The Nitroblue Tetrazolium (NBT) Test
A methodological and clinical study

AKADEMISK AVHANDLING

som med vederbörligt tillstånd av
Medicinska Fakulteten vid Umeå universitet
för avläggande av medicine doktorsexamen
kommer att offentligen försvaras i
Samhällsveterhuset, sal D,
torsdagen den 30 maj 1974 kl. 9 f.m.

av

BENGT BJÖRKSTÉN
Med. lic.
THE NITROBLUE TETRAZOLIUM (NBT) TEST

A methodological and clinical study

by

BENGT BJÖRKSTÉN
This dissertation is based on the following six papers:


V. Björkstén, B. & de Chateau, P.: Use of the Nitroblue Tetrazolium (NBT) Test in the Differentiation of Pyelonephritis from Cystitis. Submitted for publication.


References to these papers will be made with the Roman numerals I - VI.
INTRODUCTION 5

Background 5

NBT test methods in current use 7

Mechanism of the NBT test 8

AIM OF THE PRESENT INVESTIGATION 10

THE INFLUENCE OF TECHNICAL FACTORS ON THE NBT TEST (I, II) 10

A. Blood samples: Venous or capillary? 10

B. Choice of anticoagulants 11

C. Influence of serum components 12

D. Transport and storage of blood samples 13

E. Performance of the NBT test 13

F. Evaluation of NBT positive and NBT negative cells in the sample 14

RECOMMENDED TEST PROCEDURE 15

THE NBT TEST VALUES IN VARIOUS GROUPS OF PATIENTS 17

A. Infections 17

   a. Bacterial and viral infections 17

   b. Other infections 22

B. Disorders of phagocyte function 23

C. Influence of other conditions and of pharmacotherapy 23

PRESENT INDICATIONS FOR THE NBT TEST IN CLINICAL PRACTICE 25

SUMMARY 26

The influence of technical factors on the NBT test 26

NBT test values in different types of infections 27

The NBT test in Chronic Granulomatous Disease 27

Conclusions 28

ACKNOWLEDGEMENTS 28

REFERENCES 30
INTRODUCTION

Background

The tétrazolium salts are easily reduced by a number of enzymes into formazan and, since this reduction often is accompanied by a change in colour, they are widely used in histochemistry (Glenner, 1969). Phagocytosis and intracellular killing of micro-organisms by normal leucocytes are accompanied by increased oxidative metabolic activity in the cells (Johnston & Baehner, 1971) and under these conditions the presence of tétrazolium salts results in intracellular formazan production. Thus Siegert et al already in 1951, using the tétrazolium salt TTC, found that the reducing capacity of whole blood was elevated in blood samples from patients with malignancy, especially when complicated with a bacterial infection. They also suggested that this increased reducing capacity of blood was due to changes in the white blood cell function.

Baehner and Nathan (1967) reported that neutrophils from patients with Chronic Granulomatous Disease (CGD) lacked the ability to reduce Nitroblue tétrazolium (NBT) into formazan and this NBT test was suggested as a diagnostic test for the disease. This condition, first described by Berendes et al in 1957, is characterized by recurrent severe bacterial infections occurring in early childhood. In 1966 Holmes et al reported that the syndrome was caused by a defect of leucocytes in their killing of certain bacteria. Since then several other granulocyte dysfunction syndromes have been described (Lancet, 1974). To confirm such defects in phagocytosis or intracellular killing of microorganisms, tests measuring the rate of killing of different microorganisms are preferred. Such tests, however, are time-consuming and living bacteria have to be used. Thus there is an obvious need for a simple test method by which patients with increased susceptibility for bacterial and fungal infections can be screened for abnormalities in granulocyte function.
In 1968 Park et al introduced a simplified NBT test showing that when venous blood from healthy persons was mixed with NBT solution in vitro, a low proportion of the granulocytes reduced the dye to nitroblue formazan. This formazan was precipitated in the cytoplasm of the cells and could easily be visualized in the light microscope. The authors also showed that the proportion of such NBT positive neutrophils was much elevated in blood from patients with untreated bacterial or fungal infections and moderately so in blood from patients with viral infections. Several investigators have later reported similar results (Matula & Paterson, 1971; Humbert et al 1971; Feigin et al 1971). However, a low proportion of NBT positive neutrophils in a patient with an acute infection could indicate either that the infection was non-bacterial or that the granulocytes were unable to reduce NBT as seen in CGD. To diagnose CGD, the stimulated NBT test was introduced by Park & Good in 1970. In this modified NBT test the blood sample is incubated with bacterial endotoxin prior to the addition of NBT solution. This incubation increases the proportion of NBT positive neutrophils in blood samples from healthy persons, while the proportion remains low in samples from patients with CGD.

The NBT test is currently used as an aid in the differentiation of various kinds of infections, where high NBT test figures should indicate an untreated bacterial, fungal or parasitic infection. The test is also used as a screening test for CGD and to detect heterozygote carriers of this disease. Although many reports on the NBT test have been published during the last 6 years, the validity of the test is still unsettled. Many investigators consider it to be of great clinical value (Park, 1971; Wollman et al, 1972; Freeman et al, 1973; Fikrig et al; 1973, Gordon et al, 1973), while others are critical (Segal et al, 1973; Steigbigel et al, 1974). These differences of opinions may partly be explained by the fact that the test methods used by different investigators have varied. Consequently,
an evaluation of the clinical usefulness of the NBT test must begin with an analysis of the technical factors that influence the test results.

**NBT test methods in current use.**

During the last few years several variations of three different NBT test methods have been suggested.

In the "quantitative" NBT test used by Baehner and Nathan (1968) a washed leucocyte suspension is incubated with NBT solution and latex particles are used to promote phagocytosis. The formazan produced by the leucocytes is extracted with pyridine and the amount of formazan recovered is then determined spectrophotometrically. With this test method the maximal amount of formazan produced by a certain number of cells is determined. This method was used in paper VI.

Gifford and Malawista (1970) introduced the "NBT slide test". One drop of capillary blood is allowed to form a clot on a glass slide in humified air at 37°C. The granulocytes adhere to the glass and, after washing away the blood clot, NBT solution is added to the glass slide, which is then further incubated at 37°C. The glass slide is then counterstained and the proportion of cells containing formazan is evaluated. Normally more than 30% of the adherent cells contain formazan. With this method patients with and carriers of CGD may be detected. A slight variation of this method was used by Kim et al (1973) for differentiation of varying types of infection. The main advantage of the "NBT slide test" would be that capillary blood in small amounts may be used. However, the reliability of the test has not yet been investigated enough.

The third NBT test method in current use is the "qualitative" method of Park et al (1968). By this method a small amount of blood with anti-
coagulant added, is incubated with NBT solution and then thin smears are made on glass slides. The glass slides are counterstained and the percentage of granulocytes with intracellular deposits of formazan, NBT positive cells, are counted. Most investigators have used this method and several modifications have been described (References see below). As this test method was considered to be the most appropriate for routine clinical work it was chosen in our laboratory when the NBT test was introduced for clinical routine purposes.

**Mechanism of the NBT test**

Although the NBT test has been recommended for routine use in clinical practice (Lancet, 1971; Feigin, 1971), the mechanisms of the test are not known. The intact cell membrane is thought to be impermeable to NBT (Park, 1971) and thus changes in cell membrane permeability or phagocytosis of the dye would be necessary to allow the intracellular deposit of reduced NBT. Recently Segal and Levi (1973), using washed buffy-coat preparations of human blood suspended in different media, found that stimulation of neutrophils with endotoxin and the presence of heparin or fibrinogen were necessary for dye reduction. They suggested, that NBT only enters neutrophils in quantities visible by light microscopy after stimulation, resulting in phagocytosis of a dye and heparin or fibrinogen macromolecular complex. The authors did not find the presence of immunoglobulins and complement necessary for the test to function. However, other investigators (Freeman & King, 1972 a; Hellum and Solberg, 1973) have stated that an intact phagocytic system, including immunoglobulins and complement, probably is necessary for the NBT test to function.

The exact mechanisms of the reduction of NBT into formazan within the cells are not known, although there are several possible explanations. In granulocytes from different patients with CGD, lacking the ability to reduce NBT,
Figure 1  Schematic representation of the enzyme reactions responsible for the burst of oxidative metabolic activity occurring in a normal neutrophil following phagocytosis. NAD, NADH, NADP and NADPH are oxidized and reduced nucleotides. GSH = reduced glutathione. GSSG = oxidized glutathione.

1. Reaction catalyzed by NADH oxidase
2. Reaction catalyzed by NADPH oxidase
3. "- glutathione peroxidase
4. "- glucose-6-phosphate
5. "- 6-phosphogluconate dehydrogenase. dehydrogenase
lowered activity of NADH-oxidase and glutathione peroxidase and an accelerated
decay of glucose-6-phosphate dehydrogenase has been reported (Johnston &
Baehner, 1971). Of these enzymes, NADH-oxidase may be responsible for the
intracellular reduction of NBT (Johnston & Baehner, 1971). NADPH-oxidase,
another pyridine nucleotide, could also catalyze this reaction. Enzyme
reactions responsible for the burst of oxidative metabolic activity which
normally occurs following phagocytosis are shown in Figure 1.

Nathan (1974) recently suggested that NBT either could substitute for oxygen
in the oxidase reactions or be reduced by superoxide radicals produced by
the oxidases. The superoxide radical is a highly reactive compound produced
when oxygen is reduced by a single electron. It is spontaneously dismutated
to hydrogen peroxide and oxygen \((2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2)\). Increased production
of superoxide by normal granulocytes during phagocytosis has been demonstrated
greatly diminished production by granulocytes from patients with CGD. Since
superoxide rapidly reduces NBT in vitro (Nishikimi et al, 1972) it may be
responsible for the formazan produced in the NBT test.

AIM OF THE PRESENT INVESTIGATION.
The purpose of this investigation was to study the influence of different
technical factors on the qualitative NBT test and to investigate the value of
the test in different clinical conditions. After this study was begun several
investigations concerning these questions have been published and they are
also reported in this paper.

THE INFLUENCE OF TECHNICAL FACTORS ON THE NBT TEST (I, II).
A. Blood samples: Venous and capillary?

In most investigations, venous blood was used, but some investigators have
recommended the use of capillary blood (Barker & Farnes, 1972; Štrukelj &
The results of NBT tests performed on venous and capillary blood drawn from 63 patients were compared in paper I. In patients with increased proportion of NBT positive neutrophils the test values were much lower in capillary than in venous blood. The difference was so great that NBT test results obtained using capillary blood cannot be compared to those obtained using venous blood. This may partly be explained by either haemolysis or by complement inactivation occurring in the capillary blood samples (I). However, neutrophils in capillary blood can be stimulated by endotoxin (I) and thus capillary blood may be used for screening for CGD. When this study was in press Randall et al (1973) reported NBT test results in simultaneously drawn venous and capillary blood samples from 26 healthy persons. They found similar NBT test values in venous and capillary blood. However, since the patients studied by them were healthy, most of the NBT test results were low in both the venous and the capillary blood samples. Thus the investigation has little relevance for the question whether capillary blood may be used in conditions with high NBT test values.

B. Choice of anticoagulants.

Park et al (1968) in the first report on the "qualitative" NBT test used 75-100 IU heparin per ml blood as anticoagulant and since then heparin has been used by most investigators. In 1972, however, Barker & Farnes reported that the NBT test results were influenced by heparin, not indicating how.

This investigation (II) shows that small changes in heparin concentration influence the NBT test. If 50 IU heparin per ml blood is used instead of 10-30 IU, elevated NBT test results may be obtained in blood from healthy persons (II). Thus, the lowest concentration of heparin preventing coagulation of the blood samples should be used. The effects of varying heparin con-
centrations may explain discrepancies in results obtained by different investigators.

While this study was performed, Hellum and Solberg (1973) reported the effect on the NBT test of 10,100 and 500 IU heparin per ml blood and concluded that standardization of the test with 10 IU heparin per ml blood should increase the applicability of the test. Hellum and Solberg (1973) also studied the influence of other anticoagulants on the NBT test, comparing 10 IU heparin per ml blood, EDTA, oxalate and sodium citrate. They found that use of any of the calcium-binding anticoagulants tested resulted in low NBT test results in blood samples from patients with bacterial infection. In contrast to the results of Park & Good (1970) and Hellum & Solberg (1973), Gordon et al (1973) reported that EDTA, together with Ficoll (a sucrose polymer), could be used as anticoagulant in the NBT test. The authors stated that duplicate samples using EDTA and heparin were compared, but they did not mention the heparin concentration used, nor did they state if there were any differences in test results using the two anticoagulants.

C. Influence of serum components.

Park & Good (1970), Freeman & King (1972 a) and Hellum & Solberg (1973) have found the NBT test to be complement dependent. This could also be supported by the results in paper I, in which fresh serum was found more efficient to obtain high NBT test results after endotoxin stimulation than preheated serum (56°C for 30 minutes). Since low NBT test values were reported in patients with hypogammaglobulinaemia the NBT test may be influenced by immunoglobulins (Freeman & King, 1972 a; Park, 1971). Segal and Levi (1973) recently reported that neither complement nor immunoglobulins were obligatory for the dye to enter the neutrophils. They found that complexes of NBT and heparin or fibrinogen could be phagocytized by neutrophils suspended in a serum-free medium. Their results however, do not exclude
that serum components are necessary when NBT tests are performed on blood samples not incubated with endotoxin. The varying results obtained by different investigators cannot be explained, since the exact mechanisms of the NBT test are not known presently.

D. Transport and storage of blood samples.

In paper II the effect of storage of blood samples was investigated. Storing for up to 8 hours at +4°C or for up to 4 hours at +23°C did not influence the test results. When the blood samples were stored at 23°C for 8 hours or more, elevated test results were obtained in some samples. The reasons for this were not investigated.

E. Performance of the NBT test.

Park et al (1968), in their original description of the NBT test, incubated the blood NBT solution mixture in air at 37°C for 15 minutes and then at room temperature for another 15 minutes. Other investigators used a water bath (Humbert et al, 1970) or a thermal block (Charette & Komp, 1972) at 37°C. Incubation time intervals of 15 (Humbert et al, 1970), 25 (Matula & Paterson, 1971), 30 (Park et al, 1968), 45 (Gordon et al, 1973) and 60 minutes (Henkel Chretien & Garagusi, 1971 a) have been used.

Baehner and Nathan (1968), using the "quantitative NBT test, found that the production of formazan was influenced by the incubation time and temperature and that the formation was maximal when the test was performed at 37°C.

In paper II the effect of incubation time on the "qualitative" NBT test was investigated and it was found that, as the incubation was prolonged, the proportion of NBT positive neutrophils rose in the samples. Elevated test values were thus obtained in 5 of 13 blood samples from healthy persons after 30 minutes' incubation and in all the samples when they were incubated for
50 minutes. In the original description of the NBT test (Park et al, 1968) the test tubes were incubated for 15 minutes at 37°C in air and then for another 15 minutes at room temperature. We measured the incubation time necessary to reach a temperature of 36-37°C in the test tubes, comparing incubation in air and in a water bath. Incubation for 15 minutes at 37°C resulted in a temperature in the test tubes of 36-37°C for 13 minutes when the test was performed using a water bath and for only 6-7 minutes when done in air. The incubation of the blood and NBT solution mixture should not be done in air, since the incubation temperature may vary under these conditions. The results indicate that differences in incubation time may explain the varying NBT test results among different investigators.

F. Evaluation of NBT positive and NBT negative cells in the sample.

Cells containing a large amount of formazan often clump together, making counting difficult. Only cells that were not clumped together were counted in this investigation.

Not only neutrophilic granulocytes but also eosinophilic granulocytes and monocytes may reduce NBT to formazan. Monocytes apparently do so more often than neutrophils, while eosinophils do so only occasionally (Humbert et al, 1971). Only neutrophils should be counted as falsely high test values otherwise may be obtained in patients with monocytosis and falsely low test values in patients with eosinophilia. Since the confidence limits of the proportion of NBT positive cells in the sample are given by the binomial distribution (II), the reading error increases with the proportion of NBT positive neutrophils. Further, the tendency of NBT positive cells to clump together also make the test results more uncertain when the proportion of NBT positive neutrophils increases. This would mean that even large differences obtained between two high test values may not be significant.
RECOMMENDED TEST PROCEDURE.

Based on the methodological studies the following test procedure is recommended. An amount of 2 ml of venous blood is drawn in a 4 ml plastic tube (Nunc Plastics, Roskilde, Denmark) containing 35 IU heparin (Vitrum AB, Stockholm). The blood samples are stored and transported at 4°C for not more than 8 hours until the NBT test is done. When performing the NBT test 0.2 ml of the blood sample is thoroughly mixed with an equal volume of 0.1 % NBT solution in sterile saline. The NBT solution is kept at 4°C for not more than 48 hours prior to use and is filtered through a cellulose filter retaining fine grained precipitates (Munktell's filter no 8, Rudolph Grave AB, Solna). The blood NBT solution mixture is incubated in a 37°C water bath for 25 minutes and thin smears of the mixture are then made on glass slides which have been washed in 70 % ethyl alcohol. The slides are fixed in methanol for 10 minutes and then counterstained with Giemsa's stain (pH 6.8) for 10 minutes. When the slides are dry, 100 neutrophilic granulocytes are counted in a light microscope (Magnification 630 x). The prevalence of cells containing discrete intracellular deposits of nitroblue formazan, larger than the normal neutrophilic granules are expressed as a percentage value. Only clearly identifiable neutrophils are counted. Monocytes and eosinophils are not included in the count. When the endotoxin stimulated NBT test is done the blood sample is incubated with 10 µg lipopolysaccharide 0111:B5 (Difco Laboratories, Detroit, Michigan) for 10 minutes in a 37°C water bath. Then the NBT test is done as described above.

With this NBT test method, one group of 95 healthy persons aged 17 to 55 years (average 28 years) and another group of 23 healthy newborns were investigated (III). The proportions of NBT positive neutrophils are normally increased in newborns (Humbert et al, 1970; Cocchi et al, 1971 a) and thus blood samples from them may serve as positive controls of the test. The NBT test values obtained in these two groups are given in Fig. 1, paper III.
Figure 2  NBT test values in 35 healthy children. The NBT test results are expressed as the proportion and the total number of NBT positive Neutrophils per cu.mm. blood.
Beyond the newborn period NBT test values of less than 13 % were considered normal, while test values of more than 19 % were considered "high". Test values of 13-19 % were classified as "intermediate".

Figure 2 shows the NBT test results in 35 children aged 1-15 years admitted to the paediatric clinic, University Hospital of Umeå. All the children were healthy on admission and none of them had any symptoms of urinary tract infection or other infections. The NBT test results are expressed both in percentage and in absolute number of NBT positive neutrophils. The absolute number was obtained by multiplying the proportion of NBT positive neutrophils with the number of neutrophils per cu.mm. blood. As shown in the figure only two of the patients had more than 500 NBT positive neutrophils per cu.mm. blood. These two patients also had intermediate proportions of NBT positive neutrophils (13 and 16 %). Consequently 500 NBT positive neutrophils per cu.mm. blood was adopted as normal upper limit in children.

THE NBT TEST VALUES IN DIFFERENT GROUPS OF PATIENTS (III, IV, V, VI).

A. Infections.

a. Bacterial and viral infections.

The first report on the use of the NBT test in the diagnosis of infections was that of Park et al in 1968. They found an average of 8.5 % NBT positive neutrophils in blood from healthy persons, an average of 46.6 % in blood from patients with untreated bacterial meningitis and an average of 9.5 % in blood from patients with viral meningitis. Similar results were reported by Humbert et al (1971) and Feigin et al (1971). However, other investigators have reported low NBT test figures in patients with bacterial infections, (false negative test results) (Eposito & de Lalla, 1972; Segal et al, 1973), or high NBT test figures in patients with viral infections (false positive test results) (Elgefors & Olling, 1972). This may seriously impair the value of the NBT test in the differentiation of the aetiology of infections.
The groups "bacterial" and "viral" infections may include widely different diseases unless the infections are characterized as to location and offending organisms. Park (1971) pointed out that "localized bacterial infections not reflected in the systemic circulation" do not cause elevated NBT test figures. No investigation comparing NBT test results in infections caused by different bacterial species has yet been carried out.

In this investigation the NBT test was evaluated in three different types of bacterial infections, i.e. in streptococcal throat infection (III), gangrenous appendicitis (IV) and urinary tract infections (V). Elevated NBT test results in streptococcal throat infections were only found in about half of the patients (III). Appendicitis did not influence the NBT test results, not even when complicated by peritonitis (IV). Pyelonephritis caused by *E. coli* usually resulted in elevated NBT test figures, while cystitis or asymptomatic bacteriuria caused by the same kind of bacteria did not (V).

To evaluate the influence of bacterial infections upon the NBT test apparently the location of the infections and the kind of bacteria involved must be considered. In an effort to do so we evaluated the records of patients with suspected infections, in which NBT tests had been performed in our laboratory. The patients were grouped according to diagnosis and bacterial aetiology. Patients receiving antibacterial treatment when blood for NBT tests was drawn were excluded. In patients with normal test results the endotoxin stimulated NBT test was performed to exclude CGD.

Table I shows the results of NBT tests performed in our laboratory on blood samples from 129 patients evaluated to have bacterial or viral infections, together with NBT test results reported by other investigators. We found high NBT test values in 49% of the 51 patients with an untreated bacterial infection and in 14% of the 78 patients with a viral infection. Thus only
<table>
<thead>
<tr>
<th>Reference</th>
<th>No of patients</th>
<th>&quot;normal&quot; (&lt;13 %)</th>
<th>&quot;intermediate&quot; 13 - 19 %</th>
<th>&quot;high&quot; &gt;20 %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>%</td>
<td>n</td>
<td>%</td>
</tr>
<tr>
<td><strong>Bacterial Infections</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Humbert et al, 1971</td>
<td>53</td>
<td>10</td>
<td>19</td>
<td>7</td>
</tr>
<tr>
<td>Matula &amp; Paterson, 1971</td>
<td>65</td>
<td>4(^a/)</td>
<td>6</td>
<td>27(^b/)</td>
</tr>
<tr>
<td>Gordon et al, 1973</td>
<td>56</td>
<td>13</td>
<td>23</td>
<td>0</td>
</tr>
<tr>
<td>Segal et al, 1973</td>
<td>39</td>
<td>15</td>
<td>38</td>
<td>5</td>
</tr>
<tr>
<td>Fikrig et al, 1973</td>
<td>36</td>
<td>2</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>Björksten</td>
<td>51</td>
<td>22</td>
<td>44</td>
<td>4</td>
</tr>
<tr>
<td><strong>Viral Infections</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Humbert et al, 1971</td>
<td>43</td>
<td>34</td>
<td>80</td>
<td>4</td>
</tr>
<tr>
<td>Gordon et al, 1973</td>
<td>48</td>
<td>42</td>
<td>88</td>
<td>5</td>
</tr>
<tr>
<td>Segal et al, 1973</td>
<td>21(^c/)</td>
<td>14</td>
<td>67</td>
<td>6</td>
</tr>
<tr>
<td>Fikrig et al, 1973</td>
<td>67</td>
<td>66</td>
<td>99</td>
<td>1</td>
</tr>
<tr>
<td>Björksten</td>
<td>78</td>
<td>59</td>
<td>76</td>
<td>8</td>
</tr>
</tbody>
</table>

a) Less than 11 % NBT positive neutrophils  
   b) Proportion of NBT positive neutrophils 11-20 %  
   c) Two of the patients had a parasitosis
about half of the patients with bacterial infection had a high NBT test value. Similar results were reported by Matula and Paterson (1971) and Segal et al (1973). Fikrig et al (1973) on the other hand found high NBT test values in 94% of 36 patients with bacterial meningitis. The proportions of NBT positive neutrophils in blood samples from patients with viral infection were low in all the investigations cited. This study showed, however, a higher proportion of high test values associated with viral infections than did other investigations.

In an attempt to evaluate the influence of different bacterial species on the NBT test, test results reported by us and by other investigators are grouped according to bacterial aetiology in Table 2. Since most investigators do not state the offending micro-organisms in the individual patients, the number of NBT test results given in the table is small and thus the question whether different bacterial species influence differently upon the NBT test cannot be answered. Infections with coliform bacteria often cause high NBT test results. However, the location of the infection may be of importance. As shown in paper IV, in perforated appendicitis, where an infection with coliform bacteria presumably is at hand, the NBT test results were normal. Further, as shown in paper V, cystitis did not cause high test values, whereas pyelonephritis did. Steigbigel et al (1974) recently found elevated test values in only 7 of 13 blood samples from patients with bacteriemia caused by gram negative bacteria.

In miliary tuberculosis and in tuberculous meningitis elevated NBT test scores are reported (Park, 1971), in contrast to the normal test results reported in other forms of active mycobacterial infection (Park et al, 1968; Matula & Paterson, 1971; Gordon et al, 1973). Streptococcal infections often do not give rise to elevated NBT test figures (Table 2, III), not even in patients with a febrile infection and an elevated ESR. Only few
Table II  NBT test results in patients with infections caused by different bacterial species.

<table>
<thead>
<tr>
<th>Reference</th>
<th>No of patients</th>
<th>&quot;Normal&quot; (≤13 %)</th>
<th>&quot;Intermediate&quot; (13-19 %)</th>
<th>&quot;High&quot; (≥20 %)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Staphylococcus aureus</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vaucher et al (1970)</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Ng et al (1972)</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Björkstén</td>
<td>6</td>
<td>2</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>4</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td><strong>Streptococci</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vaucher et al, 1970</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Randall et al, 1973</td>
<td>37</td>
<td>29</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Shapera &amp; Matsen, 1973</td>
<td>20</td>
<td>20</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Björkstén (III)</td>
<td>18</td>
<td>8</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td>Björkstén</td>
<td>11</td>
<td>7</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>89</td>
<td>64</td>
<td>7</td>
<td>18</td>
</tr>
<tr>
<td><strong>Pneumococci</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vaucher et al (1970)</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Eposito &amp; de Lalla (1972)</td>
<td>5</td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ng et al (1972)</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Wollman et al (1972)</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Björkstén</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>8</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td><strong>Coliforms</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vaucher et al (1970)</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Freeman &amp; King (1972 a)</td>
<td>3</td>
<td>0</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Wollman et al (1972)</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Gordon et al (1973)</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Björkstén (V) pyelonephritis</td>
<td>14</td>
<td>3</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>Björkstén (V) cystitis</td>
<td>23</td>
<td>20</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>22a)</td>
<td>4a)</td>
<td>2a)</td>
<td>16a)</td>
</tr>
<tr>
<td><strong>Meningococci</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vaucher et al (1970)</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Eposito &amp; de Lalla (1972)</td>
<td>7</td>
<td>7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Gordon et al (1973)</td>
<td>3</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>10</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td><strong>Salmonella</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vaucher et al (1970)</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Ng et al (1972)</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Björkstén</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>3</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td><strong>Haemophilus influenzae</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Park et al (1968)</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>10b)</td>
</tr>
<tr>
<td>Feigin et al (1971)</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Fikrig et al (1973)</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Björkstén</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>3</td>
<td>0</td>
<td>12</td>
</tr>
<tr>
<td><strong>Mycoplasma pneumoniae</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Freeman &amp; King (1972 b)</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Gordon et al (1973)</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Björkstén (III)</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Björkstén</td>
<td>7</td>
<td>1</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>1</td>
<td>4</td>
<td>7</td>
</tr>
</tbody>
</table>

a) Patients with cystitis excluded  
b) More than 17 % NBT positive neutrophils.
NBT test results in patients with pneumococcal and meningococcal infections are reported and most of them were normal, while the NBT test results were elevated in most of the few reported patients with *H. influenzae* and *M. pneumoniae* infections.

As shown in Tables 1 and 2 false negative test results are common in bacterial infections. The clinical value of the NBT test in the diagnosis of infections does not depend on even highly significant differences in mean test values in groups of patients with bacterial or viral infections, but on the frequency of overlap in test values between the two kinds of infections. There is a considerable overlap in test values obtained in patients with viral and bacterial infections. This was true also in the present investigation using a standardized technique.

**b. Other infections.**

High NBT test results have been found in patients with *Candida albicans* septicaemia (Park et al, 1968) and in malaria (Andersen, 1971; Pujol-Moix, 1971). We found NBT test results of 30-45% on several occasions in the single patient with malaria we have investigated. Henkel Chretien & Garagusi (1971 b) reported high NBT test scores in 3 patients with loiasis, trichinosis and amoebic liver abscess respectively. An elevated NBT test result has also been reported in cutaneous leishmaniasis (Rubin & Tramont, 1972). Recently Campos et al (1973) reported a NBT test score of 66% in a patient with Rocky Mountain spotted fever. Rubin & Tramont (1972) found normal NBT test results in hookworm and pneumocystis carini infection, in cryptococcosis and in tertiary syphilis (one patient with each disease).

Apparently elevated NBT test scores can be expected in patients with infections caused by *Candida albicans* and in malaria. Too few reports are published on the NBT test results in patients with other parasitic or
rickettsial infections to allow any definite conclusions.

B. Disorders of phagocyte function.

In Chronic Granulomatous Disease (CGD) the neutrophils lack the ability to reduce NBT into nitroblue formazan (Windhorst et al, 1967; Baehner & Nathan, 1968). In this disease the neutrophil phagocytosis is normal while there is a defect in intracellular killing of certain, i.e. catalase-producing, bacterial species.

Although the lack of formazan production in the granulocytes is not conclusive evidence of a defect in the intracellular bactericidal function, the NBT test is accepted as a diagnostic test for CGD instead of more time consuming and technically difficult bactericidal tests. For the diagnosis of the syndrome the quantitative NBT test of Baehner & Nathan (1968), the "slide test" of Gifford & Malawista (1970) or the stimulated NBT test of Park & Good (1970) may be used. The last two modifications of the NBT test would also detect serious impairment in phagocytic function, independent of whether this is due to cellular dysfunction or due to lack of necessary serum components. In paper VI the quantitative NBT test was used to confirm the diagnosis of CGD in a 10-year old girl and to investigate her family. It was shown that neutrophils from the patient lacked the ability to reduce NBT, while the neutrophils of her mother and a brother had a moderately decreased capacity to reduce the dye. These results would be compatible with a dominant inheritance with a variable expressivity. Such mode of inheritance has not as far as we know been described earlier.

C. Influence of other conditions and of pharmacotherapy.

High test figures have been reported in osteogenesis imperfecta (Humbert et al, 1971), acute myocardial infarction (Lauter et al, 1973), haemophilia (Humbert et al, 1971), thalassemia (Liakakos & Viachos, 1973; Tan et al,
and after intramuscular injection of typhoid vaccine (Grush & Mauer, 1969). Tan et al. (1973) reported increased NBT test values in a number of haematologic disorders, including sickle cell anemia, idiopathic thrombocytopenic purpura, multiple myeloma, polycythemia vera, penicious anemia, iron deficiency anemia, anemia of uremia and in leukemia. Further they found high NBT test values in carcinoma and in systemic lupus erythematosus.

Apart from conditions mentioned above we have found high NBT test values in the absence of other signs of infection in one patient with Wegeners granulomatosis on five different occasions, in both of two patients with generalized dermal urticaria, and on three different occasions in one of three investigated patients with ulcerative colitis. Thus a number of disorders may possibly cause high NBT test values and this may seriously impair the clinical value of the test. High test values have also been found in healthy newborn babies during the first 4 weeks (III, Cocchi et al, 1971; Humbert et al., 1970) and in pregnant women (Drysdale, 1972).

Low test figures in the presence of bacterial infections may be obtained in a number of conditions when the phagocytosis system of the host is not functioning normally (Park, 1971; Tan et al., 1973).

After institution of antibacterial treatment elevated NBT test results rapidly normalize, usually within 48 hours. This has been attributed to the effectiveness of therapy (Park, 1971) but recently Rubinstein & Pelet (1973) reported low NBT reducing capacity in vitro of granulocytes in the presence of therapeutic concentrations of tetracycline or high concentrations of other antibiotics. Thus at least some antibiotics may influence the NBT test.

Treatment with cytostatics and corticosteroids in patients with bacterial infections are reported to give rise to false negative test results (Park,
Corticosteroids also depress the NBT test response of the neutrophils in vitro (Henkel Chretien & Garagusi, 1971 a). Norden & Reese (1972) found elevated NBT test figures in blood from healthy women on oral contraceptives. However, we were not able to confirm their results (Björkstén & Solheim, 1973). Apparently several drugs may influence the test results.

PRESENT INDICATIONS FOR THE NBT TEST IN CLINICAL PRACTICE.

The early optimistic reports on the NBT test as a valuable tool in the differentiation of bacterial, fungal and parasitic infections from viral infections and other diseases causing fever and/or leucocytosis have not been fulfilled. An evaluation of the NBT test results obtained by different investigators is however complicated by the fact that methodological factors may have influenced the test results.

False negative test results in the presence of bacterial infections involving the systemic circulation are common and so are, although to a smaller extent, false positive test results in viral infections. A number of non-infectious diseases, pregnancy, corticosteroids, cytostatics, some antibiotics and possibly other drugs may influence the test results. A careful evaluation of how the NBT test is influenced by different infections in different parts on the body may reveal its precise indications. Until this has been done the test should not be introduced as a routine test in the aetiologic differentiation of fever and leucocytosis of unknown origin.

So far the NBT test seems to be of value; a) as a screening test for Chronic Granulomatous Disease and b) in the differentiation of pyelonephritis from lower urinary tract infections (V). Further, other investigators have shown the test to be of value c) in the differentiation of bacterial and viral meningitis (Fikrig et al, 1973) and d) in monitoring patients with an increased susceptibility to bacterial infections (Wollman et al, 1972).
SUMMARY

The nitroblue tetrazolium (NBT) test, introduced by Park et al in 1968, by which neutrophilic granulocytes may be divided into NBT positive and NBT negative cells, depending on whether they reduce the dye into nitroblue formazan or not, has been recommended as an aid in the differentiation of bacterial and fungal infections from viral infection. Further the test has been used as a screening test for granulocyte dysfunction syndromes.

The aim of this investigation was to study the influence of different technical factors on the NBT test and to investigate its clinical value in different clinical conditions.

The influence of technical factors on the NBT test.

This was studied in papers I and II. The presence of serum was found necessary for the test to function and fresh serum was more efficient than heat-in-activated serum (I). Capillary blood was not suitable for demonstrating an elevated proportion of NBT positive neutrophils. This could at least in part be explained by the fact that haemolysis was shown to have an inhibitory effect (I). Blood samples could be stored at 4°C for at least 8 hours without this influencing the test results (II). Storage at 23°C for 8 hours or more, often resulted in elevated NBT test figures (II). High concentrations of heparin caused elevated NBT test figures (II). The blood NBT solution mixture should be incubated for a constant time interval (25 minutes), using a 37°C water bath (II).

A suitable test procedure for the performance of the NBT test is given. Using this NBT test method normally less than 13% of the neutrophils are NBT positive (III). The total number of NBT positive neutrophils is normally not more than 500 per cu.mm. blood.
NBT test values in different types of infections

In paper III the value of the NBT test in the aetiological diagnosis of acute throat infections was investigated in 40 patients. High NBT test figures were found in about 50% of patients with streptococcal infections but in none of the patients with viral infection. A high NBT test figure would be an indication of streptococcal infection, while on the other hand a normal NBT test result does not exclude infection with that micro-organism.

The NBT test response was investigated in 95 patients with acute abdominal symptoms; forty of which had focal, gangrenous or perforated appendicitis (IV). The test was of no value in the preoperative diagnosis of appendicitis.

In paper V the NBT test was used for level diagnosis in 37 patients with urinary tract infections. Elevated proportions of NBT positive neutrophils were found in 11 of 14 patients with pyelonephritis, but only in 3 of 23 patients with lower urinary tract infection. The total number of NBT positive neutrophils was 1000 or more per cu.mm. blood in 12 of 14 patients with pyelonephritis, while it was 800 or less in all the investigated patients with lower urinary tract infections. Thus the NBT test was found to be of value in the differentiation of lower urinary tract infections from pyelonephritis.

The results of NBT tests in 129 patients with a variety of bacterial and viral infections were evaluated. High test values were obtained in 49% of the patients with bacterial infections and in 14% of those with viral infections.

The NBT test in Chronic Granulomatous Disease.

The NBT test was used to confirm the diagnosis of Chronic Granulomatous Disease (CGD) in a girl suffering from recurrent severe bacterial infections since early childhood (VI). Investigation of relatives showed abnormal NBT test
results in her mother and a brother. These findings could possibly be ex-
plained by autosomal dominant inheritance with variable expressivity. This
has not been described previously.

Conclusions
Since the NBT test is influenced by a number of technical factors, the test
method must be carefully standardized and each laboratory must determine
the normal NBT test values. Even with a standardized technique there is a
considerable overlap in the test results obtained in blood samples from
patients with different types of infections. Further the test is influenced
by pharmacotherapy and several non-infectious diseases. The test should at
present not be introduced as a routine test in the differentiation of fever
and leucocytosis of unknown origin. However, the NBT test is useful a) as
a screening test for Chronic Granulomatous Disease, b) in the differentiation
of pyelonephritis from lower urinary tract infections, c) in the differentia-
tion of bacterial and viral meningitis and d) in monitoring patients with
an increased susceptibility to bacterial infections.

ACKNOWLEDGEMENTS
I wish to express my sincere gratitude to:

Karl Martin Lundmark, M.D., for directing my interest to granulocyte function,
for his support and for many hours of stimulating discussions.

Professor Bertil Hoorn for his interest, helpful advice and generosity in
placing resources of his department at my disposal.

Professor Jan Winberg for his invaluable advice and generous support as well
as for many inspiring suggestions.

Gunhild Beckman, Ph.D., professors Lars Beckman, Jan Carlsson and Stig Holm
as well as the staffs in the departments of Bacteriology and Paediatrics
for valuable criticism, inspiring interest and valuable help in many ways.
Bertil Cedergren, M.D. and his staff for valuable contribution with samples from blood donors.

Mrs Monica Nimrodsson, Mrs Åsa Nordvall and Mr Håkan Persson as well as all the other members of the staff at the department of Virology, for invaluable technical assistance.

Mrs Birgitta Backman, B.A., for revising the English manuscript.

Miss Katrine Andersson for skilful typing of the manuscript and for valuable help with the figure drawings and Miss Gunilla Boström for skilful typing.

The investigation was supported by a grant from the Faculty of Medicine, University of Umeå.
REFERENCES


