within cells or artificially in vitro, converts it from a pale yellow water-soluble compound to a dark blue water-insoluble formazan, visible by light microscopy, and under certain circumstances by electron microscopy, which does not diffuse from the site of reduction (Nachlas et al, 1957); when entrapped within liposomes and then reduced, the dye should remain localised to the liposome because of its insolubility in both water and lipid.

5.2. METHODS

5.2.1. Preparation of liposomes

Liposomes were prepared by the method of Gregoriadis et al (1971). Lecithin (40.0 μM), cholesterol (11.4 μM) and phosphatidic acid (5.7 μM), giving a molar ratio of 7.2:1, were dissolved in chloroform (5 ml) in a 100 ml round bottomed flask (Quickfit). Rotary evaporation at 37°C (Büchi rotavapor-R, speed 7) for approximately 2 minutes resulted in the formation of a thin film of lipid on the walls of the flask. The flask was gassed with O_2 free N_2, 2.0 ml of the aqueous solution to be entrapped was added, the flask regassed with N_2 and shaken vigorously until the lipid film was dispersed in the aqueous phase. The milky suspension was kept at room temperature for 2 hours and then sonicated 4 times, each time for a duration of 15 seconds (150 Watt MSE ultrasonic disintegrator) at an interpeak amplitude of 8 microns, with the 2.0 cm titanium probe placed just below the surface of the liquid and the bottom of the flask resting on ice.

The flask was regassed with N_2 and kept at room temperature for 3 hours. Liposomes were separated from non-entrapped material by centrifugation at 100,000 G for 30 minutes or by molecular sieve chromatography on a Sepharose 6B column (1 x 25 cm) equilibrated with 6.6 mM phosphate buffer, pH 7.2, containing 0.15 M NaCl.

5.2.2. Entrapment of the dye within liposomes

NBT was entrapped in liposomes in two ways. Free NBT (2 and 10 mg/ml in aqueous solution) was entrapped in the liposomes which were then sonicated and allowed to stand at room temperature for 3 hours. After centrifugation, the pellet was washed with and resuspended in 0.15M saline.

Liposomes containing reduced NBT were obtained by first entrapping an aqueous mixture of NBT (2 mg/ml) and NADH (10 mM) as described above, and then promoting intraliposomal reduction of the dye by the addition, 3 hours after sonication, of a portion (5% of final volume) of an aqueous solution of phenazine methosulphate (10 mM). The dark blue suspension was allowed to stand at room temperature for 16 hours, after which the liposomes were separated from the unentrapped formazan by molecular sieve chromatography, centrifiuged at 100,000 G for 30 minutes, and resuspended in 1.0 ml of 0.15 M NaCl.

To ensure that the formazan was entrapped within liposomes and not attached to non-liposomal lipids, 0.1 ml of an aqueous solution of ^{131}I (γ activity: 6×10^5 cpm) was added to the mixture of NBT and NADH before entrapment. A comparison

was made between the radioactivity, counted using a well-type sodium iodide detector, and formazan concentration, measured as absorbance at 515 nm after extraction with pyridine, on fractions eluted from the column.

The amount of NBT entrapped within liposomes was measured by reduction of the NBT to formazan by the addition of NADH (5 mM) and phenazine methosulphate (5 mM) followed by sonication. The formazan was then extracted into pyridine, the absorbance measured at 515 nM against a blank containing a mixture of water and pyridine, and the concentration extrapolated from a standard curve for NBT reduced and extracted in the same way.

Liposomes containing NBT and formazan were examined by electron microscopy.

5.2.3. Animal experiments

Male albino rats (Wistar) weighing 100-120 G were injected intravenously with:

(i) 1.0 ml of a suspension of liposomes containing 40.0 mg of lipids and; 5.0 mg (3 animals) or 1.0 mg of unreduced NBT (6 animals); or 1.0 mg of formazan (2 animals);

(ii) 1.0 ml of solution containing 1.0 mg (4 animals); or 5.0 mg (5 animals) of NBT in 0.36 M dextrose;

(iii) 1.0 ml of a mixture of equal volumes of a suspension of liposomes (80.0 mg of lipid/ml) and non-entrapped

aqueous NBT (10.0 mg/ml) in 0.36 M dextrose (1 animal).

(iv) 1.0 ml of a mixture of rat serum and 1.25 mg of formazan. This was made by mixing an aqueous solution of NBT (2.5 mg/ml) and NADH (10 mM) with 0.9 ml of heterologous rat serum (to prevent precipitation of the formazan) after which 0.1 ml of phenazine methosulphate (10 mM) was added to promote reduction of the NBT. The mixture was then dialysed against 2 litres of 0.15 M saline for 16 hours at 4°C across a cellulose membrane.

5.2.3.a Light microscopy

The animals that survived the injection were decapitated from 15 minutes to 20 hours after injection and specimens of brain, myocardium, lung, liver, kidney, pancreas, inguinal lymph node, small and large gut and spleen were immediately fixed in 10% formaldehyde for 24 hours, embedded in wax, sectioned and examined unstained and after staining with a saturated solution of tartrazine in 2-ethoxyethanol. Smears of blood and bone marrow were also examined unstained and after staining with Leishman's stain.

5.2.3.b Electron microscopy

Specimens of liver and spleen from animals injected with 1.0 mg of NBT in solution (2 animals), or entrapped within liposomes (2 animals) and decapitated at 2 hours, and from control animals, were immediately fixed in a mixture of glutaraldehyde (2.5%) and sodium cacodylate (0.1 M) at pH 7.4 followed by 1.0% osmium tetraoxide in phosphate buffer (pH 7.4), dehydrated through a graded series of alcohols and embedded in epoxy resin.

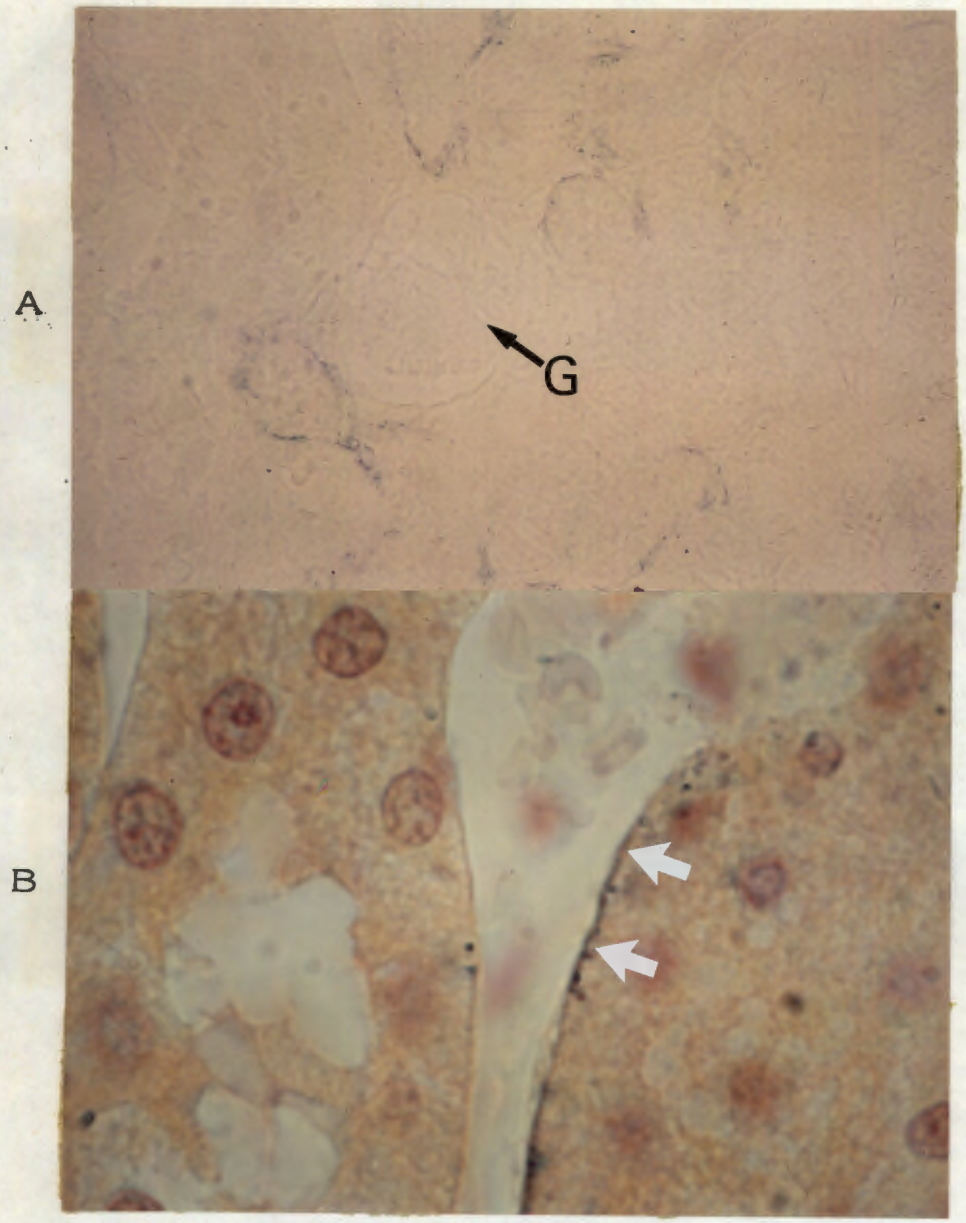


FIGURE 5.2.

Light micrograph of kidney 15 minutes after the injection of free NBT showing the dye (arrows) localised to the basal layers of the distal convoluted tubules. A glomerulus (G) can be seen in Fig.A.

(A) Unstained x 300

(B) Tartrazine x 1,000

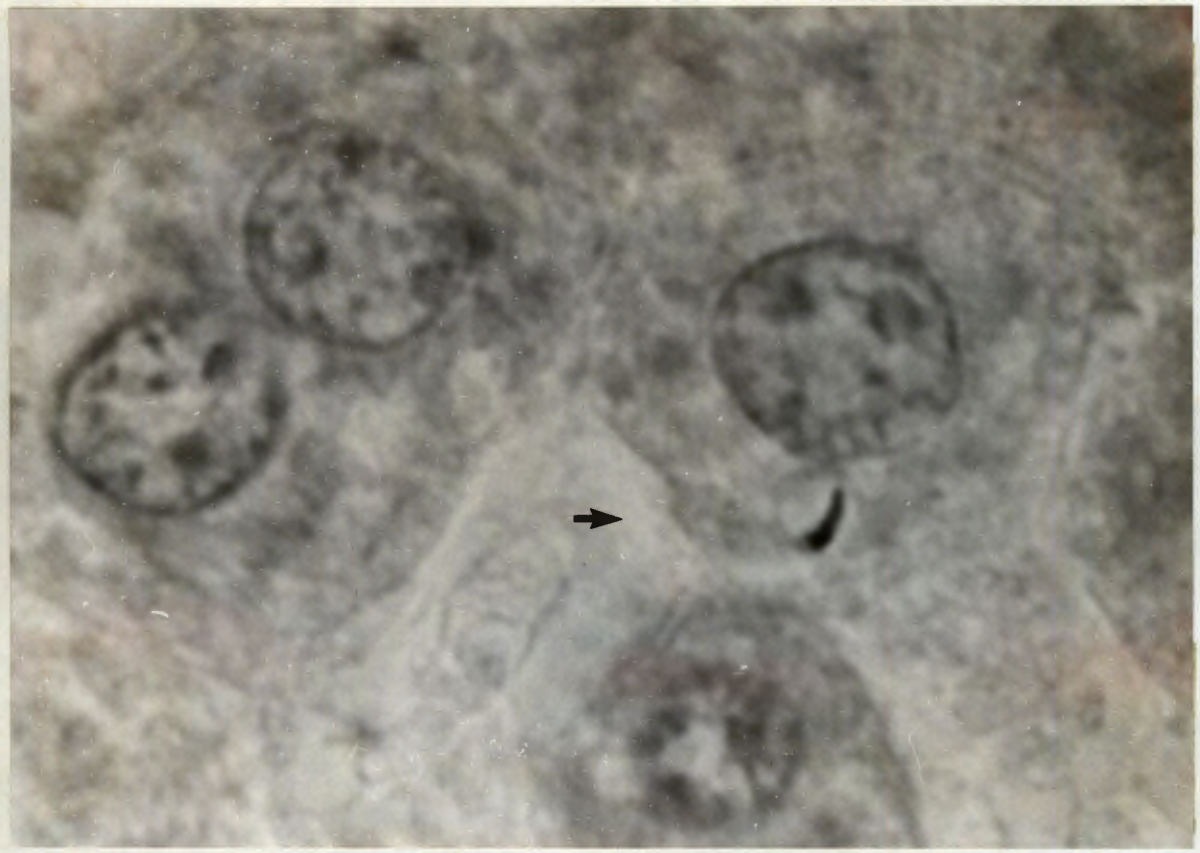


FIGURE 5.3.

Light micrograph of liver 20 hours after the injection of free NBT, an intravacuolar needle-shaped formazan crystal is visible within an hepatocyte (arrow).

Tartrazine x 4,000

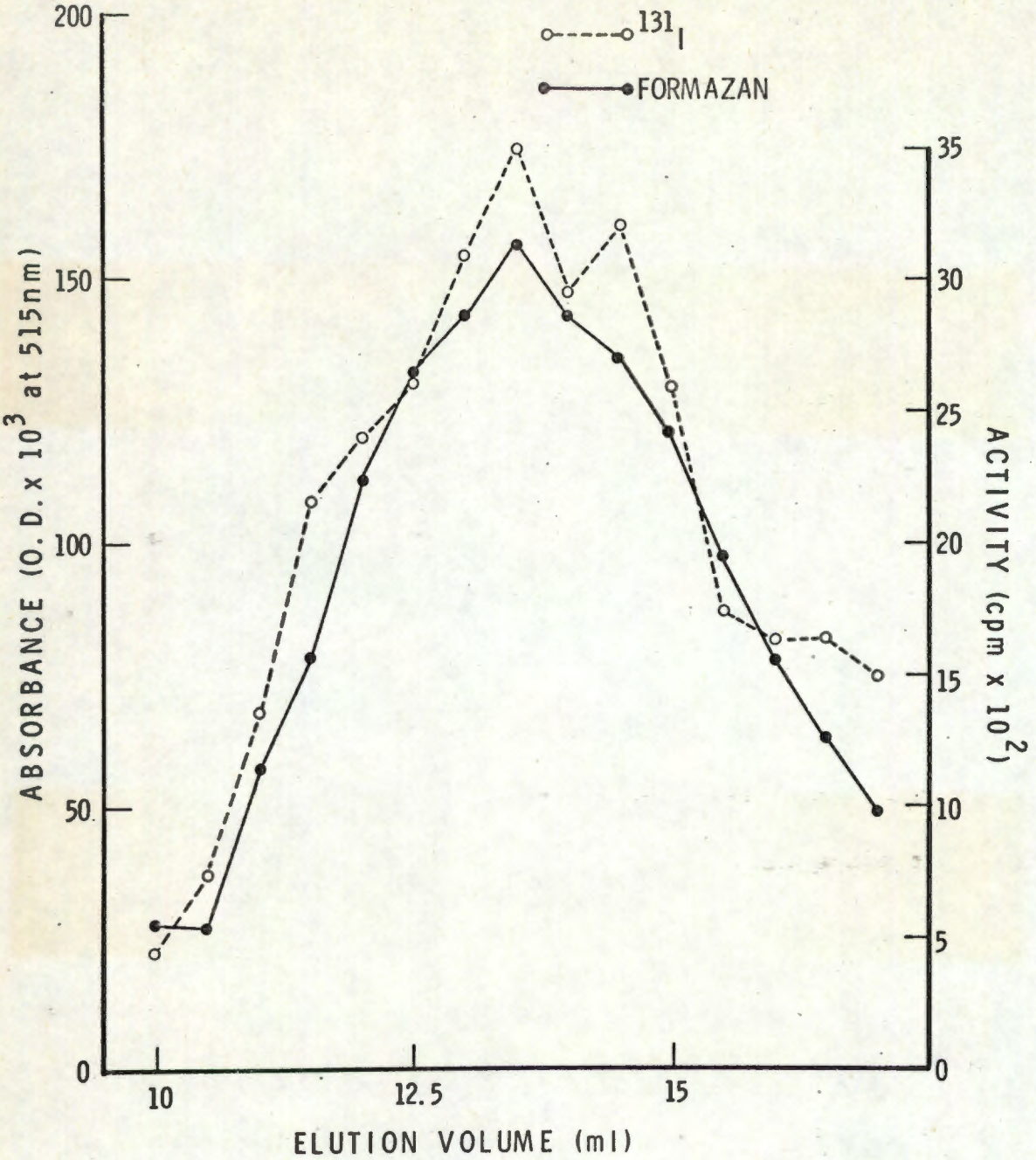


FIGURE 5.4

The association between the entrapment of formazan, measured as absorbance at 515 nM, and the gamma activity of entrapped ^{131}I, indicating co-entrapment of these substances within the liposomes.

Thin sections were examined unstained or after staining with lead citrate (Reynolds, 1963) and uranyl acetate.

Control liposomes and liposomes containing entrapped formazan were examined unstained and after negative staining with 2.0% sodium silico tungstate.

5.3. RESULTS (Table 5.1)

i. Unreduced NBT unentrapped in liposomes

All 5 rats injected with 5.0 mg of NBT in aqueous solution and the single animal injected with 5.0 mg of NBT together with but unentrapped in liposomes, convulsed and died almost immediately. Rats injected with 1.0 ml of NBT did not show side effects of injection.

Light microscopy

Punctate formazan deposits were observed sub-endothelially in the distal convoluted tubules of the kidney (identified by staining with Periodic Acid Schiff) in the rat killed 15 minutes after injection (Fig. 5.2). In the rat killed 20 hours after injection the formazan was scattered throughout the cytoplasm of these cells and very occasional needle-shaped crystals were found in hepatocytes (Fig. 5.3) and splenic macrophages.

ii. Formazan entrapped in liposomes

The close association between the radioactivity and the absorbance at 515 nM after column chromatography of the liposome and formazan mixture (Fig. 5.4) indicated co-entrapment of ^{131}I and formazan within liposomes.

TABLE 5.1 The light microscopic localisation of reduced NBT at various times after the intravenous injection of NBT in the oxidised and reduced forms, associated and unassociated with liposomes, into rats

PREPARATION ADMINISTERED	QUANTITY OF NBT 5 mg	1 mg Number of animals	TIME (hrs)	Liver Site	Kidney Site	Spleen Site	Bone Marrow Site
NBT	5		0	IMMEDIATE DEATH			
		1	0.25	0	++ Distal convoluted tubules	0	0
		1	20	+ Hepatocytes	+	± Macrophages	0
NBT in liposomes	1	1	0.5	0	+	0	0
	1	1	1	0	++ Distal convoluted tubules	0	0
	1	1	3	0 Kupffer cells	++	+ Macrophages	+ Macrophages
		1	20	+ (Neutrophils)	+	+++ (Neutrophils)	+ (Neutrophils)
Reduced NBT in liposomes		2	1	++ Kupffer cells (Neutrophils)	0	+++ Macrophages (Neutrophils)	+ Macrophages (Neutrophils)
Reduced NBT No liposomes		1	1	0	0	0	0

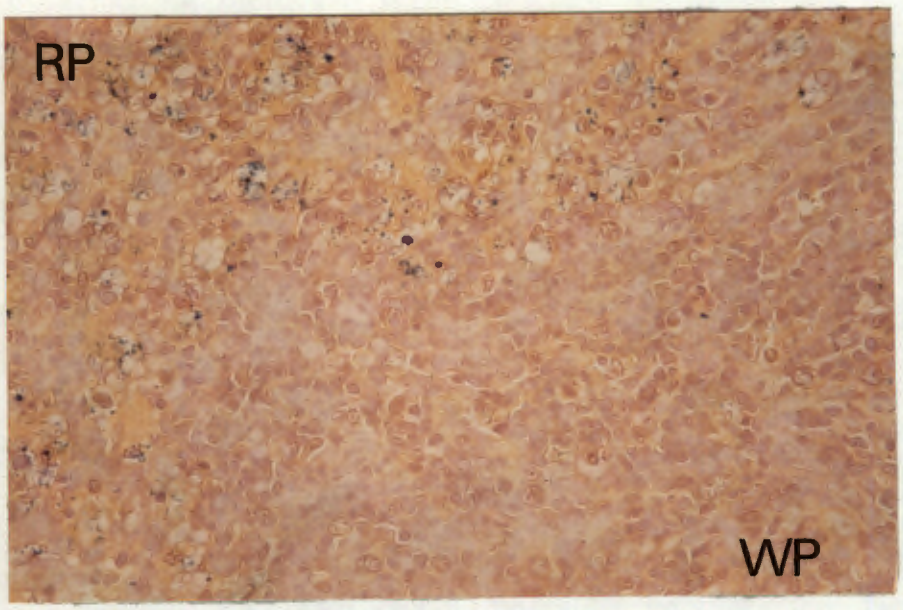


FIGURE 5.5.

Light micrograph of spleen 1 hour after the injection of liposomes with entrapped formazan. Abundant deposits of formazan are present within macrophages and neutrophils, in the red pulp (RP) but not in the white pulp (WP).

Tartrazine x400

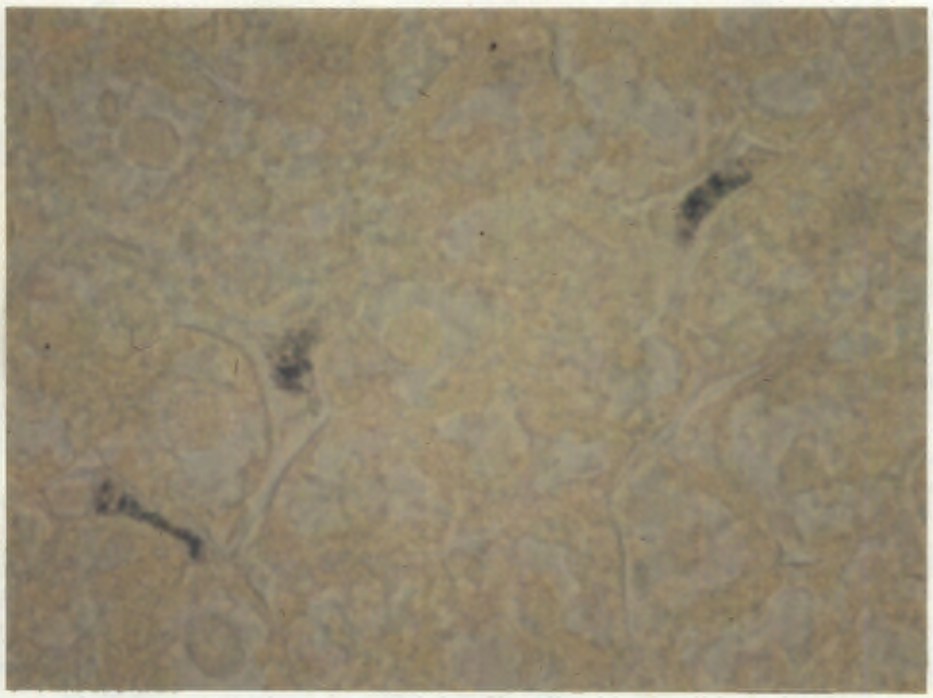


FIGURE 5.6.

Light micrograph of liver 1 hour after the injection of liposomes containing entrapped formazan. Formazan deposits are present in and localised to, Kupffer cells.

Tartrazine x1,000

None of the rats injected with liposome-entrapped formazan showed any side effects of the injection.

Light microscopy

Abundant deposits of formazan were present in splenic macrophages and neutrophils (Fig. 5.5) and Kupffer cells and neutrophils in the liver (Fig. 5.6), and in smaller quantities in bone marrow macrophages and neutrophils, of animals that received liposome-entrapped formazan and were sacrificed 1 hour after injection. Formazan deposits were observed in very occasional peripheral blood neutrophils and macrophages - the extreme rarity of such cells did not allow accurate quantitation.

iii. Unreduced NBT entrapped in liposomes

All animals that received 5.0 or 1.0 mg of NBT were free of side effects after injection.

Light microscopy

Liposome-entrapped NBT gave rise to a picture which was a hybrid of that obtained with free NBT and liposome-entrapped formazan. Punctate deposits were present in the distal convoluted tubules of the kidney after 30 minutes. Formazan deposits were also found in the spleen, and to a lesser extent in the bone marrow, in macrophages and neutrophils after 3 hours, and in Kupffer cells and neutrophils of the liver after 20 hours. The concentration of the visible formazan deposits was unrelated to the concentration of entrapped NBT and is probably a reflection of the number of particles rather than the total quantity of dye.

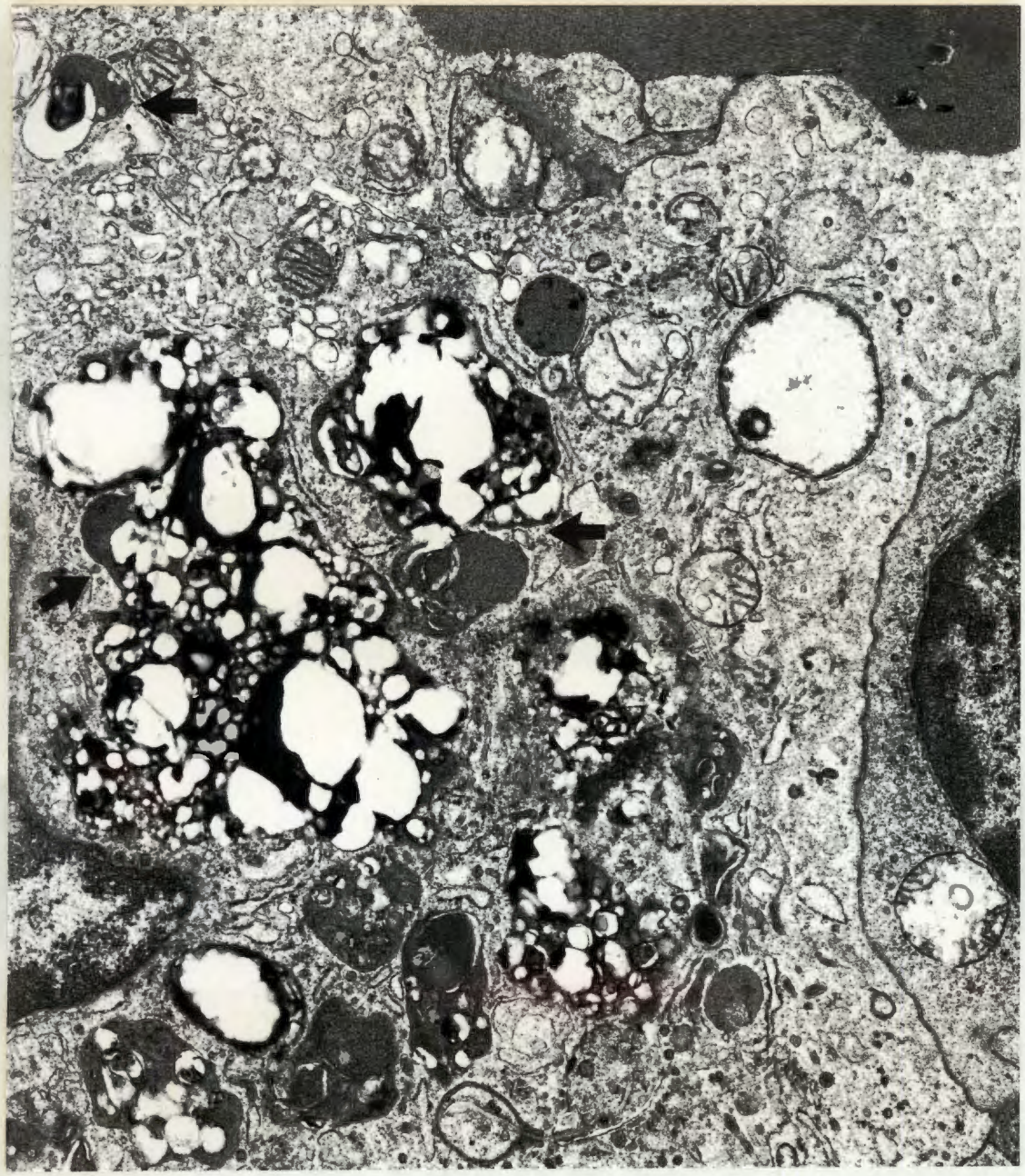


FIGURE 5.7.

Electron micrograph of splenic macrophage 2 hours after the injection of liposome entrapped NBT. Lysosomes can be seen in close proximity to and actually fusing with (arrows) phagocytic vacuoles containing liposomal remnants.

Uranyl acetate and lead citrate x15,000

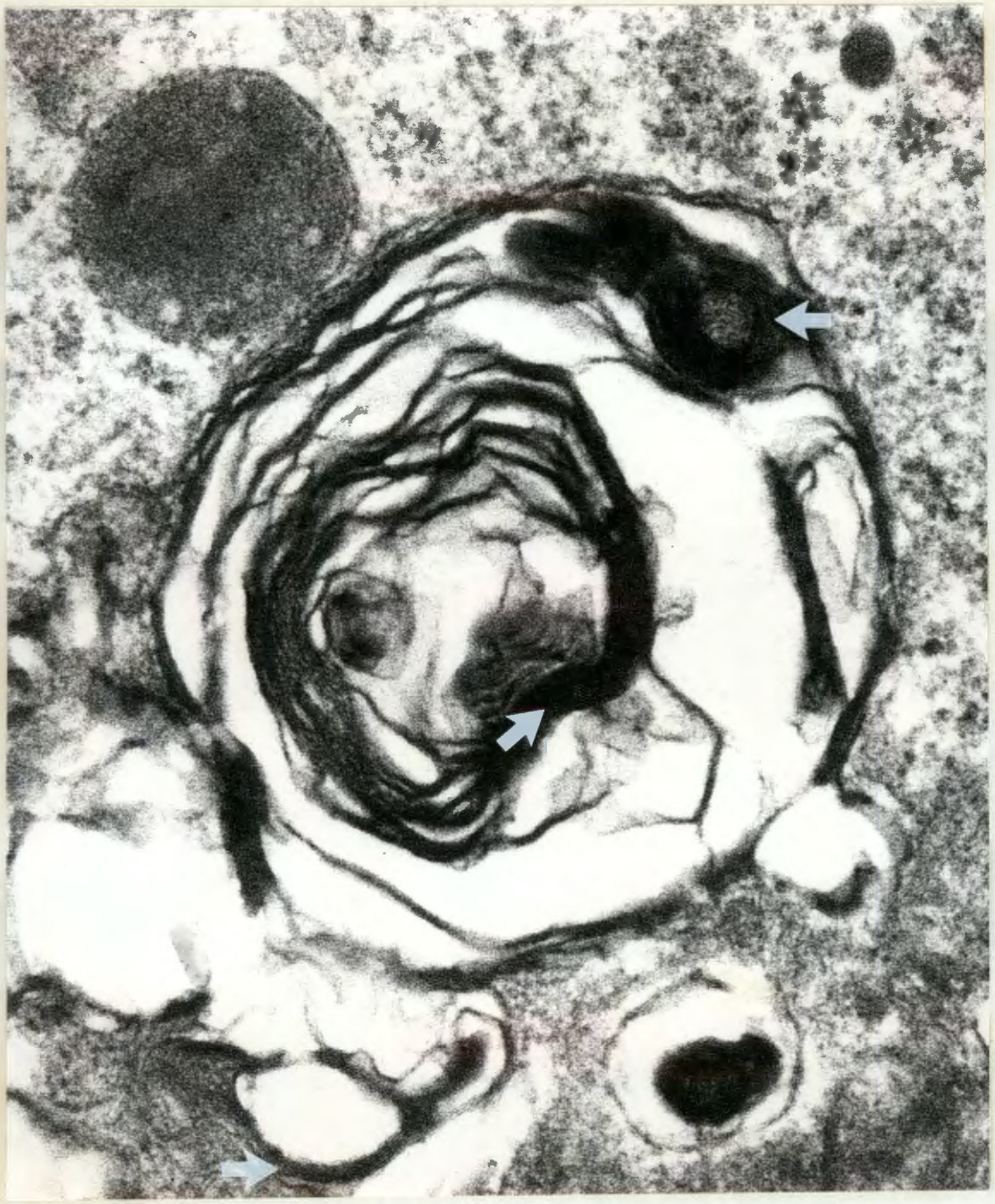


FIGURE 5.8.

Splenic macrophage 2 hours after the injection of liposome-entrapped NBT, showing a phagocytic vacuole containing multilamellated liposomes (arrows).

Uranyl acetate and lead citrate x200,000.

FIGURE 5.9.

Unstained electron micrograph of splenic macrophage 2 hours after the injection of liposome-entrapped NBT, showing electron dense formazan crystals within phagocytic vacuoles. x15,000.

iv. Formazan unentrapped in liposomes

The animal injected with formazan in the absence of liposomes did not show side effects of the injection.

Light microscopy

No deposits of formazan were observed.

5.3.1. Electron microscopy

5.3.1.a Spleen

Numerous lamellated myelin figures were observed within membrane bounded vacuoles in macrophages and neutrophils of animals injected with liposome-entrapped NBT (Fig. 5.7).

Fusion of lysosomes with the phagocytic vacuoles was clearly visible and the lamellae of the myelin figures had roughly the same periodicity as those of the intact liposome (Figs. 5.1 and 5.8).

In unstained sections formazan crystals were clearly visible within the phagocytic vacuoles (Fig. 5.9).

In comparison with liposomes the myelin figures appeared distorted and irregular.

No such changes were observed in control animals or animals injected with NBT alone.

5.3.1.b Liver

Identical myelin figures were abundant in Kupffer cells and in occasional neutrophils and portal tract macrophages of animals injected with liposome-entrapped NBT. No such changes were observed in the livers of control animals and animals injected with NBT alone.

5.3.1.c Liposomes

Liposomes containing formazan (Fig. 5.1) were identical in size and conformation to control liposomes. The formazan produced by intraliposomal reduction of NBT was electron translucent.

5.4. DISCUSSION

Liposomes are phagocytosed by cells of the reticulo-endothelial system and by neutrophils after intravenous administration. When prestained with formazan, deposits of this material were observed by light microscopy almost exclusively in Kupffer cells and neutrophils of the liver (Fig. 5.6) and macrophages and neutrophils in the spleen (Fig. 5.5) and, to a lesser extent, the bone marrow and blood. Electron microscopy showed large concentrations of liposomes in the same cells in the liver and spleen (Fig. 5.7).

After the administration of free NBT, formazan deposits were found predominantly in the distal convoluted tubules of the kidney (Fig. 5.2). Accumulation at this site occurs very rapidly and could result from pinocytosis by these cells. Trace amounts of formazan also occurred as needle-shaped crystals within vacuoles in hepatocytes (Fig. 5.4) and splenic macrophages.

A hybrid picture was obtained with liposomes associated with unreduced NBT, which probably resulted from the injection of a mixture of liposome-entrapped NBT, and free NBT, sequestered between the liposomes during centrifugation and released by resuspension and intravenous injection. Of interest is the delay before reduction of this NBT. Macrophages

and neutrophils reduced dye very rapidly after endocytosis (Nathan et al, 1969), which suggests that the lag period is a measure of the rate of liposomal degradation within the cell. Structural disorganisation of ingested liposomes is apparent on electron microscopy and is suggested by the observation of formazan crystals within phagocytic vacuoles; when reduced within liposomes the dye is electron translucent and thus must be released from the liposome in the oxidised form to allow crystal formation. Degradation of liposomes probably results from the action of lipases released from lysosomes, which were seen to fuse with and discharge their contents into phagocytic vacuoles containing liposomes (Fig. 5.8).

The protective action of liposomes was demonstrated by the immediate death of all the animals to whom a large dose (5 mg) of unreduced NBT was administered alone or in the presence of, but unentrapped in, liposomes, whereas the animals which were given the same amount of NBT entrapped within liposomes were unaffected.

Formazan is insoluble in water, but it remains in solution in serum in which it is bound to lipoproteins (4.3). Its circulation in this form when administered without liposomes, could account for the absence of focal tissue deposition.

CGDC was initially thought to represent "a generalised disease of the reticuloendothelial system" (Bridges et al, 1959) because of the widespread distribution of granulomata,

consisting predominantly of macrophages and epitheloid cells, and abscesses containing neutrophils and macrophages, throughout the reticuloendothelial system. It is now known that the primary defect in this disease results in a decreased capacity of neutrophils from these patients to kill certain micro-organisms (Quie et al, 1967) after phagocytosis.

Bacteria are removed from the circulation by phagocytosis, and the patients do not develop overwhelming septicaemia (Bridges et al, 1969, Thompson and Soothill, 1970), however, the bacteria remain viable, sequested from the lethal effects of antibody and most antibiotics , and capable of prolonging the infection upon release from the cells.

There are three possible methods of eradicating the infecting organism. A neutropenia can be induced to prevent neutrophils from harbouring the infecting agent, thereby allowing antibiotics to gain access to them. This form of therapy has been used in two patients by Thompson and Soothill (1970), who employed busulphan to promote neutropenia, with limited success. Although a theoretical possibility, this approach to therapy appears dangerous because of the risk of super infection by organisms resistant to the antibiotic therapy, and because of the non-infective complications of cytotoxic therapy (Calabresi and Welch, 1962).

Another approach to therapy in these patients is the use of antibiotics capable of gaining access to and killing organisms within neutrophils. Rifampin is concentrated in neutrophils, unlike penicillin which does not gain access to

intracellular sites, and is the only one of a large number of commonly used antibiotics to kill intracellular bacteria (Mandell, 1973). The use of this drug would appear to be the most logical treatment of patients with CGDC and its use is at present being evaluated by Professor J.F. Soothill (Department of Immunology, Institute of Child Health, London; personal communication).

The third approach to the treatment of these patients is the introduction of bactericidal agents or systems directly into the leukocyte. The defect in leukocytes of patients with CGDC has been partially corrected in vitro with H_2O_2 producing drugs such as phenazine or methylene blue (Lehrer, 1969) or H_2O_2 generating systems such as latex particles coated with glucose oxidase (Johnston and Baehner, 1970), but although these techniques are helpful in establishing the bactericidal action of H_2O_2, they appear impractical for therapeutic use.

In this study, liposomes were shown to localise predominantly in the reticuloendothelial system, in Kupffer cells and occasional neutrophils in the liver, and macrophages and neutrophils in the spleen and, to a lesser extent, the bone marrow. The reason for the scarcity of liposomes in circulating leukocytes is unknown. It is possible that the injection of liposomes as a bolus resulted in mixing of the liposomes with only a small proportion of the circulating leukocytes and that most of the liposomes were removed by a single passage of the blood through

the organs in which they were later located. It is also possible that having phagocytosed particulate matter, leukocytes rapidly leave the circulation and enter the tissues.

Liposomes localise in the anatomical areas and cells in which pathological lesions are observed in CGDC. It has been shown that macrophages migrate to areas of inflammation after the phagocytosis of particulate matter (Ginsberg et al, 1969), a process which might result in the accumulation of liposome containing cells in inflammatory tissue. These vehicles may therefore be of use in the transport of bactericidal agents or systems into neutrophils and macrophages.

It is unlikely that liposomes would be toxic to cells as they are composed of naturally occurring lipids and there is evidence in this and other (Gregoriadis and Buckland, 1973) studies that they are degraded within the phagocytic vacuole. Nevertheless, toxicity studies on liposomes and any entrapped therapeutic agents should be performed in animals before use in humans, unless treatment employing these agents could possibly cure an otherwise rapidly fatal disease.

In vitro experimentation on the bactericidal and toxic effects of liposome-entrapped compounds after uptake by phagocytic cells of CGDC patients or in the model system of CGDC (Mandell et al, 1970) should be informative and simple

to perform. Agents that should be tried include: penicillin and other antibiotics in case the rifamycins are ineffective in vivo, or for the treatment of infections by organisms resistant to therapy with these agents; various enzymes implicated in the pathogenesis of CGDC; ascorbic acid and other systems capable of the formation of H_2O_2 or super oxides. The generation of H_2O_2 or other oxidising compounds within the phagocytic vacuoles of phagocytes may be toxic to cells with a poor capacity for producing reducing equivalents, as cytoplasmic free radical scavengers (Slater, 1972b) may be incapable of detoxifying these compounds. This possible toxic effect should be carefully investigated.

Even if liposomes or other bactericidal mechanisms were to prove effective in killing bacteria, this does not mean that the granulomata would regress. It is possible that granulomata occur as a result of ineffective digestion of antigenic bacterial material, which may be a completely separate process from that of bacterial killing (Ginsberg, 1972). Little is known of the digestive processes and as a result, no dignostic tests or clinical abnormalities of this system have been described.

Liposomes may be useful in the study and possibly in the treatment of other disorders of neutrophil function, such as the Chediak-Higashi syndrome and myeloperoxidase deficiency. They may also be useful for the investigation and selective treatment

of disorders of the reticuloendothelial system. Diseases such as Gaucher's disease, in which there is a disorder of the metabolism of glucosyl ceramides (Fredrickson and Sloan, 1972) might eventually be amenable to therapy with liposome-entrapped enzymes. Histeocytosis X, a granulomatous lesion, composed predominantly of histiocytic cells (Wintrobe and Boggs, 1970) and histiocytic medullary reticulosis, a neoplasm of erythrophagocytic histocytes (Scott and Robb-Smith, 1939), would seem to be ideal conditions for therapy with liposome-entrapped cytotoxic agents.

5.5. SUMMARY

The cellular destination of liposomes has not been previously demonstrated, and their proposed in vivo therapeutic use was thus highly theoretical despite successful implementation in a model system.

In this study, intravenously injected liposomes were observed to localise predominantly in splenic macrophages and neutrophils, and in Kupffer cells of the liver and, to a lesser extent, in bone marrow macrophages and neutrophils. This suggests that liposome - entrapped agents would be suitable for experimentation upon, or as therapy for, inherited enzyme deficiencies and infective disease, as in CGDC, or neoplastic disease, of phagocytic cells. The scope and advantages of so highly selective an approach to therapy are great.

CHAPTER 6. Conclusions

6.1. The NBT test

i. The NBT test, whether performed by the method of Park et al (1968) or Gordon et al (1973), was found to be unreliable in distinguishing between pyogenic infection and other diseases.

ii. There was no relationship between the result of tests performed by the two different methods.

iii. There is an appreciable observer error in the interpretation of the slide preparations. This error seemed to be reduced by experience in the interpretation of these specimens.

iv. NBT precipitates and complexes with heparin and/or fibrinogen and acts as a histochemical marker of the phagocytosis of these complexes by neutrophils. The NBT test is therefore a measure of the phagocytic activity of neutrophils.

v. Neutrophil uniformly exposed to the complexed NBT exhibit a variable phagocytic response. In patients with pyogenic infection, the extent of this phagocytic response appeared to be related to the severity of the illness.

vi. Experiments conducted with an in vitro model system, simulating the NBT test, indicated that a humoral factor might be responsible for the enhanced NBT reduction observed in sick patients.

vii. Serum concentrations of a number of compounds are known to be elevated in acutely ill patients. Of those tested, only α1-acid glycoprotein, endotoxin and immunoglobulins enhanced NBT reduction,

any of which, singly or in combination, could be responsible for positive NBT tests in ill patients.

viii. Evidence for the enhancement of NBT reduction by a humoral factor and by α^1-acid glycoprotein was strongest in tests performed on blood anticoagulated with EDTA. Heparin itself could be responsible for the poor correlation between these factors and the NBT score of tests performed on blood in which it was used as anticoagulant.

ix. The physiological roles of heparin, α^1-acid glycoprotein and fetuin, a closely related acid glycoprotein present in the foetal serum of certain animals, are unknown. They all promote the phagocytosis of precipitates of fibrinogen and heparin, and might function as a non-specific opsonising system to enhance the phagocytosis of exogenous material and autologous debris, both locally and within the circulation.

x. False-negative test results occur in patients with diseases such as CGDC, in which the neutrophils have a diminished ability to reduce the dye. These conditions are extremely rare and false-negative results would appear to be most commonly related to the replacement of mature circulating neutrophils by immature cells, which have a normal capacity to reduce the dye, but in which phagocytosis of complexed NBT is diminished. Under unusual circumstances, immune complexes could also result in false-negative tests.

xi. An in vitro model system, in which endotoxin is used as an artificial stimulus of phagocytosis, can be used to measure the capacity of neutrophils to phagocytose and to reduce the dye. In this system it was shown that there was no difference in the dose response to endotoxin of neutrophils from normal subjects and patients with Crohn's disease, diabetes mellitus and hypogammaglobulinaemia, geriatric patients and patients receiving therapy with prednisone and sodium aurothiomalate.

xii. Complement and immunoglobulins are not obligatory for dye reduction.

xiii. The NBT test does not seem to have wide application as a diagnostic test for bacterial infection, however, an in vitro stimulation test appears to be of potential value in the assessment of phagocytosis and dye reduction, which seems closely related to microbicidal mechanisms, by neutrophils, and as an indicator of the maturity of circulating neutrophils and of circulating immune complexes.

6.2. The quantitative NBT test

i. The quantitative NBT test has a different underlying mechanism and different application.

ii. NBT is toxic to neutrophils, and dye reduction by these cells is closely related to the release of cytoplasmic constituents from the cell, probably as a result of enhanced contact between intracellular reducing compounds and NBT at the damaged outer membrane of the cell.

iii. In neutrophils from patients with CGDC, the amount of dye reduction is abnormally low when compared with the release of cytoplasmic constituents. This suggests that NBT does not exert its toxic effect purely by the consumption of reducing equivalents, and that the factor, or factors, responsible for the reduction of NBT is absent from the cytoplasm of these cells.

iv. The expression of NBT reduction by cells as a factor of LDH release, should allow more accurate standardisation of the quantitative NBT test.

v. The main application of the quantitative NBT test is in the diagnosis of CGDC. It is also of use in distinguishing between inefficient phagocytosis and defective dye reduction by cells which exhibit diminished dye reduction in a stimulated NBT test.

6.3. Lipoproteins

i. Reduced NBT binds specifically to and is solubilised by serum lipoproteins. The mechanism of this binding is unknown.

ii. This property allows the use of reduced NBT as a specific prestain for the identification of lipoproteins after separation by electrophoresis.

iii. The separation of NBT prestained serum lipoproteins by polyacrylamide gel electrophoresis was found to be simple and reproducible, and of value in the qualitative identification of the various hyperlipidaemias. A number of minor slow α lipoprotein

bands were present in all normal sera. A band with electrophoretic mobility intermediate between that of the β and pre-β bands was present in a significant proportion of normal subjects. This band, referred to as the 'inter-β' band, could represent a minor (Sf 12-20) component of LDL. It might also represent the 'broad β' lipoprotein. If broad β hyperlipoproteinaemia is shown to be clearly distinguishable by this technique, it would greatly simplify the diagnosis of this condition.

iv. There is urgent need of a simple method for the quantitation of lipoproteins that measures protein concentration rather than the concentrations of protein-bound lipids. If the binding of reduced NBT is found to be related to the concentration of proteins, rather than to that of any particular lipid, it should allow the quantitation of these proteins by densitometry.

v. Reduced NBT could be a useful marker for lipoproteins in other separation techniques.

6.4. Liposomes

i. Liposomes are potentially useful vehicles for the selective transport of therapeutic agents to particular intracellular targets.

ii. The whole principle of the use of these microbodies as a therapeutic tool hinges upon their exact cellular and subcellular localisation after intravenous administration. These facts were unknown.

iii. Liposome-entrapped NBT and formazan were traced by light and electron microscopy to determine the fate of liposomes after intravenous administration. They were found to localise predominantly in Kupffer cells in the liver and splenic macrophages and to a lesser extent in neutrophils in these organs, and in macrophages and neutrophils in the bone marrow.

iv. These findings suggest that liposome-entrapped agents should be useful for experimentation upon, and therapy for, diseases involving phagocytic cells.

NBT has been a useful tool in the studies described in this thesis. In view of its unique properties it should be of value, and should find wide application, in many other fields of experimental biology and clinical medicine.

SOURCE OF MATERIALS

Analar grade materials were used throughout this study. Special materials, materials which are not freely available from the major pharmaceutical suppliers, or materials, the source of which is of specific importance, are described below.

Compound	Source or Specification	Supplier
α^1-Acid glycoprotein	Human	Kindly donated by Dr.A.Tavill Northwick Park Hospital

This protein was isolated from human serum by ion exchange chromatography on diethylaminoethyl cellulose (DEAE, DE 52, Whatman), followed by separation by polyacrylamide (8%) electrophoresis and elution from the gel. Its purity was confirmed by Laurell electrophoresis.

Adenosine diphosphate (ADP)	Equine muscle	Sigma, London
Antisera and standard antigens:		
Anti-whole human serum	Sheep	Burroughs Wellcome, Beckenham, England.
Anti α_1-acid glycoprotein	Rabbit	Behringwerke, Lahn, Germany.
Anti β-lipoprotein	"	" "
Anti α_1-lipoprotein	"	" "
Partagen-plates	"	" "
Standard α^1-acid glycoprotein	Human	" "
Standard whole human serum containing CRP	"	" "

Compounds	Source or Specification	Supplier
Cyclic adenosine monophosphate (AMP)	Equine muscle	Sigma, London.
Cytochalasin B		Imperial Chemical Industries, Pharmaceutical Division, Cheshire.
Endotoxin	Escherichia coli (0127 : B8)	Difco, Detroit, U.S.A.
Fetuin	Bovine	Grand Island Biological Co., Grand Island, New York.
Fibrinogen	Human (Grade L)	Kabi, Stockholm, Sweden.
Hank's solution		Oxoid, London
Heparin	1,000 i.u./ml containing 0.15% Chlorocresol B.P.	Weddel Pharmaceuticals, London.
Immunoglobulins	Pooled human gammaglobulins	Lister Institute, Elstree, Herts.
Latex particles	0.81 μ in diameter	Difco, Detroit, U.S.A.
Lipids:		
Lecithin	Egg	Lipid Products, Redhill, Surrey.
Phosphatidic acid		" "
Cholesterol		British Drug Houses, London.
Lysolecithin		Koch-Light, Colnbrook, Bucks.
Nitroblue tetrazolium		Sigma, London.

Compounds	Source or Specification	Supplier
Neuraminidase	Vibrio cholerae 500 i.u./ml	Behringwerke, Lahn, Germany.
Phospholipase C		Sigma, London.
Prostaglandins	E_1, A_1, $F_2\alpha$	Upjohn Pharmaceuticals, Michigan, U.S.A.
	Kindly donated by John E. Pike.	
Radiolabelled compounds		Radiochemical Centre, Amersham.
Retinol	All trans.	Sigma, London.
Thrombin	Bovine	Parke-Davis, England.

INDEX TO FIGURES

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* L.M. refers to light micrograph

E.M. refers to electron micrograph

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CONTENTS OF APPENDIX

APPENDIX

APPENDIX A

Details of normal subjects (group I)

GROUP NO.	STUDY NO.	AGE	SEX	E.S.R.	W.B.C.	PERCENTAGE NEUTROPHILS	PLATELET COUNT	SHIFT TO THE LEFT	TOXIC GRANULATIONS	C.R.P. mg/%	α_1 - acid GLYCOPROTEIN mg/%
I	43	24	F	-	6.7	64	-	0	0	-	-
2	62	29	M	-	6.7	66	-	0	0	-	-
3	64	I6	F	-	5.7	52	-	0	0	-	-
4	65	2I	F	-	7.0	78	-	0	0	-	-
5	66	2I	F	-	6.8	60	-	0	0	-	-
6	82	3I	M	-	5.9	70	-	0	0	-	-
7	95	25	M	3	6.3	68	-	0	0	-	37
8	96	28	F	9	5.2	66	-	0	0	-	90
9	97	22	M	2	5.3	80	-	0	0	-	75
I0	98	2I	F	I7	5.9	74	-	0	0	-	90
II	II5	45	F	4	5.3	70	2	0	0	-	-
I2	II6	50	F	I0	I8.8	85	I	0	0	-	85
I3	I26	29	M	I	4.4	53	I	0	0	-	-
I4	I28	37	F	23	8.2	63	2	0	0	-	-
I5	I39	I6	F	7	5.0	60	2	0	0	-	-
I6	I40	I0	F	6	4.3	35	2	0	0	-	-
I7	I66	46	F	I2	6.4	44	-	0	0	-	40
I8	I73	23	M	2	7.4	42	-	0	0	-	-
I9	I96	53	M	9	6.5	59	I	0	0	-	-
20	I97	46	F	I7	6.I	62	2	0	0	-	-
2I	2I0	70	F	II	4.9	5I	I	0	0	-	80
22	250	36	F	I3	9.6	62	-	0	0	-	55
23	25I	46	F	I3	6.3	54	-	0	0	-	II2
24	252	63	M	I2	5.3	67	-	0	0	-	85
25	257	29	M	2	7.4	65	I	0	0	-	I45
26	258	28	F	3	9.5	55	2	0	0	-	82

NBT Score					
EDTA/FICOLL			HEPARIN		
OBSERVER			OBSERVER		
I	2	3	I	2	3
33	20	9	0	6	2
2	I	0	35	22	I5
I	0	I	I	I	I
I	3	I	9	4	I
0	I	3	3I	I9	7
0	2	5	32	I3	II
2	0	I	43	2I	2
I	I	2	I0	I0	3
0	0	I	I0	I0	I0
I	I	2	30	I3	8
2	3	II	I5	I2	8
4	4	7	I5	2I	7
0	4	6	0	8	5
7	I4	5	44	39	38
7	4	6	6	I	6
2	5	2	I5	I4	7
0	7	2	2	7	3
I	4	4	5	I2	6
0	I	0	6	5	4
I	3	4	I8	I8	2I
2	8	I	3	II	6
I0	II	-	5	I3	-
I2	7	-	2	2	-
5	2	-	0	2	-
I2	II	-	20	20	-
5	9	-	I	I2	-

APPENDIX B

Details of patients with untreated pyogenic infections (group 2)

GROUP NO:	STUDY NO.	AGE	SEX	DIAGNOSIS	ORGANISM ISOLATED
I	263	34	M	Liver abscess	9,I0
2	253	2I	F	Pharyngitis	II
3	245	7I	M	Multiple abdominal abscesses after surgery	8
4	244	49	F	Fungal endocarditis	I2
5	236	57	M	Appendix abscess	Nil
6	234	25	M	Abdominal wound abscess	Nil
7	23I	5	M	Lung abscess	3,7
8	227	7	M	Otitis media	Nil
9	2I7	80	F	Pneumonia	2
I0	2I6	2	M	Otitis media, pneumonia	Nil
II	2I3	2	M	Pneumonia	Nil
I2	20I	70	F	Wound abscess	2,I3
I3	I99	7I	M	Wound abscess	4,7,9
I4	I93	67	F	Wound abscess	Nil
I5	I92	66	M	Liver abscess	I
I6	I87	I4	M	Appendicitis	Nil
I7	I78	64	F	Infected burn of scalp	7
I8	I59	50	F	Pneumonia	Nil
I9	I58	5	M	Erysipelas	II
20	I44	4I	F	Wound abscess	2

Key to organisms:

I : Bacteroides species
2 : Escherichia coli
3 : Haemophilus influenza
4 : Proteus vulgaris
5 : Pseudomonas aeruginosa
6 : Staphylococcus albus
7 : Staphylococcus aureus
8 : Streptococcus faecalis
9 : Streptococcus non-haemolytic
I0 : Salmonella species
II : Streptococcus β haemolytic
I2 : Candida albicans
I3 : Diptheroids

DURATION OF ILLNESS (DAYS)	E.S.R.	W.B.C.	PERCENTAGE NEUTROPHILS	PLATELET COUNT	SHIFT TO THE LEFT	TOXIC GRANULATIONS	C.R.P. mg/%	α' - acid GLYCOPROTEIN mg/%	SEVERITY OF ILLNESS	NBT Score					
										EDTA/FICOLL			HEPARIN		
										OBSERVER			OBSERVER		
										I	2	3	I	2	3
3	II8	7.2	90	-	2	2	-	625	3	60	65	-	33	29	-
4	4	6.8	74	-	0	0	-	95	I	8	23	-	20	28	-
5	25	I7.5	95	2	2	0	-	360	2	I2	4	-	28	22	-
>30	72	6.0	40	2	0	0	-	60	I	2	2	-	3	3	-
2	64	I0.5	89	2	0	0	II4	350	2	22	9	-	57	I7	-
3	30	6.9	60	2	0	0	60	I55	I	I4	I0	-	4	3	-
I4	68	9.5	85	2	2	2	-	355	2	6	I0	5	44	II	I0
2	20	3.I	42	I	0	0	-	-	3	I2	I2	8	I2	I8	I5
2	27	I6.6	69	-	0	0	-	3I5	I	2	I	2	-	-	-
3	27	7.9	70	-	0	0	-	-	I	8I	64	85	63	7I	8I
3	67	28.2	96	2	I	I	-	-	I	68	50	48	67	83	85
2	57	I6.5	69	2	0	0	75	250	I	II	6	8	5	0	3
4	83	II.0	78	2	0	0	-	-	I	5	9	0	I9	II	I3
3	58	6.9	7I	2	0	0	-	235	I	6	2	2	I2	5	4
I	80	I2.0	86	2	0	I	-	290	3	46	35	I2	86	39	I3
I	I5	I3.7	-	I	0	0	-	-	2	7	I3	6	I2	I6	I2
4	2I	7.9	77	-	0	0	-	225	I	0	3	I	5	II	3
2	60	6.2	70	-	0	0	-	-	2	73	54	27	0	5	5
2	30	I5.0	90	-	0	0	-	-	2	40	65	52	33	40	40
3	88	I3.2	82	-	I	I	-	-	I	0	5	I3	44	25	I9

GROUP NO.	STUDY NO.	AGE	SEX	DIAGNOSIS	ORGANISM ISOLATED
2I	I42	20	F	Wound abscess	Nil
22	I4I	46	F	Pyelonephritis	2
23	I23	62	F	Wound abscess	4,7,8
24	II7	6	M	Appendicitis	Nil
25	II3	74	F	Wound abscess	2,4
26	III	44	M	Perianal abscess	Nil
27	II0	54	F	Urinary tract infection	2
28	I0I	30	F	Infected uterus	Nil
29	I00	83	F	Wound abscess	2,8
30	9I	25	M	Urinary tract infection	3,I2
3I	86	2	M	Pneumonia	3
32	78	27	M	Perianal abscess	7
33	77	22	M	Perianal abscess	Nil
34	75	9	M	Pyonephrosis	Nil
35	59	89	F	Urinary tract infection	2
36	57	65	F	Wound abscess	Nil
37	53	I8	M	Portal pyaemia	Nil
38	48	5/I2	F	Gastroenteritis	2
39	38	6I	M	Wound abscess	4
40	29	79	F	Subphrenic abscess	7,9

Key to organisms:

I : Bacteroides species
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6 : Staphylococcus albus
7 : Staphylococcus aureus
8 : Streptococcus faecalis
9 : Streptococcus non-haemolytic
I0 : Salmonella species
II : Streptococcus β haemolytic
I2 : Candida albicans
I3 : Diptheroids

DURATION OF ILLNESS (DAYS)	E.S.R.	W.B.C.	PERCENTAGE NEUTROPHILS	PLATELET COUNT	SHIFT TO THE LEFT	TOXIC GRANULATIONS	C.R.P. mg/%	α^1- acid GLYCOPROTEIN mg/%	SEVERITY OF ILLNESS	NBT Score					
										EDTA/FICOLL			HEPARIN		
										OBSERVER			OBSERVER		
										I	2	3	I	2	3
3	II	7.6	58	-	0	0	-	-	I	I0	5	4	43	39	36
3	II3	7.I	66	-	0	0	-	-	2	3	5	4	5	7	6
4	77	9.8	58	2	0	0	-	-	I	9	8	8	I3	I3	I7
I	42	4.7	39	2	0	0	-	I90	2	5	4	II	27	I5	7
3	35	I3.0	83	2	0	0	-	-	I	0	3	6	24	26	I9
3	32	9.4	68	2	0	0	-	I55	I	9	20	I4	2	I	3
7	I7	9.3	88	I	3	I	-	I35	I	4I	40	I2	I7	I7	9
4	20	I3.5	84	2	0	0	-	-	I	7	5	2	25	8	I4
4	9I	I2.I	86	2	0	0	-	395	I	5	0	6	6	3	9
2	I5	I3.0	86	-	0	0	-	-	I	52	I0	7	I9	28	I7
4	-	II.6	48	-	0	0	-	-	2	-	-	-	II	6	I
4	-	I0.I	80	-	I	0	-	I50	2	7	I	I	36	40	4
4	-	I0.8	70	-	0	0	-	-	2	20	6	3	3I	5	I
I0	-	7.I	54	-	0	0	-	-	I	49	29	4	8	3	4
5	-	5.9	58	-	0	0	-	-	I	0	I	5	I7	9	7
4	-	6.0	48	-	0	0	-	-	I	2	0	3	I	3	I
3	-	I5.3	90	-	0	0	-	-	2	8	-	7	24	I4	7
3	-	9.3	78	-	0	0	-	-	2	-	-	-	26	II	7
4	-	32.9	86	-	2	0	-	-	2	30	I2	I2	27	5	9
5	-	II.0	72	-	I	2	-	-	I	6	3	2	24	7	4

APPENDIX C

Details of patients with treated pyogenic infection (group 3)

GROUP NO.	STUDY NO.	AGE	SEX	DIAGNOSIS	BACTERIA ISOLATED	ANTIBIOTIC TREATMENT
I	32	2	M	Acute osteitis femur	5*	H,D
2	35	52	F	Abdominal wound abscess	I,2,9	I
3	39	50	F	Abdominal wound abscess	I,2,9	I
4	40	30	F	Abdominal wound abscess	I,4,8	F
5	52	60	F	Oesophagitis	3	A
6	67	25	M	Infective arthritis knee	6	D
7	I24	35	M	Abdominal wound abscess	2,5,8	G,H
8	I33	38	F	Chronic pyelonephritis	Nil	F
9	I43	27	M	Suppurative prostatitis	7*	B
I0	I74	50	F	Post-operative septicaemia	6*	C
II	I85	50	F	Abdominal wound abscess	Nil	A
I2	200	35	M	Generalised peritonitis	2,5	J
I3	202	74	M	Cholecystitis	Nil	A
I4	205	45	M	Septicaemia from decubitus ulceration	9*	A,C,G,J
I5	206	I5	F	Appendix abscess	2	C
I6	2I4	2	F	Otitis media	Nil	B
I7	2I9	53	M	Subphrenic abscess, lymphoma	Nil	C,G
I8	222	2I	M	Appendix abscess	I	A
I9	224	23	F	Tubo ovarian abscess	2	A,F
20	226	8	M	Bronchopneumonia	Nil	F
2I	238	65	F	Cellulitis of leg, S.L.E.	5	H
22	24I	2I	M	Resolving liver abscess	I	E
23	243	7I	F	Cellulitis of leg	Nil	H
24	246	54	M	Abdominal wound abscess	4,7	A
25	249	39	M	Generalised peritonitis	Nil	C,G
26	26I	22	F	Pharyngeal abscess	Nil	E

Key to chemotherapy:

A : Ampicillin
B : Cephalixin
C : Cephaloridine
D : Cloxacillin
E : Clindamycin
F : Co-trimoxazole
G : Gentamicin
H : Penicillin
I : Sulphadimidine
J : Tetracycline

Site of isolation:
* = blood culture - all others isolated from the site of disease.

DURATION OF TREATMENT (DAYS)	E.S.R.	W.B.C.	PERCENTAGE NEUTROPHILS	PLATELET COUNT	SHIFT TO THE LEFT	TOXIC GRANULATIONS	C.R.P. mg/%	α^1 - acid GLYCOPROTEIN mg/%	SEVERITY OF ILLNESS	NBT Score					
										EDTA/FICOLL			HEPARIN		
										OBSERVER			OBSERVER		
										I	2	3	I	2	3
7	-	I0.0	62	-	0	0	-	-	I	59	44	I6	I2	6	0
3	-	6.2	79	-	0	0	-	230	I	5	0	8	4	2	I
4	-	8.7	74	-	0	0	-	-	I	6	3	4	5	4	I
4	-	9.I	80	-	0	0	-	-	I	I6	2	5	37	II	7
3	-	8.3	62	-	0	0	-	-	2	-	I0	-	2	5	I
I4	-	II.2	79	-	0	0	-	-	2	3	5	8	20	I	7
4	III	I2.2	78	4	0	0	-	-	2	0	3	9	4	2	5
>30	95	5.4	52	2	0	0	-	-	I	II	9	I3	4	I	4
4	37	4.I	72	-	0	0	-	215	3	39	23	I5	59	27	45
I	2	6.0	86	-	2	0	-	-	3	I6	9	4	65	34	5
2	II2	II.3	64	2	0	0	-	-	I	26	20	2I	8	9	I
2	60	7.5	82	2	0	0	I6I	I80	3	46	35	I4	25	48	22
4	4	24.7	9I	2	0	0	-	-	2	0	I	I	I6	5	II
>30	I0I	II.9	79	3	0	0	-	-	2	3	I	I	28	6	9
I	56	I3.8	85	2	0	0	7I	I65	3	I2	I2	3	5I	32	20
2	-	I0.5	75	2	0	I	-	-	I	48	48	36	20	I7	27
2	I2	8.8	5I	-	0	0	-	I70	2	I2	I4	6	0	I	5
9	I06	I6.2	79	3	0	2	I70	290	2	54	49	66	39	54	37
4	I08	I4.3	-	3	-	-	25	300	2	57	59	82	67	36	2I
0.5	7	4.2	38	-	0	0	-	-	I	25	28	26	3	4	2
0.5	93	I2.3	96	-	0	0	-	-	2	35	II	-	2	I	-
I0	75	7.4	83	-	0	0	-	270	I	3	I	-	0	0	-
4	I35	II.0	89	2	0	0	-	225	I	5	6	-	2	2	-
2	34	5.5	64	I	0	0	-	I57	I	39	30	-	2	5	-
0.5	50	I6.7	68	-	0	0	-	245	3	26	2I	-	27	28	-
I	55	I5.3	82	-	0	0	-	I20	2	36	22	-	I0	25	-

Key to organisms:

I : Bacteroides species
2 : Escherichia coli
3 : Haemophilus influenza
4 : Proteus vulgaris
5 : Pseudomonas aeruginosa
6 : Staphylococcus albus
7 : Staphylococcus aureus
8 : Streptococcus faecalis
9 : Streptococcus non-haemolytic
I0 : Salmonella species
II : Streptococcus β haemolytic
I2 : Candida albicans
I3 : Diptheroids

APPENDIX D

Details of patients with tuberculosis (group 4)

GROUP NO.	STUDY NO.	AGE	SEX	SITE OF LESION	TREATMENT
I	30	I6	F	Cervical adenitis	Nil
2	I05	34	M	Miliary	Nil
3	I36	50	M	Pulmonary	Nil
4	I37	33	M	Distal ileum	Nil
5	I54	33	F	Distal ileum	Nil
6	22I	40	F	Cervical adenitis	Nil
7	242	44	F	Axillary adenitis	Nil

E.S.R.	W.B.C.	PERCENTAGE NEUTROPHILS	PLATELET COUNT	SHIFT TO THE LEFT	TOXIC GRANULATIONS	C.R.P. mg/%	α' - acid GLYCOPROTEIN mg/%	NBT Score					
								EDTA/FICOLL			HEPARIN		
								OBSERVER			OBSERVER		
								I	2	3	I	2	3
-	4.I	78	-	0	0	-	-	II	4	0	8	6	6
50	8.4	78	2	I	I	-	-	3	I	3	40	9	II
27	8.0	5I	2	0	0	-	-	I6	9	5	I0	7	9
37	6.3	68	I	I	0	-	-	8	I3	8	0	I0	5
66	9.7	82	-	0	0	-	295	48	60	36	46	24	26
I7	6.3	63	2	0	0	-	-	48	42	4I	II	27	I3
34	6.0	62	-	0	0	-	I05	52	37	-	0	3	-

APPENDIX E

Details of patients with non-bacterial infection or infestation (group 5)

GROUP NO.	STUDY NO.	AGE	SEX	DIAGNOSIS	DIAGNOSTIC CRITERIA
I	76	7	F	Mumps. Polyarthritis	Serology
2	79	32	F	Infectious mononucleosis	Serology
3	II2	30	F	Rubella	Clinical
4	I20	5I	F	Erythema nodosum after smallpox vaccination	Clinical
5	I55	I8	M	Massive ascaris lumbricoides infestation	Barium meal Microscopy of stool
6	I6I	27	F	Mild enterovirus	Serology
7	I62	42	F	Strong enterovirus	Serology
8	I63	37	F	ECHO I and rhinovirus	Serology
9	I64	23	F	Strong enterovirus and rhinovirus	Serology
I0	I65	45	F	Influenza virus	Serology
II	I67	20	F	Rhinovirus	Serology
I2	I68	23	F	Strong enterovirus and rhinovirus	Serology
I3	I69	27	F	Strong enterovirus and rhinovirus	Serology
I4	I70	5I	M	Influenza virus	Serology
I5	I7I	50	F	Rhinovirus	Serology
I6	I72	45	F	Rhinovirus	Serology
I7	80	29	M	Hepatic schistosomiasis	Liver biopsy
I8	225	I0	F	Rubella	Clinical
I9	240	I8	F	Viral meningitis (L.C.M. virus)	Serology
20	248	67	F	Herpes zoster	Clinical
2I	260	6	M	Infectious mononucleosis	Serology

DURATION OF ILLNESS (DAYS)	E.S.R.	W.B.C.	PERCENTAGE NEUTROPHILS	PLATELET COUNT	SHIFT TO THE LEFT	TOXIC GRANULATIONS	C.R.P. mg/%	α^1 - acid GLYCOPROTEIN mg/%	NBT Score					
									EDTA/FICOLL			HEPARIN		
									OBSERVER			OBSERVER		
									I	2	3	I	2	3
3	-	8.9	36	-	0	0	-	-	I	5	0	2	I	7
I4	-	8.5	56	-	0	0	-	-	38	36	9	50	48	I9
3	II	I4.2	60	-	0	0	-	-	4	3	9	I0	7	I
2	I9	7.6	43	2	0	I	50	-	0	3	6	I4	20	I3
30	4	5.3	50	-	0	0	-	-	4	I	4	I3	20	4
5	I0	7.I	72	-	0	0	-	-	8	3	6	I7	4	7
5	I0	7.0	74	-	0	0	-	-	2	5	6	I0	2	3
5	6	9.3	52	-	0	0	-	-	2	6	5	I4	I2	8
5	I0	7.8	52	2	0	0	-	-	4	0	3	4	8	I
5	2	6.6	54	3	0	0	-	-	2	0	I	2	3	I
5	6	7.I	50	-	0	0	-	-	2	I	3	5	5	I
5	5	9.0	68	-	0	0	-	-	0	8	4	II	6	3
5	8	6.8	60	-	0	0	-	-	0	I	6	0	2	3
5	2	9.4	68	-	0	0	-	85	0	3	3	I0	5	6
5	I7	7.6	50	-	0	0	-	-	0	0	0	8	4	5
5	2	8.2	60	-	0	0	-	-	I	0	I	I9	7	5
30	-	I0.4	62	-	0	0	-	-	0	2	I	II	7	5
2	26	5.I	52	0	2	0	-	-	4I	30	29	I5	26	I2
I	29	6.9	54	-	0	0	-	II0	I2	II	-	3	I	-
3	27	5.3	68	-	0	0	-	2I0	7	7	-	0	3	-
4	-	I0.7	72	-	0	0	-	-	25	33	-	2	7	-

APPENDIX F

Details of patients with non infective conditions (group 6)

GROUP NO.	STUDY NO.	AGE	SEX	DIAGNOSIS	DRUG THERAPY
I	3I	42	F	Fibroadenosis of the breast	Nil
2	33	4	M	Bronchospasm (acute)	Bronchodilators
3	34	2	M	Paraffin inhalation	Nil
4	36	7I	M	Haemorrhoids	Nil
5	37	64	M	Rheumatoid arthritis	Steroids, salicylates
6	4I	75	F	Multiple myeloma	Nil
7	42	36	M	Duodenal ulcer with dyspepsia	Alkalis
8	44	22	F	Pregnant	Nil
9	45	I9	F	Diabetes	Insulin
I0	47	63	F	Temporal arteritis	Nil
II	49	0.05	F	Jaundice	Nil
I2	5I	49	F	Crohn's disease	Nil
I3	58	69	F	Carcinoma of the colon	Nil
I4	60	I9	F	Crohn's disease	Nil
I5	6I	53	M	Cirrhosis of the liver with ascites	Ampicillin
I6	63	64	F	Cervical spondylosis	Diazepam
I7	68	66	M	Chronic lymphatic leukaemia	Nil
I8	69	68	F	Cervical spondylosis	Nil
I9	70	32	F	Cholelithiasis	Nil
20	7I	I9	F	Systemic lupus erythematosus	Prednisone I0 mg/day
2I	72	82	F	Multiple myeloma	Melphelan
22	73	7I	F	Multiple myeloma	Melphelan
23	74	4I	F	Hashimoto's thyroiditis	Nil
24	83	68	F	Diabetes	Insulin
25	84	40	F	Diabetes	Insulin
26	89	50	F	Post cholecystectomy. No complications.	Nil
27	90	50	F	Post cholecystectomy. No complications.	Nil
28	92	60	F	Myocardial infarct	Nil
29	93	75	F	Acute pancreatitis	Nil
30	94	27	F	Oral contraceptive	Nil
3I	99	64	M	Diabetes	Tolbutamide
32	I02	54	M	Post laparotomy. No complications.	Nil
33	I03	84	F	Carcinoma of the colon	Nil

DURATION (DAYS)	E.S.R.	W.B.C.	PERCENTAGE NEUTROPHILS	PLATELET COUNT	SHIFT TO THE LEFT	TOXIC GRANULATIONS	C.R.P. mg/%	α^1- acid GLYCOPROTEIN mg/%	NBT Score					
									EDTA/FICOLL			HEPARIN		
									OBSERVER			OBSERVER		
									I	2	3	I	2	3
> 30	-	5.9	64	-	I	I	-	-	0	0	44	33	I5	5
0.5	-	I2.7	82	-	2	I	-	-	50	28	II	58	I5	I6
I	-	I2.6	62	-	0	0	-	-	26	I2	3	29	I0	I
> 30	-	8.5	57	-	0	0	-	-	50	35	I3	40	I7	2
> 30	-	7.5	86	-	0	0	-	-	5	2	6	I5	I2	7
> 30	-	4.4	40	-	0	0	-	-	30	I0	I0	I7	I0	II
2	-	8.3	76	-	0	0	-	-	I	-	I0	2	I8	2
84	-	6.I	59	-	I	0	-	-	2	0	5	5	4	5
> 30	-	3.8	62	2	0	0	-	-	-	-	3	I0	9	9
> 30	-	9.8	66	-	0	0	-	-	-	-	7	I9	9	7
7	-	I2.5	35	-	0	0	-	-	-	-	-	39	22	23
> 30	-	9.3	82	-	2	2	9I	-	2	2	6	44	I3	35
> 30	-	8.2	82	-	I	0	-	-	3	2	3	-	-	-
> 30	-	I0.3	92	-	I	I	83	300	I9	3	3	66	I5	5
> 30	-	8.2	86	-	I	0	-	-	4	5	5	50	5	7
> 30	-	5.7	76	-	0	0	-	-	I8	II	6	0	0	0
> 30	-	I6.6	I0	-	0	0	-	-	I	I	3	I	5	2
> 30	-	5.9	4I	-	0	0	-	-	6	5	5	28	3	9
> 30	-	6.I	56	-	0	0	-	-	30	I4	2	50	I8	I3
> 30	-	7.9	74	-	0	0	-	-	2	2	I	0	0	0
> 30	-	2.7	74	-	0	0	-	-	67	33	5	I3	I2	7
> 30	-	6.9	52	-	0	0	-	-	4	4	3	II	7	I
7	-	8.I	60	-	0	0	-	-	3I	I9	6	I7	3	8
> 30	-	6.8	38	-	0	0	-	-	-	2	2	I5	9	8
> 30	-	4.7	68	-	0	0	-	-	0	0	3	8	8	2
3	7I	5.0	63	-	0	0	-	-	2I	I4	5	55	27	I0
3	43	9.0	70	-	0	0	-	-	4	I	6	2	0	I
0.5	I8	6.2	79	-	0	0	-	-	0	I	0	3	3	2
I	I4	II.8	70	-	0	0	-	-	I	I	I	I	0	4
> 30	4	8.8	66	-	0	0	-	70	3	2	0	48	I9	8
> 30	I7	5.3	68	-	0	0	-	-	I	0	5	30	20	I4
2	7	I5.4	82	2	0	0	-	-	2	6	9	4	4	6
> 30	26	8.4	73	2	0	0	-	-	3	4	I	7	2	3

Details of patients with non infective conditions (group 6)

GROUP NO.	STUDY NO.	AGE	SEX	DIAGNOSIS	DRUG THERAPY
34	I04	I8	F	Post laparotomy. No complications	Nil
35	I07	65	F	Abdominal pain, constipation	Nil
36	I08	72	M	Disseminated abdominal adenocarcinoma	Nil
37	II4	60	F	Post cholecystectomy. No complications.	Nil
38	II8	34	F	Pregnant	Nil
39	II9	52	M	Hypertension	Methyl dopa Bethanadine
40	I2I	27	F	Ulcerative colitis	Salazopyrin Codeine phosphate
4I	I22	69	F	Myelofibrosis	Nil
42	I25	34	F	Erythema nodosum, hilar lymphadenopathy	Nil
43	I27	78	F	Myxoedema, pernicious anaemia	Nil
44	I29	59	M	Rheumatoid arthritis	Indomethacin Penicillamine
45	I30	50	F	Rheumatoid arthritis	Penicillamine
46	I3I	52	M	Histiocytic medullary reticulosis*	Nil
47	I34	62	F	Adenocarcinoma colon	Nil
48	I35	62	M	Carcinoma bronchus	Nil
49	I45	40	M	Ulcerative colitis	Salazopyrin
50	I46	I9	M	Crohn's disease	Prednisone
5I	I47	69	F	Ulcerative colitis	Codeine phosphate
52	I48	72	F	Chronic lymphatic leukaemia	Nil
53	I49	32	M	Crohn's disease	Salazopyrin Prednisone
54	I50	62	M	Rheumatoid arthritis	Gold, Ibuprofen
55	I5I	43	M	Myelofibrosis	Nil
56	I52	72	F	Myelofibrosis	Nil
57	I53	52	F	Erythema nodosum	Nil
58	I56	I8	M	Crohn's disease	Prednisone
59	I57	35	F	Disseminated abdominal adenocarcinoma	Prednisone
60	I60	I3	M	Abdominal pain, constipation	Nil
6I	I75	72	M	Post hemicolectomy. No complications.	Ampicillin

DURATION (DAYS)	E.S.R.	W.B.C.	PERCENTAGE NEUTROPHILS	PLATELET COUNT	SHIFT TO THE LEFT	TOXIC GRANULATIONS	C.R.P. mg/%	α_1 - acid GLYCOPROTEIN mg/%	NBT Score					
									EDTA/FICOLL			HEPARIN		
									OBSERVER			OBSERVER		
									I	2	3	I	2	3
3	20	8.1	62	2	0	0	-	-	I0	6	6	33	I5	8
3	I8	5.7	55	2	0	0	-	-	2	9	I2	2	I	3
>30	95	2I.5	78	2	I	0	-	-	26	I9	2	I0	8	I0
4	50	4.3	47	0	0	0	-	I80	I3	I5	4	38	2I	I7
+I96	34	7.5	70	0	0	I	-	-	3	3	8	4	I0	2
> 30	I3	8.4	82	-	0	0	-	I80	2	0	4	25	23	I4
>30	5	7.7	69	2	0	0	54	-	3	0	4	40	40	50
>30	88	7.6	58	I	I	0	-	-	2	6	7	37	25	25
I4	75	9.7	72	2	0	0	I87	260	48	44	25	58	4I	2I
>30	30	6.4	64	2	0	0	-	-	3	I	5	3	6	8
>30	73	7.8	73	2	0	0	-	-	9	6	8	37	I2	23
>30	63	6.4	68	2	0	0	-	-	2	5	3	I0	II	5
>30	75	2.2	54	I	0	0	50	-	4	7	6	6	7	I0
>30	58	7.4	69	2	0	0	-	-	I2	I3	6	26	I7	II
>30	8	I7.5	76	I	I	0	-	-	I7	25	I0	I8	I7	8
>30	3	8.2	40	-	0	0	-	-	50	34	22	I0	9	8
>30	8	6.4	72	-	0	0	I0I	-	I5	II	8	2I	25	33
>30	20	8.4	80	-	0	0	42	-	I	3	4	I	7	I
>30	I4	30.0	II	-	0	0	-	-	0	I	2	0	0	5
>30	62	2I.2	94	-	2	0	-	-	4	I2	2	I7	I3	I5
>30	89	8.2	90	I	0	0	42	220	40	44	26	40	I5	I5
>30	I8	3.9	72	-	0	0	45	-	2	3	4	II	23	7
>30	33	7.0	82	-	0	0	50	-	6	9	7	65	42	53
>30	57	6.3	56	-	0	0	-	-	9	I2	9	6	0	4
>30	48	I6.2	7I	-	2	3	46	-	6	9	4	6	8	3
>30	8I	I5.2	85	-	I	I	-	-	9	6	I3	I5	9	I0
I	5	5.5	-	I	-	-	-	-	60	46	29	0	I	I
5	2I	9.6	74	2	0	0	-	-	0	I	4	I	2	I

Details of patients with non infective conditions (group 6)

GROUP NO.	STUDY NO.	AGE	SEX	DIAGNOSIS	DRUG THERAPY
62	I76	52	M	Lymphoma*, post splenectomy	Nil
63	I77	64	M	Abdominal wound haematoma	Nil
64	I8I	53	M	Chronic lymphatic leukaemia	Nil
65	I82	39	M	Crohn's disease	Salazopyrin
66	I83	35	M	Chronic lymphatic leukaemia	Chlorambucil
67	I84	25	F	Acute myeloid leukaemia	Busulphan
68	I86	72	M	Multiple myeloma	Hydrocortisone Calcitonin
69	I89	27	M	Backache (non specific)	Nil
70	I9I	I8	M	Crohn's disease	Nil
7I	I94	22	F	Pregnant	Nil
72	I95	50	F	Rheumatoid arthritis	Indomethacin Aloxiprin
73	I98	39	F	Pregnant	Nil
74	203	3I	F	Cyclical fever	Nil
75	204	46	M	Carcinoma bronchus	Nil
76	207	85	M	Benign gastric ulcer	Alkalis
77	208	I5	F	Acute myeloid leukaemia	Busulphan
78	209	73	F	Chronic lymphatic leukaemia	Nil
79	2II	82	F	Multiple myeloma	Nil
80	2I2	70	F	Chronic lymphatic leukaemia	Nil
8I	2I5	76	M	Polyarteritis nodosum	Prednisone
82	220	70	F	Ulcerative colitis	Ferrous sulphate
83	229	59	F	Scleroderma	Prednisone
84	230	69	M	Ulcerative colitis	Nil
85	232	49	F	Crohn's disease	Nil
86	235	66	F	Oesophageal stricture, dehydration	Nil
87	237	30	M	Non specific abdominal pain	Nil
88	239	65	F	Glioma	Nil
89	247	7	F	Diabetic precoma	Insulin
90	255	6	M	Diabetic precoma	Insulin
9I	256	6	F	Diabetic precoma	Insulin
92	262	I8	F	Systemic lupus erythematosus	Nil

* Same patient

DURATION (DAYS)	E.S.R.	W.B.C.	PERCENTAGE NEUTROPHILS	PLATELET COUNT	SHIFT TO THE LEFT	TOXIC GRANULATIONS	C.R.P. mg/%	α_1 - acid GLYCOPROTEIN mg/%	NBT Score					
									EDTA/FICOLL			HEPARIN		
									OBSERVER			OBSERVER		
									I	2	3	I	2	3
5	I	8.5	76	-	0	0	-	-	4	4	3	9	II	8
3	49	II.8	6I	-	0	0	-	285	6	7	I	5	I	2
>30	2	7.6	30	0	0	0	-	-	6	4	5	I2	9	9
>30	64	I0.3	84	3	0	0	65	-	II	3	6	I4	6	8
>30	I8	9.0	34	0	0	0	-	-	6	9	2	I5	I4	5
>30	40	35.7	II	0	2	I	-	2I0	I	-	-	3	-	-
>30	I50	9.I	68	2	0	0	45	220	I	4	7	I	3	5
7	9	5.6	45	I	0	0	-	-	3	5	2	23	9	5
>30	29	8.2	8I	2	0	0	62	290	8	5	5	3I	I5	7
±90	I0	9.3	73	2	0	0	-	-	3	3	4	2	3	I
>30	97	5.I	80	2	0	0	83	240	3	I	2	0	I	I
±30	5	7.0	57	2	0	0	-	-	II	8	5	4	4	9
>30	7I	7.5	60	-	0	0	-	-	24	30	I0	3	I	2
>30	64	7.6	74	-	I	0	45	-	6	6	5	2	0	2
>30	I8	6.6	56	-	0	0	-	I20	3	6	4	I4	2	I4
>30	5	3.5	65	0	0	0	-	5	4	2	I	2	I	4
>30	I3	I6.2	II	0	0	0	-	50	I	I	I	2	2	I
>30	32	3.4	75	2	0	0	44	II2	7	4	5	0	6	3
>30	30	8.4	34	2	0	0	-	I60	I0	I0	3	9	8	7
>30	58	I3.5	89	2	0	0	-	337	26	23	20	I2	8	6
>30	I6	5.0	56	2	0	0	-	I45	4I	66	50	5I	43	44
>30	83	I2.5	90	3	0	0	-	3I0	30	I7	I9	0	0	I
>30	I06	I9.4	76	-	-	-	-	-	82	72	7I	36	25	26
>30	64	9.0	63	2	I	I	-	440	48	44	34	I0	4	8
2	5	I0.7	73	2	0	0	-	I50	I4	4	-	9	6	-
I	I3	6.4	44	2	0	0	-	95	32	3I	-	8	6	-
>30	I6	6.8	64	-	0	0	-	II7	38	23	-	5	9	-
2	30	9.0	60	-	0	0	-	-	33	20	-	34	36	-
2	26	6.0	50	-	0	0	-	-	69	74	-	28	32	-
2	2	I8.4	74	-	0	0	-	-	24	I2	-	2I	32	-
>30	5	5.5	70	-	0	0	-	II2	I	0	-	5	2	-

APPENDIX G

Details of patients with undiagnosed disease (group 7)

GROUP NO.	STUDY NO.	AGE	SEX	UNCONFIRMED DIAGNOSIS
I	46	I5	F	Infectious mononucleosis
2	50	62	F	Pyrexia of unknown origin
3	54			Details lost
4	55			Details lost
5	8I	43	M	Cholecystitis
6	85	3I	F	Abdominal pain - urinary tract infection
7	I09	59	M	Pyrexia of unknown origin
8	I32	72	M	Subacute bacterial endocarditis
9	I38	36	F	Details lost
I0	I79			Details lost
II	I80	9	M	Infectious mononucleosis
I2	I88	I6	F	Infectious mononucleosis
I3	I90	74	M	Pyrexia and abdominal pain - ? cholecystitis
I4	2I8	83	M	Pneumonia
I5	223	83	M	Pulmonary consolidation - ? pneumonia ? infarction
I6	228	53	M	Pyrexia of unknown origin
I7	233	25	F	Pyrexia of unknown origin

E.S.R.	W.B.C.	PERCENTAGE NEUTROPHILS	PLATELET COUNT	SHIFT TO THE LEFT	TOXIC GRANULATIONS	C.R.P. mg/%	α^1 - acid GLYCOPROTEIN mg/%	NBT Score					
								EDTA/FICOLL			HEPARIN		
								OBSERVER			OBSERVER		
								I	2	3	I	2	3
-	I0.5	48	-	0	0	-	-	I4	I6	I6	34	I5	I0
-	I0.I	72	-	0	0	-	-	2	0	3	0	I	3
-	I5.6	90	-	2	0	-	-	I	2	I	I4	I4	9
-	9.6	50	-	0	0	-	-	2	3	3	I4	I2	6
-	5.2	20	-	0	0	-	-	4	6	0	8	0	9
-	5.I	66	-	0	0	-	-	0	4	8	24	3	2
90	I4.7	72	I	0	0	-	-	25	22	I0	II	I2	8
2I	I4.9	77	I	0	0	-	-	6	I0	8	4	0	2
7	4.2	64	2	0	0	-	-	4	5	I	4	7	3
7	7.5	-	I	-	-	-	-	37	22	II	28	24	23
II	8.6	59	2	0	0	-	-	I6	I4	I6	I	4	I
I0	5.I	52	I	0	0	-	-	I0	9	I0	23	22	8
80	9.8	87	2	0	0	-	-	23	4	5	I7	5	9
56	9.0	66	-	0	0	-	400	36	26	36	8	I0	9
59	8.0	76	3	0	0	-	-	35	4I	5I	39	2I	40
I06	I0.5	82	3	0	0	-	-	I4	7	7	8	2	I
4	5.6	50	I	0	0	-	30	I8	I5	6	7	I0	3

APPENDIX H

Details of patients with non-infective disease (group 6)

DISEASE	NUMBER
'Auto-immune' disease	
Rheumatoid arthritis	5 ...
Systemic lupus erythematosus	2
Hashimoto's thyroiditis	I
Myxoedema and pernicious anaemia	I
Polyarteritis nodosum	I
Scleroderma	I
Temporal arteritis	I
Malignant disease	
Carcinoma	
Colonic	3 ...
Bronchial	2
Disseminated abdominal adenocarcinoma	2
Glioma	I
Lymphoma	I
Haematological disease	
Chronic lymphatic leukaemia	6 ...
Myeloma	5 ...
Myelofibrosis	3 ...
Acute myeloid leukaemia	2
Inflammatory bowel disease	
Crohn's disease	8 ...
Ulcerative colitis	5 ...
Postoperative period	6 ...
Miscellaneous conditions	
Abdominal pain (non specific)	3
Backache	I
Bronchospasm (acute)	I
Cervical spondylosis	2
Cirrhosis of the liver with ascites	I
Cholelithiasis	I
Cyclical fever	I
Diabetes	4 ...
Diabetic precoma	3 ...
Duodenal ulcer	I
Erythema nodosum	I
Erythema nodosum with hilar lymphadenopathy	I
Fibroadenosis of the breast	2
Gastric ulcer (benign)	I
Haemorrhoids	I
Hypertension	I
Jaundice (physiological)	I
Myocardial infarct	I
Oesophageal stricture and dehydration	I
Oral contraceptive pill recipient	I
Pancreatitis (acute)	I
Paraffin inhalation	I
Pregnancy	4 ...
Wound haematoma	I
TOTAL :	91

Results are expressed as the mean ± S.E. NBT Score

EDTA-FICOLL SCORE SUBGROUP	GROUP	HEPARIN SCORE SUBGROUP	GROUP
... 8.8(±3.9)		.. 19.4(±6.2)	
	12.5(±3.9)		12,8(±3.5)
... 6.0(±2.4)			
	13.1(±3.8)		11.1(±2.6)
... 4.0(±1.5) .. 16.0(±11.4) ... 3.3(±1.1)	7.4(±3.9)	... 6.5(±2.4) ... 5.4(±2.4) .. 37.7(±12.7)	11.5(±4.1)
.. 12.8(±4.5) .. 35.4(±13.6)	20.9(±6.4)	.. 23.7(±6.4) .. 27.6(±8.5)	25.1(±5.1)
... 9.6(±3.3)		.. 22.2(±9.5)	
.. 42.0(±11.2)		.. 15.8(±4.3) .. 27.7(±3.1)	
... 4.8(±1.8)		... 3.8(±0.5)	

APPENDIX I

Details of NBT stimulation test on normal subjects.

GROUP NUMBER	AGE	SEX	DATE	NBT Score /ENDOTOXIN/ g/ml									
				-4 IxI0	-5 IxI0	-6 IxI0	-7 IxI0	-8 IxI0	-9 IxI0	-I0 IxI0	-II IxI0	-I2 IxI0	0
I	20	F	-	32	26	27	2I	-	-	-	-	-	0
2	I8	F	-	33	28	I3	22	-	-	-	-	-	0
3	20	F	-	I8	29	24	3I	-	-	-	-	-	0
4	I8	F	-	8	I5	9	5	-	-	-	-	-	0
5	22	M	-	-	-	-	36	33	II	2	0	0	0
6	26	M	-	I0	22	I3	-	-	-	-	-	-	0
7	25	M	-	43	26	20	-	-	-	-	-	-	0
8	7	M	-	-	-	I6	I5	20	I5	0	-	-	0
9	40	M	-	-	I4, I5	-	-	-	-	-	-	-	0
I0	22	F	-	-	-	4	-	-	-	-	-	-	-
II	22	F	-	-	-	32	-	-	-	-	-	-	-
I2	30	M	-	-	-	24	-	-	-	-	-	-	-
I3	35	M	-	-	-	I2	-	-	-	-	-	-	-
I4	25	F	-	-	-	3I	-	-	-	-	-	-	-
I5	22	F	-	-	-	23	-	-	-	-	-	-	-
I6	22	M	-	-	-	-	3I	I9	6	0	0	-	0
I7	28	M	-	-	-	40	-	-	-	-	-	-	-
I8	40	F	-	-	-	28	-	-	-	-	-	-	-
I9	35	M	-	-	-	I9	25	20	2I	I	-	-	0

20	29	M	2.I2.7I	-	-	-	43	-	25	32	-	-	0
			23.I2.7I	-	-	-	-	24	I8	2	3	2	I
			4.I.72	-	-	-	25	25	24	8	I	-	2
			2I.3.72	-	-	-	64	I7	I	0	0	-	I
			20.8.73	30	34	38	-	-	-	-	-	-	0
2I	28	M	-	-	-	-	-	I3	5	0	I	2	4
22	25	F	23.I2.7I	-	-	-	II	I2	II	0	-	-	0
			I0.3.72	-	-	22	-	-	-	-	-	-	0
23	I8	F	23.I2.7I	-	-	-	I0	4	4	3	I	0	I
			23.3.72	-	-	-	I0	5	3	I	0	-	0
24	60	M	-	-	-	-	44	33	8	I	I	-	0
25	3I	M	-	-	-	-	34	I3	7	0	I	-	0
26	I9	F	4.I.72	-	-	-	32	I2	3	0	0	-	0
			23.3.72	-	-	-	29	I0	4	0	0	-	0
27	I8	F	-	-	-	-	4I	48	I7	0	0	-	9
28	22	F	23.I2.72	-	-	-	I9	32	I5	5	7	-	7
			2I.3.72	-	-	-	64	35	I2	7	5	-	I
29	22	M	-	-	-	-	I7	I3	3	I	0	-	0
30	2I	M	23.I.72	-	-	-	I8	I2	7	3	0	-	0
			23.3.72	-	-	-	24	I4	5	2	I	-	0
3I	35	F	23.I.72	-	-	-	23	2I	24	I	0	-	0
			2I.3.72	-	-	-	23	23	2	3	0	-	0
32	45	M	-	-	-	33	3I	2	5	0	-	-	0
33	40	F	-	-	-	24	I5	3	I	0	-	-	0
34	I9	M	-	-	-	35	I9	4	0	I	-	-	0
35	34	F	-	-	-	I0	I6	I3	6	6	-	-	0
36	40	M	-	-	-	28	I4	I0	4	4	-	-	0

APPENDIX J

Details of NBT stimulation test on patients with various diseases.

Subgroup A - Patients receiving prolonged prednisone therapy.

APPENDIX NUMBER	AGE	SEX	DISEASE	PREDNISONE THERAPY (mg/day)	OTHER THERAPY	NBT Score (ENDOTOXIN g/ml)					
						-7 IxI0	-8 IxI0	-9 IxI0	-I0 IxI0	-II IxI0	0
I	40	M	Polyarteritis nodosum	60	-	23	23	8	8	4	0
2	82	F	Carcinoma cervix	20	-	6	3	0	I	2	0
3	I7	M	Idiopathic thrombocytopenic purpura	20	Cyclophosphamide	20	44	3	0	0	I
4	20	F	Wergener's granuloma	I5	-	26	27	5	5	6	0
5	54	F	Miliary tuberculosis Aplastic anaemia	I0	Isoniazid Streptomycin	44	I0	23	25	I0	I
6	54	F	Rheumatoid arthritis Amyloidosis	8	-	82	46	8	3	I	0

Subgroup B - Patients with Crohn's Disease

			TREATMENT	DURATION						
7	40	F	Tetracycline	I year	I5	I3	2	3	0	0
8	43	M	Nil		I2	28	3	4	I	0
9	64	M	Chlorpropamide	years	33	24	6	3	0	0
I0	38	M	Salazopyrin	years	27	2I	I2	3	0	0
II	30	M	Nil		24	3I	I9	I	0	0
I2	73	M	Ampicillin	2 weeks	46	35	I9	0	3	2

Subgroup C - Patients with rheumatoid arthritis receiving chronic therapy with sodium aurothiomalate

I3	55	F	23	6	I	0	0	I
I4	57	F	30	25	3	I	0	2
I5	65	F	II	II	0	I	I	4
I6	6I	F	28	I6	I0	I	I	0
I7	54	F	29	30	-	4	-	I
I8	55	F	7	I	I	6	2	4
I9	43	F	32	I5	5	0	3	I
20	64	M	53	36	I6	3	2	0

Subgroup D - Geriatric patients receiving no treatment

2I	88	F	58	34	I4	2	4	I
22	72	M	42	I8	3	0	0	I
23	92	F	30	4	5	2	I	0
24	9I	F	2I	I3	I	4	0	0
25	77	F	27	27	I0	2	0	0
26	90	F	40	40	24	2	0	0

Subgroup E - Diabetic patients

			INSULIN TREATMENT (i.u./day)						
27	52	M	34	33	28	6	I	0	I
28	49	M	22	36	32	27	0	2	I
29	26	F	56	30	2I	9	I	3	I
30	72	F	20	9	20	9	2	0	0
3I	66	F	I5	I4	I5	0	0	0	0

APPENDIX K

Details of NBT stimulation test on patients with immune deficiency disease.

GROUP NUMBER	PATIENT	AGE	SEX	HYPOGAMMAGLOBULINAEMIA	CLINICAL CONDITION	ASSOCIATED DISEASES
I	D.C.	4	M	Sex linked	Well	-
2	J.F.	I4	F	Childhood onset	Slightly unwell	Bronchiectasis
3	A.G.	I2	M	Sex linked	Very sick	Viral meningo encephalitis
					"	"
					"	"
					"	"
					"	"
4	S.G.	9	M	Sex linked	Well	Bronchitis
5	M.G.	I5	M	Sex linked	Well	Recurrent bronchitis
6	A.G.	I3	M	Sex linked	Well	-
7	G.S.	I4	M	Childhood onset	Very sick	Septic arthritis knee
					Well	Convalescent
					Well	Cured
8	D.W.	2	M	Congenital	Well	Previous septic arthritis
9	C.L.	I2	M	Sex linked	Well	-
I0	B.H.	25	M	Childhood onset	Well	-
II	H.J.	I5	M	Childhood onset	Well	-
I2	B.S.	26	M	Sex linked	Well	-
I3	A.H.	8	M	Childhood onset	Unwell	Hepatitis ? cause
I4	D.G.	I9	M	Sex linked	Well	-

[IMMUNOGLOBULIN]			NBT Score							DATE OF TEST
			[ENDOTOXIN] g/ml							
IgM	IgG	IgA mg/%	$IxIO^{-6}$	$IxIO^{-7}$	$IxIO^{-8}$	$IxIO^{-9}$	$IxIO^{-10}$	$IxIO^{-11}$	0	
<2	120	0	0	0	-	-	-	-	-	29.1.73
8	180	0	28	14	5	4	0	-	0	14.8.72
4	210	9	-	0	0	0	2	1	0	5.4.72
-	-	-	1	1	1	1	-	-	0	23.6.72
-	-	-	1	-	-	-	-	-	-	27.6.72
4	360	9	1	-	-	-	-	-	-	2.8.72
-	-	-	0	0	2	0	0	-	0	2.11.72
<2	190	0	7	4	3	0	0	-	0	14.8.72
7	360	0	35	39	8	2	0	-	0	21.8.72
7	385	102	-	27	31	10	1	0	0	21.8.72
0	385	0	-	6	8	1	0	0	0	29.12.71
<2	250	0	-	12	9	0	0	0	0	13.3.72
5	270	0	15	16	9	5	0	-	0	30.10.72
0	515	0	-	5	4	5	0	0	0	5.4.72
<2	230	0	35	19	4	0	1	-	0	20.2.73
15	0	0	18	31	16	3	2	0	0	31.7.72
4	270	0	27	31	10	1	0	-	0	31.7.72
0	440	0	25	15	5	9	2	-	12	5.9.72
200	145	0	46	39	28	11	0	-	0	18.1.73
33	900	0	-	20	5	4	0	0	0	18.1.73

GROUP NUMBER	PATIENT	AGE	SEX	HYPOGAMMAGLOBULINAEMIA	CLINICAL CONDITION	ASSOCIATED DISEASES
15	C.H.	25	M	Adolescent onset	Unwell	Borrelia
					"	Hepatitis
					Penicillin	"
					therapy	"
					Well	Nil
16	C.B.	62	M	Adult onset	Well	Bronchiectasis
17	M.H.	42	M	Adult onset	Well	-
18	M.J.	60	F	Adult onset	Well	-
19	J.W.	43	F	Adult onset	Unwell	Bronchiectasis
20	M.T.	35	F	Adult onset	Well	-
21	B.L.	30	F	Adult onset	Unwell	Recurrent bronchitis
22	R.F.	29	M	Adult onset	Well	-
23	C.C.	40	M	Adult onset	Unwell	Chronic sinusitis
24	W.A.	35	F	Adult onset	Unwell	Chronic bronchitis
25	C.E.	3	M	OTHER DISEASES Chediak-Higashi Syndrome	Well	Nil
26	K.L.	4	M	C.G.D.C.	Unwell	Nil

/IMMUNOGLOBULIN/			NBT Score /ENDOTOXIN/ g/ml							DATE OF TEST
IgM	IgG	IgA mg/%	$IxI0^{-6}$	$IxI0^{-7}$	$IxI0^{-8}$	$IxI0^{-9}$	$IxI0^{-10}$	$IxI0^{-11}$	0	
< 2	< 2	< 2	-	I	3	I	I	2	2	8.3.72
8.5	280	0	-	2	6	2	0	0	2	I3.3.72
			3	8	2	I	2	-	0	I3.6.72
			3	8	2	I	2	-	0	I7.7.72
			8	8	4	0	0	-	0	I6.9.73
I4.5	345	0	-	I7	32	8	2	2	3	8.3.72
25	400	0	34	40	I4	7	0	-	0	I4.8.72
5	I30	0	-	22	24	3	I	0	0	2.5.72
4	350	0	-	32	I9	9	I	0	0	8.3.72
0	0	0	35	35	29	24	-	-	0	I3.6.72
< 2	I80	0	-	37	I9	0	0	0	2	5.4.72
< 2	60	0	34	II	I2	2	2	0	0	I8.I.73
I2	445	0	50	46	40	4	0	-	0	I7.7.72
5	360	< 2	I6	23	I	I	0	-	0	2I.8.72
60	420	I48	43	ANTICOAGULANT EDTA (2.4mg/ml)					32	I0.8.73
			60						36	I0.8.73
			80	HEPARIN (50i.u./ml)					50	I0.8.73
			70						54	
-	-	-	0	EDTA (2.4mg/ml)					0	I0.8.73

APPENDIX L

The effect of hormones on NBT reduction by neutrophils. Results are expressed as the NBT score (%). The concentration of heparin is shown and the concentration of the additive is shown in brackets. Duplicate values are indicative of two studies.

ADDITIVE		ADRENALINE	CORTISONE ACETATE	HYDROCORTISONE HEMISUCCINATE
[HEPARIN] units/ml		I2	I2	50
NBT Score %	ADDITIVE (CONCENTRATION)	48 ($I0^{-3}_{M}$)	- ($I0^{-I}_{M}$)	0,0
		I0 ($I0^{-4}_{M}$)	0 ($I0^{-3}_{M}$)	49,47
		5 ($I0^{-5}_{M}$)	68 ($I0^{-5}_{M}$)	46,48
		4 ($I0^{-6}_{M}$)	65 ($I0^{-7}_{M}$)	52,50
		5 ($I0^{-7}_{M}$)	59 ($I0^{-9}_{M}$)	44,48
		4 ($I0^{-8}_{M}$)	- ($I0^{-II}_{M}$)	57,52
		4 ($I0^{-9}_{M}$)	-	-
	PBSA CONTROL	4 (0)	-	57,56
	+ ENDOTOXIN I0μg/ml	74 (0)	-	74,86
COMMENTS			PARTICULATE: No reduction in supernate after I8 hours at concentration of $I0^{-3}_{M}$	No reduction in supernate after I8 hours at concentration of $I0^{-I}_{M}$

HEXOESTROL PROPIONATE	NORADRENALINE	ISOPRENALINE	PROSTAGLANDIN
			E^{I} A^{I} $F^{2}\alpha$
50	50 I2	I2	I2 I2 I2
I ($I0^{-4}_{M}$) 30	3 ($I0^{-3}_{M}$) 29		2I I0 30 (IμG/ml)
44 ($I0^{-6}_{M}$) 35	22 ($I0^{-5}_{M}$) 4		23 2I 36 (I00NG/ml)
29 ($I0^{-8}_{M}$) 4I	30 ($I0^{-7}_{M}$) I4		
4I ($I0^{-I0}_{M}$) 55	54 ($I0^{-9}_{M}$) 5		
34 ($I0^{-I2}_{M}$) 40	-	-	
43 ($I0^{-I4}_{M}$) 37	-	-	
-	- -	-	
53	43 -	-	37
-	- -	-	45
	Decreased reduction in supernate at concentration of $I0^{-3}_{M}$	Direct reduction of NBT in supernate	

The effect of lysolecithin, phospholipase C, retinol, α^{1}- acid glycoprotein and fetuin on NBT reduction by neutrophils. Results are expressed as the NBT Score. Anticoagulants and heparin concentration are shown and the concentration of the additive is shown in brackets. Multiple results indicate different studies.

ADDITIVE		LYSOLECITHIN	PHOSPHOLIPASE C (0.2µg/ml causing lysis of 50% R.B.C.s mpH 7.4)	RETINOL 25mg/ml in Ethanol	
ADDITIVE (H=HEPARIN i.u./mg)		H 50		H 2	35
NBT Score	ADDITIVE (CONCENTRATION)	38 (I00µg/ml) -		3	I4
				0	I6
		36 (I0µg/ml) 57		0	25
				I	23
		43 (Iµg/ml) 37		0 5	30 72
				- 0	- 65
		35 (I00Ng/ml) 33		2 -	33 -
				- 0	- 48
		49 (I0Ng/ml) 26		0 2	23 70
				-	-
		-	-	0	63
		-	-		
	PBSA CONTROL	40		8 0,I	I 60,63
	+ ENDOTOXIN (I0ug/ml)	9I		- -	- -
COMMENTS		Lysolecithin I-I00µg/ml Phospholipase C I00Ng-I0ug/ml resulted in punctate formazan deposits which appeared to be on the outer membrane of the cell		I	I5 32
				Inhibition at higher concentration due to high Ethanol concentration (± I0mg/ml)	

RETINOL			α^{I}- acid GLYCOPROTEIN		FETUIN	
I.0mg/ml in Ethanol						
50	EDTA		H 50	EDTA	H 50	EDTA
	-	(250ug/ml)	57	26(3.3mg/ml)	39 (I0mg/ml)	22
	-	(200ug/ml)				
	-	(I50ug/ml)	42	43(I.7mg/ml)	38 (2mg/ml)	3I
	-	(I00ug/ml)				
	-	(50ug/ml)			30 (400ug/ml)	37
	-	(30ug/ml)				
	-	(25ug/ml)			2I (80ug/ml)	I0
	-	(I0ug/ml)				
	-	(5ug/ml)			43 (I6ug/ml)	2
0	0	(2.5ug/ml)				
-	-	(I.0ug/ml)				
I2	-	(0.5ug/ml)				
40	-		23	0	23	4
87	-		-	-	-	-
		I% Ethanol				

The effect of platelet clot, lactate and pH variations, sterile pus and Triton X 100 on the reduction of NBT by neutrophils. Results are expressed as the NBT Score (%). Anticoagulants and Heparin concentration are shown and the concentration of pH of the additive is shown in brackets. Duplicate values indicate two studies.

ADDITIVE	PLATELET CLOT				LACTATE		
	SUPERNATE	HOMOGENATE	SUPERNATE	HOMOGENATE			
ANTICOAG. (HEPARIN i.u./ml)	H 50		EDTA		H 50	2	EDTA
NBT Score ADDITIVE (CONCENTRATION)	32	I	4	3	I9	0	0 ($I\bar{0}^{I}_{M}$)
	-	-	-	-	II	0	0 ($I\bar{0}^{2}_{M}$)
	-	-	-	-	I9	0,2	0 ($I\bar{0}^{3}_{M}$)
	-	-	-	-	I9	I,0	0 ($I\bar{0}^{4}_{M}$)
	-	-	-	-	II	0	0 ($I\bar{0}^{5}_{M}$)
	-	-	-	-	-	-	-
	-	-	-	-	-	-	-
PBSA CONTROL	44	44	5	5	I8	I,I	0
+ ENDO. (I0ug/ml)	-	-	-	-	-	4I	23
COMMENTS		Reaction mixture clotted. NBT reduction seen in clot	No clotting				

PHOSPHATE BUFFER		STERILE PUS			TRITON X 100		
		SUPERNATE		HOMOGENATE			
H 2	EDTA	EDTA			EDTA	H 2	
0 (5.5^{pH})	0	0	($3.3x10^{-1}$ % by volume)	0	0	0.05%	0
0 (6.0)	0	0	($3.3x10^{-2}$ % by volume)	0	2	0.01%	I
0 (6.5)	0	0	($3.3x10^{-3}$ % by volume)	0			
0 (6.75)	0	0	($3.3x10^{-4}$ % by volume)	0			
0 (7.0)	0						
0 (7.25)	0						
0 (7.5)	0						
-	-	0			0		I
-	-	-			23		4I
	Excess clumping of fibrinogen in all tubes						

The effect of ADP and Cyclic AMP on the reduction of NBT by neutrophils. Results are expressed as the NBT Score (%). Anticoagulants and the Heparin concentration are shown and the concentration of the additives are shown in brackets.

ADDITIVE		ADENOSINE DIPHOSPHATE		CYCLIC AMP
ANTICOAGULANT (H=HEPARIN, i.u./ml)		H 50	EDTA	H 50
NBT Score	ADDITIVE (CONCENTRATION)	55 (Img/ml)	3	I7 (I.75µg/ml)
		48 (I00µg/ml)	-	2I (I7.5Ng/ml)
		44 (I0µg/ml)	0	3I (I75pg/ml)
		57 (Iµg/ml)	-	-
		65 (I00Ng/ml)	0	-
		54 (I0Ng/ml)	-	-
		46 (INg/ml)	0	-
	PBSA CONTROL	48	0	22
	+ ENDOTOXIN (I0µg/ml)	-	-	54
COMMENTS				

APPENDIX M

NBT scores from blood anticoagulated with heparin (5 studies), and the concentrations of ^{125}I fibrinogen and ^{35}S heparin in the plasma supernate (2 studies) after incubation with varying concentrations of NBT followed by centrifugation.

[NBT]mg/ml	NBT Score						RADIOACTIVITY IN SUPERNATE						
							γ activity (C.P.M.)				β activity (C.P.M.)		
	Subjects					Mean	Recorded	Background	Actual	Mean Actual	Recorded	Corrected for γ activity	Mean
	1	2	3	4	5								
2.0							754 470	110 112	644 358	501	620 587	513 524	518
1.0	69	16	22	46	41	39	2486 1524	115 113	2371 1411	1891	3216 3750	2823 3502	3163
0.50	59	12	5	47	50	35	4592 3286	112 112	4480 3174	3827	14190 13034	13448 12470	12959
0.25	49	9	0	13	3	15	5857 4598	110 120	5747 4478	5112	23296 18872	22368 18085	20227
0.125	1	0	0	0	3	1	6200 5742	120 134	6080 5608	5844	24742 24926	23735 23941	23838
0	0	0	0	0	0	0	6254 5284	113 90	6141 5194	5677	24976 24595	23959 23683	23821
NBT Control (No ^{125}I)											24 28		
Control (No NBT)							6544	60	6484		1074		

NBT scores from EDTA anticoagulated blood (5 studies), and concentrations of ^{125}I fibrinogen and fibrinogen in the plasma supernate (duplicate values) after incubation with varying concentrations of NBT followed by centrifugation. The fibrinogen concentration was measured by the tanned red cell haemagglutination inhibition immunoassay (Merskey, et al, 1969).

[NBT] mg/ml	NBT Score						Radioactivity in Supernate (cpm)				Radioactivity precipitated (%)	[Fibrinogen] in plasma mg/ml
	Subjects											
	1	2	3	4	5	Mean	Recorded	Background	True	Mean		
1.0	23	30	58	44	27	36	1280	107	1173			0.01
							1247	107	1140	1156	91.7	0.01
0.75	23	29	42	38	24	31	1641	107	1534			0.04
							1527	107	1420	1420	89.3	0.02
0.50	21	24	41	35	16	29	3040	107	2933			0.32
							3055	107	2948	2940	78.8	0.16
0.375	0	44	18	9	10	16	6784	107	6677			1.28
	0						6013	107	5906	6241	54.9	-
0.250	0	11	0	0	0	2	13542	107	13435			2.56
							13655	107	13548	13496	2.5	5.12
0.125	0	2	0	0	1	1	13567	107	13460			5.12
							14122	107	14015	13737	0.8	5.12
0.063	0	0	0	0	0	0	13857	107	13750			5.12
							13991	107	13884	13812	0.25	10.24
0	0	0	0	0	0	0	13878	107	13771			10.24
							14031	107	13924	13847	0	10.24

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THE MECHANISM OF THE ENTRY OF DYE INTO NEUTROPHILS IN THE NITROBLUE TETRAZOLIUM (NBT) TEST

A. W. SEGAL AND A. J. LEVI

Department of Gastroenterology, Northwick Park Hospital and Clinical Research Centre, Harrow, Middx.

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SUMMARY

1. Washed buffy-coat preparations of human blood suspended in different media were exposed to the Nitroblue Tetrazolium (NBT) and the mechanism of entry of the dye into neutrophil polymorphonuclear leucocytes was investigated.

2. Stimulation of neutrophils with endotoxin and the presence of heparin and/or fibrinogen in the suspending medium were necessary for dye reduction.

3. NBT complexed with and precipitated heparin and/or fibrinogen from solution. The percentage of cells reducing the dye and the degree of precipitation of fibrinogen and heparin depended on the concentration of dye; the critical concentration of NBT necessary for each effect was the same.

4. Electron microscopy of stimulated neutrophils revealed the presence of amorphous material, which was probably complexed NBT, outside the cell, in the process of endocytosis and within membrane-bound vacuoles within the cytoplasm.

5. Neither complement nor immunoglobulins were obligatory for dye reduction in this system.

6. It is suggested that NBT only enters neutrophils in quantities visible by light microscopy, after stimulation which produces phagocytosis of a macromolecular complex of the dye and heparin and/or fibrinogen.

Key words: infection, granulocytes, phagocytosis, fibrinogen, tetrazolium salts.

The Nitroblue Tetrazolium (NBT) test is a rapid, simple non-specific method of distinguishing bacterial, fungal and protozoal infections from other disease processes such as viral infections, autoimmune diseases, neoplasms and rejection of allografts (Park, Fikrig & Smithwick, 1968; Matula & Paterson, 1971; Anderson, 1971; Wollman, David, Brennan, Lewy, Stenzel, Rubin & Miller, 1972). The test is performed by incubating a small volume of heparinized blood with a dilute solution of Nitroblue Tetrazolium. This dye is water-soluble and light yellow in colour

Correspondence: Dr A. W. Segal, Northwick Park Hospital, Harrow, Middx. HA1 3UJ.

but forms a dark-blue water-insoluble formazan compound on reduction. When the test is performed on the blood from a normal person, a small proportion of the neutrophil polymorphonuclear leucocytes contain formazan deposits in their cytoplasm. When more than 10% of neutrophils are found to contain formazan deposits, the test is classified as positive and this strongly suggests the presence of a bacterial or other treatable infection.

The mechanisms of the entry of the NBT into the cell and of its provocation by infection are unknown. It has been postulated that the finding of formazan deposits in neutrophils is related to their phagocytic activity and that the increased metabolic activity associated with phagocytosis is responsible for the reduction of NBT to formazan (Park *et al.*, 1968). Park (1971) holds the view that a change of membrane permeability is necessary to allow the entry of NBT into the cytoplasm. The activation of complement has been implicated as a result of the decrease of sensitivity of the test when ethylenediaminetetra-acetate (EDTA) is used as anticoagulant instead of heparin (Park & Good, 1970). It has been suggested further, that immunoglobulins have a functional role because of the finding of false negative results in infected hypogammaglobulinaemic patients (Freeman & King, 1972).

METHODS

Preparation of cells

Blood samples were collected from healthy volunteers between the ages of 18 and 30 years. Blood samples from different individuals were used for each experiment.

A portion (25 ml) of heparinized blood (50 units/ml) was centrifuged at 450 *g* for 10 min at 15°C. The buffy coat (approximately 5 ml) was aspirated with a Pasteur pipette and washed three times in 50 ml of Hanks balanced salt solution at 4°C. After each wash, the suspension was centrifuged at 200 *g* for 10 min at 15°C. After the final wash, most of the supernatant was discarded and the washed cells were mixed by gentle shaking. A portion (0·4 ml) of the washed cell preparation was mixed with 0·6 ml of the suspending media.

Suspending media

The washed cells from four individuals were suspended in each of the following solutions: (i) autologous serum; (ii) autologous serum containing disodium EDTA (4·0 mg/ml); (iii) autologous serum containing mucous heparin (Weddel Pharmaceuticals) (70 units/ml); (iv) Hanks solution (Oxoid); (v) Hanks solution containing mucous heparin (10 units/ml); (vi) Hanks solution containing disodium EDTA (4·0 mg/ml). Fibrinogen (Kabi) in sodium chloride solution (0·15 mol/l) was added to aliquots of each of suspensions (ii)–(vi) to give a final concentration of 5 mg/ml. Additional studies were done with solutions (i) and (ii).

Neutrophil stimulation

To cause stimulation, 10 μg of *Escherichia coli* endotoxin (type 0127 : B8, Difco) in 0·05 ml of phosphate (0·075 mol/l)–saline (0·15 mol/l) mixture (pH 7·2) was added to a 1·0 ml sample of each of the above suspensions in a disposable plastic test tube (Luckham L/P 3S) and the tubes were incubated for 10 min at 37°C. This amount of endotoxin was used because in a dose–response study it was the minimum necessary to give a maximal response. Similar amounts of suspension were incubated without endotoxin.

Incubation with NBT

To 0·5 ml of the stimulated and unstimulated cell suspensions was added 0·5 ml of a 0·1% solution of NBT (Sigma) in phosphate (0·075 mol/l)–saline (0·15 mol/l) mixture (pH 7·2), which was centrifuged at 1000 *g* for 10 min immediately prior to use. The mixture was gently shaken and incubated at 37°C for 30 min.

Staining and counting of neutrophils

Thin smears were made on glass slides, air-dried and stained with Leishman's stain. Then 100 neutrophils were counted on each slide and those containing a densely staining formazan deposit at least the size of one lobe of the nucleus of the cell were classified as positive. All counts were performed by the same person (A.W.S.). Twenty slides were re-examined and the standard deviation of a single count (percentage of positive cells) was estimated to be 2·2.

Precipitation of fibrinogen by NBT

To 0·6 ml samples of fibrinogen (5 mg/ml) in Hanks solution with and without EDTA (4·0 mg/ml) and to plasma from blood anticoagulated with EDTA (2·4 mg/ml) was added 1·0 ml of 0·1% solution of NBT in phosphate-buffered saline. These mixtures were gently shaken and incubated in a water bath at 37°C for 30 min. After centrifugation at 500 *g* for 10 min, the deposit was washed three times with distilled water. The washed deposit was dissolved in Hanks solution by incubation in a water bath at 37°C for 30 min. The solution was then centrifuged at 1000 *g* for 10 min. Thrombin (bovine, Parke–Davis, 50 units/ml) was added to the supernatant and the mixture observed for the presence of a clot. Precipitates produced by the addition of NBT to plasma anticoagulated with EDTA were redissolved in a similar manner. Electrophoresis of these redissolved precipitates and a standard solution of fibrinogen (Kabi) in saline (5·0 mg/ml), against whole human antiserum (Burroughs Wellcome) was performed by a modified Laurell technique (Minchin Clarke & Freeman, 1968).

Precipitation of heparin by NBT

To 0·6 ml of a solution of heparin (70 units/ml) in Hanks solution and 0·6 ml of plasma from blood anticoagulated with heparin (50 units/ml) was added 1·0 ml of a 0·1% solution of NBT in phosphate-buffered saline. The solutions were examined microscopically, using ordinary and polarized light, for precipitated particles, as were control solutions of heparin in Hanks solution and plasma, and the 0·1% solution of NBT. Reduction of the solutions was performed by the addition of phenazine methosulphate (BDH) and NADH (Boehringer). To investigate whether or not the precipitates obtained above contained NBT, they were dialysed against water for 72 h using a cellulose membrane and then reduced by the addition of phenazine methosulphate and NADH and examined for blue coloration.

Measurement of the relationship between the concentration of NBT, the precipitation of fibrinogen and heparin, and the percentage of positive neutrophils

A portion (1·0 ml) of solutions of NBT in phosphate-buffered saline in concentrations of 0·1–0·0032 g/100 ml was added to 1·0 ml of whole blood anticoagulated with EDTA (2·4 mg/ml) or heparin (50 units/ml). Endotoxin stimulation, incubation, staining and counting were carried out as described above. Portions (1·0 ml) of solutions of NBT in phosphate-buffered

saline in concentrations of 0·2–0·0032 g/100 ml were added to 0·6 ml of plasma to which was added 0·03 μCi of ^{125}I-labelled fibrinogen (The Radiochemical Centre, Amersham) and to 0·6 ml of plasma from heparinized blood (50 u/ml) containing 0·03 μCi of ^{125}I-labelled fibrinogen plus 0·28 μCi of heparin [^{35}S] sulphate (corresponding to 60 μg, The Radiochemical Centre, Amersham). After incubation for 30 min at 37°C the mixtures were centrifuged at 4000 *g* for 30 min. The gamma activity in the supernatant was counted using a well-type sodium iodide detector. The samples containing no NBT gave about 14 000 c.p.m. and those with the highest concentration of NBT gave about 1200 c.p.m., the background being 20 c.p.m. The ^{35}S activity was counted in a liquid-scintillation system, the corresponding count rates being about 25 000 c.p.m. to about 1000 c.p.m. with a background count of 25 c.p.m. Each sample was counted for 100 s with a cut-off at 10 000 counts. The observed beta counts were corrected for the contribution due to ^{125}I, which amounted to 13·6% of the observed gamma counts.

Effect of centrifugation of the NBT solution on the reduction of NBT by neutrophils

A solution of 0·1% NBT in phosphate-buffered saline was centrifuged at 30 000 *g* for 16 h. A portion (1·0 ml) of this solution was added to 1·0 ml of blood anticoagulated with either EDTA (2·4 mg/ml) or heparin (50 u/ml) with and without prior stimulation with endotoxin as described above. Incubation, staining and counting were performed as above. After centrifugation the solution of NBT was examined microscopically, using ordinary and polarized light, for the presence of particles.

Electron-microscopic studies of leucocytes after exposure to NBT

A portion (1·0 ml) of 0·1% NBT was added to 1·0 ml of whole blood, using EDTA (2·4 mg/ml) and heparin (50 u/ml) as anticoagulant after prior incubation of the blood with endotoxin (10 μg/ml) at 37°C for 10 min. The mixture was incubated in a water bath at 37°C for 15 min, centrifuged at 400 *g* for 60 min and the buffy coat aspirated with a Pasteur pipette, fixed by the method of Hirsch & Fedorko (1968), embedded in Epon (G. T. Gurr and Searle Scientific Supplies, Bucks.). Thin sections were cut and examined unstained and after staining with uranyl acetate and lead citrate (Renolds, 1963) in an A.E.I. EM6B electron microscope. Control samples were prepared by excluding the NBT or endotoxin from the above mixtures.

RESULTS

Reduction of NBT by stimulated neutrophils suspended in various media

A small percentage of neutrophils were found to be NBT-positive when suspended in serum or Hanks solution with or without the addition of disodium EDTA (Table 1). Prior stimulation with endotoxin did not affect these results. The addition of fibrinogen resulted in a small increase in the percentage of positive cells in the absence of stimulation but in a marked increase with stimulated cells. In the presence of heparin, a large percentage of the neutrophils suspended in serum or Hanks solution became positive regardless of whether or not fibrinogen was added. The discrepancy between the percentage of positive cells found when stimulated cells were suspended in serum plus heparin (70 units/ml) or Hanks solution plus heparin (10 units/ml) can be attributed to the different concentrations of heparin added to the two solutions, a lower concentration being added to Hanks solution to prevent the clumping of cells caused by higher concentrations.

Precipitation of fibrinogen by NBT

Solutions of fibrinogen in Hanks solution with or without added EDTA, or plasma anticoagulated with EDTA, all yielded a precipitate after incubation with NBT. The precipitates were dissolved and subsequently formed clots upon the addition of thrombin, indicating the presence of fibrinogen. Immunoelectrophoresis of the redissolved precipitate from plasma showed that fibrinogen, identified by its electrophoretic mobility, formed the major protein component. A number of minor protein peaks were also seen. These probably result from entrapment of protein within the fibrinogen precipitate but could be due to precipitation of other proteins by NBT on a smaller scale than in the case of fibrinogen.

TABLE 1. Effects of the addition of endotoxin and/or fibrinogen to cells suspended in autologous sera and various media. Results are expressed as the percentage (mean and range) of NBT-positive cells

Additions		Suspending medium					
Endotoxin	Fibrinogen	Serum	Serum +EDTA	Serum +heparin	Hanks solution	Hanks solution+ EDTA	Hanks solution+ heparin
−	−	0 (0–3)	0 (0–1)	10 (0–37)	0 (0)	1 (0–1)	1 (0–4)
+	−	1 (0–3)	0 (0–2)	38 (9–73)	1 (0–2)	0 (0–1)	12 (0–32)
−	+	−	2 (0–2)	8 (2–121)	3 (0–7)	5 (0–10)	7 (0–11)
+	+	−	14 (7–36)	30 (18–39)	16 (10–25)	28 (18–45)	50 (39–58)
No. of experiments		10	6	4	4	4	4

Precipitation of heparin by NBT

Numerous small precipitated particles could be seen on microscopy with both ordinary and polarized light after the addition of NBT to the solution of heparin. These particles developed a dark-blue colour upon reduction.

Demonstration of NBT in the precipitates of fibrinogen and heparin

Unreduced NBT was found to be freely dialysable across a cellulose membrane. After dialysis against water for 72 h the precipitates of fibrinogen and heparin were incubated with a reducing solution. The development of a deep-blue coloration of these precipitates indicated the presence of formazan. Thus NBT was not dialysed off the precipitates.

Effect of decreasing the concentration of NBT on the precipitation of fibrinogen and heparin and on the reduction of NBT by stimulated neutrophils

In EDTA anticoagulated blood and plasma, concentrations of NBT greater than 0·05% caused almost total precipitation of fibrinogen and resulted in a significant percentage of stimulated neutrophils becoming NBT-positive. Concentrations of NBT of 0·025% or less resulted in little fibrinogen precipitation or NBT reduction by neutrophils. The critical con-

centration of NBT necessary for reduction by a significant percentage of neutrophils and for marked fibrinogen precipitation was similar (Fig. 1). In heparinized plasma, NBT caused the precipitation of both fibrinogen and heparin. The precipitation of fibrinogen and heparin and the percentage of positive cells were decreased in parallel by dilution of the dye (Fig. 2).

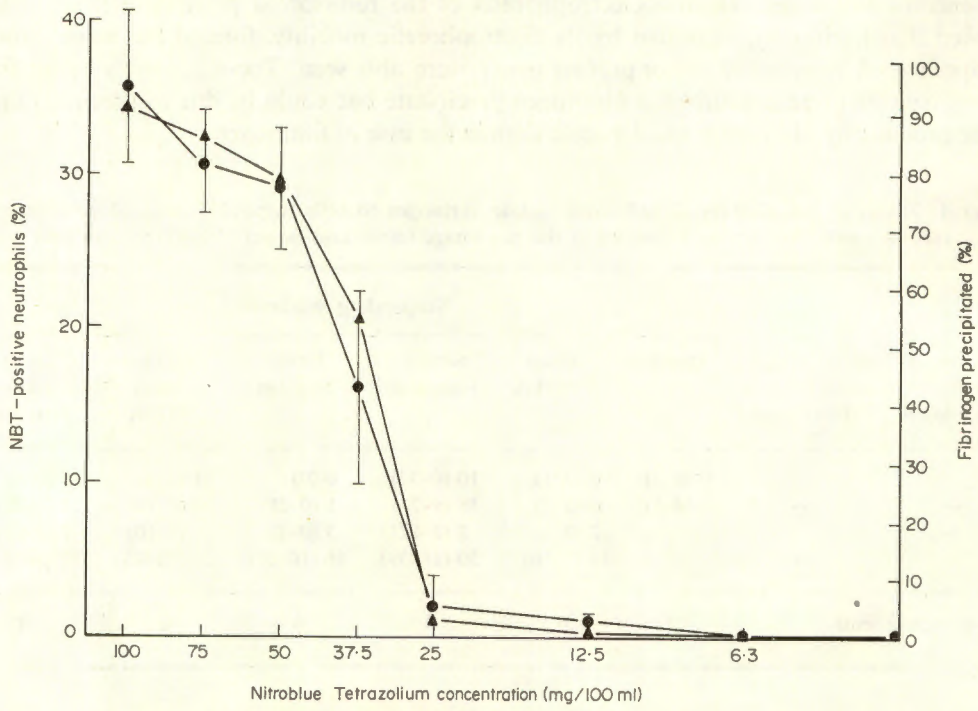

FIG. 1. Relationship between the percentage of NBT-positive neutrophils in blood anticoagulated with EDTA and stimulated with endotoxin (●, mean of five studies ±1 SEM) and the percentage of ^{125}I-labelled fibrinogen precipitated from EDTA-treated plasma upon the addition of varying concentrations of dye (▲, mean of duplicate values shown).

Effects of centrifugation of the NBT solution

No particulate matter could be detected on microscopic examination of the solution of NBT after centrifugation at 30 000 *g* for 16 h. Use of this NBT solution did not decrease the percentage of NBT-positive neutrophils in stimulated blood when compared with solutions of NBT prepared in the standard manner.

Electron-microscopic studies of leucocytes after exposure to NBT

Membrane-bounded vacuoles, containing densely staining amorphous material, were found in the cytoplasm of a large proportion of the monocytes and neutrophils from blood exposed to both NBT and endotoxin. Similar amorphous less densely staining material was also seen both extracellularly and undergoing phagocytosis (Fig. 3).

The neutrophils appeared swollen and their outer membranes were partially ruptured. The

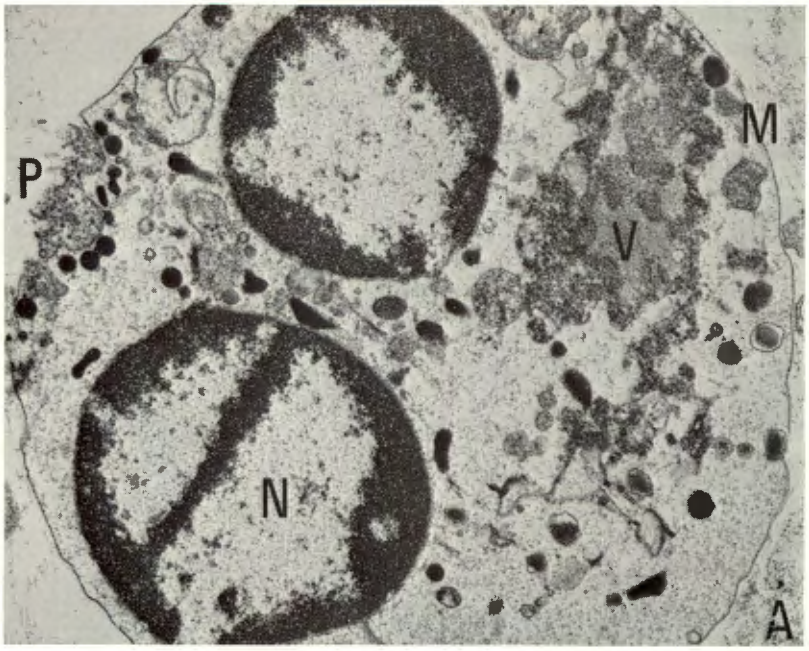

FIG. 3. Stained electron micrograph of a neutrophil after exposure to NBT and endotoxin, showing membrane-lined vacuoles containing amorphous material (V), amorphous material outside the cell (A) and undergoing phagocytosis (P), nucleus (N) and outer membrane (M). Magnification $\times 12\,000$.

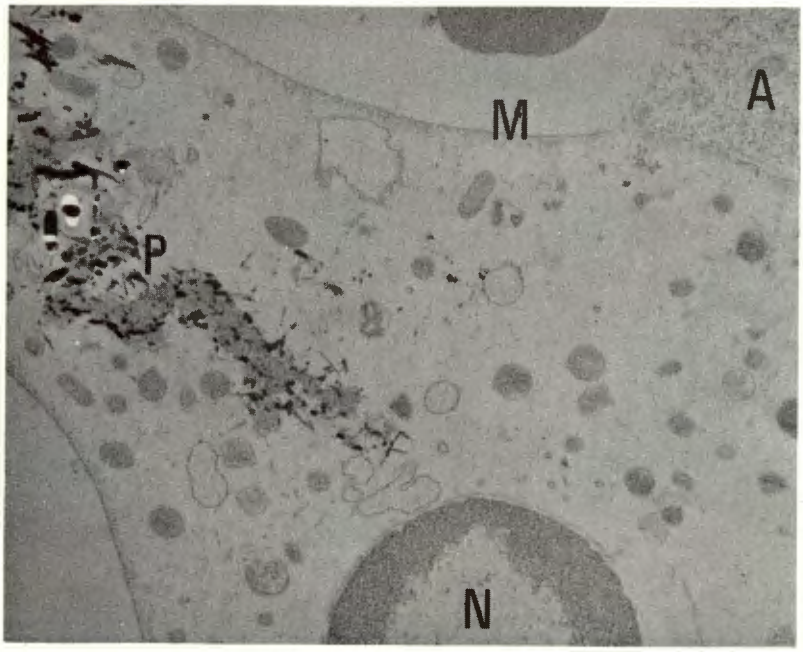

FIG. 4. Unstained electron micrograph of a neutrophil after exposure to NBT and endotoxin showing amorphous material outside the cell (A), amorphous material containing electron-dense particles within the cell (P), nucleus (N) and outer membrane (M). Magnification $\times 12\,000$.

(*Facing p.* 822)

amorphous material was seen purely extracellularly in blood exposed to NBT alone but was not seen after exposure to endotoxin in the absence of NBT. Neither cellular swelling nor abnormalities in the outer membrane of neutrophils were found in cells that were not exposed to a combination of both endotoxin and NBT. Unstained sections showed the amorphous material to contain electron-dense, elongated, somewhat needle-shaped particles approximately 150–500 nm in length. These particles were most numerous when the amorphous material was within phagocytic vesicles and could represent formazan deposits (Fig. 4).

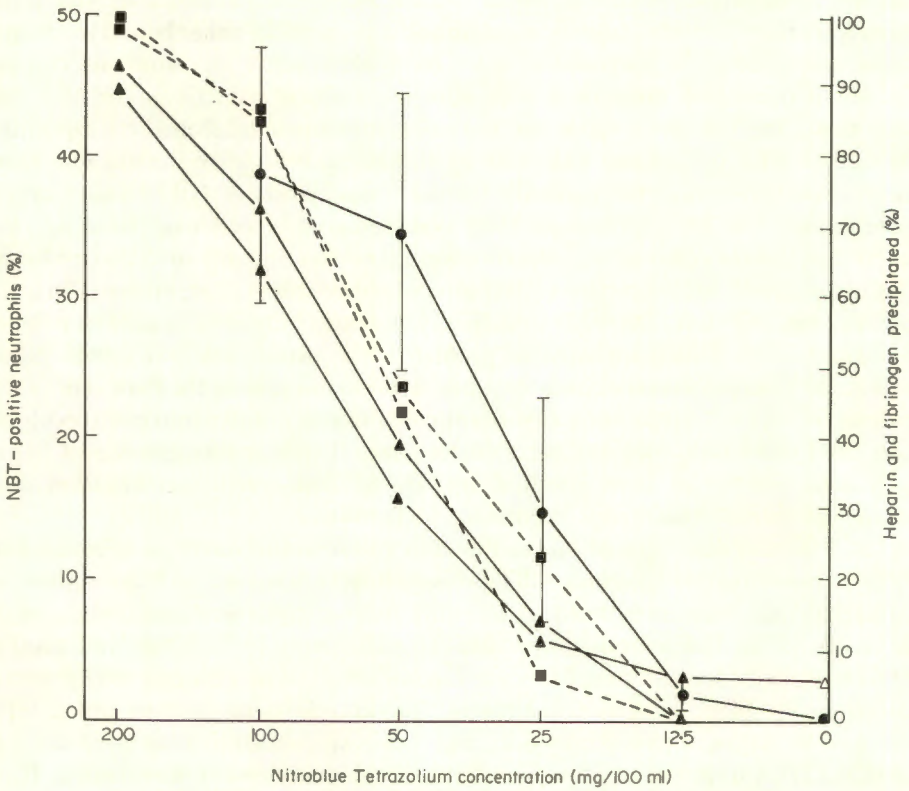

FIG. 2. Relationship between the percentage of NBT-positive neutrophils from heparinized blood after endotoxin stimulation (●, mean of five studies ±1 SEM) and the precipitation of ^{125}I-labelled fibrinogen (▲) and ^{35}S-labelled heparin (■) from heparinized plasma (individual experiments) upon the addition of varying concentrations of NBT.

DISCUSSION

The availability of a rapid indicator of bacterial infection has important clinical application. It gives direction to further lines of investigation and helps safeguard against indiscriminate therapeutic manoeuvres. An understanding of the mechanism of such a test delineates exactly what it is that is being measured and makes the interpretation of the results obtained more

logical. In order to investigate this a model system was used. This system is basically similar to the standard NBT test, but differs in that endotoxin was used as an artificial stimulus of neutrophil activity.

The present study shows that NBT has a dual action. It causes the precipitation of a macromolecular complex, the nature of which depends upon the anticoagulant used. In the case of heparin, both heparin and fibrinogen are precipitated, whereas in the presence of EDTA, fibrinogen alone is precipitated. The cationic precipitation of heparin (Scott, 1968) and fibrinogen (Surgenor, 1952) have been described and although precipitation of these substances by NBT has not been investigated, this would seem a likely mechanism. The NBT could not be dialysed from the macromolecular precipitate and it is therefore either bound to or enmeshed within this precipitate. Phagocytosis of the precipitated material results in concomitant phagocytosis of dye which turns dark blue after intracellular reduction. The blue colour is easily seen under the light microscope and the cell containing it is labelled NBT-positive.

The failure of NBT uptake and reduction by stimulated cells when the dye is presented to them in solution emphasizes the necessity for the phagocytosis of NBT in association with particulate matter. The phagocytosis of these complexes with their associated dye explains how the NBT enters the cell, and why the formazan 'granules' assume such bizarre formations within the cytoplasm. The neutrophils cluster around particulate matter, probably in attempted phagocytosis, and this clumping leads to difficulty in making accurate counts of positive cells in a blood smear. This is the main technical problem in the test of Park *et al.* (1968). NBT tends to crystallize out of solution in the concentrations in which it is used in the Park *et al.* (1968) test. Phagocytosis of NBT crystals is not a significant mode of entry of dye into neutrophils because significant NBT reduction does not occur in the control tests in the absence of heparin or fibrinogen and removal of these residual crystals by high-speed centrifugation does not decrease the number of neutrophils becoming NBT-positive.

When the NBT test is performed on the blood of patients with bacterial infection a smaller percentage of the neutrophils become NBT-positive if EDTA is used as anticoagulant instead of heparin. This has been interpreted as resulting from complement inactivation by EDTA (Park & Good, 1970). The demonstration that the addition of ficoll to the NBT solution can reverse this effect (Gordon, Rowan, Brown & Carson, 1973), that a normal NBT response can be obtained in the blood of C6 (complement fraction)-deficient rabbits and C4-deficient guinea-pigs (A.W. Segal, unpublished work), and in cells suspended in a balanced salt solution, suggests that EDTA does not mediate its effect through complement inactivation. It is more likely that the nature of the precipitates presented to the neutrophils for phagocytosis determines the degree of dye reduction.

Similarly, as washed cells can reduce NBT when suspended in Hanks solution in the presence of heparin, it is unlikely that soluble immunoglobulins are essential for NBT reduction. Although these experiments do not completely exclude the possibility that adsorbed immunoglobulins play a role, in another study, using similar methods, it was found that in only four of twenty hypogammaglobulinaemic patients was a negative NBT test obtained after endotoxin stimulation *in vitro*. The reduction of NBT was unrelated to the pattern of immunoglobulin deficiency and occurred despite immeasurably low (less than 2 mg/100 ml) serum levels of IgG, IgA or IgM (immunoglobulins G, A and M respectively). When neutrophils from those patients in whom impaired NBT reduction was observed were suspended in normal plasma the deficient reduction was not corrected. Nor was NBT reduction decreased in normal cells

suspended in their hypogammaglobulinaemic plasma (Segal & Webster, unpublished work). These facts imply that the primary defect of NBT reduction by the neutrophils of hypogammaglobulinaemic patients is cellular rather than humoral.

A positive NBT test depends upon enhanced phagocytic activity of neutrophils. This may result from the presence of bacteria, or products thereof, in the peripheral circulation (Cohn & Morse, 1960). The minimum concentration of *E. coli* endotoxin necessary to produce a positive test *in vitro* in this model system is 1 ng/ml (Segal, unpublished work), which is in the concentration range found in endotoxaemia *in vivo* (Levin, Poore, Zauber & Oser, 1970; McGill, Porter & Kass, 1970). Circulating bacterial products could be the natural stimulus of phagocytosis that is demonstrated by a positive NBT test in patients with bacterial infection. The percentage of positive cells never reaches 100% under any of the experimental circumstances investigated even when a large excess of endotoxin was used *in vitro*. This raises the possibility that there may be more than one functional population of neutrophils.

The conversation of a neutrophil into an NBT-positive cell encompasses a wide spectrum of neutrophil function, from the ability to be stimulated to phagocytic activity, through phagocytosis, to reduction of the dye by functional intracellular reductase enzyme systems. Thus an *in vitro* stimulation test is a good screening test of normal neutrophil function, and quantitation of such a test has enabled the demonstration of partial defects of neutrophil function in a series of patients (Segal, unpublished work).

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RE-EVALUATION OF NITROBLUE-TETRAZOLIUM TEST

A. W. SEGAL SHEILA F. TRUSTEY
A. J. LEVI

Divisions of Clinical Investigation and Hospital Infection, Northwick Park Hospital and Clinical Research Centre, Harrow HA1 3UJ, Middlesex

Summary The nitroblue tetrazolium (N.B.T.) test has been widely recognised as a useful method of distinguishing between pyogenic bacterial infection and other disease processes. This test, done by two different methods, has been re-evaluated on 223 individuals including healthy controls and patients with a wide spectrum of disease. There was a wide overlap in the results obtained from controls, patients with pyogenic infection, and patients with other diseases; and the test had no diagnostic relevance. The N.B.T. result in patients with pyogenic infection was found to correlate with the severity of the illness. There was poor agreement between the results obtained from the two methods used for the test. Significant observer error was found in interpretation of the results and this was related to observer experience. The test has not upheld its early promise as a simple accurate method of distinguishing bacterial infection from other disease processes.

Introduction

THE nitroblue tetrazolium (N.B.T.) test was developed in 1968 by Park et al.[1] as a non-specific in-vitro diagnostic test for bacterial infection. Heparinised blood is incubated with a dilute solution of N.B.T., and the percentage of neutrophils containing deposits of dark-blue formazan, the reduced product of N.B.T., within their cytoplasm is counted. In patients with bacterial or fungal infections the percentage of neutrophils containing formazan, called N.B.T.-positive cells, was greater than 10%; in healthy controls and patients with non-pyogenic diseases the percentage of N.B.T.-positive cells was less than 10%. These findings have been confirmed in children[2,3] and adults,[4,5] and the list of diseases resulting in a positive test has been

expanded to include malaria [6] and helminthic [7] and *Mycoplasma* infections.[8] Occasional false-negative results have been recorded, and these have been attributed to an inability of the neutrophils to respond to the stimulus of infection. To distinguish true and false-negative results an in-vitro stimulation test, using *Escherichia coli* endotoxin, was developed [9] to indicate the ability of neutrophils to respond to stimulation.

One of the technical difficulties of the test, as described by Park, is the tendency of neutrophils to clump around particles, since shown to be precipitated fibrinogen and heparin.[10] This makes identification and counting difficult. Clumping can be reduced by the use of ethylenediamine tetra-acetic acid (E.D.T.A.) instead of heparin as anticoagulant, but this reduces the sensitivity of the test; Gordon et al.[11] showed that the addition of the sucrose polymer 'Ficoll' to the incubation mixture prevented this loss of sensitivity and clumping of the cells.

It thus appeared that a very powerful tool for the specific diagnosis of pyogenic infection had been developed and that a major technical difficulty in its application had been circumvented.

False-negative tests on patients with major [12–14] and with minor bacterial infections [15] and false-positives on patients with viral meningitis,[16] neonates,[17] recipients of oral contraceptives,[18] and various disease processes [8,13] have been recorded.

We have attempted to clarify the value of the N.B.T. test as a diagnostic test of pyogenic infections, by doing it in two ways using modifications of the methods of Park et al. and of Gordon et al. on blood from 197 patients with a wide spectrum of disease, and on 26 healthy controls. The findings were correlated with the total white-blood-cell-count, neutrophil-count, erythrocyte-sedimentation rate (E.S.R.), and the serum concentration of C-reactive protein (C.R.P.).

Patients, Controls, and Methods

Patients and Controls

The numbers, ages, sex, and classification of the patients and controls are shown in table 1.

Group 1.—Healthy hospital staff.

Groups 2 and 3.—Patients with pyogenic infection. Those in group 3 had received antimicrobial chemotherapy at the time of the test; 4 patients had received therapy for longer than 1 week; the mean period of

TABLE I—DETAILS OF PATIENTS AND CONTROLS

—	Patients and controls studied							
	Healthy controls (1)	Untreated pyogenic infection (2)	Treated pyogenic infection (3)	Tuberculosis (4)	Non-bacterial infection (5)	Non-infective disease (6)	Undiagnosed disease (7)	Total
No.	26	40	26	7	21	91	14	225
No. of males	9	20	14	3	5	37	8	96
Mean age (yr.)	33·3	41·5	37·9	35·7	31·2	48·5	50·8	42·4
s.d. of age (yr.)	15·3	24·9	20·2	22·2	16·6	22·2	24·7	21·3

treatment for the remainder was 2·2 days. The criteria for inclusion of patients in these groups was the clinical diagnosis supported by either bacteriological identification of the organism, in 38 of the patients, the detection of significant quantities of pus in patients with abscesses, or the histological picture of acute suppuration in appendicitis. These patients were clinically subdivided into three grades of severity according to the systemic manifestations of the disease process. These were absent or minimal in patients classified as grade I, which included patients with small localised abscesses of postoperative wound sepsis. Grade-III patients were severely ill with infections of the order of severity of septicæmia or liver abscess. Grade-II patients had illnesses of intermediate severity.

Group 4.—Patients with bacteriologically proven active tuberculosis.

Group 5.—Patients with non-bacterial infections: 1 with hepatic schistosomiasis, 1 with massive ascaris infestation, and 19 with viral disease of which 16 were confirmed serologically; a clinical diagnosis was made in 2 cases of rubella and in 1 of herpes zoster.

Group 6.—Hospital patients with a diverse range of non-infective diseases.

Group 7.—14 patients in whom bacterial infection was suspected but not proven.

2 patients were included in group 2 and in group 6.

N.B.T. Tests

Blood was taken by venepuncture into a disposable plastic syringe and 1·0 ml. was transferred to plastic test-tubes containing 0·05 ml. of a heparin solution containing 1000 I.U. per ml. (50 I.U. per ml.) and 5·0 ml. to glass bottles containing 12 mg. of disodium E.D.T.A. solution (2·4 mg. per ml.). All tests were done immediately or within 1 hour of venepuncture after storage of blood samples at 4°C.

For the test with heparinised blood, 0·2 ml. was incubated at 37°C for 10 minutes in a disposable plastic test-tube (Luckham LP/3S). 0·2 ml. of a 0·1% solution of N.B.T. (Sigma) in phosphate-buffered saline, pH 7·2, was then added. The mixture was gently shaken, then incubated at 37°C for 15 minutes, then at room temperature (±25°C) for another 15 minutes. The sample was applied to glass slides by running a drop of the test mixture down the slide as described by Wollman et al.[19] This method is here referred to as the heparin method.

The E.D.T.A. blood was tested as above, except that the N.B.T. solution contained ficoll (Pharmacia, 200 mg. per ml.); the mixture was incubated at 37°C for 30 minutes and then at room temperature for 15 minutes; and the sample was applied to glass slides by smearing. This

method is referred to as the E.D.T.A./ficoll method.

Smears were air-dried and stained with Leishman's stain (British Drug Houses).

Counting

The smears were counted independently by three observers who did not know the patient details; one (observer 1) was very experienced (having previously counted 3000 such preparations), one was relatively experienced (observer 2), and the third had no previous experience with this test (observer 3). One hundred neutrophils were counted on each slide; cells containing formazan deposits at least the size of one lobe of the nucleus were recorded as N.B.T. positive and the percentage of N.B.T.-positive cells was designated as the "N.B.T. score". The 45 slides in which the greatest counting difference between the two more experienced observers was obtained were recounted by both observers to obtain an artificially correct result for comparison with other data. 20 slides prepared by the heparin method and 20 prepared by the E.D.T.A./ficoll method were randomly

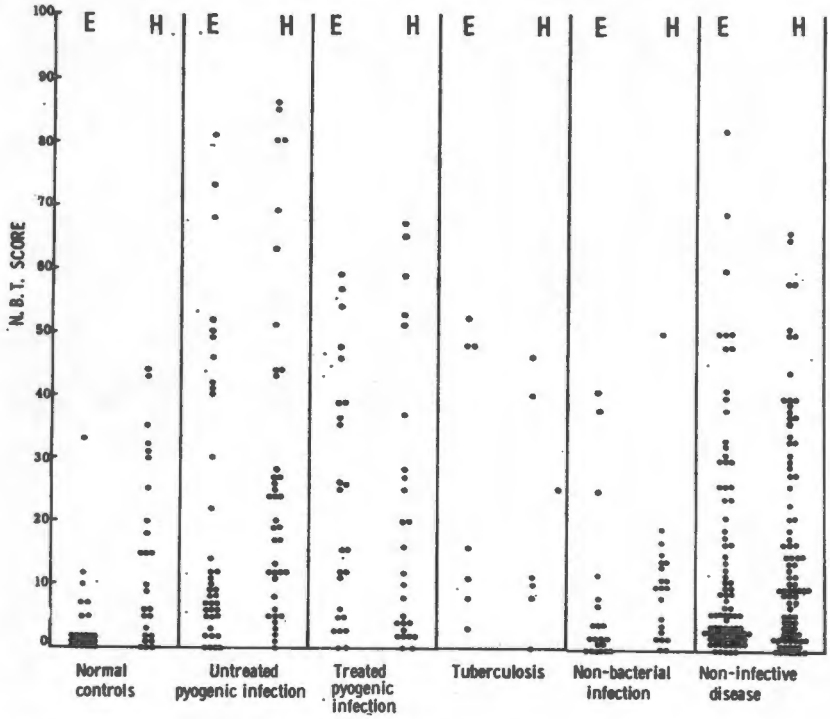

Fig. 1—N.B.T. scores obtained with E.D.T.A./ficoll (E) and heparin (H) methods.

TABLE II—THE EFFECT OF SEVERITY OF ILLNESS ON N.B.T. SCORES IN BACTERIAL INFECTIONS: MEAN SCORES (ANGULAR TRANSFORMATIONS) WITH STANDARD ERROR SHOWN

—	Severity of illness					
	Grade 1		Grade 2		Grade 3	
	No.	N.B.T. score	No.	N.B.T. score	No.	N.B.T. score
Untreated pyogenic infection						
E.D.T.A./ficoll method	23	19·8 (±3·4)	13	25·4 (±4·5)	3	34·6 (±9·4)
Heparin method	22	25·1 (±3·3)	15	31·8 (±3·9)	3	47·6 (±8·8)
Treated pyogenic infection						
E.D.T.A./ficoll method	11	26·0 (±4·9)	9	23·3 (±5·4)	5	30·7 (±7·3)
Heparin method	11	14·5 (±4·6)	10	21·1 (±4·8)	5	42·2 (±6·8)

selected and recounted to assess the observer error.

The scores obtained by observer 1, modified in the light of observer 2's results, were regarded as the test result for intergroup comparison and for relating to the other measurements.

Other Investigations

The cell-counts were performed on a Coulter counter model S. Blood-smears were examined by an experienced technician. The E.S.R.s were determined by the Westergren method in disposable plastic tubes ('Dispette'). Serum-C.R.P. was measured by the radial immunodiffusion technique on 'Partagen' plates (Boehringer) on 18 patients from groups 2 and 3 and 18 patients from group 6.

Results

The controls gave consistently low N.B.T. scores by the E.D.T.A./ficoll method. In all other groups there was considerable overlap of the N.B.T. scores (fig. 1). Consistently high scores were obtained in patients from group 6 with inflammatory bowel disease, erythema nodosum, myelofibrosis, and diabetic precoma.

A comparison of the clinical assessment of severity of illness of patients in groups 2 and 3 with the N.B.T. scores showed an increased N.B.T. score with increasing severity. This was more striking for the heparin than the E.D.T.A./ficoll method (table II).

The total number of N.B.T.-positive neutrophils per c.mm. (i.e., N.B.T. score × neutrophil-count) showed even less group specificity than the N.B.T. score. Attempts at the separation of patients with bacterial infection from other groups by the use of nomograms constructed by Feigin et al.[2] did not result in greater accuracy in the interpretation of the results of the N.B.T. tests.

The discrepancies between duplicate counts tended to be smaller when the counts were themselves small, and to counteract this the counts were transformed to angles before (Fisher and Yates, table X^{20}) analysis. In these units the error variances were 20·5 and 15·1, and 17·6 and 20·3 for counts performed by observers 1 and 2 on slides prepared by the E.D.T.A./ficoll and heparin methods, respectively. The average variance is 18·4, corresponding in the original units to a standard deviation of $\pm 7{\cdot}5\%$ at a count of 50%. After allowing for counting errors the correlation between observers 1 and 2 was very high (figs. 2 and 3), estimated at 1·00 and 0·99 for preparations made by the

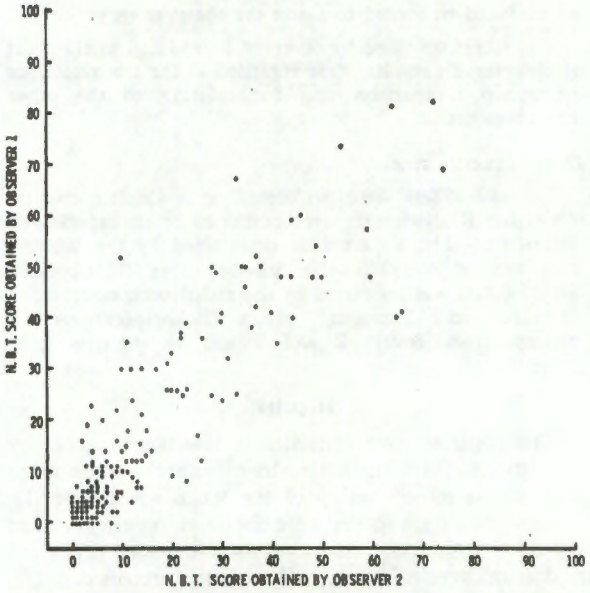

Fig. 2—E.D.T.A./ficoll method: relation between duplicate counts obtained by two experienced observers.

E.D.T.A./ficoll and heparin methods, respectively. Despite these close correlations there was an inter-observer difference in the absolute results obtained, observer 1 consistently counting higher than observer 2 on the preparations from the heparin tests. There was closer agreement of the counts performed on slides made by the E.D.T.A./ficoll method (figs. 2 and 3). There was only a low correlation between the scores obtained from the two methods performed on the same individual (fig. 4). Using angular transformations we made comparisons between the scores obtained by observer 1 and observer 3. The correlation coefficients were 0·32 and 0·57, and 0·58, and 0·53 for the first 100 and last 100 scores by the E.D.T.A./ficoll and heparin methods, respectively. Thus the counting error of observer 3 is reduced with experience with regard to the E.D.T.A./ficoll but not the heparin preparations.

Comparisons were made between the N.B.T. score obtained by both methods of performing the test and the E.S.R., and white-blood-cell and neutrophil counts in the various groups. Group 4 was excluded because of small numbers. Apart from the correlation coefficients of 0·40 and 0·45 ($P<0·01$) obtained in group

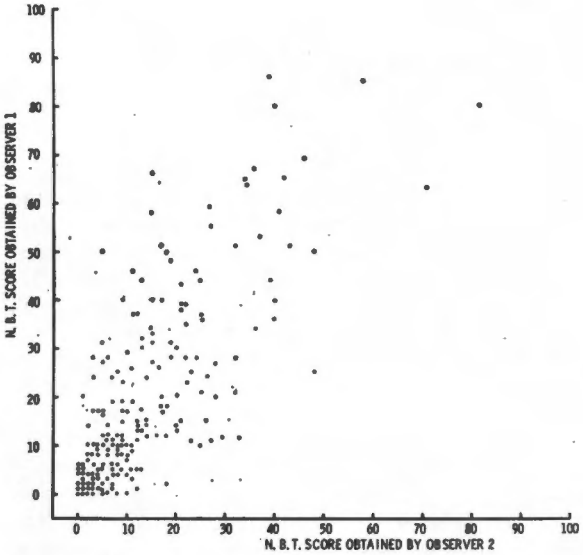

Fig. 3—Heparin method: relation between duplicate counts obtained by two experienced observers.

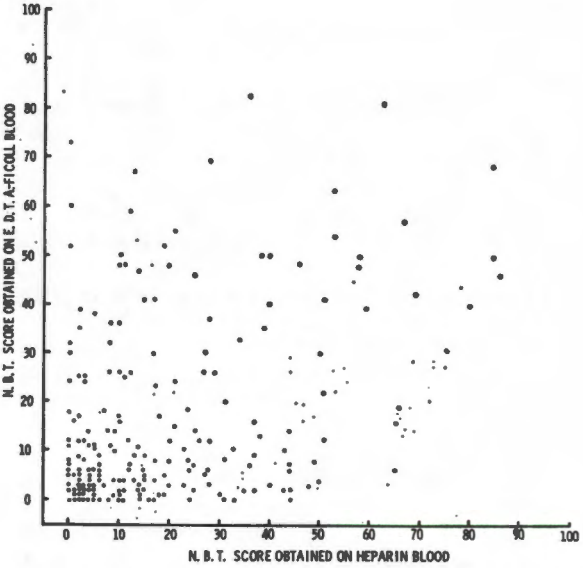

Fig. 4—Relation between the N.B.T. scores obtained when the test was done simultaneously on the same individuals by the E.D.T.A./ficoll and heparin methods.

2 between the heparin scores and the W.B.C. and neutrophil counts, respectively, there was no correlation between the N.B.T. score and other variables. No correlation was obtained between the N.B.T. scores and the serum-C.R.P. concentration.

Discussion

To have broad clinical application a diagnostic test should give few false-negative or false-positive results. This is not our finding with the N.B.T. test. There was a wide overlap in the results from patients with pyogenic infection—whether or not antimicrobial treatment had commenced—in non-pyogenic infection, and in non-infective disease. In addition, a wide range of values was obtained in healthy controls when the test was done with heparinised blood. False-positive tests were seen with inflammatory bowel disease, non-pulmonary tuberculosis, and, as previously described,[13] in myelofibrosis. High results were also found in children with acute non-inflammatory diseases such as acute bronchospasm, diabetic precoma, and paraffin inhalation. This finding, the false-negative results in patients with localised sepsis, and the positive correlation between the N.B.T. score and the white-blood-cell and neutrophil counts in patients with untreated pyogenic infection suggests that the test may reflect an acutely stressful situation rather than its underlying cause. If so, false-negative results might be expected in critically ill patients with pyogenic infection, as has been recorded in fatal meningitis.[13] Abnormalities of neutrophil function are known to occur in overwhelming infection,[21] and the possibility that neutrophils become refractory to similar repetitive stimuli is being investigated. The presence of defective neutrophil function secondary to infection would account for the false-negative results recorded by Park and Good, in which the "N.B.T. test stimulated" was found to be negative.[9] No such failure of stimulation was found by us when the test was done on the blood from 50 healthy individuals.

Immune complexes decrease N.B.T. reduction by neutrophils [22] and could cause the false-negative results obtained in systemic lupus erythematosus and meningococcal meningitis, both diseases in which circulating immune complexes are known to occur.[23] Wollman et al.[19] found the N.B.T. test to be useful in patients after renal transplantation, uniformly low

results being found in patients with rejection, whereas infected patients gave high results. It is possible that the low results obtained in the patients with the rejection phenomena were the more abnormal, resulting from a secondary abnormality of neutrophil function, induced by immune complexes, which inhibited the high N.B.T. result we have found in acutely ill patients.

The difference between our results and those of other studies is difficult to explain. It may be partly due to different methods of patient selection, but the wide range of results found in healthy controls when the test is done on heparinised blood indicates that some other factor is involved.

In many cases the two tests gave different results. This suggests a basic difference in the mechanism underlying the two tests. The dye enters stimulated neutrophils complexed with precipitated heparin and/or fibrinogen,[10] so the difference in the mechanism of the two tests is likely to lie in the interaction of neutrophils with these complexes.

The counting error has not been previously commented upon. The result recorded by any one observer is subjective and depends on the interpretation not only of the amount of formazan within a cell necessary to qualify the cell as N.B.T. positive, but also the interpretation of whether or not a cell is in fact a neutrophil; large deposits of formazan within a cell may make identification difficult. Taking into account the individual counting error and the subjective nature of counting, observations that N.B.T. counts above 10% are universally positive[5] seem unfounded. Our results also indicate that experience is important for an accurate assessment of the result and that the test is not suitable for irregular performance by untrained staff.

The N.B.T. test is valuable in the investigation of neutrophil physiology and function and may be of value in specific clinical situations[19,24] and in monitoring the clinical course of patients in whom the pretreatment status of the test is known.[25,26] However, as a simple specific test for pyogenic infection in adults it does not seem to have fulfilled its early promise because of the large overlap of results with those obtained in healthy people and in patients with diseases other than pyogenic infection, both microbial and non-microbial, and because of the variation between the results of different observers.

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Short Communication

NITROBLUE TETRAZOLIUM — A NEW LIPOPROTEIN STAIN

A. W. SEGAL, D. PUTMAN AND H. G. MINCHIN CLARKE
Clinical Research Centre, Watford Road, Harrow, Middlesex, HA1 3UJ (Great Britain)
(Received June 4th, 1973)

SUMMARY

The di-formazan of nitroblue tetrazolium (NBT) is presented as a new, specific pre-stain for lipoproteins. NBT requires no organic solvent and is shown to be effective in agarose, cellulose acetate and polyacrylamide gel. When added in the correct concentrations, NBT does not appear to alter the electrophoretic migration or antigen antibody reaction of the lipoproteins.

Key words: *Disc electrophoresis – Laurell electrophoresis – Lipoprotein pre-stain – Monorocket immunoelectrophoresis – Nitroblue tetrazolium*

The lipoproteins, concerned with transport of fats and fat-soluble substances, may be separated and quantitated by ultracentrifugation but for clinical investigation the simpler, less expensive, electrophoretic separation techniques have been exploited.

Accurate characterisation depends upon combining a separation technique giving good resolution with a sensitive indication of the various components. In this study it will be shown that the di-formazan of nitroblue tetrazolium (NBT), which needs no organic solvent, stains the lipoproteins a dark blue-purple colour. NBT, when added to serum samples prior to electrophoresis, gives satisfactorily stained lipoprotein bands or precipitates in each of the media tested and does not appear to alter the electrophoretic properties of the lipoproteins when added in the correct concentrations.

MATERIALS AND METHODS

Blood samples from normal subjects were allowed to coagulate at room temperature and electrophoresis of the separated serum was performed as soon as possible. To 1 ml of each serum was added 0.5 ml of a solution of NBT (Sigma) in water, in concentrations from 0.05% to 0.80% and 0.5 ml of an aqueous solution (10 mg/ml) of the reduced form of nicotinamide adenine dinucleotide (NADH (Boehringer). Unless otherwise described, a concentration of 0.15% NBT was used. After gentle mixing, reduction of the NBT was brought about by the addition of approximately 100 μg of solid phenazine methosulphate (B.D.H.). This will be referred to as a "pre-stained" sample. Unadulterated sera were used as control samples except in immunoelectrophoretic systems where the serum was diluted with an equal volume of physiological saline.

The efficiency of reduced NBT as a pre-stain for lipoproteins was tested on four electrophoretic systems. In three, the pre-stained sample was run together with the control sample, the lipoproteins in the latter being stained with Sudan black (saturated solution in 60% ethanol) after electrophoresis; in the case of the fourth, using polyacrylamide gel, the control sample was pre-stained with Sudan black.

Conventional electrophoresis

(a) on cellulose acetate (Millipore),

(b) on agarose, using the "Agaroslide system" (Millipore).

In both these systems sera were pre-stained with concentrations of NBT from 0.05%–0.8%.

Antigen-antibody crossed electrophoresis (Laurell electrophoresis)

Was performed according to Clarke and Freeman[1] using anti-whole human serum (Burroughs Wellcome).

"Monorocket" immunoelectrophoresis

Was performed by the method of Laurell[2] using anti-whole human serum (Burroughs Wellcome).

Polyacrylamide disc electrophoresis

The method used was reported by Frings *et al.*[3]. Twenty-five μl serum pre-stained with Sudan black and 50 μl serum pre-stained with Sudan black and 50 μl serum pre-stained with concentrations of NBT from 0.05%–0.8% were run simultaneously. The use of NBT as a pre-stain allowed the sample gel to be dispensed with and after the addition of a small amount of solid sucrose to the sample it was layered on the polymerised concentrating gel layer.

RESULTS

The addition of phenazine methosulphate to the serum sample containing NBT

and NADH resulted in blue colouration of the mixture. The colouration developed within a few seconds and reached maximum intensity within 5 minutes.

Conventional electrophoresis

The pattern of lipoprotein separation was similar whether pre-stained with NBT or post-stained with Sudan black provided that the concentration of NBT did not exceed 0.15%. The experimental time using NBT was reduced by the time necessary for staining the sample after electrophoresis; this time was approximately 120 minutes for both cellulose acetate and the Agaroslides.

Antigen-antibody crossed electrophoresis

Blue colouration was found in the precipitates previously identified as alpha- and beta-lipoproteins (Fig. 1). The size, shape and position of the lipoprotein arcs did not differ appreciably from those produced when the control serum was run simultaneously and stained afterwards with Amido black or Sudan black.

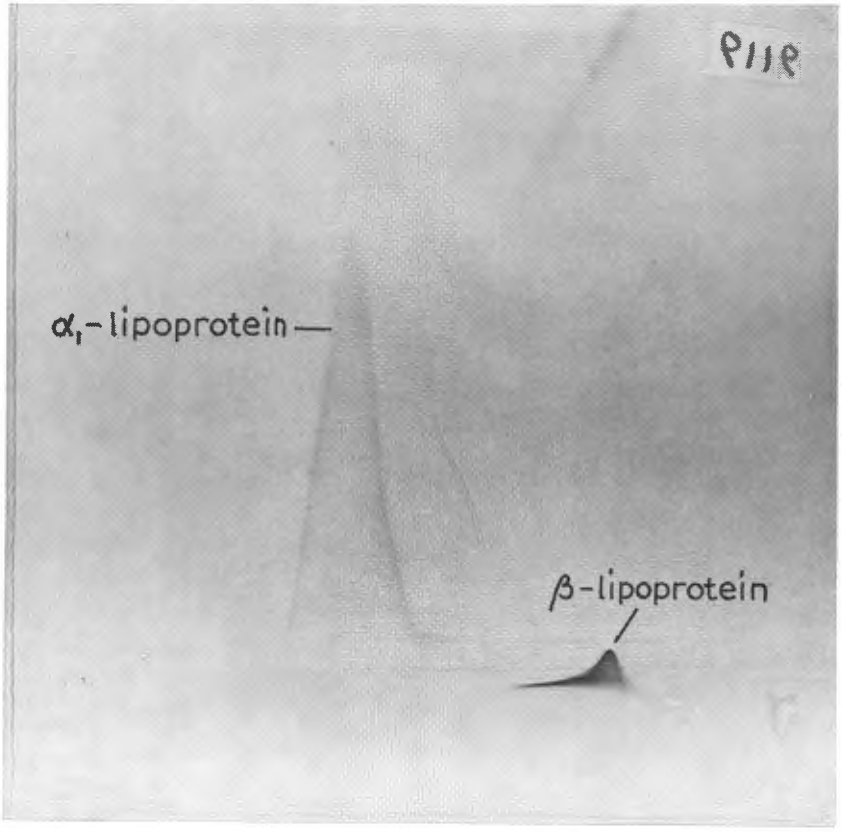

Fig. 1. Laurell electrophoresis of serum pre-stained with nitroblue tetrazolium.

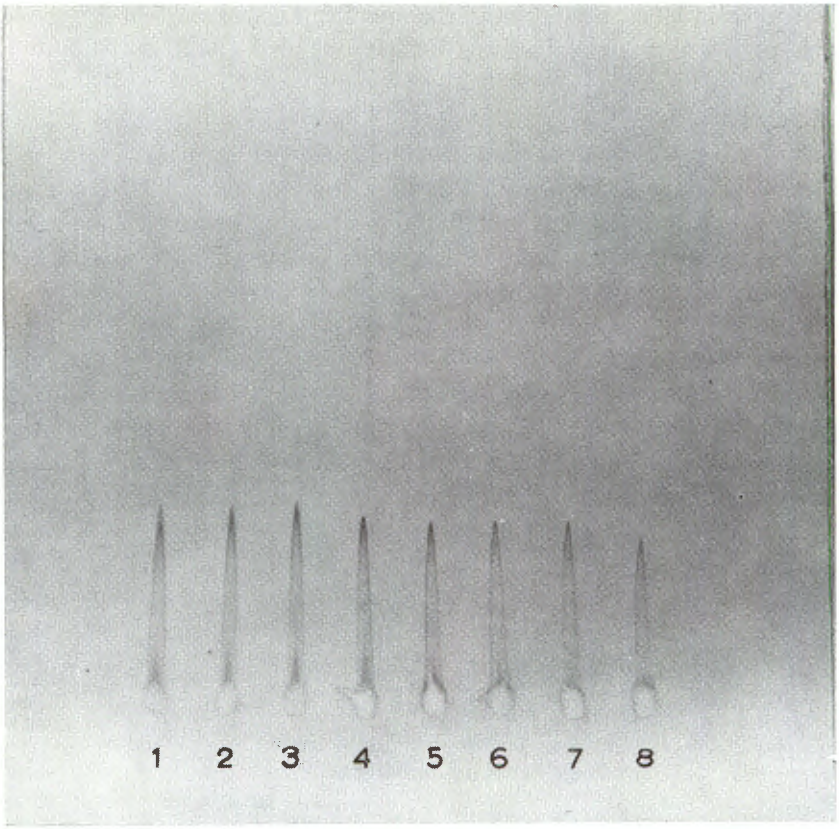

Fig. 2. "Monorocket" electrophoresis of serum: samples 1–4 pre-stained with nitroblue tetrazolium; samples 5–8 post-stained with Sudan black.

"Monorocket" immunoelectrophoresis

Repeated single volume samples, pre-stained with NBT in concentrations less than 0.15%, resulted in rockets of constant height (standard error 0.16) which matched the rockets obtained from equal volumes of serum post-stained with Sudan black (Fig. 2). Increasing concentrations of NBT greater than 0.15% resulted in a progressive reduction in the height of the rockets.

Acrylamide disc electrophoresis

Compared to Sudan black as a pre-stain, NBT gave a greater intensity of colour. The pre-beta and beta-lipoprotein bands were comparable in appearance but additional bands were demonstrated in the alpha region (Fig. 3). The absence of a sample gel layer did not affect reproduceability.

DISCUSSION

Pre-staining of samples before electrophoresis is the ideal method of revealing

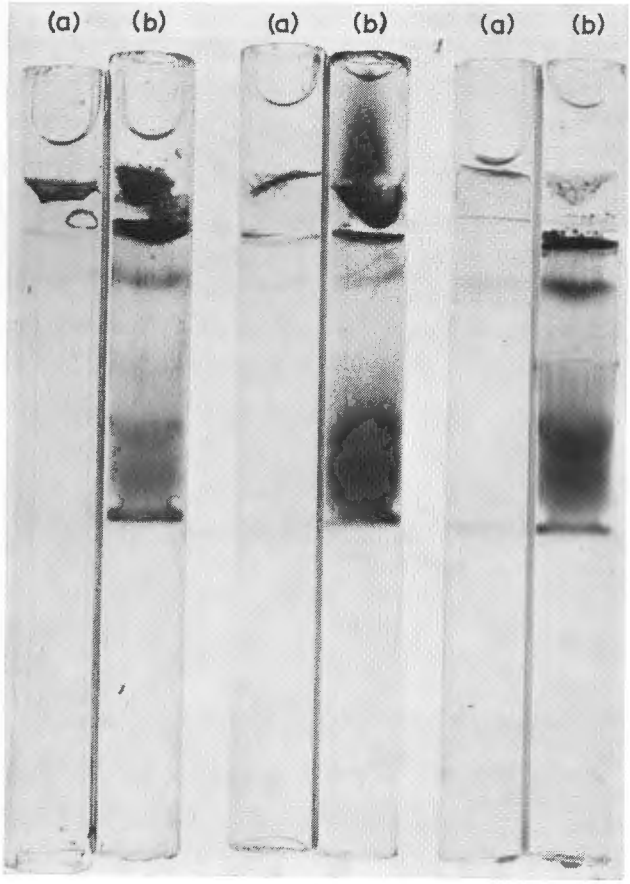

Fig. 3. Disc electrophoresis of three normal sera pre-stained with (a) Sudan black and (b) nitroblue tetrazolium.

substances with a specific affinity for the indicator. It allows observation of the progress of the electrophoretic migration, immediate interpretation of the separation and eliminates background staining.

Although the mechanism is still uncertain, NBT has been shown by means of antigen-antibody crossed electrophoresis to be a specific pre-stain for lipoproteins. The pattern of staining in all but polyacrylamide gel is the same as that found after staining with conventional lipid stains and, in suitable concentrations, there is no evidence of interference with electrophoretic responses or antigen–antibody reactions. The additional alpha-bands on polyacrylamide may have become visible due to the intensity of the stain.

The advantages of using NBT are clearly shown in the Millipore Agaroslide system. The electrophoresis time can be varied by direct observation of the migration of the lipoproteins and the lipoprotein pattern can be directly recorded by obtaining an immediate tracing from a densitometer. The entire process takes 30 minutes com-

pared to 150 minutes necessary to obtain a result by the prescribed method.

Pre-staining of serum with NBT should also simplify lipoprotein separation by column chromatography and high speed centrifugation as the lipid-rich fractions appear blue and could be localised spectrophotometrically utilising the absorbance of light by formazan at a wavelength of 580 nm.

In the correct concentrations NBT causes intense pre-staining of lipoproteins without changing their electrophoretic or immunoelectrophoretic characterisation in the systems tested. It has the advantages of being inexpensive, rapid, simple to apply and active in the absence of organic solvents. In the rapidly expanding field of lipid research, with all its clinical applications, it is hoped that the introduction of NBT as a pre-stain will resolve some of the difficulties of lipoprotein characterisation.

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