SOME ASPECTS OF LEUCOCYTE METABOLISM : "IN VITRO" EFFECTS OF DRUGS INDUCING AGRANULOCYTOSIS ON SEPARATED HUMAN LYMPHOCYTES AND POLYMORPHONUCLEAR LEUCOCYTES

Thesis

Presented for the degree of

Doctor of Philosophy

in the University of Edinburgh

by

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July, 1968

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AGRANULOCYTOSIS

INTRODUCTION

The blood dyscrasias produced by adverse reactions to drugs are a diverse group. They take the form of cytopenias occurring infrequently and generally unpredictably. To date more than 350 drugs have been associated with haematological disorders (American Medical Association Registry on Adverse Reactions).

It is beyond the scope of this work to consider these dyscrasias in detail, though the five major ones will be considered briefly. These are:-

1. Thrombocytopenia

This is characterised by a depressed platelet count. The normal platelet count is 250,000 - 350,000 per cmm. falling in thrombocytopenia to 40,000 per cmm. or less when the symptoms of thrombocytopenic purpura appear, these being localised or general haemorrhage. This condition can be induced by such drugs as Sedormid (allylisopropylacetylurea) and quinidine. Thrombocytopenia has been shown to be an immune reaction by the demonstration of drug-dependent antibodies against platelets. (Sedormid antibodies - Ackroyd 1953, quinidine antibodies - Bolton 1956).

2. Haemolytic anaemia

This condition is characterised by a persistent anaemia

with a resulting reticulocytosis. Haemolytic anaemia is in some instances an immune reaction as evidenced by the demonstration of drug-dependent Coombs - positive red cell antibodies. An example of a drug acting in this way is Stibophen (Snapper et al 1953, Harris 1956).

Haemolytic anaemia as a drug-induced blood dyscrasia is also relatively frequent among individuals who have a heritable deficiency of the enzyme glucose-6-phosphate dehydrogenase. This deficiency renders the individual sensitive to the toxic actions of certain oxidant drugs such as pamaquin (Carson 1956, Tarlov 1962).

3. Megaloblastic anaemia

A state of macrocytosis is found in this condition. The bone marrow picture is similar to that found in folic acid deficiency. It is thought that drugs inducing megaloblastic anaemia e.g. hydantoin derivatives, interfere with folic acid metabolism in the bone marrow (Flexner 1960).

4. Aplastic anaemia

This is manifested by a reduction in the formative elements of the bone marrow, though the degree of depression of the various cell lines is variable. About one half of the reported cases are considered to be idiopathic, though many

may have resulted from undisclosed exposure to drugs or other noxious agents. Two drugs commonly associated with aplastic anaemia are benzene (Hunter, 1939) and chloramphenicol (Registry). Drug-dependent antibodies have not been demonstrated.

Aplastic anaemia has the highest fatality rate of all blood dyscrasias.

5. Agranulocytosis (granulocytopenia).

In this condition there is a decrease in the number of circulating and marrow granulocytes. It is the most frequent blood dyscrasia constituting 40% of all cases reported to the Registry on Adverse Reactions. Table 1 shows the number of cases of agranulocytosis due to certain drugs. Almost all cases have resulted from long-term therapy with these drugs.

Since this work has been concerned with those drugs inducing agranulocytosis this condition will be considered in more detail.

Table 1. Incidence of agranulocytosis

Drug	Cases of agranulocytosis	
	Drug alone*	Total +
Chlorpromazine	83	194
Thiouracil	19	25
Amidopyrine	14	65
Phenylbutazone	17	44
Chloramphenicol	18	75
Tetracycline	1	47
Sulphisoxazole	9	48

* Drug inducing agranulocytosis was the only drug administered.

+ All cases of agranulocytosis, due to the drug alone and also where other known toxic drugs were administered along with the named drug.

AGRANULOCYTOSIS

with particular reference to amidopyrine.

1. The symptoms of agranulocytosis

Agranulocytosis as a clinical entity was first described by Schultz in Germany in 1922. It is characterised by a

marked leucopenia with a white cell count falling as low as 500/cmm. (normal range 5,000 - 10,000/cmm.). The percentage of granulocytes falls until none may be present. The bone marrow generally shows a virtual absence of granular cells and their precursors. These symptoms are generally accompanied by sore throat, fever and prostration. In fulminating cases the patient dies within a few days from toxaemia and septicaemia.

2. The occurrence of agranulocytosis

The incidence of this dyscrasia is more frequent in women than in men, 70-80% of cases occurring in women generally in the age group of 40-60 years. The patient generally has a history of having taken a drug occasionally or for prolonged periods. (Plum and Thomsen 1940, Borglin and Mansson 1951).

Using rats, Borglin and Mansson (1951) showed that castrated females were more susceptible to the central toxic actions of amidopyrine (convulsions with subsequent death) than normal females, normal males, and castrated males. Administration of oestrogens to castrated females restored their resistance to the toxic actions of amidopyrine to the level of normal females. It was therefore suggested that

the higher incidence of agranulocytosis in women aged 40-60 years might be due to the decreased oestrogen levels at that period of life.

3. Theories of the aetiology of agranulocytosis - historical

a) Bacterial toxins In 1924 Lovett examined a patient with diagnosed diphtheria who had a negligible granulocyte count. He was able to isolate Bacillus pyrocyaneus from the patient's blood; on injecting a culture of this micro-organism into guinea pigs he observed a granulocytopenia to develop after five days. He therefore suggested that agranulocytosis could be due to:-

a) a local infection with some specific micro-organism which had a direct toxic action on the bone marrow;

b) an injury to the granulocytes during the maturation period which led to a consequent lack of resistance to infection.

Dennis (1933) found he could produce a sustained leukopenia in rabbits by inserting a plastic capsule containing a culture of Streptococcus viridans into the abdominal cavity, this organism having been isolated from a case of fatal agranulocytosis. The culture remained in the capsule while the toxins diffused out. He obtained inconclusive results using cultures of Staphalococcus aureus, Streptococcus

haemolyticus and Bacillus proteus, all these organisms being isolated from the same case of agranulocytosis. Since the pathogenic factor was diffusable, he suggested that a leucocidin rather than a specific micro-organism was responsible for the phenomenon of agranulocytosis.

A bacterial aetiology for agranulocytosis is unlikely since it has been clearly shown (Roberts and Kracke 1930) that the basic pathology is:-

a) a disappearance of granulocytes, and

b) a subsequent bacterial invasion.

b) Glandular dysfunction Corey and Britton (1932) observed that the removal of the adrenals in cats and rabbits led to a marked leucopenia, though not to a characteristic agranulocytosis. The leucopenia could be corrected by administration of cortico-adrenal extract. It was suggested that agranulocytosis was associated with adrenal insufficiency.

c) Vitamin deficiency On feeding monkeys a diet deficient in vitamin G (B_2), Day et al (1935) found a slight leucopenia to occur, the granulocytes being reduced more than the other cell types. Since a typical agranulocytosis was not obtained they were unconvinced that the vitamin deficiency was the cause of the leucopenia.

d) Bone marrow idiosyncrasy In the course of fatal agranulocytosis, the leucopenic state, with an absence of granulocytes, persists until death, although the marrow may be hyperplastic (Fitz-Hugh and Krumbhaar 1932). This finding led to the conclusion that the agent causing agranulocytosis either:-

a) arrested the maturation of the granulocytes at their formative centres, or

b) produced degenerative changes in the cells before they were sufficiently developed for their normal migration into the blood stream.

e) A chemical aetiology In 1910 Selling observed that persons working in a tin can factory, where large quantities of benzene were used, showed a high incidence of leucopenia. He was able to produce similar leucopenic states in rabbits by the injection of benzene. He concluded that the leucopenia was the results of a direct toxic action of the benzene on the blood cells.

Somewhat later (Kracke 1931), a case of recurrent agranulocytosis was observed in a woman who had a history of taking large doses of coal tar derivatives. It was postulated that the probable aetiological factor in this case was a substance containing benzene or one of its derivatives. Further investigations were made (Kracke and Parker 1934 a, b) it

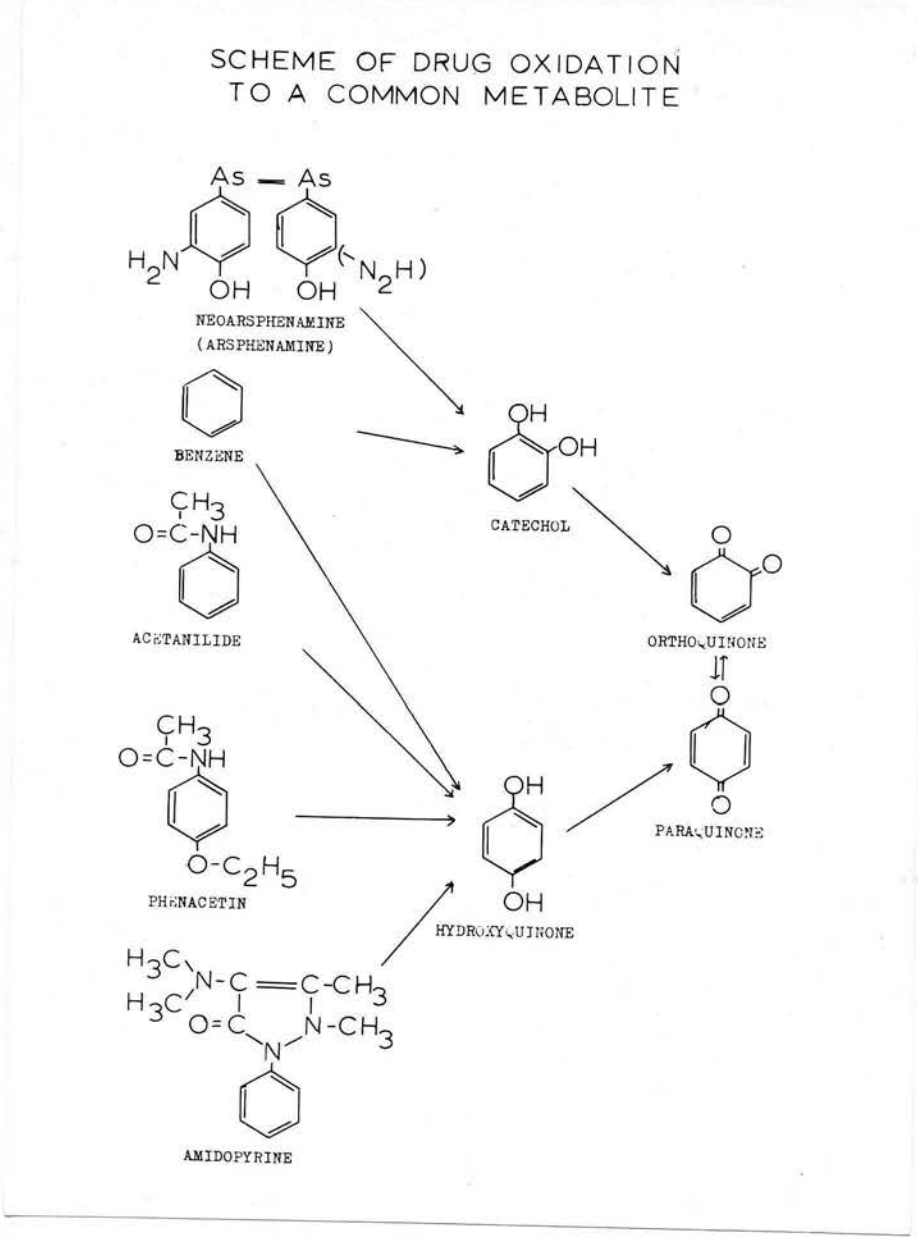

SCHEME OF DRUG OXIDATION
TO A COMMON METABOLITE
As — As
H_2N
OH
OH
N_2H
NEOARSPHENAMINE
(ARSPHENAMINE)
BENZENE
OH
OH
CATECHOL
CH_3
O=C-NH
ACETANILIDE
ORTHOQUINONE
CH_3
O=C-NH
$O-C_2H_5$
PHENACETIN
OH
OH
HYDROXYQUINONE
PARAQUINONE
H_3C
H_3C
N-C
C-CH_3
O=C
N-CH_3
N
AMIDOPYRINE

Figure 1.

being shown that the increasing incidence of agranulocytosis could be correlated with the increasing usage of such drugs as amidopyrine, acetanilide, arsphenamine and neoarsphenamine. All these drugs contain a benzene ring. The theory was advanced that the common factor between these drugs when degraded in the body was an oxidation product of benzene (Fig. 1), and that it was this oxidation product which was the agent responsible for inducing agranulocytosis. In support of this idea was the observation that an apparent state of agranulocytosis could be produced in rabbits by the injection of catechol or quinone.

4. The aetiology of agranulocytosis - Current ideas

a) The drug acting as a hapten In 1931 Pepper noticed that almost all of his patients who experienced agranulocytosis had a history of some type of allergy, suggesting that some type of allergic reaction was involved in agranulocytosis.

Madison and Squier (1934; Squier and Madison, 1935) while supporting the idea that an oxidation product of benzene was the aetiological factor responsible for the occurrence of agranulocytosis, put forward the view that this adverse reaction was not due to a direct toxic action but rather to an allergic or anaphylactoid hypersensitivity reaction. This idea stemmed from their observations that when a test dose of amidopyrine

was administered to an amidopyrine-sensitive patient, a rapid and profound leucopenia developed which subsequently recovered spontaneously.

Similar results were obtained by Benjamin and Biederman (1934). Since scratch tests, intracutaneous tests, and passive transfer tests using amidopyrine-sensitive serum all yielded negative results, they proposed that agranulocytosis was due to a drug hypersensitivity reaction with the haemopoietic system acting as the shock organ.

Leucocyte agglutinins were first demonstrated by Moeschlin and Wagner (1952). Using serum from an amidopyrine-sensitive patient who had just received a test dose of the drug they ~~were~~ were able to show 'in vitro' agglutination of leucocytes taken from both the sensitive patient and normal donors. They also demonstrated that 500 ml. of whole blood taken from an amidopyrine-sensitive patient immediately following a test dose, when transfused into a normal donor, resulted in a pronounced fall in the circulating granulocyte count of the recipient within 30 minutes, followed by a spontaneous recovery in around four hours.

The mechanism of leucocyte agglutination is as yet not fully understood. Following ingestion of the drug there are two ways in which antibody (Ab) formation might occur:

i) the drug itself may act as the antigen (Ag); this is unlikely due to the small size of drug molecules;

ii) the drug molecule may act as a hapten which, when combined with a protein, forms an Ag which in turn induces Ab formation. This protein is a soluble plasma protein and not a leucocyte protein since, firstly, although the drug will combine with the leucocyte the bond between the two is so weak that a quick wash will remove the drug. For a hapten-protein complex to induce Ab formation the bond between the two must be very stable (Shulman, 1964), and, secondly, agglutination tests with strongly positive sera produce agglutination of heterologous as well as of homologous leucocytes (Moeschlin, 1958).

The anti-amidopyrine Ab appears to be a thermostable gamma globulin, whose antigenic activity is directed against the phenazone group in amidopyrine, and also but to a lesser extent in similar compounds which contain a phenazone group. (Thierfelder et al 1964).

Moeschlin (1958), from his studies of amidopyrine-induced agranulocytosis, considered that after induction of the Ab by the haptenic determinant of the drug-protein complex, the Ab became fixed to the cells; reintroduction of the drug caused an Ag-Ab

reaction, with agglutination of the leucocytes and their subsequent destruction.

Shulman (1964) in his review of quinine and quinidine-induced purpura and of Stibophen-induced haemolytic anaemia, considered that following Ab induction and reintroduction of the drug, that the first reaction was of the Ab with the Ag. The Ab-Ag complex was then adsorbed nonspecifically by the cells, the adsorption being determined by the size, configuration, and charge of the Ag-Ab complex and of the cellular adsorption sites. Agglutination followed adsorption of the complex.

Much more is known about antibodies against platelets and red cells than against leucocytes. The information concerning all three cell types is, in the main, very similar.

Leucocyte agglutination results in massive peripheral leucocyte destruction which stimulates the release of mature, and later of immature, granulocytes from the marrow, the marrow eventually becoming hypoplastic.

Leucocytes agglutinated 'in vivo' are removed from the circulation by the lung capillaries (Bierman et al 1951; Weisberger and Heinle 1951).

Not all cases of amidopyrine-induced agranulocytosis are accompanied by detectable antibodies. It seems possible that most cases

are due to Ab formation since very few instances are known where an attack has begun in less than 5-7 days which is the time required for Ab formation. The fact that Ab's frequently cannot be demonstrated may well be a fault in the methods of Ab detection.

b) An abnormally high drug concentration If abnormally high concentrations of a drug in the blood were produced, for instance by increased absorption, decreased excretion, or decreased detoxification, then normal cells might be damaged. Alternatively, if a cell were sensitive to a drug at a normal therapeutic blood concentration, the same effect would be observed. This toxic mechanism is found in individuals who have a heritable deficiency of erythrocytes glucose-6-phosphate dehydrogenase, which renders the erythrocytes susceptible to the toxic actions of certain oxidant drugs such as pamaquin.

c) A metabolic abnormality It is conceivable that a susceptible individual could metabolise a drug to an abnormal metabolite or else to a metabolite which is not toxic to normal subjects. An abnormal metabolite might have unusually potent antigenic properties. A metabolic abnormality has not been demonstrated.

d) Drug acting as an antagonist A drug or one of its

metabolites could antagonise a metabolic pathway which is vital in a sensitive person, but which is not vital in normal individuals due to the availability of other pathways. This has not been demonstrated.

None of these proposals for the aetiology of agranulocytosis can explain all cases. It is probable that a number of factors in combination render an individual sensitive to the toxic actions of a drug.

THE PRESENT WORK

Only a small proportion of the population is liable to experience agranulocytosis, this blood dyscrasia being induced by one of a large number of drugs. It is not known what factors predispose these individuals to this drug-induced condition. Since polymorphs are affected to a much greater extent than lymphocytes by these drugs, it was decided to investigate the actions of some drugs known to induce agranulocytosis on certain 'in vitro' metabolic reactions of human peripheral leucocytes, to determine if there were any metabolic differences between these two leucocyte types.

Initially it was hoped to observe the effects of the drugs on bone marrow maturation as determined by changes in cell morphology. However, on reading the literature it soon became

evident that a method for culturing marrow 'in vitro', such that maturation proceeds as 'in vivo', does not exist, despite the efforts of many workers over the last thirty years. The use of peripheral leucocytes was considered justifiable since there is little evidence to suggest that the metabolism of circulating granulocytes is basically different from their metabolism in the marrow, (Seitz, 1965). The use of peripheral blood had the advantage that normal blood is readily available, whereas normal marrow is not easily obtained, and also that leucocytes can be separated into their constituent cell types largely free from other cell types, while marrow is a heterogeneous mixture of cells. Recently rat marrow has been partially separated by means of a glasswool column to remove most of the granulocytes, followed by centrifugation of the remaining cells through a dextran density gradient system. At the moment this method yields only lymphoid cells, but it should be possible to develop it such that myeloid cells can be recovered (Morrison 1967, Morrison et al 1968).

When proposing reasons why some individuals are more sensitive to the toxic actions of these drugs than are other individuals, certain factors common to all cases of agranulocytosis must be borne in mind:

i) the incidence of agranulocytosis is low for all drugs;

ii) drug-induced agranulocytosis occurs after the administration of normal therapeutic doses over a period of time varying from a week to several months;

iii) agranulocytosis is not a side-effect since

a) its occurrence is unpredictable, and

b) its occurrence is in no way related to the normal pharmacological actions of the drugs.

One possible explanation which could account for these observations is that there is a genetic factor inducing susceptibility to agranulocytosis, but as yet there is no evidence for this. Hereditary factors have been shown to be involved in certain other conditions such as the heritable deficiency of erythrocyte glucose-6-phosphate dehydrogenase, and the heritable deficiency of pseudocholinesterase which renders individuals highly sensitive to the actions of suxamethonium (Lehmann and Liddell, 1962). A familial incidence of aplastic anaemia due to chloramphenicol has also been reported (Rosenthal and Blackman, 1956).

Before any hereditary metabolic abnormality could be demonstrated it was necessary to determine the normal metabolic characteristics of leucocytes, and also the effects of drugs thereon. This was the primary objective of the

present work. If it could be demonstrated that leucocytes obtained from drug-sensitive patients, and also from their siblings, had different metabolic characteristics with regard to the effects of the sensitising-drugs, then some evidence for a hereditary factor inducing susceptibility to agranulocytosis would be suggested.

SECTION 2

SEPARATION OF LEUCOCYTES AND THE MEASUREMENT OF LEUCOCYTE OXYGEN UPTAKE

THE METABOLIC PARAMETERS TO BE STUDIED

Following the decision to use peripheral blood leucocytes it was necessary to find suitable metabolic parameters for investigation. There were two alternative approaches to this question:-

a) investigate the effects of the drugs on specific enzyme systems, or

b) investigate the effects of drugs on metabolic processes involving a number of enzymes.

The second method was used ~~in the initial studies~~ since it was thought more likely that a drug effect would be observed on a number of enzymes rather than on a single enzyme.

The first metabolic reaction selected for investigation was the cell respiration, this parameter being chosen because it is an indicator of the general metabolic integrety of the cell, and also because it is fairly easily measured.

A number of preliminary experiments were performed to check that the leucocyte respiration was measurable, to determine the best method for expressing the results, and to determine if any drugs showed a noticable effect on leucocyte respiration.

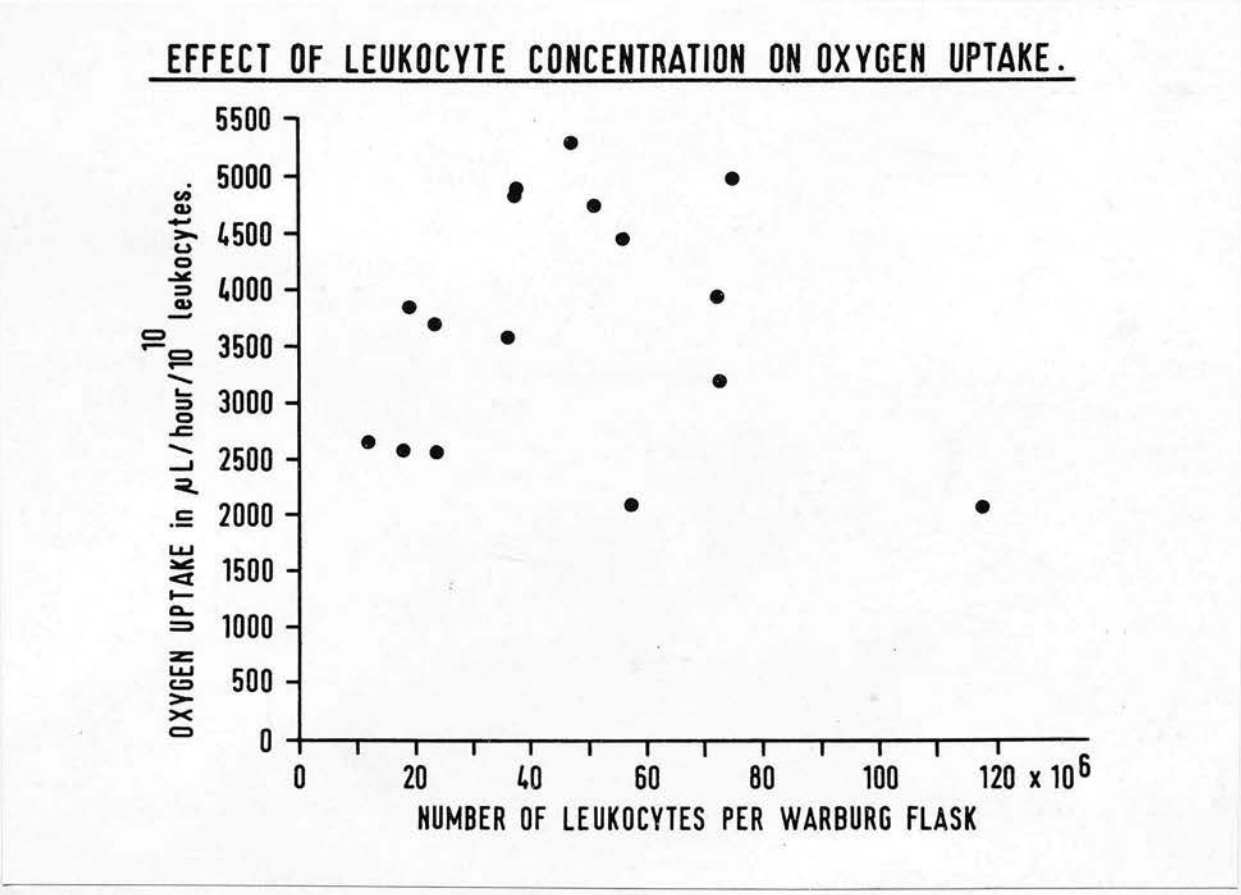

EFFECT OF LEUKOCYTE CONCENTRATION ON OXYGEN UPTAKE.
OXYGEN UPTAKE in µL/hour/10^10 leukocytes.
5500
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
0
20
40
60
80
100
120 x 10^6
NUMBER OF LEUKOCYTES PER WARBURG FLASK

Figure 2.

Rabbit leucocytes were used in these experiments, leucocyte-rich suspensions being prepared using the buffy-coat technique, the respiration being measured in a conventional Warburg apparatus. The results of these experiments were as follows:-

a) Using leucocyte concentrations of 12-118 x 10^6 leucocytes/Warburg flask the rate of oxygen uptake (= respiration) was found to range from 4-37 µL/hour which was quite measurable.

b) If the oxygen uptake was proportional to the number of leucocytes being used it would be legitimate to express all the results in the same units. However if the oxygen uptake was not proportional to the leucocyte concentration all experiments would have to be performed using the same leucocyte concentration to allow the results to be expressed in terms of the same units. A number of experiments were done to estimate the oxygen uptake using varying leucocyte concentrations. A graph was then plotted of the oxygen uptake in µL/hour/10^{10} leucocytes against the number of leucocytes/Warburg flask (Fig. 2). No apparent crowding effect was observed, that is as the leucocyte concentration increased the oxygen uptake/hour/10^{10} leucocytes did not decrease. It thus seemed legitimate to

express all the results in the same units regardless of the number of leucocytes per flask.

c) Chloramphenicol at 4.0×10^{-3} M was found to inhibit the respiration by about 60%, while 1.0×10^{-3} M inhibited by about 33%. This indicated that inhibitory effects of the drugs should be measurable.

The second metabolic parameter which was investigated was the leucocyte lactic acid production. This parameter was chosen because leucocytes are known to exhibit the Pasteur effect, that is when the respiration is depressed, for instance by a reduced oxygen tension, then the lactic acid production is stimulated (Hedeskov and Esmann, 1966; Bird, 1951; this work Section 3, page 72, Fig. 20).

SEPARATION OF LEUCOCYTES FROM WHOLE BLOOD 'IN VITRO'

It was mentioned on page 19 that chloramphenicol was found to inhibit leucocyte respiration. However since blood contains two main leucocyte types - polymorphonuclear leucocytes (polymorphs or PMN) and lymphocytes - it was not possible to determine whether both cell types were being affected, or whether only one type was being inhibited. It was therefore necessary to find a method for separating these two cell types.

In the past many observations on the biochemical behaviour of human leucocytes have been made on normal unseparated suspensions because it was not possible to separate the cell types. Leukaemic leucocytes have also been used since it was possible to obtain relatively pure suspension of these cells. However these are not normal cells. For instance the respiration of normal leucocytes is greater than the respiration of chronic myeloid leucocytes which in turn is greater than the respiration of chronic lymphocytic leucocytes (Beck and Valentine 1952)

Many different methods for isolating leucocytes from whole blood have been used with varying degrees of success in the past. Very briefly a few of these methods are:-

a) allowing the anticoagulated blood to sediment giving a leucocyte rich supernatant;

b) centrifuging the blood to give the so-called "buffy-coat" where the leucocytes are layered on top of the erythrocytes;

c) centrifuging the blood sample in a waisted tube of such a size that the leucocytes collect in the narrow section of the tube in three distinct layers, these being, from top to bottom, lymphocytes, PMN, and erythrocytes (Haight and Rossiter 1950, Ventzke et al 1959).

d) centrifugation through a sucrose gradient (Archdeacon et al 1964, ~~Stollof~~ Stoloff and Weiss 1963).

e) centrifugation through bovine serum albumin of specific gravity about 1.079, the leucocytes sinking to the bottom (Vallee et al 1947).

f) addition of high molecular weight substances such as dextran, fibrinogen, or phytohaemagglutinin, causing rouleaux formation of the erythrocytes with a subsequent rapid sedimentation of these cells. Dextran and fibrinogen are more efficient than phytohaemagglutinin (Skoog and Beck 1956). Phytohaemagglutinin is frequently used in chromosome studies since it promotes mitosis.

Animal leucocytes may be obtained in almost pure suspensions by the injection of certain substances into the abdominal cavity. Saline causes the migration of PMN (Rossi and Zatti 1964), and turpentine the migration of lymphocytes (Allison et al 1964). It is doubtful if these leucocytes are entirely normal since their migration is the result of a stress reaction. Animal lymphocytes can also be obtained in pure suspensions from the thoracic lymph ducts (Bollman et al 1948).

SEPARATION OF LEUCOCYTES BY THE GLASS BEAD COLUMN METHOD

A considerable advance in the technique of cell separation was made by Johnson and Garvin in 1959. They found that if heparinised human blood was passed through a column of siliconed glass wool contained in a glass tube, the PMN were retained while the lymphocytes and erythrocytes passed straight through. Garvin (1961) investigated this phenomenon and found that if glass beads were used instead of glass wool, the PMN were still retained but could be recovered by washing the column with a calcium-and magnesium-free solution containing 0.02% of ethylene diamine tetraacetic acid, disodium salt (EDTA). The optimum conditions for PMN retention were found to be

i) a long glass bead column,

ii) a slow flow rate when eluting the column to remove lymphocytes and erythrocytes (eluting fluid was plasma),

iii) pH 7.4,

iv) a temperature of 37^{o}C, and

v) calcium and magnesium must be present.

This method for separating PMN from lymphocytes was quantitated by Rabinowitz in 1964. This method with some modifications has been used in this work.

DIAGRAM OF COLUMN USED
FOR LEUKOCYTE SEPARATION

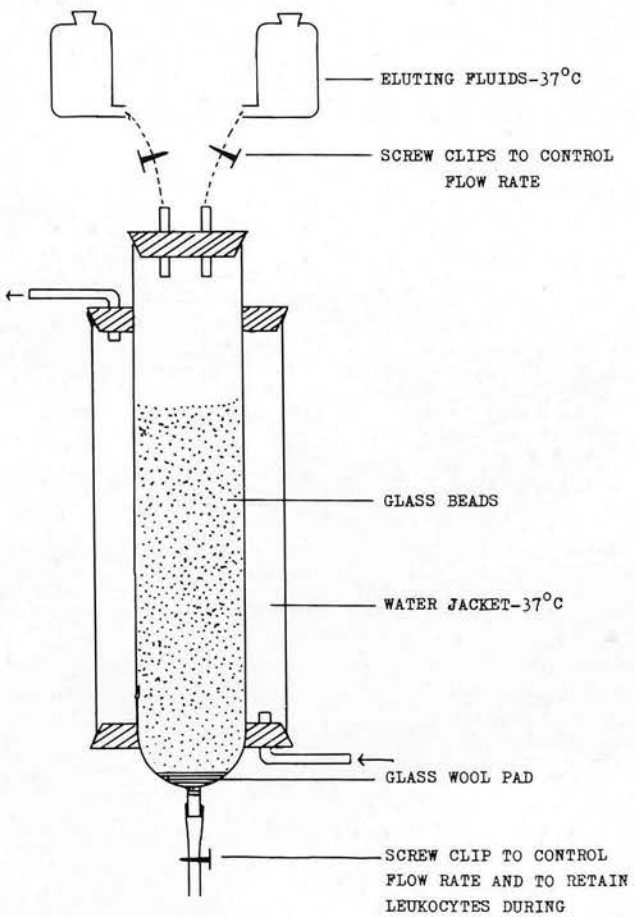


Figure 3.

1. Principles of the separation procedure

A leucocyte-rich suspension was prepared using the buffy-coat technique, the leucocytes being resuspended in plasma to a volume suitable for the column being used. The leucocyte suspension was added to the column and incubated at 37°C for 30 minutes. The leucocytes were removed from the column by washing with

i) 80% Hanks BSS + 20% plasma, pH 7.4, giving erythrocytes and lymphocytes;

ii) a Ca^{++}- and Mg^{++}- free solution containing 0.02% EDTA, pH 7.4, giving PMN.

2. Materials

Blood in 20 ml and 100 ml volumes was obtained from haematologically normal volunteers. Large volumes (400 ml) were obtained from the Blood Transfusion Service, Royal Infirmary, Edinburgh. This blood was generally from donors with a past history of infectious hepatitis, the haematological picture being normal. Preservative-free heparin (10U/ml) was used as the antieoagulant.

Fig. 3 shows the apparatus used. The glass column was made from Pyrex tubing, its size being 1.0 cm. diameter x 20 cm height for 20 ml. blood samples, 1.5 x ~~26~~ 26, or 2.5 x 35 cm.

columns were used for the larger blood samples. The column was surrounded by a water jacket.

The glass beads[a] had a mean diameter of 0.2 mm., a volumn of 4.19×10^{-6} cc., and a surface area of 1.26×10^{-3} cm^2. Each centimeter of the 1.0 x 20 cm. column contained 106,000 beads (Garvin, 1961). A ~~samll~~ small pad of glass wool was placed at the bottom of the column to retain the beads. The beads were discarded after use.

All glassware with which the blood came into contact was siliconed. Initially Repelcote[b] was used but this appeared to give a poorly siliconed surface. For this reason the silicone was changed to M441[c] for all the glass except the beads which were siliconed with the water-soluble silicone Siliclad[d]. After siliconing, all glassware was washed 10 times in tap water and 10 times in glass-distilled water. After use siliconed glass was soaked in Decon 75[e] overnight, followed by 20 tap water washes and 10 distilled water washes. After three experiments the silicone was removed with chromic acid, the glass then being re-siliconed, All other glassware was cleaned with chromic acid after each experiment and washed as indicated above.

All centrifugation was carried out in an M.S.E. Super Multex swinging-arm centrifuge.

Erythrocyte and nucleated white cell counts were ~~done~~ made using an Improved Neubauer counting chamber.

Differential leucocyte counts were initially performed using Leishman stain. It was later found that the differential counts could be made at the same time that the nucleated cell counts were done. Not only was this easier than making smears, but the results were more reproducible and were within 1% of counts made with stained smears.

a Ballatoni grade number 11 : Jencons (Scientific) Ltd., Hemel Hempstead, Herts.

b Repelcote; Hopkins and Williams Ltd.

c M 441 Silicone : I.C.I.

d Siliclad : Clay-Adams Inc. , New York. Distributed by Arnold R. Horwell Ltd., London.

e Decon 75 : Medical Pharmaceutical Developments Ltd.

3. Solutions

1) Hanks buffered salt solution (hereafter Hanks) :

Sodium chloride	8.00 gm./litre
Potassium chloride	0.40 gm./litre
Calcium chloride,6H20	0.14 gm./litre
Magnesium sulphate.7H20	0.20 gm./litre
Disodium hydrogen ortho phosphate	0.12 gm./litre
Potassium dihydrogen orthophosphate	0.06 gm./litre
Sodium bicarbonate	0.35 gm./litre
Glucose	1.00 gm./litre
Distilled water	to 1000 mls.

This solution when mixed with 20% plasma was used as the lymphocyte (and erythrocyte) eluting fluid.

2) 0.02% EDTA solution:

Ethylene diamine tetraacetic acid, disodium salt (EDTA)	0.20 gm./litre
Sodium chloride	8.00 gm./litre
Potassium chloride	0.20 gm./litre
Disodium hydrogen orthophosphate	1.15 gm./litre
Potassium dihydrogen orthophosphate	0.20 gm./litre
Glucose	0.20 gm./litre
Distilled water	to 1000 mls.

This solution was used as the PMN eluting fluid. Plasma was added to the collecting tubes to a final concentration of 20%.

3.) Adjustment of pH

The pH of both of these solutions was adjusted to ~~pH~~ 7.4 using 0.5 N hydrochloric acid or 0.5 N sodium hydroxide.

4. Separation of Leucocytes - Procedure

a) Human Blood The leucocyte separation method was tested using 20 mls. human peripheral blood obtained via the intracubital vein. In these experiments the leucocyte-rich suspension was prepared using the buffy-coat technique since this method has been used successfully by numerous workers (e.g. Rubini et al 1961, Schultz and Kaminker 1962, Ohta 1964). The leucocytes were resuspended in 2-3 mls. plasma and applied to a 1.0 x 20 cm. glass bead column. A gentle positive pressure at the top of the column was required to induce the suspension onto the column. After leaving the suspension on the column for 30 minutes, the lymphocytes (and erythrocytes) and PMN were eluted from the column using the solutions detailed above; a gentle positive pressure was required. Table 2 shows the

TABLE 2. LEUCOCYTE YIELD AFTER SEPARATION - HUMAN BLOOD

Expt. no.	Total no. of leucocytes added to column	Differential counts: % polymorphs (% PMN) : % lymphocytes (%L)	Polymorphs			Lymphocytes		
			Number added to column	Number recovered from column	Yield	Number added to column	Number recovered from column	Yield
	$\times 10^6$	%PMN : %L	$\times 10^6$	$\times 10^6$	%	$\times 10^6$	$\times 10^6$	%
6	35.7	66.1 : 33.9	24.9	14.3	57.5	10.8	8.8	81.6
9	200.2	66.3 : 33.7	133.0	45.0	33.8	67.2	48.2	71.8
11	220.0	71.7 : 28.3	158.0	79.0	50.0	62.0	55.6	89.7
15	149.1	60.0 : 40.0	89.6	37.7	42.2	59.5	51.1	86.0
17	97.9	55.2 : 44.8	54.1	20.9	38.6	43.8	33.1	75.5
22	140.0	58.0 : 42.0	81.2	65.1	80.3	58.8	30.1	51.2
				Mean :	50.4			76.0
				S.E. :	6.90			5.63

results of these experiments. There would appear to be a 50% loss of PMN but only a 25% loss of lymphocytes.

b) Rabbit blood A number of separations were attempted using animal blood, the potentional advantage over human blood being that the effects of administration of the drugs over a period of time could be investigated.

It was felt inadvisable to use small animals such as rats, since the volume of blood obtainable from a single animal would be small, and so would necessitate mixing the blood from several animals. For this reason rabbits of 2-3 Kg were used. These animals yielded up to 100 mls. blood by cardiac puncture under nembutal anaesthesia (60 mg./Kg.) administered via the ear vein. A 20 gauge needle on a polythene canula was inserted into the heart, the blood being allowed to flow into a tube containing 10U/ml. heparin. The first 50 ml, came rapidly, the second 50 ml. requiring gentle suction from a syringe on the end of the canula. On attempting to perform a separation of the leucocytes in an identical manner to that successfully used for the human blood, the leucocyte-rich suspension was found to clot on the column. Increasing the heparin concentration to 50U/ml. appeared to prevent this

Table 3. LEUCOCYTE YIELD AFTER SEPARATION - RABBIT BLOOD

Expt. no.	Total no. of leucocytes added to column	Differential counts:- % polymorphs (%PMN) : % lymphocytes (%L)	Polymorphs			Lymphocytes		
			Number added to column	Number recovered from column	Yield	Number added to column	Number recovered from column	Yield
	x 10^6	%PMN : %L	x 10^6	x 10^6	%	x 10^6	x 10^6	%
0.02% EDTA								
7	102.4	28.1 : 71.9	28.7	0.1	0.4	73.7	43.8	59.4
8	77.4	45.9 : 54.1	35.6	0.9	2.4	41.8	19.5	46.4
10	100.0	55.2 : 44.8	-	-	-	45.0	22.6	50.2
18	284.8	38.9 : 61.1	111.0	7.5	6.8	173.8	156.5	90.2
				Mean:	3.2			61.2
				S.E.:	1.87			9.91
0.07% EDTA								
14	26.9	30.1 : 69.9	8.1	→0	0.0	18.8	14.8	78.8
16	64.9	25.3 : 74.7	16.4	0.924	5.6	48.5	43.7	90.5
20a	172.0	15.8 : 84.2	27.2	12.2	44.8	144.0	72.9	50.6
20b	172.0	15.8 : 84.2	27.2	8.3	30.5	144.0	62.3	43.3
21	145.8	15.0 : 85.0	21.8	2.0	9.18	124.0	54.0	43.5
				Mean:	17.8			61.3
				S.E.:	2.70			9.78

Table 4. RECOVERY OF LEUCOCYTES DURING COLUMN SEPARATION

Aliquot volume (ml)	Number of leucocytes / aliquot x 10^6	Differential count, % polymorphs : % lymphocytes	Polymorphs		Lymphocytes	
			Recovery x 10^6	Yield %	Recovery x 10^6	Yield %
EXPT. A. Leucocyte suspension Hanks:	1004.0	58.0 : 42.0	594		410	
0 - 10	155.0	15.7 : 84.3	24.3	4.1	130.7	31.8
10 - 20	111.0	5.3 : 94.7	5.9	1.0	105.1	25.6
20 - 30	66.0	7.0 : 93.0	4.6	0.8	61.4	15.0
30 - 40	25.2	13.0 : 87.0	3.3	0.6	21.9	5.4
40 - 50	11.6	14.5 : 85.5	1.7	0.3	9.9	2.4
50 - 60	8.5	32.6 : 67.4	2.8	0.5	5.7	1.4
60 - 70	7.4	31.1 : 68.9	2.3	0.4	5.1	1.2
70 - 80	-	-	-	-	-	-
80 - 90	-	-	-	-	-	-
90 -100	-	-	-	-	-	-
100 -110	6.9	60.0 : 40.0	4.1	0.7	2.8	0.7
110 -120	-	-	-	-	-	-
120 -130	5.5	16.4 : 83.6	0.9	0.2	4.7	1.1
EDTA:						
0 - 10	8.0	83.4 : 16.6	6.6	1.1	1.4	0.3
10 - 20	196.8	97.2 : 2.8	191.0	32.2	5.8	1.4
20 - 30	93.6	94.2 : 5.8	88.2	14.8	5.4	1.3
30 - 40	35.6	87.4 : 12.6	31.1	5.2	4.5	1.1
40 - 50	21.2	73.0 : 27.0	15.5	2.6	5.7	1.4
50 - 60	14.1	95.8 : 4.2	13.5	2.3	0.6	0.1
60 - 70	8.7	93.0 : 7.0	8.1	1.4	0.6	0.1
70 - 80	5.6	80.5 : 19.5	4.5	0.8	1.1	0.3
80 - 90	7.4	89.4 : 10.6	6.6	1.1	0.8	0.2
EXPT. B. Leucocyte suspension Hanks:	330.0	37.7 : 62.3	124		206	
0 - 10	73.0	0.0 :100.0	0.0	0.0	73.0	35.4
10 - 20	35.2	0.0 :100.0	0.0	0.0	35.2	17.1
20 - 30	17.6	0.0 :100.0	0.0	0.0	17.6	8.5
30 - 40	9.9	5.0 : 95.0	0.5	4.0	9.4	4.6
40 - 50	1.5	9.3 : 90.7	0.1	→ 0	1.4	0.7
50 - 60	1.3	5.0 : 95.0	0.1	→0	1.2	0.6
60 - 70	0.9	2.0 : 98.0	→ 0	→ 0	0.9	0.4
70 - 80	1.0	6.3 : 93.7	→ 0	→0	1.0	0.5
80 - 90	-	-	-	-	-	-
90 -100	-	-	-	-	-	-
100-110	0.6	4.8 : 95.2	→ 0	→ 0	0.5	0.3
EDTA:						
0 - 10	1.4	92.0 : 8.0	1.3	1.1	→ 0	→ 0
10 - 20	41.2	96.0 : 4.0	39.6	32.0	1.6	0.8
20 - 30	22.1	95.0 : 5.0	21.3	17.2	0.8	0.4
30 - 40	4.3	95.0 : 5.0	4.1	3.3	0.2	0.1
40 - 50	1.8	76.0 : 24.0	1.4	1.1	0.4	0.2
50 - 60	1.1	100.0 : 0.0	1.1	0.9	0.0	0.0
60 - 70	0.4	78.0: 22.0	0.3	0.2	0.1	→ 0
70 - 80	1.2	88.0 : 12.0	1.1	0.9	0.1	→ 0
80 - 90	1.0	91.0 : 9.0	0.9	0.7	0.1	→ 0

clotting. 0.02% EDTA proved unsuccessful in eluting the PMN from the column, so this concentration was increased to 0.07%. The results of these experiments are given in table 3. On comparing this table with table 2, it can be seen that human and rabbit lymphocytes give similar yields, while the PMN behave quite differently, human PMN being recoverable, rabbit PMN being unrecoverable.

Since it was not possible under these conditions to recover rabbit PMN, it was decided to use only human leucocytes.

5. The Eluting Volume Required to Effect Separation

In order to determine the volume of ~~eluting~~ fluid required to elute human PMN and lymphocytes, two experiments were done using a 1.5 x 26 cm. column and blood volumes of 50 and 80 mls. Following buffy-coat separation of the erythrocytes and incubation of the leucocyte-rich suspension on the column, 130 mls. of Hanks/plasma solution, and 100 mls. of the 0.02% EDTA solution were run through the column, the eluate being collected in 10 ml. aliquots. Nucleated and differential cell counts were made on each aliquot. Table 4 shows the leucocyte recoveries in each aliquot. A histogram (Fig. 4) was plotted of the mean percentage leucocyte recovery against the eluate volume, these

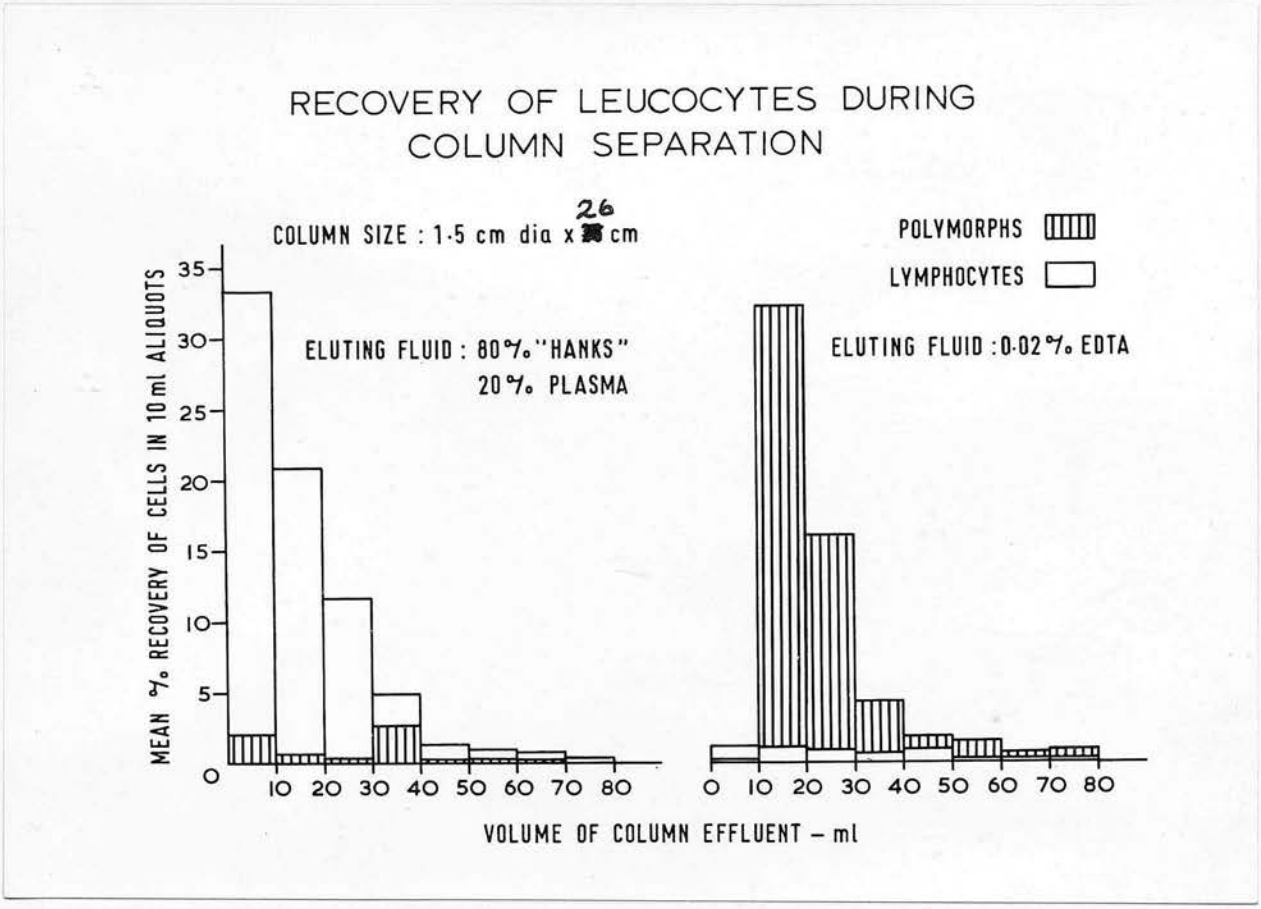

RECOVERY OF LEUCOCYTES DURING COLUMN SEPARATION
COLUMN SIZE : 1·5 cm dia x 26 cm
ELUTING FLUID : 80% "HANKS" 20% PLASMA
ELUTING FLUID : 0·02% EDTA
POLYMORPHS
LYMPHOCYTES
MEAN % RECOVERY OF CELLS IN 10 ml ALIQUOTS
0 5 10 15 20 25 30 35
10 20 30 40 50 60 70 80
0 10 20 30 40 50 60 70 80
VOLUME OF COLUMN EFFLUENT – ml

Figure 4.

Table 5a. Yields of leukocytes during column separation

Eluting fluid	Aliquot (mls.)	Polymorphs : % yield			Lymphocytes : % yield		
		Table 4a	Table 4b	Mean	Table 4a	Table 4b	Mean
80% Hanks+ 20% plasma.	0-10	4.1	0.0	2.05	31.8	34.5	33.6
	10-20	1.0	0.0	0.5	25.6	17.1	21.35
	20-30	0.8	0.0	0.4	15.0	8.5	11.75
	30-40	0.6	4.0	2.3	5.4	4.6	5.0
	40-50	0.3	→ 0	0.15	2.4	0.7	1.55
	50-60	0.5	→ 0	0.25	1.4	0.6	1.0
	60-70	0.4	→ 0	0.2	1.2	0.4	0.8
	70-80		→ 0	0		0.5	0.5
	80-90						
	90-100						
	100-110	0.7	0.0	0.35	0.7	0.3	0.5
	110-120						
	120-130	0.2		0.2	1.1		1.1
0.02 % EDTA.	0-10	1.1	1.1	1.1	0.3	→ 0	0.15
	10-20	32.2	32.0	32.1	1.4	0.8	1.1
	20-30	14.8	17.2	16.0	1.3	0.4	0.85
	30-40	5.2	3.3	4.25	1.1	0.1	0.65
	40-50	2.6	1.1	1.85	1.4	0.2	0.85
	50-60	2.3	0.9	1.6	0.1	→ 0	0.05
	60-70	1.4	0.2	0.8	0.1	→ 0	0.05
	70-80	0.8	0.9	0.85	0.3	→ 0	0.15
	80-90	1.1	0.7	0.9	0.2	→ 0	0.1
	90-100						

Table 5b. Contamination of leukocytes during column separation

	Polymorph suspension		Lymphocyte suspension	
	Number recovered x 10^6	Differential Count *	Number recovered x 10^6	Differential Count *
Table 4a	425.1	94.5 :5.5	347.3	12.5:87.5
Table 4b	70.1	99.96:0.04	139.7	0.7:99.3
Mean	247.6	97.23:2.77	243.5	6.6:93.4

* Differential count expressed as % polymorphs: % lymphocytes.

values being shown in table 5a. Table 5b shows the mean contamination of the pooled aliquots. It can be seen that the bulk of the lymphocytes are recovered in the first 40 mls.; a similar result was obtained for the PMN. In all subsequent experiments using this column size the eluting volumes were both 50-60 mls.

THE OXYGEN UPTAKE OF SEPARATED LEUCOCYTES

1. Method

The apparatus used for the measurement of oxygen uptake was a standard Warburg respirometer (Umbreit et al ~~1957~~ 1964). The flask volume was approximately 25 mls.

A leucocyte-rich suspension was prepared from 80-100 mls. of human blood using the buffy-coat technique. Following column separation the leucocyte suspensions were centrifuged down, the leucocyte pellets being resuspended in Hanks. The composition of the Warburg flasks was

leucocyte suspension in Hanks	1.0 mls.
plasma	0.8 mls.
~~medium 199~~ Hanks	2.2 mls.

giving a final composition of 1 part plasma : 4 parts Hanks. All the flasks were set up in duplicate. The centre well contained 0.3 mls. of 10% KOH on a piece of filter paper. The flasks were

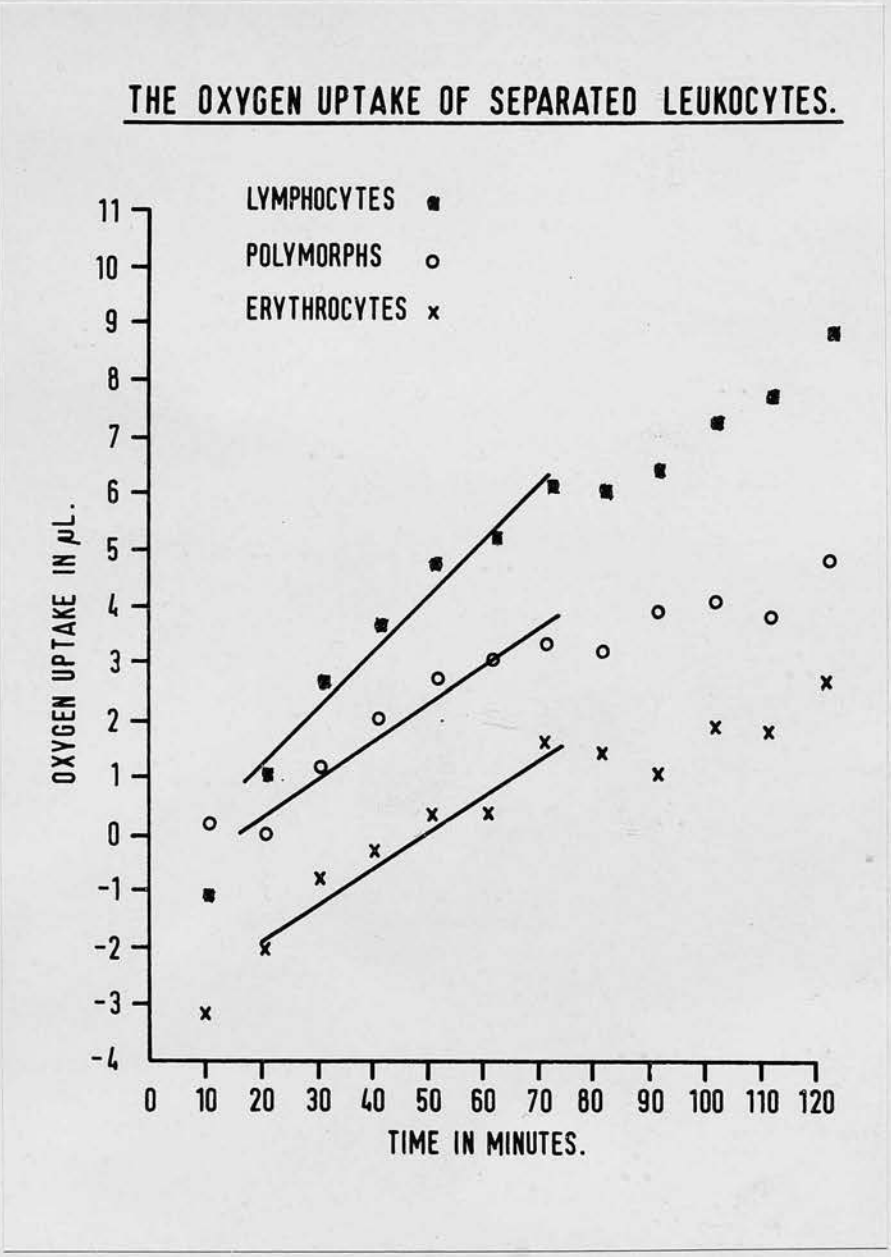

THE OXYGEN UPTAKE OF SEPARATED LEUKOCYTES.
LYMPHOCYTES
POLYMORPHS
ERYTHROCYTES
OXYGEN UPTAKE IN µL.
11
10
9
8
7
6
5
4
3
2
1
0
-1
-2
-3
-4
0
10
20
30
40
50
60
70
80
90
100
110
120
TIME IN MINUTES.

Figure 5.

gassed with 100% oxygen for 5 minutes then equilibrated in the water bath at 37°C for 15 minutes before taking any manometer readings.

~~Manometer~~ Readings were taken every 10 minutes for 2-3 hours. The flasks were shaken at 120-130 oscillations per minute.

The flasks were set up as follows:

flask 1 thermobarometer, 4.0 mls. water;

flask 2, 3 controls, medium but no leucocytes;

flask 4, 5 erythrocyte suspension obtained from the buffy-coat residue;

flask 6, 7 lymphocyte suspension;

flask 8, 9 PMN suspension.

The erythrocyte suspensions were included to allow for a correction to be made in the calculations of the oxygen uptake of the two leucocyte suspensions, due to the erythrocyte contamination of these suspension.

2. Calculation of the oxygen uptake

A graph was plotted of the total oxygen uptake per flask in µl against the time in minutes (e.g. Fig. 5). The rate of uptake was determined by drawing the best straight line through the 20-70 minute points - the 10 minute point was not included since it was invariably very low i.e. the oxygen uptake was very high during the first 10 minutes. Similar results have been observed by other workers (Soffer and Wintrobe 1932).

Knowing the rate of oxygen uptake for the erythrocyte suspension a correction was made for the erythrocyte contamination of the two leucocyte suspensions. Since the differential counts of the PMN and lymphocyte suspensions were known, the oxygen uptake could be calculated as follows:

1) if the composition of the PMN suspension is X PMN + Y lymphocytes, and the rate of oxygen uptake is Z µl/hour, and

11) if the composition of the lymphocyte suspension is X' PMN + Y' lymphocytes, and the rate of oxygen uptake is Z' µl/hour,

then the following relationships exists:

X PMN + Y lymphocytes = Z µl/hour,

X' PMN + Y' lymphocytes = Z' µl/hour.

On solving these two equations simultaneously, the oxygen uptake of the two cell types ~~were~~ was obtained, the results being expressed as

oxygen uptake of PMN (ΔO_2 PMN) = A µl/hour/10^{10} PMN

oxygen uptake of lymphocytes (ΔO_2 L) = B µl/hour/10^{10} lymphocytes

3. Results

The results of a number of separations and determinations of ΔO_2 of separated leucocytes are given in tables 6a (yields), 6b (ΔO_2's) and 7 (contamination of the suspensions). In

Table 6a. LEUCOCYTE YIELD AFTER SEPARATION

Experiment no.	Total no. of leucocytes added to column x 10^6	Differential counts:- % polymorphs (%PMN) : % lymphocytes (%L) %PMN : %L	Polymorphs			Lymphocytes		
			Number added to column x 10^6	Number recovered from column x 10^6	Yield %	Number added to column x 10^6	Number recovered from column x 10^6	Yield %
0.07% EDTA								
25	498	57.8 : 42.2	288	155	53.8	210	83.2	39.6
26	723.9	56.5 : 43.5	409	148.4	36.3	314.9	192.3	61.1
27	720	59.0 : 61.0	425	257.2	60.6	295	206	69.8
28	326	34.7 : 65.3	113	106.3	94.2	213	100.9	47.4
				mean :	61.2			54.5
				S.E. :	12.12			6.7
0.02% EDTA								
30	589.4	50.0 : 50.0	294.7	145	49.4	249.7	206	70.0
34	642	55.1 : 44.9	353	146	41.4	289	180.6	62.5
35	661.3	55.2 : 44.8	364	168	46.2	297.3	100.6	33.9
				mean :	45.7			55.5
				S.E. :	2.33			11.0

Table 6b. OXYGEN UPTAKE OF SEPARATED LEUCOCYTES

Experiment no.	Number of cells added to Warburg flasks			Oxygen uptake in µl/hour/10^{10} cells		
	Polymorphs x 10^6	Lymphocytes x 10^6	Erythrocytes x 10^9	Polymorphs	Lymphocytes	Erythrocytes
0.07% EDTA						
25	16.6	30.9	5.6	1600	2478	10.6
26	29.6	37.1	3.2	0	3150	-00.31
27	41.2	64.3	5.5	569	1041	6.45
28	20.3	26.6	6.4	52	3546	9.1
			mean :	555.34	2553.8	6.5
			S.E. :	334.92	549.63	2.415
0.02% EDTA						
30	45.8	41.5	6.9	36	1315	5.8
34	45.1	36.6	8.2	343.3	1957	7.94
35	25.2	41.9	5.9	114.6	1425	6.5
			mean :	164.6	1565.7	6.7
			S.E. :	92.17	198.21	0.63

Table 7. CONTAMINATION OF LEUCOCYTE SUSPENSIONS AFTER SEPARATION

Experiment no.	Number of leucocytes x 10^6*	Number of polymorphs x 10^6*	Number of lymphocytes x 10^6*	Number of erythrocytes x 10^6*	Differential count **	
					% PMN	%L
0.07% EDTA	Polymorphs Suspension					
25	33.2	30.9	2.3	100	93.0	7.0
26	39.1	37.1	2.0	140	94.8	5.2
27	69.2	64.3	4.9	800	92.9	7.1
28	29.0	26.6	2.4	112	91.8	8.2
				mean :	93.1	6.9
				S.E. :	0.62	0.62
0.02% EDTA						
30	44.1	41.5	2.6	93	94.4	5.6
34	44.6	36.6	8.0	224	82.0	18.0
35	50.8	41.9	8.9	77	82.5	17.5
				mean :	86.3	13.7
				S.E. :	4.05	4.05
0.07% EDTA	Lymphocyte Suspension					
25	18.9	2.3	16.6	4.9 x 10^9	12.0	88.0
26	30.4	0.8	29.6	6.5 x "	2.7	97.3
27	44.5	3.3	41.2	17.5 x "	7.4	92.6
28	21.9	1.6	20.3	4.95 x "	7.2	92.8
				mean :	7.3	92.7
				S.E. :	1.90	1.90
0.02% EDTA						
30	47.1	1.3	45.8	6.15 "	2.7	97.3
34	46.7	1.6	45.1	5.8 "	3.3	96.7
35	25.8	0.6	25.2	25.3 "	2.5	97.5
				mean :	2.8	97.2
				S.E. :	0.24	0.24

* Number of leucocytes per Warburg flask

** Differential count expressed as % polymorphs (%PMN) :
% lymphocytes (%L).

experiments 25-28 the PMN eluting fluid was 0.07% EDTA due to an error in making up the fluid. The experiments were repeated using 0.02% EDTA (nos. 30, 34, 35). It can be seen that this resulted in a slightly lower PMN yield. The ΔO_2 values appeared lower, but this may just be due to the great variation in ΔO_2.

4. Conclusions

These results were unsatisfactory since they were extremely variable, and also they were very low. The results can be compared with only two other published papers, since only two groups of workers have used this method of spearation.

ΔO_2 PMN :	this work	164.6	μl/hour/10^{10} PMN
	Rabinowitz (1964)	3600	
	Hedeskov and Esmann (1966)	2600	
ΔO_2 L :	this work	1566	μl/hour/10^{10} lymphocytes.
	Rabinowitz	4100	
	Hedeskov and Esmann	2600	

POSSIBLE FACTORS INFLUENCING LEUCOCYTE RESPIRATION

Since the observed values for ΔO_2 of both leucocyte types were so low, an investigation of the various factors which could

have been responsible was made. The possibilities considered were that:

i) the leucocytes were being damaged by centrifugation;

ii) the glass bead separation method was having an adverse effect upon the leucocytes;

iii) for some reason the leucocytes were not viable;

iv) the pH during the oxygen uptake incubation period was changing from the initial value of 7.4;

v) the medium being used for the measurement of ΔO_2 was unsatisfactory.

It was also possible that the number of leucocytes used in these experiments was too low. When there are few leucocytes in the Warburg flasks, the oxygen uptake is necessarily low with a consequent inaccuracy in reading the manometer levels.

1. Damage to leucocytes due to centrifugation

Up to this stage the leucocyte-rich suspensions had been prepared using the buffy-coat technique, which necessitated centrifuging at about 300 G. Hedeskov and Esmann centrifuged their separated cells at 600 G.

Rabinowitz states that centrifugation at more than 150 G is damaging to the leucocytes. It was therefore decided to try using dextran for the sedimentation of the erythrocytes. Two

ables 8a & 8b.

YIELD AFTER SEPARATION

Polymorphs			Lymphocytes		
Number added to column x 10^6	Number recovered from column x 10^6	Yield %	Number added to column x 10^6	Number recovered from column x 10^6	Yield %
195	135	69.0	187	85	45.6
343	162	47.3	137	76	55.3
152	74	48.7	190	114	60.0
298	89	30.0	135	73	53.7
341	159	46.7	187	96	51.2
269	126	46.9	92	54	58.3
507	226	44.6	209	93	44.4
173	41	23.5	128	60	46.8
	mean :	44.6			51.9
	S.E. :	4.80			2.09

EN UPTAKE OF SEPARATED LEUCOCYTES

d to	Oxygen uptake in µl/hour/10^{10} cells		
Erythrocytes x 10^9	Polymorphs	Lymphocytes	Erythrocytes
4.0	429	568	9.6
3.5	875	1690	21.2
7.5	1391	996	11.9
3.8	812	937	23.3
7.4	772	1008	16.2
10.4	501	1768	25.7
7.5	349	1190	9.6
10.6	1550	1462	19.6
mean:	834.9	1202.4	17.2
S.E.:	154.63	145.10	2.22

Table 9. CONTAMINATION OF LEUCOCYTE SUSPENSIONS

Experiment no.	Number of leucocytes x 10^6*	Number of polymorphs x 10^6*	Number of lymphocytes x 10^6*	Number of erythrocytes x 10^6*	Differential count **	
					%PMN	%L
Polymorph Suspension						
43	39.1	33.6	5.5	48	86.0	14.0
44	57.0	51.8	5.2	66	90.8	9.2
45	35.2	29.7	5.5	21	84.3	15.7
47	36.8	33.1	3.7	33	89.8	10.2
50	74.9	66.1	8.8	90	88.3	11.7
51	59.5	50.4	9.1	51	84.8	15.2
52	103.2	90.2	13.0	10	87.4	12.6
53	18.8	15.9	2.9	8	84.6	15.4
				mean :	87.0	13.0
				S.E. :	0.88	0.88
Lymphocyte Suspension						
43	24.6	2.2	22.4	400	8.6	91.4
44	27.1	3.4	23.8	348	12.3	87.7
45	48.0	2.5	45.5	262	5.3	94.7
47	31.2	2.2	29.0	130	7.0	93.0
50	32.9	3.0	29.9	480	9.0	91.0
51	28.4	7.1	21.3	560	24.5	75.5
52	45.0	2.8	42.2	585	6.2	93.8
53	28.1	0.8	27.3	248	3.0	97.0
				mean :	9.5	90.5
				S.E. :	2.36	2.36

* Number of leucocytes per Warburg flask

** Differential count expressed as % polymorphs (%PMN) : % lymphocytes (%L)

types were tried, these being B.D.H. dextran grade B, molecular weight 150,000 - 200,000, and Pharmacia dextran 250, mol. wt. 250,000. The Pharmacia dextran was found to give a slightly higher leucocyte yield than the B.D.H. dextran; it also sedimented the erythrocytes nearly twice as fast. For these reasons only Pharmacia dextran was used.

Using dextran a number of determinations of ΔO_2 were made. The erythrocytes were sedimented by addition of one part of 5% dextran in Hanks, pH 7.4, to 5 parts whole blood, and standing for 30-40 minutes at room temperature. The resulting erythrocyte-poor supernatant was removed and centrifuged at about 130 G for 10 minutes, the leucocytes being resuspended in the supernatant to the required volume. The remainder of the separation procedure was as indicated previously. The Warburg procedure was as usual with the exception that air was used as the gas phase since it has been shown that high oxygen tensions can exert an inhibitory action on a number of tissue enzymes (Dickins, 1946).

The results are given in tables 8a (yields), 8b(ΔO_2's) and 9 (contamination of the suspensions). It can be seen that there has been an improvement; although the ΔO_2's are still low they are more consistent than previously.

2. The effect of glass bead column separation on leucocyte respiration

It was considered that the passage of the leucocytes through the glass bead column could be affecting the leucocytes adversely. An experiment was set up to find if this was so.

The ΔO_2's of three leucocyte suspensions were determined, the suspensions being

i) a dextran-sedimented mixed leucocyte suspension,

ii) a column-separated PMN suspension, and

iii) a column-separated lymphocyte suspension,

all three suspensions originating from the same blood sample. The ΔO_2's were found to be

i) mixed leucocytes - 1016
ii) PMN - 830 *
iii) lymphocytes - 1115 **

) μl/hour/10^{10} leucocytes

(* cf. table 8b where mean = 834.9; ** cf. table 8b where mean = 1202.4)

The differential count of the mixed leucocyte suspension was 57.4% PMN : 42.6% lymphocytes. The calculated ΔO_2 of a suspension with this differential count using the ΔO_2 values obtained for the separated leucocytes is

PMN contribution = 0.574 x 830 = 476

lymphocyte contribution = 0.426 x 1115 = 476

Total = 952 μl/hour/10^{10} leucocytes.

This value for ΔO_2 (952) is 93.6% of the value obtained for the mixed leucocyte suspension, comparing favourably with the figure of 91% found by Rabinowitz. This experiment was repeated somewhat later, identical results being obtained (93.9%).

It therefore seemed that the column separation procedure was not responsible for the low ΔO_2 values.

3. Leucocyte viability tests

It was possible that both the separated and unseparated leucocytes were only partially viable. If so, that would explain the experiment above where the ΔO_2 was the same before and after separation. To test this suggestion, viability stains were tried. The theory behind these stains is that viable cells exclude the stain while non-viable cells become stained. Two methods were attempted:

(i) Trypan blue method (Rabinowitz 1964). Three drops of a leucocyte suspension are added to one drop of a 1% trypan blue solution in Hanks at pH 7.4 After incubation at 37°C for 30 minutes, a normal leucocyte count is made, the counted leucocytes consisting of unstained viable leucocytes and stained

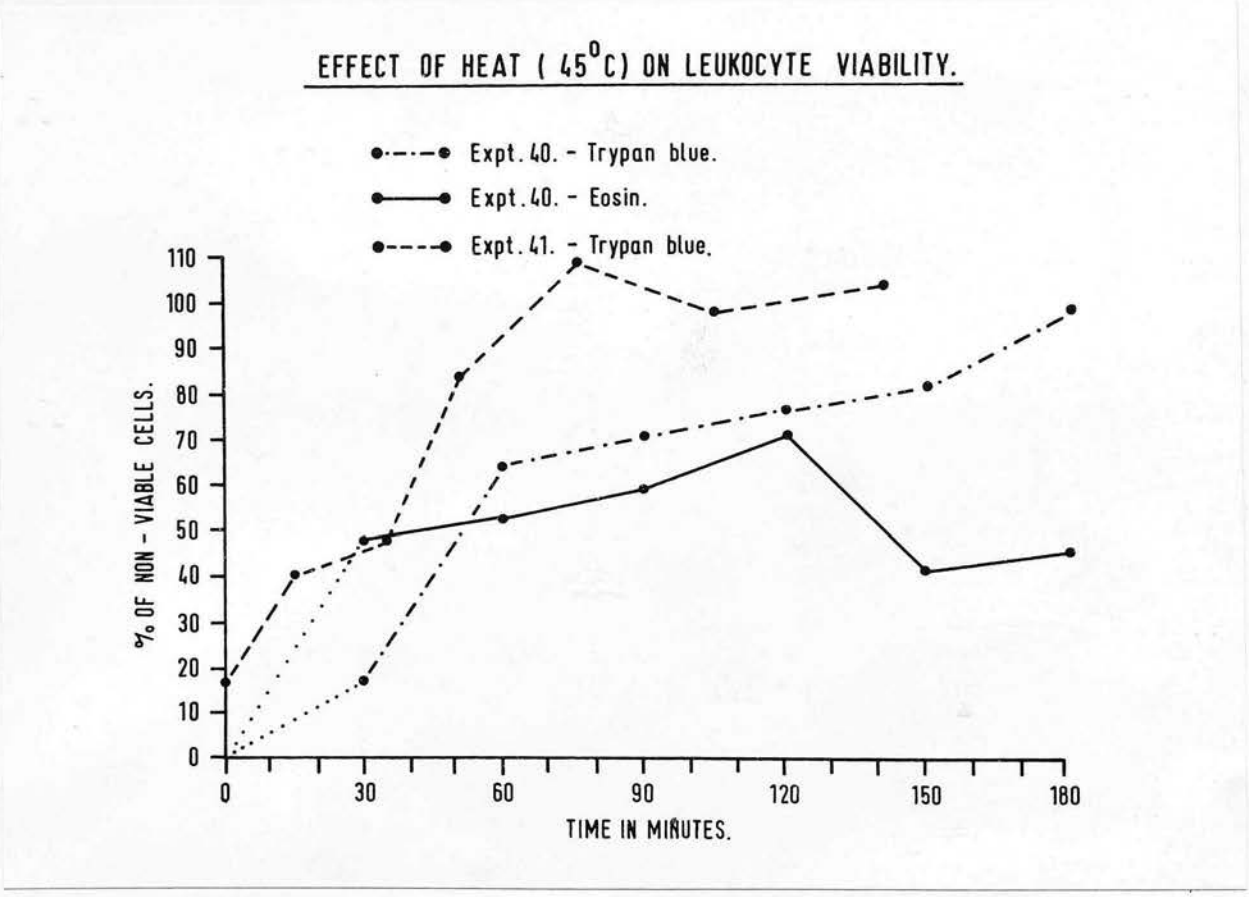

EFFECT OF HEAT (45°C) ON LEUKOCYTE VIABILITY.
Expt. 40. - Trypan blue.
Expt. 40. - Eosin.
Expt. 41. - Trypan blue.
% OF NON - VIABLE CELLS.
110
100
90
80
70
60
50
40
30
20
10
0
0
30
60
90
120
150
180
TIME IN MINUTES.

Figure 6.

TABLE 10. EFFECT OF INCREASED TEMPERATURE (45 C) ON LEUKOCYTE VIABILITY

Experiment 40

Time in minutes	leukocyte count of suspension x 10^6 /ml.	Eosin count		Trypan blue count	
		Number staining x 10^6/ml	Non-viable cells %	Number staining x 10^6/ml	Non-viable cells %
0	11.9				
30	11.2	5.3	47.3	1.2	10.7
60	9.4	4.9	52.2	6.0	63.8
90	9.0	5.3	59.2	6.3	70.4
120	9.9	7.1	71.7	7.6	76.8
150	8.9	3.8	42.7	7.3	82.0
180	7.5	3.5	46.7	7.4	98.7

Experiment 41

Time in minutes	leukocyte count of suspension x 10^6/ml.	Trypan blue count	
		Number staining x 10^6/ml	Non-viable cells %
0	13.6	2.2	16.1
15	12.3	4.9	39.8
35	11.7	5.6	47.8
50	10.4	9.7	93.3
75	9.7	10.5	108.5
105	10.4	10.2	98.3
140	8.0	8.3	103.8

non-viable leucocytes.

(ii) Eosin method (Hanks and Wallace 1958). A 1:1000 dilution of eosin in Tyrode solution, pH 7.4, is made up. A normal leucocyte count is made using this solution as the diluting fluid. The viable cells are unstained, the non-viable cells appearing pink.

Since some difficulty was experienced when attempting to decide whether a leucocyte was taking up the stain, an experiment (Expt. 40) was set up such that a leucocyte suspension would become completely non-viable over a period of time. 5 mls. blood were sedimented with dextran, the erythrocyte-poor supernatant being removed. This leucocyte suspension was then incubated at 45°C in a water bath, viable counts being made at intervals using trypan blue and oesin. The experiment (Expt. 41) was repeated using trypan blue. The results are given in table 10 and Fig. 6. As can be seen from Fig. 6 there is a general trend towards non-viability with time, though the degree of non-viability is variable. It was found to be extremely difficult to distinguish between stained and unstained leucocytes, there being a high degree of subjectivity in the numbers of stained/unstained leucocytes counted.

When applying these viability stains to column separated leucocytes it invariably appeared that either all or none of the leucocytes were being stained . Since Rabinowitz found 0.2% of column separated leucocytes to stain (i.e. to be non-viable), while

Table 11. pH CHANGES DURING OXYGEN UPTAKE DETERMINATIONS

Experiment number	Leucocyte suspension (unseparated)		Polymorph Suspension		Lymphocyte Suspension		Erythrocyte suspension	
	Final pH*	ΔO_2+	Final pH*	ΔO_2+	Final pH*	ΔO_2+	Final pH*	ΔO_2+
52	not done		8.1) 8.1)	349	9.2) 9.2)	1190	7.8) 9.1)	9.6
53	9.4) 9.0)	1016	7.9) 8.1)	830	9.1) 8.9)	1115	7.8) 7.8)	19.6

* Initial pH = 7.4

+ ΔO_2 = oxygen uptaken in µl/hour/10^{10} leucocytes

Hedeskov and Esmann found a value of 1%, this suggests that essentially all (or none) of the leucocytes separated in this work were viable.

4. pH changes during the oxygen uptake incubation period

Since the separation procedure seemed to be working correctly, the next step to be investigated was the respiration determinations. Firstly, the pH was checked to ascertain if it had changed significantly from the initial value of 7.4. Two experiments were done, the pH being measured at the termination of the two hour incubation period. The results are shown in table 11. It can be seen that the pH rises in all the suspensions. There are two possible causes:

i) KOH contamination of the flask contents. This is unlikely since there was no free KOH in the centre-well. Occasionally the flask fluid splashed into the centre-well this presumably killing the leucocytes. To avoid this detrimental effect on the leucocytes on the odd occasion when splashing occurred, the filter paper / KOH was placed in the side arm.

ii) Since Hanks solution contains bicarbonate, it is possible that CO_2 was being lost. However Rabinowitz used the same medium and did not find the pH to rise.

5. The effect of different media on the pH changes during the oxygen uptake incubation period.

In an endeavour to prevent the pH rising five different media were compared;

i) 100% plasma, pH 7.4;

ii) 50% plasma + 50% Hanks, pH 7.4;

iii) 50% plasma + 50% 'old' Hanks, pH 7.4. Normal Hanks was kept overnight at 37°C. The pH went up, but was restored to 7.4 before use.

iv) 50% plasma + 50% medium 199, pH 7.4;

v) 50% plasma + 50% 'phosphate' Hanks, pH 7.4. The composition of this medium is similar to Hanks, except that the bicarbonate has been omitted and the phosphate buffer concentration increased:

'Phosphate' Hanks solution:-

Sodium chloride	8.0 gm/litre
Potassium chloride	0.4 gm/litre
Calcium chloride.$6H_2O$	0.14 gm/litre
Magnesium sulphate.$7H_2O$	0.20 gm/litre
Potassium dihydrogen orthophosphate	1.78 gm/litre
Disodium hydrogen orthophosphate	9.55 gm/litre
Glucose	1.0 gm/litre
Distilled water	to 1000 mls.

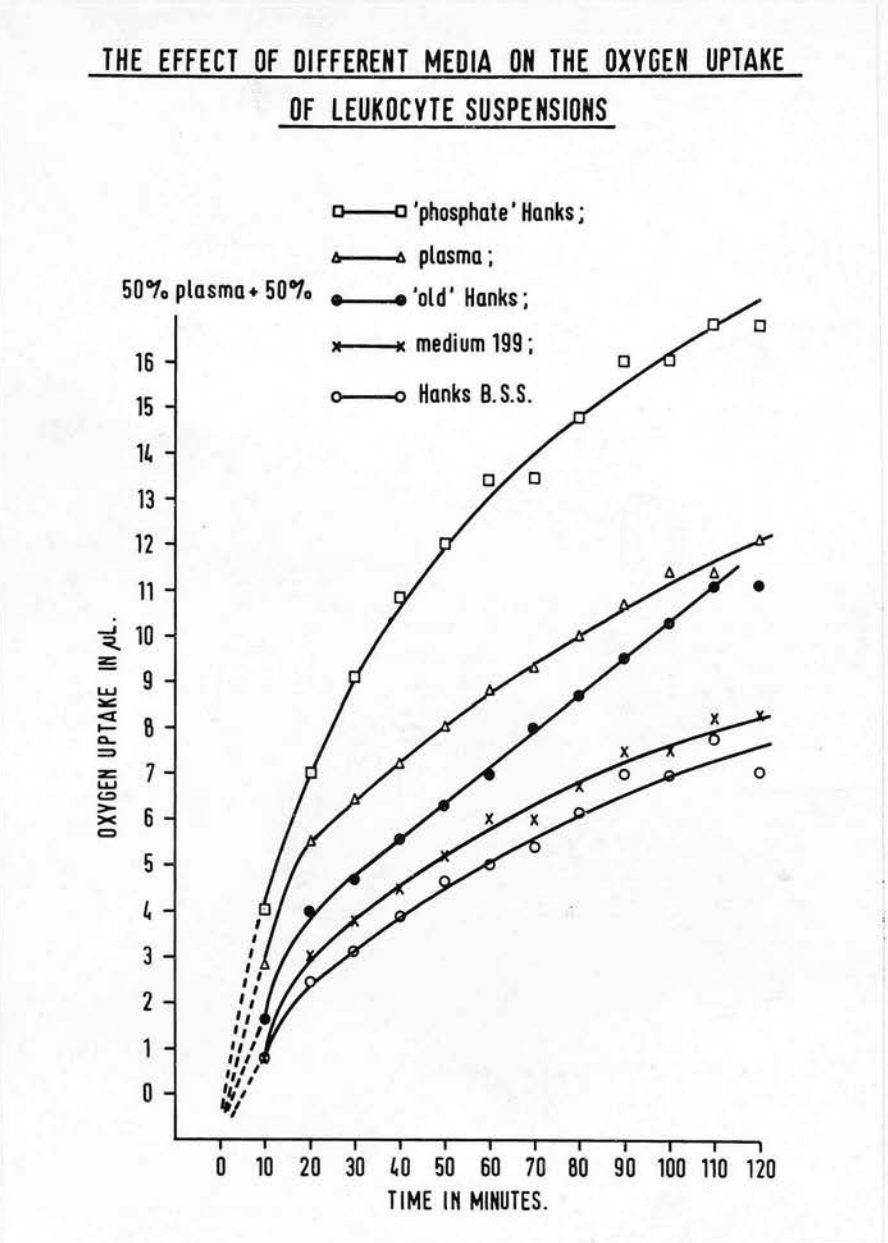

THE EFFECT OF DIFFERENT MEDIA ON THE OXYGEN UPTAKE OF LEUKOCYTE SUSPENSIONS
'phosphate' Hanks;
plasma;
50% plasma + 50% 'old' Hanks;
medium 199;
Hanks B.S.S.
OXYGEN UPTAKE IN µL.
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
0 10 20 30 40 50 60 70 80 90 100 110 120
TIME IN MINUTES.

Figure 7.

TABLE 12. EFFECT OF DIFFERENT MEDIA ON pH CHANGES DURING OXYGEN UPTAKE MEASUREMENTS

Medium: 50% plasma plus 50% : -	pH					
	Initial (before incubation)			Final (after 2 hours incubation)		
	Expt. 54	Expt. 55	Mean	Expt. 54	Expt. 55	Mean
Plasma	7.4	7.36	7.38	9.0	9.1	9.05
Hanks BSS	7.38	7.38	7.38	8.9	9.0	8.95
'Old' Hanks	7.38	7.40	7.39	8.9	9.0	8.95
Medium 199	7.37	7.36	7.37	8.9	8.9	8.9
'Phosphate' Hanks	7.37	7.39	7.38	8.0	8.0	8.0

TABLE 13. EFFECT OF DIFFERENT MEDIA ON LEUCOCYTE OXYGEN UPTAKE

Medium (pH 7.4) 50% plasma + 50% : -	ΔO_2 : $\mu l/hr/10^{10}$ leucocytes				ΔO_2 expressed as a % of the 'phosphate' Hanks value.
	Expt. 54	Expt. 55a	Expt. 55b	Mean.	
'Phosphate' Hanks	3625	3955	3767	3782.3	(100)
Plasma	1537	2920	2785	2414.0	62.8
Hanks BSS	1486	2400	~~3215~~ 2315	2067.0	54.7
Medium 199	1505	2230	2250	1995.0	52.8
'Old' Hanks	1537	2104	2125	1922.0	50.8

Since there were insufficient leucocytes from 100 mls. blood to compare these media on separated leucocytes simultaneously, mixed leucocyte suspensions were used. The results are given in tables 12 (pH changes) and 13 (ΔO_2's). It will be observed that the 'phosphate' Hanks kept the pH down better than the other media, and also that it gave much higher ΔO_2 values. A modification to the plasma was found to keep the pH down to about 7.7 - 7.8; some of the blood was removed before the addition of dextran. This blood sample was centrifuged hard, the leucocyte-free plasma being aspirated. The plasma was acidified to pH 6.5 and a negative pressure applied to remove dissolved CO_2. After about 30 minutes the pH was restored to 7.4 (method of MacLeod and Rhoads, 1939). This treatment had little effect on the ΔO_2 values.

Fig. 7 shows the results of one experiment, demonstrating that the 'phosphate' Hanks medium is preferable to the other media.

THE USE OF LARGE BLOOD VOLUMES

In order to find the optimum conditions for the leucocyte respiration, it was necessary to increase the blood volume to have sufficient leucocytes for the comparison of two treatments. 400 mls. of whole blood were collected into siliconed blood bottles containing 20 mls. of saline and preservative-free heparation in a final

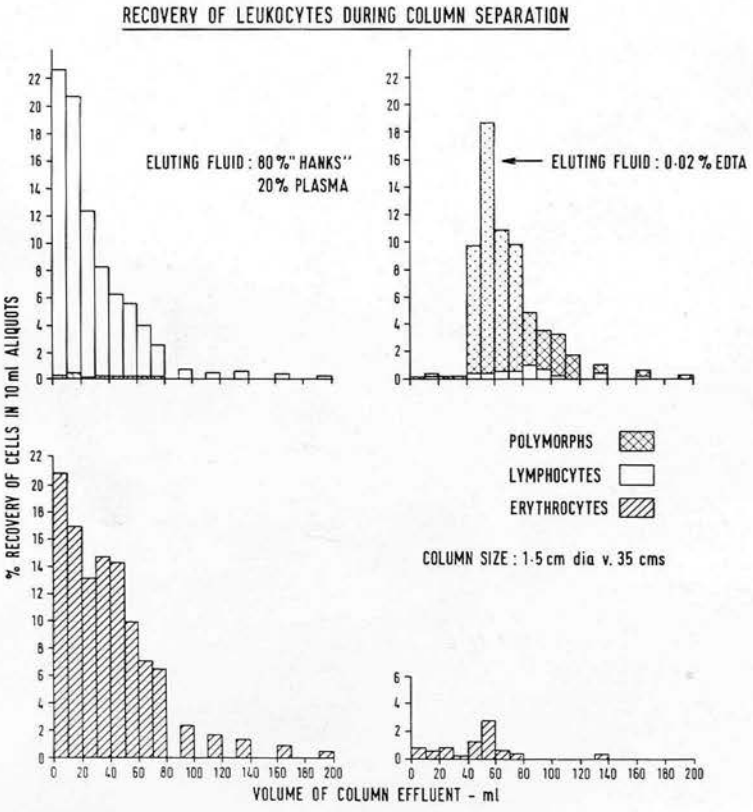

RECOVERY OF LEUKOCYTES DURING COLUMN SEPARATION
ELUTING FLUID : 80% "HANKS" 20% PLASMA
ELUTING FLUID : 0·02 % EDTA
POLYMORPHS
LYMPHOCYTES
ERYTHROCYTES
COLUMN SIZE : 1·5 cm dia v. 35 cms
% RECOVERY OF CELLS IN 10 ml ALIQUOTS
VOLUME OF COLUMN EFFLUENT - ml

Figure 8.

Table 14. RECOVERY OF LEUCOCYTES DURING COLUMN SEPARATION

Aliquot and Solution (mls)	No. of leucocytes /aliquot x 10^6	Polymorphs			Lymphocytes			Erythrocytes	
		Differential count % PMN	Recovery x 10^6	Yield %	Differential count % Lymphocytes	Recovery x 10^6	Yield %	No. in aliquot x 10^6	Yield %
Leucocyte Suspension	1004	79.3	795		20.7	209		3630	
HANKS:									
0 - 10	49	23.5	1.5	0.2	76.5	47.5	22.7	760	20.9
10 - 20	45.5	4.4	2.0	0.3	95.6	43.5	20.8	620	17.1
20 - 30	26.5	1.9	0.5	0.1	98.1	26.0	12.4	480	13.2
30 - 40	18.6	5.2	1.0	0.1	94.8	17.6	8.4	536	14.8
40 - 50	14.2	5.6	0.8	0.1	94.4	13.4	6.4	520	14.3
50 - 60	12.6	4.5	0.6	0.1	95.5	12.0	5.8	366	10.1
60 - 70	9.3	8.0	0.8	0.1	92.0	8.6	4.1	260	7.2
70 - 80	5.9	6.7	0.4	0.1	93.3	5.5	2.6	204	5.6
80 - 90	-	-	-	-	-	-	-	-	-
90 - 100	2.2	18.6	0.4	0.1	81.4	1.8	0.8	90	2.5
100 - 110	-	-	-	-	-	-	-	-	---
110 - 120	1.7	21.2	0.4	0.1	78.8	1.3	0.6	64	1.8
120 - 130	-	-	-	-	-	-	-	-	-
130 - 140	2.0	20.0	0.4	0.1	80.0	1.6	0.8	52	1.4
140 - 150	-	-	-	-	-	-	-	-	-
150 - 160	-	-	-	-	-	-	-	-	-
160 - 170	1.5	24.1	0.4	0.1	75.9	1.1	0.5	34	0.9
170 - 180	-	-	-	-	-	-	-	-	-
180 - 190	-	-	-	-	-	-	-	-	-
190 - 200	0.9	11.8	0.1	→0	88.2	0.8	0.4	20	0.6
EDTA:									
0 - 10	1.1	81.8	0.9	0.1	18.2	0.2	0.1	32	0.9
10 - 20	2.1	62.0	1.3	0.2	38.0	0.8	0.4	16	0.4
20 - 30	1.5	66.7	1.0	0.1	33.3	0.5	0.2	32	0.9
30 - 40	1.8	75.0	1.4	0.2	25.0	0.5	0.2	6	0.2
40 - 50	79.8	98.5	78.7	9.9	1.5	1.1	0.5	48	1.3
50 - 60	150.0	99.3	149.0	18.7	0.7	1.0	0.5	100	2.8
60 - 70	87.0	98.7	85.8	10.8	1.3	1.2	0.6	-	-
70 - 80	79.8	98.5	78.6	9.9	1.5	1.2	0.6	-	-
80 - 90	42.0	94.3	39.6	5.0	5.7	2.4	1.2	-	-
90 - 100	31.2	94.3	29.4	3.7	5.7	1.8	0.9	-	-
100 - 110	28.2	98.0	27.6	3.5	2.0	0.6	0.3	-	-
110 - 120	14.4	100.0	14.4	1.8	0.0	0.0	0.0	-	-
120 - 130	-	-	-	-	-	-	-	-	-
130 - 140	9.5	88.5	8.4	1.1	11.5	1.1	0.5	12	0.3
140 - 150	-	-	-	-	-	-	-	-	-
150 - 160	-	-	-	-	-	-	-	-	-
160 - 170	6.0	90.8	5.4	0.7	9.2	0.5	0.3	-	-
170 - 180	-	-	-	-	-	-	-	-	-
180 - 190	-	-	-	-	-	-	-	-	-
190 - 200	2.9	91.4	2.7	0.3	8.6	0.3	0.1	-	-

concentration of 10 U/ml. The erythrocytes were sedimented with 1 volume of 5% dextran to 5 volumes of blood. The glass bead column was 2.5 cm x 35 cm. height. The volume of the leucocyte suspension applied to the column was approximately 45 mls. The volume of eluting fluids required to wash the leucocytes from the column was determined in one experiment by collecting 10 ml. aliquots of the effluent and determing the number of erythrocytes, PMN, and lymphocytes in each aliquot. The results of this experiment are given in table 14. A histogram (Fig. 8) shows the percentage of each cell type recovered in each aliquot. The overall differential counts were:

i) lymphocyte eluate - 4.7% PMN : 95.3% lymphocytes, with 1 leucocyte : 21 erythrocytes;

ii) PMN eluate - 97.3% PMN : 2.7% lymphocytes, with 1.9 leucocytes : 1 erythrocyte.

The volume of eluting fluid required to obtain the lymphocytes was 200 mls., while the volume for the PMN was 160 mls.

This size of column was found to behave differently from the smaller columns. Previously the leucocyte suspension had been induced onto the column by a slight positive pressure. When using this procedure with the large column the leucocyte suspension was found to clot. This occurrence could be avoided by applying

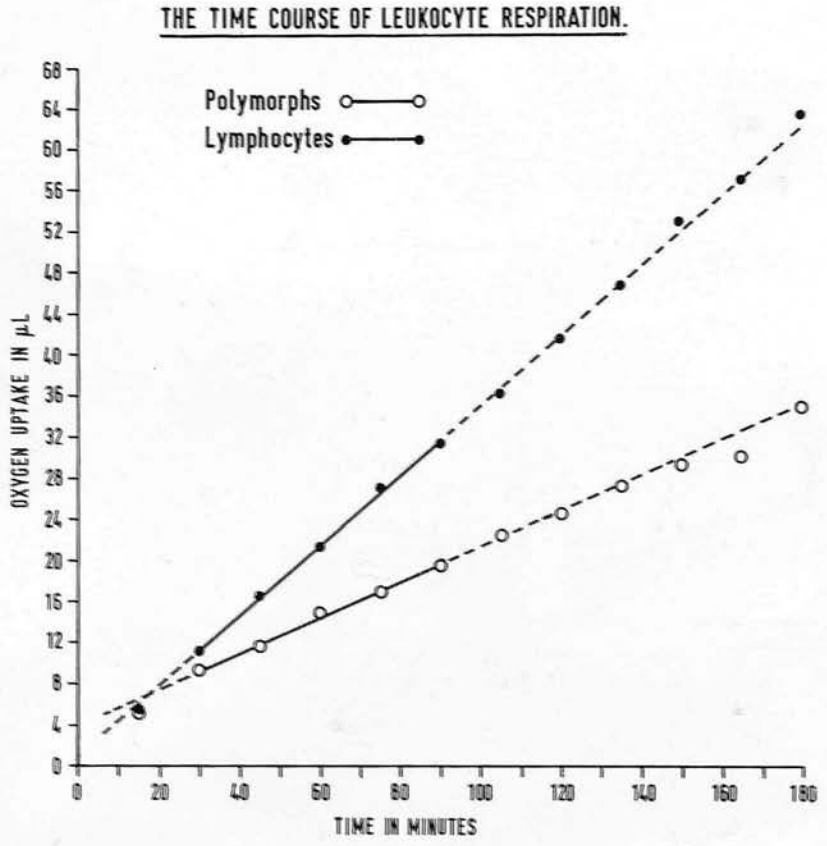

THE TIME COURSE OF LEUKOCYTE RESPIRATION.
Polymorphs
Lymphocytes
OXYGEN UPTAKE IN µL
TIME IN MINUTES
0
4
8
12
16
20
24
28
32
36
40
44
48
52
56
60
64
68
0
20
40
60
80
100
120
140
160
180

Figure 9.

a gentle negative pressure to the bottom of the column, taking about 3-4 minutes to fill the column. Using this procedure no clotting was ever observed.

The oxygen uptake was measured over a two hour period, manometer readings being taken every 10 minutes. Fig. 9 shows the time course for the respiration of the two leucocyte suspensions. After about 30 minutes the rate of oxygen uptake became linear; consequently all determinations of the rate were made over the 30-90 minute time interval, giving seven points through which the line was drawn (in Fig. 9 there are only five points since readings were taken here every 15 minutes for 3 hours to show linearity over that time). The best line drawn through all the points including the 10 and 20 minute points did not always pass through the origin, for the following reason: the oxygen uptake at any time was calculated as the difference between the manometer readings at time zero and the readings at 10 minute intervals thereafter. Thus any error in the manometer reading at time zero was reflected in all the calculations of the oxygen uptake, with the result that the curve through all the points was displaced either up or down on the y-axis. However, this displacement did not effect the gradient of the line drawn through the 30-90 minute time interval. Any error in reading the thermobarometer and control manometers at time zero also caused a shift of the curve relative

to the y-axis.

Since it had been observed that the use of 'phosphate' Hanks gave higher ΔO_2 values than normal Hanks, a few separations were tried using 'phosphate' Hanks to make up the dextran and the lymphocyte eluting fluid. This was found to be unsatisfactory, since both leucocyte suspensions were very contaminated with the other cell type.

OXYGEN UPTAKE - THE EFFECT OF DIFFERENT CONDITIONS

In an attempt to increase the ΔO_2's further, a number of experiments were conducted to test the effects of varying conditions on ΔO_2.

1. The effect of 'phosphate' Hanks compared to normal Hanks.

The separation and Warburg procedures were as indicated previously. The medium consisted of 50% plasma + 50% 'phosphate' or normal Hanks. The results are given in table 15.

Table 15. The effect of 'phosphate'/normal Hanks on leucocyte ΔO_2

Expt. no.	Cell type	Normal Hanks ΔO_2 Value	Normal Hanks ΔO_2 Mean ± S.E.	'Phosphate' Hanks ΔO_2 Value	'Phosphate' Hanks ΔO_2 Mean ± S.D.	P*
61	PMN	555		1029		
63		300		1044		
64		816	637.8	1142	1074.5	0.05
65		880	± 132.73	1083	± 25.22	
61	lympho-cytes.	2703		2642		
63		1634		1825		
64		1889	2021.5	2606	2163.5	n.s.+
65		1860	± 234.20	1581	± 270.60	

* P determined using Students 't' test. + n.s. = not significant.

There was a significant difference in PMN ΔO_2, the 'phosphate' Hanks medium giving the greater values. This medium also gave more consistent results. As far as the lymphocytes ~~are~~ were concerned there ~~is~~ was no difference between the media.

All further experiments were therefore conducted using a medium consisting of 50% plasma + 50% 'phosphate' Hanks, pH 7.4.

2. The effect of siliconed compared to unsiliconed Warburg flasks

All glassware with which the blood was coming into contact was siliconed up to this point. It was thought possible that this coating might be having an adverse effect on the leucocyte ΔO_2. To test this idea two experiments were done using siliconed and unsiliconed Warburg flasks. The results are given in table 16.

Table 16. The effect of siliconed and unsiliconed Warburg flasks on leucocyte ΔO_2.

Expt. no.	Cell types	Unsiliconed flasks ΔO_2		Siliconed flasks ΔO_2		P*
		Value	Mean ± S.E.	Value	Mean ± S.E.	
67 68	PMN	648 746	697.0 ± 49.00	1121 1274	1197.5 ± 76.72	0.05
67 68	lympho-cytes	1468 1581	1524.5 ± 55.88	1825 1621	1723.0 ± 102.45	0.15

* P determined using Students 't' test.

There was a significant difference in PMN ΔO_2, the siliconed flasks giving the higher values. The lymphocytes were scarcely affected by the presence of silicone.

3. The effect of additional calcium and magnesium

Since EDTA, which was present in the PMN eluting fluid, chaelates divalent ions, it was feasible that the leucocytes, and in particular, the PMN, were lacking in these ions. Four experiments were therefore performed with double the normal calcium and magnesium concentrations. These results are shown in table 17.

Table 17. THE EFFECT OF DOUBLED CALCIUM AND MAGNESIUM CONCENTRATIONS ON LEUCOCYTE ΔO_2

Expt. no.	cell types	Normal Ca^{++} and Mg^{++} concentrations		Double Ca^{++} and Mg^{++} concentrations		P*
		Value	Mean ± S.E.	Value	Mean ± S.E.	
61	PMN	1029	1074.5 ± 25.22	1466	953.3 ± 223.30	0.1
63		1044		648		
64		1142		746		
65		1083				
61	lymphocytes	2643	2233.5 ±226.05	1304	1451.0 ± 69.64	0.025
63		1825		1581		
64		2606				
65		1860				

* P determined using Students 't' test.

Table 18. INFLUENCE OF ERYTHROCYTE CONTAMINATION ON THE OXYGEN UPTAKE OF LEUCOCYTE SUSPENSIONS

Experiment no.	Polymorph suspension : rate* of oxygen uptake due to			Lymphocyte suspension : rate* of oxygen uptake due to		
	Leucocytes alone	Erythrocytes alone	% **	Leucocytes alone	Erythrocytes alone	%**
61	9.9	0.02	0.20	25.0	0.21	0.84
63	9.8	0.0	0.0	12.5	0.0	0.0
64	10.4	0.01	0.L	23.7	0.24	1.01
65	17.0	0.0	0.0	14.4	0.0	0.0
		Mean :	0.08		Mean :	0.46

* Rate of oxygen uptake expressed as µl/30-90 min. period

** Rate of oxygen uptake of the erythrocytes expressed as a % of the rate of oxygen uptake of the leucocytes.

There was little difference in PMN ΔO_2 with the two media, normal Ca^{++} and Mg^{++} concentrations giving slightly higher values of ΔO_2. Lymphocytes, however, showed a greatly reduced rate of respiration when the Ca^{++} and Mg^{++} concentrations were increased.

THE CORRECTION FACTOR IN LEUCOCYTE ΔO_2 DUE TO ERYTHROCYTE CONTAMINATION.

Up to this point the erythrocyte ΔO_2 had been determined in all experiments to allow a correction factor to be applied for the erythrocyte contamination of the two leucocyte suspensions; in each determination of ΔO_2 flasks had been included which contained erythrocytes obtained from the dextran sediment with negligible leucocyte contamination. The erythrocyte concentration was adjusted to approximately that present in the lymphocyte suspension. Thus, knowing the erythrocyte ΔO_2 and also the erythrocyte contamination in the two leucocyte suspensions, the erythrocyte contribution to the oxygen uptake of the leucocyte suspensions was calculated. Table 18 shows the results of four experiments, the conditions being "phosphate" Hanks, normal Ca^{++} and Mg^{++}, and with the flasks being siliconed. The erythrocyte contribution to the oxygen uptake in the PMN flasks - 0.08% - is negligible, and even that in the lymphocyte flasks - 0.5% - is too small to exert any

Table 19. EFFICIENCY OF THE GLASS BEAD SEPARATION PROCEDURE

Experiment no.	Total no. of leucocytes added to column x 10^6	Differential count of the leucocyte suspension *	Polymorph suspension			Lymphocyte suspension		
			Total added to column x 10^6	Recovery from column x 10^6	Yield %	Total added to column x 10^6	Recovery from column x 10^6	Yield %
61	1846	48.7 : 51.3	898	396	44.0	948	394	41.6
62	1970	74.3 : 25.7	1464	371	25.4	506	249	49.2
63	1958	71.5 : 28.5	1397	359	25.7	561	301	53.6
65	2848	71.3 : 28.7	2030	647	31.9	818	403	49.3
66	1395	62.5 : 37.5	872	286	32.8	523	175	33.5
67	1720	33.8 : 66.2	582	267	45.9	1138	536	47.1
68	2068	58.8 : 41.2	1218	340	27.9	850	441	51.9
				mean :	33.4		mean :	46.5
				S.E. :	3.21		S.E. :	2.62

* Differential count expressed as % polymorphs : % lymphocytes

Table 20. CONTAMINATION OF LEUCOCYTE SUSPENSIONS

Experiment no.	Polymorph Suspension					
	Number of leucocytes x 10^6*	Number of polymorphs x 10^6*	Number of lymphocytes x 10^6*	Number of erythrocytes x 10^6*	Differential count **	
					%PMN	%L
61	95.2	93.8	1.4	4.4	98.7	1.3
62	88.4	83.4	5.0	24.0	94.4	5.6
63	84.8	72.7	12.1	80.0	85.8	14.2
64	78.7	69.7	9.0	25.6	88.6	11.4
65	64.0	155.8	8.2	20.0	95.0	5.0
66	77.2	69.1	8.1	0.4	89.5	10.5
67	75.6	68.1	7.5	12.0	90.1	9.9
68	92.0	78.9	13.1	16.0	85.8	14.2
mean:	94.5		mean:	22.8	91.0	9.0
			S.E.:		1.64	1.64

leucocyte:erythrocyte ratio = 94.5 : 22.8

$\simeq$ 4.1 : 1

Experiment no.	Lymphocyte Suspension					
	Number of leucocytes x 10^6*	Number of polymorphs x 10^6*	Number of lymphocytes x 10^6*	Number of erythrocytes x 10^6*	Differential count **	
					%PMN	%L
61	95.6	2.8	92.8	600	3.0	97.0
62	72.4	13.3	59.1	1260	18.3	81.7
63	71.0	5.9	65.1	630	8.3	91.7
64	92.8	4.6	88.2	876	5.0	95.0
65	94.8	5.2	89.6	860	5.5	94.5
66	56.8	16.0	40.8	640	28.1	71.9
67	118.0	17.0	101.0	800	14.5	85.5
68	124.8	22.0	102.8	800	17.7	82.3
mean :	90.7		mean : 808.25	mean:	12.6	87.54
				S.E.:	3.05	3.05

leucocyte:erythrocyte ratio = 90.68:808.25

$\simeq$ 1: 8.9

* Number of leucocytes per Warburg flask

** Differential count expressed as % polymorphs (%PMN) :
% lymphocytes (%L)

Table 21. CONTAMINATION AND YIELD OF LEUCOCYTES USING DIFFERENT SIZES OF COLUMN

Data from tables	column size (cms)	*						
			Yield from column %	Polymorphs in suspension %	Lymphocytes in suspension %	Yield from column %	Polymorphs in suspension %	Lymphocytes in suspension %
2	1.0 x 20	M S.E.	50.4 6.90			76.0 5.63		
6,7	1.5 x 2	M S.E.	61.2 12.12	91.2 0.62	6.9 0.62	54.5 6.77	7.3 1.90	92.7 1.90
	1.5 x 2	M S.E.	45.7 2.33	86.3 4.05	13.7 4.05	55.5 11.00	2.8 0.24	97.2 0.24
8,9	1.5 x 2	M S.E.	44.6 4.80	87.0 0.88	13.0 0.88	51.9 2.09	9.5 2.36	90.5 2.36
19,20	2.4 x 35	M S.E.	33.4 3.21	91.0 1.64	9.0 1.64	46.5 2.62	12.6 3.05	87.5 3.05

* M = mean, S.E. = standard error

significant effect.

It was therefore considered unnecessary in future work to make any allowance for the erythrocyte contamination of the leucocyte suspensions.

THE EFFICIENCY OF THE COLUMN SEPARATION OF LEUCOCYTES

The efficiency of the column separation procedure in this series of experiments is summarised in tables 19 and 20. Table 19 shows the yields of the leucocytes, 33.4% of the PMN and 46.5% of the lymphocytes being recovered from the number added to the column. Table 20 shows the contamination of the suspensions with the other leucocyte type, the average differential counts being:

for the PMN suspension 91.0% PMN : 9.0% lymphocytes,

and

for the lymphocyte suspension 12.6% PMN : 87.4% lymphocytes.

A comparison of the efficiencies of the separations using different column sizes is shown in table 21. There appear to be trends, though these are not great, for

i) the yields of both PMN and lymphocytes to decrease as the column size increases, and

ii) for the purity of the two leucocyte suspensions to decrease as the column size increases.

CONDITIONS FOR SEPARATION AND DETERMINATION OF OXYGEN UPTAKE OF LEUCOCYTES TO BE OBSERVED IN FUTURE WORK.

The separation procedure seemed to be working satisfactorily at this stage. Although the values for ΔO_2 were low they were reasonably constant. Since there did not seem to be any obvious modifications for either the separation or oxygen uptake measurements, a study of the effects of drugs on leucocyte respiration was begun. The conditions adhered to were:

1) for the separation procedure:
   - i) the erythrocytes were sedimented with Pharmacia dextran 250 in Hanks solution at pH 7.4;
   - ii) all centrifugation was carried out at less than 150G.
   - iii) leucocyte suspensions were added to the column using a gentle negative pressure, taking 3-4 minutes to fill the column;
   - iv) the lymphocyte eluting fluid was 80% Hanks + 20% plasma, the volume being about 200 mls.;
   - v) the PMN eluting fluid was 0.02% EDTA, the volume being about 160-180 mls. Plasma was added to the collecting tubes, the final concentration being 20%;

vi) the separated leucocyte suspensions were made to the required volume with 'neutralised' plasma.

2. for the oxygen uptake determinations:

i) all flasks were siliconed;

ii) air was used as the gas phase;

iii) the medium was 50% plasma + 50% 'phosphate' Hanks; PH 7.4

iv) KOH was placed on filter paper in the flask side-arm;

v) no erythrocyte suspensions were included;

vi) the flasks were shaken in the water bath at 120-130 oscillations per minute;

vii) the rate of oxygen uptake was determined over the 30-90 minute period.

SUMMARY OF 'NO-DRUG' RESULTS.

When the effects of drugs on leucocyte respiration were being investigated, the procedure was to divide the separated leucocyte suspensions into two parts; the drug was added to one half, the other half being used as a 'no-drug' control. Consequently, over a period of time the number of 'no-drug' results accumulated. Taking these results together it was possible to obtain some reasonably accurate averages for the efficiency of the separation procedure, and also for the PMN and lymphocyte ΔO_2's.

Table 22. EFFICIENCY OF DEXTRAN SEDIMENTATION

Expt. no.	Differential count of whole blood		Differential count of the Leucocyte suspension		Total no. of leucocytes in whole blood	Total no. of leucocytes in the leucocyte suspension	Yield of leucocyte after dextran sedimentation
	%PMN*	%L*	%PMN*	%L*	x 10^6	x 10^6	%
87	-	-	-	-	3010	1720	57.2
88	70.0	30.0	66.3	33.7	2590	1850	71.5
89	63.3	36.7	67.7	32.3	2810	1390	49.5
90	59.3	40.7	60.0	40.0	2980	1420	47.7
91	61.8	38.2	67.6	32.4	2880	1568	54.4
92	72.0	28.0	68.0	32.0	2805	1462	52.2
93	-	-	-	-	3315	2230	67.3
94	67.4	32.6	68.3	31.7	3620	2240	61.8
95	-	-	-	-	3455	2555	73.9
96	58.2	41.8	58.5	41.5	2290	1236	54.1
97	71.0	29.0	72.3	27.7	1138	904	79.5
98	70.2	29.8	75.8	24.2	3450	2620	76.0
100	60.6	39.4	60.5	39.5	2158	1610	74.8
101	61.3	37.8	63.5	36.5	3020	1280	42.4
102	65.0	35.0	70.2	29.8	2860	1860	65.3
103	58.2	41.8	63.0	37.0	2510	1099	43.8
104	64.5	35.5	65.6	34.4	3160	2024	64.2
105a	59.8	40.2	71.2	28.8	2010	1425	70.8
106a	66.7	33.7	78.8	21.2	2110	1738	82.4
105b	61.0	39.0	65.8	34.2	4070	2120	52.1
106b	55.0	45.0	64.6	35.4	3185	1650	51.8
108	59.6	40.4	62.3	37.7	1468	1125	76.8
109	76.3	23.7	68.8	31.1	3465	2416	69.7
110	68.8	31.2	76.5	23.5	2468	1250	~~60.7~~ 50.7
113	61.0	39.0	63.0	37.0	2830	1788	63.3
114	64.6	35.4	67.3	32.7	1210	975	80.6
115	64.2	35.8	73.5	26.5	4020	3190	79.5
116	68.0	32.0	78.3	21.7	2890	2210	76.5
117	63.9	37.1	64.6	35.4	1550	862	55.5
118	70.4	29.6	67.8	32.2	1812	1555	85.8
119	65.2	34.8	65.3	34.7	-	-	-
120	64.8	35.2	69.9	30.1	2250	1638	72.8
121	67.0	33.0	68.7	31.3	~~3457~~ 3475	2730	78.6
122	59.1	40.9	69.2	30.8	2375	1440	60.7
123	72.4	27.6	77.3	22.7	3333	1662	49.8
124	75.0	25.0	77.5	22.5	2670	1647	61.7
125	70.2	29.5	68.2	31.8	2275	915	40.3
126	59.7	40.3	65.4	34.6	2693	1382	51.4
127	71.9	28.1	64.4	35.6	1444	708	49.2
128	72.4	27.6	75.3	24.7	2220	1179	53.2
129	56.3	43.7	70.6	29.4	1952	1310	67.3
130	61.2	38.8	67.2	32.8	2015	1480	73.5
Mean	65.06	34.94	68.44	31.56			62.18

* %PMN = % polymorphs

%L = %lymphocytes

1. Yield of leucocytes following dextran sedimentation

Dextran sedimentation not only reduced the number of erythrocytes, but also reduced the number of leucocytes:

I) Table 22 shows a) the total number of leucocytes in whole blood,

b) the total number of leucocytes in the leucocyte suspension after dextran sedimentation, and

c) the percentage yield of leucocytes after dextran sedimentation.

It can be seen that only 62.2% of the leucocytes were recovered. This is the recovery which would be expected. Initially the erythrocytes and leucocytes are distributed uniformly throughout the blood sample. After dextran sedimentation the volume of the sedimented erythrocytes is about one third of the total volume. Assuming no leucocytes sediment, one would expect to recover about 66% of the leucocytes from the erythrocyte-poor supernatant.

II) Table 22 also shows a) the differential count of whole blood, and

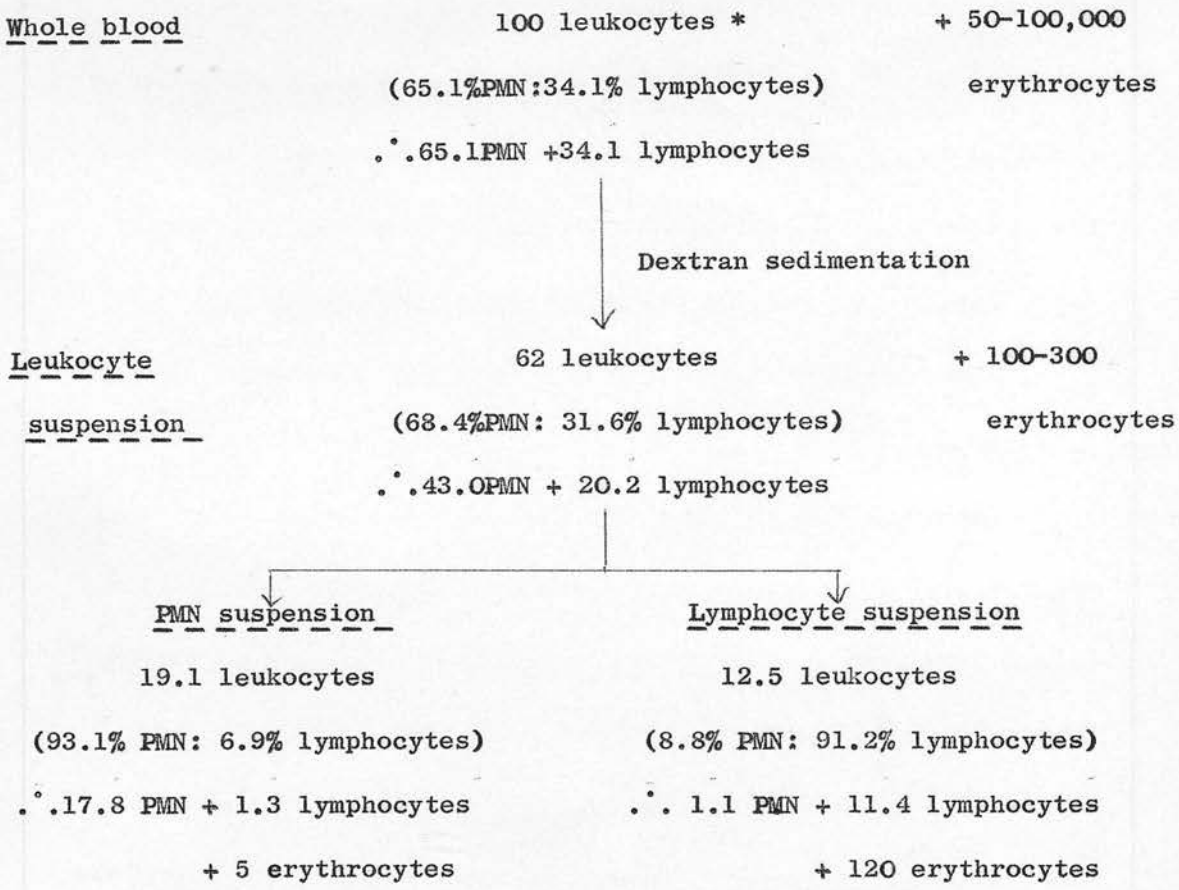
b) the differential count of the leucocyte suspension.

The mean differential count changed from 65.1% PMN : 34.9% lymphocytes to 68.4% PMN : 31.6% lymphocytes, i.e. there

Table 23. LEUCOCYTE YIELDS AND CONTAMINATION FOLLOWING GLASS BEAD SEPARATION

Expt. no.	Total leucocytes x 10^6	% leucocytes recovered from				Differential counts of the leucocyte suspension*			
		Whole blood		Column		Polymorph suspension		Lymphocyte suspension	
		PMN	L	PMN	L	%PMN	%L	%PMN	%L
		A	B	C	D	E	F	G	H
86	-	-	-	28.1	48.8	93.8	6.2	4.1	95.9
87	3010	-	-	21.8	35.0	83.4	16.6	9.0	91.0
88	2590	15.0	44.5	22.1	55.3	92.8	7.2	7.3	92.8
89	2810	12.0	20.1	22.7	45.0	94.0	6.0	10.8	89.2
90	2980	22.4	25.2	46.5	54.0	88.9	11.1	8.6	91.4
91	2880	21.2	35.1	35.9	63.7	92.6	7.4	5.5	94.5
92	2805	37.8	35.1	76.8	59.3	91.0	9.0	12.5	87.5
93	3315	28.0	25.7	32.2	59.8	92.5	7.5	8.3	91.7
94	3620	37.9	51.0	60.5	60.2	91.8	8.2	9.1	90.9
96	3455	-	-	21.0	66.0	92.2	7.8	13.4	86.6
97	2290	18.8	34.0	34.6	63.5	90.3	9.7	6.8	93.2
98	1138	42.8	40.8	53.0	53.6	91.5	8.5	6.3	93.7
100	3450	31.7	39.9	38.7	65.0	92.9	7.1	13.4	86.6
101	2158	33.0	40.4	45.2	54.0	90.0	10.0	9.6	90.4
102	3020	35.1	30.4	79.8	77.8	91.5	8.5	5.6	94.4
103	2860	18.8	29.5	26.7	52.9	91.2	8.8	8.2	91.8
104	2510	30.1	18.8	64.3	47.3	92.8	7.2	6.4	93.4
105a	3160	22.5	29.8	32.1	54.2	90.3	9.7	4.2	95.8
105b	2110	22.9	17.5	23.5	33.4	94.3	5.7	8.0	92.0
106a	2010	40.7	38.1	48.3	74.8	93.4	6.6	12.8	87.2
106b	4070	27.4	31.7	49.8	69.2	90.2	9.8	6.5	93.5
108	3185	31.3	28.8	51.3	70.2	96.4	3.6	4.4	95.6
109	1468	31.7	58.2	39.5	81.7	92.9	7.1	7.4	92.6
110	3468	37.9	57.2	60.0	62.7	94.6	5.4	3.0	97.0
111	2468	23.1	24.2	41.0	63.5	89.8	10.2	11.0	89.0
113	2830	26.4	28.8	40.5	47.8	91.2	8.8	8.0	92.0
114	1210	28.2	44.8	33.7	60.2	93.5	6.5	5.2	94.8
115	4020	36.6	44.0	40.2	74.8	95.0	5.0	8.5	91.5
116	2890	56.0	29.8	63.0	58.0	95.9	4.1	7.5	92.5
117	1550	24.4	38.7	43.4	70.9	92.9	7.1	5.7	94.3
118	1812	40.1	42.4	48.4	45.7	94.9	5.1	7.1	92.9
119	2970	29.9	34.2	48.8	55.9	97.0	3.0	4.5	95.5
120	2250	41.5	27.5	52.9	43.8	96.7	3.3	8.5	91.5
121	3475	62.0	50.7	77.2	67.8	93.5	6.5	10.0	90.0
122	2375	47.7	41.2	67.2	89.7	95.0	5.0	6.0	94.0
123	3333	16.6	19.4	19.8	47.5	94.5	5.5	6.7	93.3.
124	2670	37.8	73.2	63.6	84.7	93.9	6.1	9.2	90.8
125	2275	24.9	36.3	34.1	59.3	95.2	4.8	15.1	84.9
126	2693	19.2	26.3	43.1	59.3	94.7	5.3	6.3	93.7
127	1444	18.9	47.7	43.1	76.8	96.3	3.7	7.8	92.2
128	2220	16.4	27.7	29.8	58.0	95.7	4.3	4.9	85.1
129	1952	30.3	25.3	36.0	55.8	93.8	6.2	6.5	93.5
130	2015	22.5	32.1	27.5	51.7	97.3	2.7	4.5	95.5
	Mean:	29.9	35.5	43.8	60.3	93.1	6.9	8.8	91.2
	S.E.:	1.77	1.87	2.47	1.99	0.40	0.40	0.69	0.69

* Differential counts expressed as %polymorphs (%PMN) : %lymphocytes (%L)



*for convenience, the initial total number of leukocytes is taken as 100

Fig. 10. GENERAL SCHEME OF GLASS BEAD SEPARATION PROCESS

TABLE 24. THE OXYGEN UPTAKE OF SEPARATED POLYMORPHS AND LYMPHOCYTES.

Experiment number.	Oxygen uptake in µL/hour/10^{10} leucocytes	
	Polymorphs	Lymphocytes
86	1163	2520
87	844	2485
88	857	2257
89	1477	3597
90	1018	3116
91	1644	3175
92	705	3936
93	1394	2485
95	2084	2750
96	2566	2613
98	1356	2193
99	860	2606
100	1342	2801
101	1568	2204
102	1777	2946
104	1592	2481
105a	1202	2492
105b	1438	2989
106	1254	2524
108	1716	2273
109	1490	2310
110	1484	3079
112	1000	3098
113	2229	2009
114	2357	2797
115	1839	2453
116	1091	3282
117	1965	2331
119	2072	3256
120	1209	3327
122	1613	2777
123	1153	2822
124	1457	3375
125	1454	2778
126	1643	1999
127	1634	2002
mean	1487.7	2726.0
S.E.	71.66	80.44

PMN : 6.9% lymphocytes. This was more contamination than was found by Rabinowitz, (99.99% PMN), but he used a greater lymphocyte eluting volume which should have removed more of the lymphocytes.

Columns G and H show the average PMN contamination of the lymphocyte suspension, the differential count being 8.8% PMN : 91.2% lymphocytes. (Rabinowitz found 2.1% : 97.7%, and Hedeskov and Esmann 1.7% : 98.3% respectively.

The results in tables 22 and 23 are summarised in Fig. 10.

4. The oxygen uptake of PMN and lymphocytes

Table 24 shows the results of 36 control determinations of the two ΔO_2's, their mean values being

ΔO_2 PMN = 1488)
ΔO_2 lymphocyte = 2726) $\mu L/hour/10^{10}$ leucocytes.

These values are somewhat lower than those obtained by Rabinowitz (3600 and 4100), and by Hedeskov and Esmann (2618 and 2618).

It was stated previously that the rate of oxygen uptake was estimated over the 30-90 minute interval. If the rate is estimated over the 0-60 or 0-120 minute interval somewhat different ΔO_2's are obtained as is indicated in Fig. 10a. Table 25

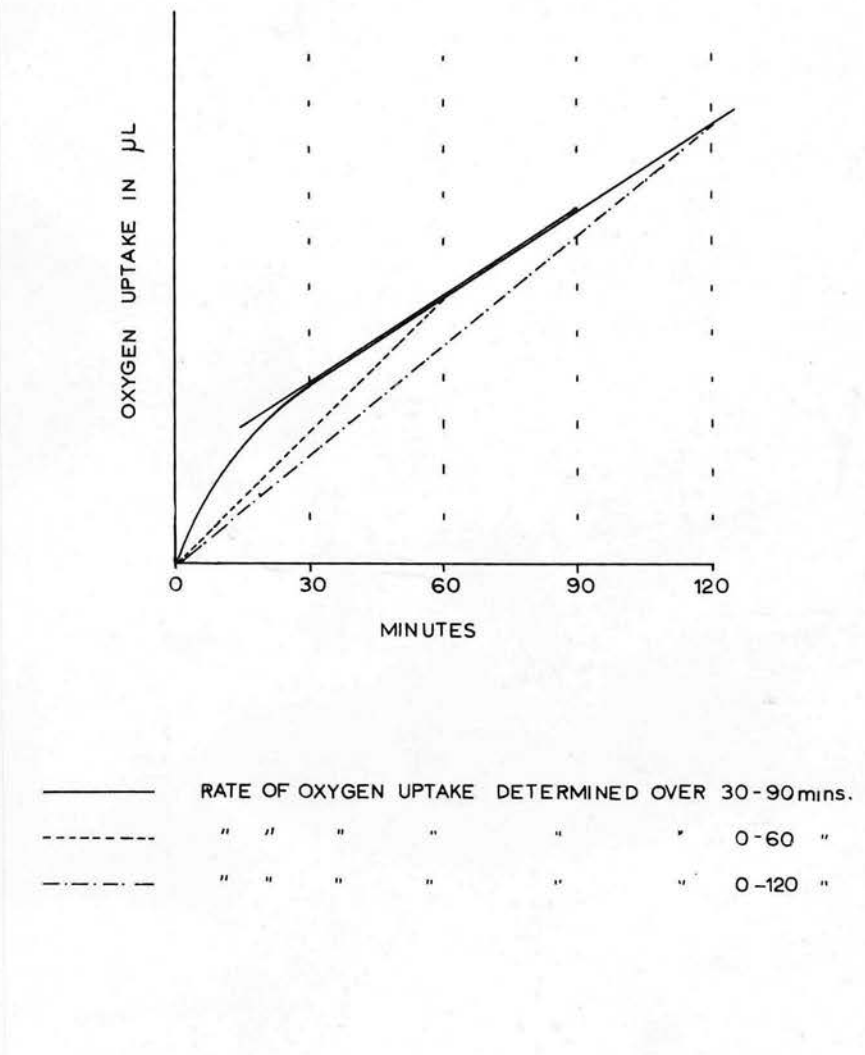


Figure 10a.

Rate of oxygen uptake measured over different time intervals.

Table 25. THE OXYGEN UPTAKE OF POLYMORPHS AND LYMPHOCYTES CALCULATED OVER DIFFERENT TIME INTERVALS

Experiment number	Oxygen uptake in μl/hour/10^{10} leucocytes calculated over the time interval			
	Polymorphs		Lymphocytes	
	0 - 60 mins.	0 - 120 mins.	0 - 60 mins.	0 - 120 mins.
88	2119	1560	4535	3430
89	4390	3055	6090	4970
93	1595	1267	3940	3245
95	3390	2865	3155	3170
98	1490	1058	3800	3030
103	3065	2200	4065	3425
105	3575	2500	4265	3155
106	2000	1710	3390	2900
107	2605	2050	3930	2995
108	2505	3130	4280	2918
109	2408	1998	3982	2840
112	2383	1733	4600	3330
114	5140	3860	4340	3360
115	3885	2670	3770	3050
116	2050	1620	5410	4260
117	2070	1940	1930	2025
118	2880	2170	6020	4910
119	2805	2475	4485	3750
120	3030	2275	6100	4470
121	2505	1787	3990	2870
122	2940	2333	5480	4110
123	1574	1417	3315	3000
124	2776	2130	5985	4630
125	1985	1790	3450	3205
126a	4640	3692	4130	3400
126b	2952	2255	3330	3025
127	3630	2730	4150	3245
128	4340	3220	6320	4635
129	4400	2940	5550	4110
130	6480	4240	8580	6150
mean:	3053.5	2355.6	4545.6	3587.1

shows the ΔO_2 values calculated over these time intervals (uncorrected for contamination with the other leucocyte type), the mean values being:

0-60 minutes : ΔO_2 PMN = 3053
: ΔO_2 lymphocytes = 4546
0-120 minutes : ΔO_2 PMN = 2356
ΔO_2 lymphocytes = 3587

$\mu L/hour/10^{10}$ leucocytes

Thus one possible explanation for the discrepancy between these results and those obtained by Rabinowitz, and by Hedeskov and Esmann, is that the rate of oxygen uptake was estimated over different time intervals. Neither of these workers states over which time interval their measurements were made, Rabinowitz stating that "measurements of oxygen uptake were made for at least two hours", while Hedeskov and Esmann say that the rate of oxygen uptake is linear for at least four hours.

5. Leucocyte crowding

It was mentioned previously(page 19)that prior to the separation procedure being attempted a number of experiments were done to see whether ΔO_2 was influenced by the number of leucocytes per Warburg flask. In those determinations of ΔO_2, no observable crowding was noticed, but this may have been due to the limited number of results.

The crowding effect has been observed by a number of workers. Barron and Harrop (1929) when determining ΔO_2 for leukaemic leucocytes found the crowding to be more marked for lymphocytes than for PMN. Hartman (1952) noticed crowding when estimating the ΔO_2 of guinea pig exudate PMN in a buffered serum medium; in a buffered saline medium very little crowding was apparent. Esmann (1964) however, found crowding in a suspension consisting mainly of PMN (human) when estimating oxygen uptake, lactic acid production, and glucose uptake in a buffered serum-free medium. Hedeskov and Esmann (1966) using column separated lymphocytes found a crowding effect which was particularly noticeable at lymphocyte concentrations below 15×10^6/ml. They found a linear relation between $\log_{10}$ (number of lymphocytes per ml.) and the corresponding $\log_{10} \Delta O_2$. It was possible to show a crowding effect in these present

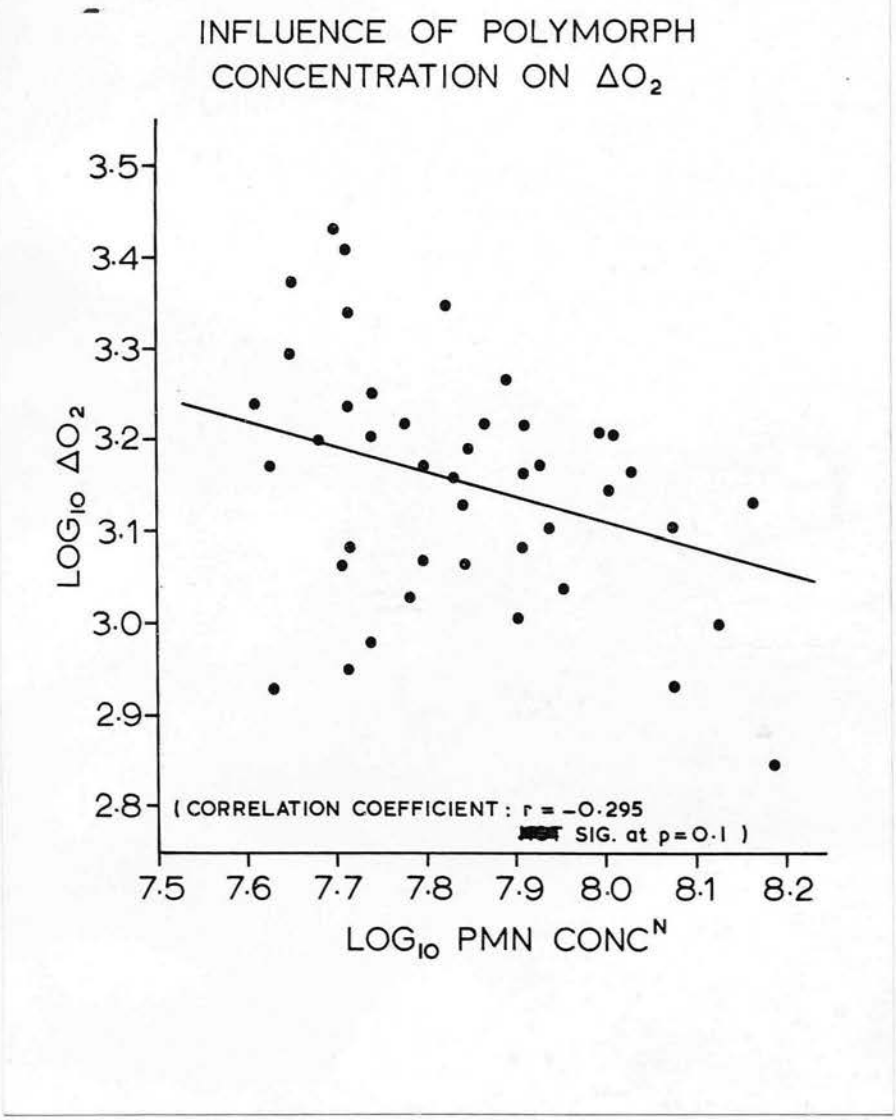

INFLUENCE OF POLYMORPH
CONCENTRATION ON ΔO_2
3·5
3·4
3·3
3·2
3·1
3·0
2·9
2·8
$LOG_{10}\ \Delta O_2$
(CORRELATION COEFFICIENT : r = −0·295
SIG. at p = 0·1)
7·5
7·6
7·7
7·8
7·9
8·0
8·1
8·2
LOG_{10} PMN $CONC^N$

Figure 11.

Table 26. INFLUENCE OF CELL CONCENTRATION ON POLYMORPH OXYGEN UPTAKE

Experiment number	Number of polymorphs / Warburg flask x 10^6 = A	$Log_{10}A$	Oxygen uptake in $\mu L/hr/10^{10}$ polymorphs = B	$Log_{10}B$
86	70.0	7.8451	1163	3.0656
87	42.5	7.6284	844	2.9263
88	54.7	7.7380	857	2.9330
89	42.6	7.6294	1477	3.1694
90	80.8	7.9074	1018	3.0076
91	74.5	7.8722	1644	3.2158
92	155.9	8.1928	705	2.8482
93	101.6	8.0068	1394	3.1443
95	70.8	7.8500	2084	3.3189
96	51.6	7.7126	2566	3.4092
98	147.6	8.1691	1356	3.1322
99	120.8	8.0820	860	2.9345
100	70.2	7.8463	1342	3.1277
101	48.4	7.6848	1568	3.1955
102	55.2	7.7419	1777	3.2497
103	61.3	7.7875	1071	3.0298
104	55.0	7.7404	1592	3.2034
105a	52.0	7.7160	1202	3.0806
105b	68.0	7.8325	1438	3.1577
106	52.6	7.7210	1254	3.0983
107	63.0	7.7993	1134	3.0697
108	51.8	7.7143	1716	3.2345
109	85.2	7.9304	1490	3.1732
110	63.0	7.7993	1484	3.1715
112	135.8	8.1329	1000	3.0000
113	66.8	7.8248	2229	3.3481
114	45.4	7.6571	2357	3.3724
115	78.3	7.8938	1839	3.2646
116	90.9	7.9586	1091	3.0378
117	44.8	7.6513	1965	3.2934
118	86.9	7.9390	1270	3.1038
119	82.0	7.9138	2072	3.2160
120	81.4	7.9104	1209	3.0823
121	120.3	8.0802	1277	3.1062
122	95.2	7.9786	1613	3.2076
123	50.8	7.7059	1153	3.0618
124	108.0	8.0334	1457	3.1635
125	81.7	7.9122	1454	3.1626
126a	60.4	7.7810	1643	3.2156
127	41.4	7.6170	1747	3.2422
128	51.8	7.7143	2186	3.3397
126b	49.6	7.6955	2682	3.4284
129	103.0	8.0128	1602	3.2046

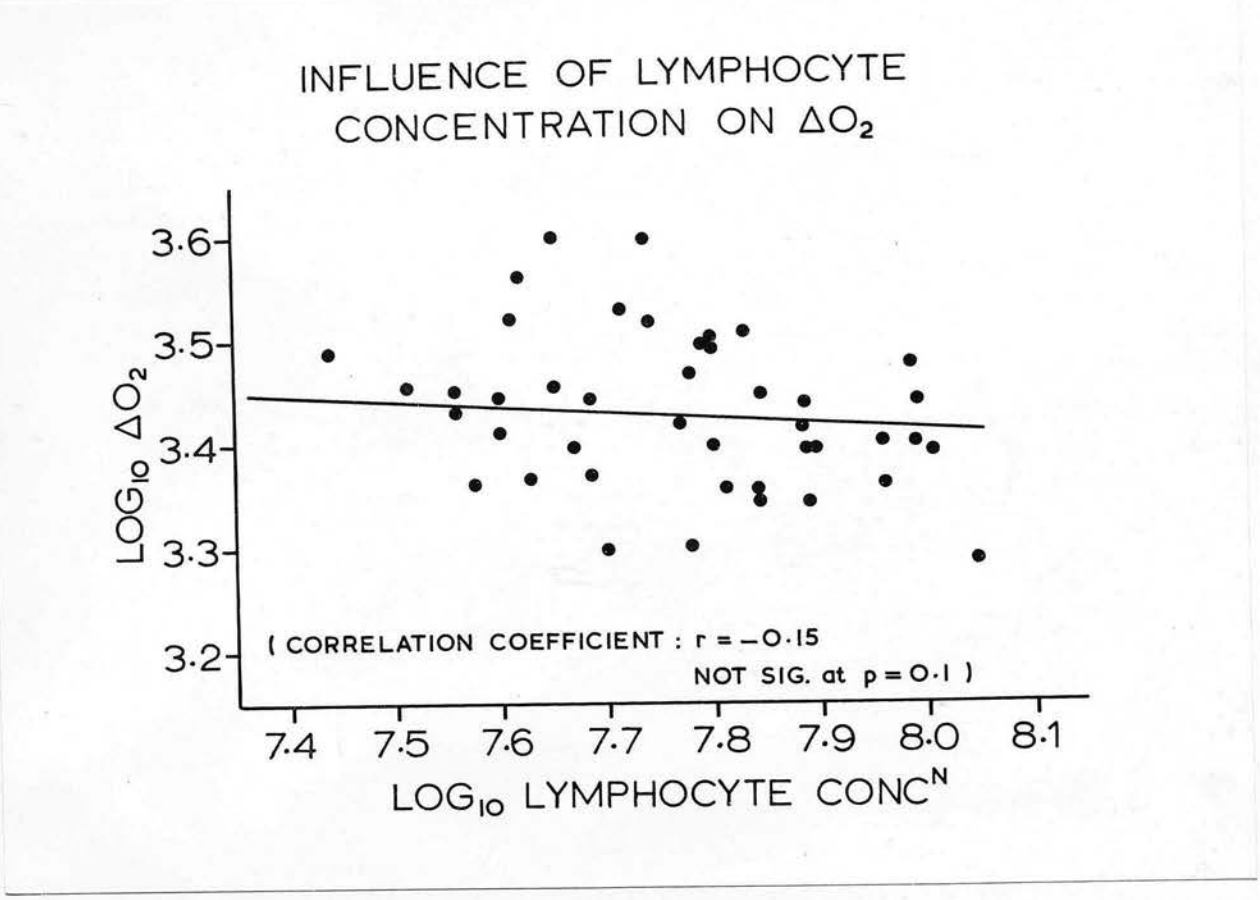

INFLUENCE OF LYMPHOCYTE CONCENTRATION ON ΔO_2
$LOG_{10}\ \Delta O_2$
3·6
3·5
3·4
3·3
3·2
(CORRELATION COEFFICIENT : r = −0·15
NOT SIG. at p = 0·1)
7·4
7·5
7·6
7·7
7·8
7·9
8·0
8·1
LOG_{10} LYMPHOCYTE $CONC^N$

Figure 12.

Table 27. INFLUENCE OF CELL CONCENTRATION ON LYMPHOCYTE OXYGEN UPTAKE

Experiment number	Number of lymphocytes / Warburg flask x 10^6 = A	Log_{10} A	Oxygen uptake in µL/hr/10^{10} lymphocytes = B	Log_{10} B
86	97.8	7.9903	2520	3.4014
87	46.5	7.6675	2485	3.3954
88	69.7	7.8432	2257	3.3535
89	41.8	7.6212	3597	3.5559
90	61.8	7.7910	3116	3.4936
91	63.5	7.8028	3175	3.5018
92	54.5	7.7364	3936	3.5951
93	77.7	7.8904	2485	3.3954
95	98.4	7.9930	2750	3.4393
96	59.2	7.7723	2613	3.4171
98	77.6	7.8899	2193	3.3410
99	76.8	7.8854	2606	3.4160
100	70.0	7.8451	2801	3.4474
101	69.6	7.8426	2204	3.3432
102	60.2	7.7796	2946	3.4692
103	39.4	7.5955	2690	3.4298
104	80.2	7.9042	2481	3.3945
105a	63.4	7.8021	2492	3.3966
105b	96.8	7.9859	2989	3.4755
106	91.4	7.9609	2524	3.4021
107	48.4	7.6848	2341	3.3694
108	67.2	7.8274	2273	3.3566
109	91.6	7.9619	2301	3.3619
110	27.4	7.4378	3079	3.4884
112	63.0	7.7993	3098	3.4911
113	60.5	7.7818	2009	3.3029
114	39.6	7.5977	2797	3.4467
115	124.6	8.0955	2453	3.3897
116	54.5	7.7364	3282	3.5162
117	42.5	7.6284	2331	3.3676
118	44.8	7.6513	3967	3.5985
119	68.0	7.8325	3256	3.5127
120	41.0	7.6128	3327	3.5220
121	111.6	8.0476	1937	3.2871
122	76.7	7.8848	2777	3.4463
123	36.2	7.5587	2822	3.4505
124	51.8	7.7143	3375	3.5283
125	4844	7.6848	2778	3.4438
126	50.2	7.7007	1999	3.3009
127	37.6	7.5752	2298	3.3613
128	32.6	7.5132	2834	3.4524
129	45.0	7.6531	2829	3.4516
130	40.0	7.6021	2574	3.4106

experiments using the results from 43 'no-drug' experiments. Tables 26 and 27 show the number of leucocytes per Warburg flask, the corresponding ΔO_2's, and their logs. Figs. 11 and 12 are the graphs of $\log_{10}$ (number of PMN/lymphocytes per flask) vs. $\log_{10}$ (of the corresponding ΔO_2). The best straight lines were calculated.

i) For PMN : equation of the line was

$$Y = -0.256X + 5.166,$$

with a correlation coefficient of $r = -0.295$ which is significant at $p = 0.1$ (at $p = 0.1$, $r = 0.2542$).

This result cannot be compared with other results since none are available for separated PMN. The nearest experiments are those of Esmann (1964) who used a suspension consisting mainly (85%) of human PMN with a concentration range of $8\text{-}40 \times 10^6$/ml. In the present work the calculations of the crowding effect were on the basis of the number of leucocytes per Warburg flask, i.e. per 4.0 mls. Thus the equivalent leucocyte concentration used by Esmann was $32\text{-}160 \times 10^6$/4.0 mls. which is a similar range to that used in these experiments. On a semi-log graph Esmann obtained a linear relation between ΔO_2 and the leucocyte concentration with $r = -0.44$ which is significant at $p < 0.001$. However in the later paper by Hedeskov and Esmann (1966) it is

stated that a log-log graph gives a linear relation between ΔO_2 and leucocyte concentration. It is thus questionable whether Esmann's value for the correlation coefficient of PMN crowding of -0.44 is valid.

ii) For lymphocytes : equation of the line was

$$Y = -0.0738X + 4.004$$

with r = -0.152 which is not significant (at p = o.1, r = ~~1~~.2542) Hedeskov and Esmann using separated lymphocytes found significant crowding ($r = -0.91$, $p < o.ool$) which does not agree with the result presented here ($p > 0.1$) the difference being difficult to explain. The cause of the crowding effect is not known. It has been demonstrated that it is not due to variation in pH, lack of glucose or organic phosphate, nor to accumulation of lactic acid (Esmann 1962).

Since it has been shown that the oxygen uptake is linear with time after the first 30 minutes (Hedeskov and Esmann 1966, this work) it is unlikely that accumulation of an inhibitor is responsible. The viscosity of blood increases at leucocyte concentrations above 40×10^6/ml. (Stephens 1936) this being readily apparent above about $60\text{-}70 \times 10^6$/ml. Since the rate of diffusion of gas through a liquid is inversely related to the viscosity of the liquid, high leucocyte concentrations would be expected to decrease the rate of O_2 diffusion through the medium.

SECTION 3. THE DETERMINATION OF THE LACTIC ACID PRODUCTION OF SEPARATED LEUCOCYTES

INTRODUCTION

A number of methods for the determination of lactic acid (LA) in blood have been developed. These include chemical and biochemical methods:

1. The chemical estimation of LA

i) Friedemann and Graeser (1933) oxidised the LA to yield an acetaldehyde bisulphite compound. The bisulphite was titrated against a dilute iodine solution and the amount of acetaldehyde calculated, the acetaldehyde concentration being proportional to the original LA concentration.

ii) Barker and Summerson (1941) oxidised the LA to acetaldehyde and obtained a coloured compound on addition of p-hydroxydiphenyl. Modifications of this method to limit the interference by impurities were made by Hullin and Noble (1953), and Mitchell and Courand (1955).

iii) In Scholander and Bradstreet's (1962) micro method semicarbazide was used to give a colour after the LA was oxidised.

iv) Gas chromatography (Hoffman et al1964) has also been used to estimate oxidised LA, this being a very sensitive method.

2. Biochemical estimation of lactic acid

i) Lundholm et al (1963a) reduced lactate with lactate

dehydrogenase in the presence of NAD to give pyruvate and $NADH^+$. The rate of loss of NAD, which was directly proportional to the rate of lactate → pyruvate, was followed in a dual-beam spectrophotometer. This method is specific for L (+) lactic acid.

Lundholm et al (1963b) compared the various methods for estimating lactic acid and concluded that:

a) the biochemical method was the most sensitive and the most specific,

b) the method of Friedemann and Graeser was generally inferior to the biochemical method;

c) Barker and Summerson's method was liable to methodological errors.

It was decided to use the method of Barker and Summerson since many workers have used this method apparently successfully, and the enzymatic method appeared somewhat complex.

THE METHOD OF BARKER AND SUMMERSON.

1. The principles of the method

The basis of the method is as follows:

i) precipitation and removal of protein;

ii) treatment with copper sulphate and calcium hydroxide to remove substances which interfere with the colour reaction, e.g. glucose, dihydroxyacetone, glyceraldehyde,

p-hydroxyphenylactic acid, malic acid, pyruvic acid, and rhamnose (Van Slyke, 1917).

iii) oxidation of lactic acid to acetaldehyde with sulphuric acid;

iv) addition of p-hydroxydiphenyl to give a purple colour.

2. Materials

10% trichloracetic acid (TCA)[a].

4% copper sulphate .12 H_2O solution ($CuSO_4$)[a].

20% copper sulphate. 12 H_2O solution ($CuSO_4$)[a].

Powdered calcium hydroxide ($Ca(OH)_2$)[b].

Concentrated sulphuric acid, sp. gr. 1.84 (H_2SO_4)[c].

1.5% p-hydroxydiphenyl[d](PP) in 0.5% sodium hydroxide[a].

Potassium lactate[e] - 50% in water.

Zinc nitrate[f].

All solutions were made up in distilled water.

The purple colour was read in

a) Unicam SP 1300 colourimeter, using a No. 4 filter with band width 510-590 mμ.

b) Unicam SP 500 spectrophotometer, wavelength 560 mμ in 1 cm light path glass cuvettes.

All pipettes were of the delivery type.

The sulphuric acid was delivered from a burette, the stopcock being lubricated with the acid.

Where 'tubes' are mentioned, these were 10 ml. graduated, stoppered Exelo tubes of 15 ml. total capacity.

---

a - Analar grade, B.D.H.

b - Extra pure grade, "

c - Microanalytical reagent, "

d - Spot-test reagent, "

e - Laboratory reagent, "

f - Laboratory chemical, May and Baker.

3. Details of the method

i) After the two hour incubation period in the Warburg flasks, when the oxygen uptake was being measured, the duplicate flask contents were pooled. 3.0 mls. were removed and added to 2.0 mls. 10% TCA in 15 ml. stoppered tubes. The tubes were shaken well and stood for 10 minutes. The precipitated protein was removed by centrifugation at 4000 rpm for 10 minutes in an MSE Minor swinging-arm centrifuge.

ii) 2.0 mls. of the protein-free supernatant were added to 7.0 mls. water and 1.0 ml. of 20% $CuSO_4$; approximately 1 gm of

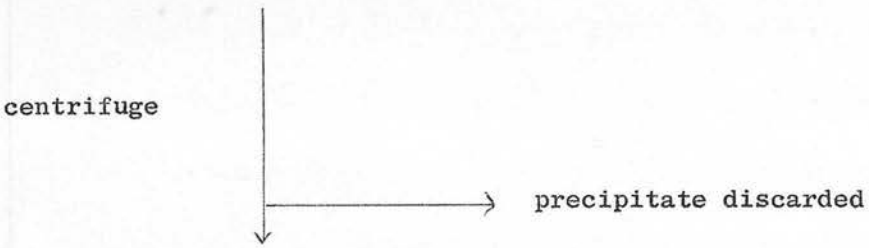
$Ca(OH)_2$ was added and the tubes shaken. After 30 minutes standing with occasional shaking the blue precipitate was removed by centrifugation as above.

iii) 1.0 ml. of the resulting supernatant was added to a fresh tube taking care to exclude the surface film precipitate. 0.05 mls. of 2.0% $CuSO_4$ were added followed by 6.0 mls. of H_2SO_4. In order to keep the temperature down to less than 70°C the addition of the H_2SO_4 was carried out in an ice bath. The tubes were then placed in a boiling water-bath for 5 minutes to complete the reaction LA → acetaldehyde, after which they were cooled to 20°C in cold water.

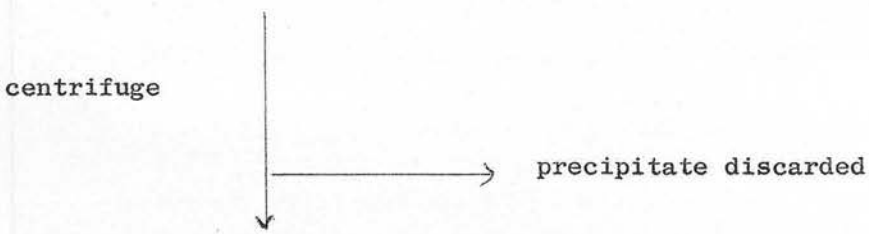
iv) 0.1 ml. p-hydroxydiphenyl ~~were~~ was then added, the resulting precipitate being dispersed by shaking. The colour was allowed to develop for 30 minutes at 30°C, the tubes being shaken every 10 minutes. At the end of this period the excess PP was dissolved by heating the tubes in a water-bath at 100°C for 90 seconds, followed by cooling to room temperature in cold water.

v) The colour intensity was read as the % transmission with reference to a blank which had been through the whole procedure, distilled water being added instead of the cell suspension. On the SP 500 and SP 1300 spectrophotometers the % transmission (%T)

3.0 mls of cell suspension from the Warburg flasks, + 2.0 ml. 10% TCA



2.0 mls. supernatant + 1.0 ml. 20% $CuSO_4$ + 1 gm. $Ca(OH)_2$.



1.0 ml. supernatant + 0.05 ml. 4.0% $CuSO_4$ + 6.0 ml. H_2SO_4.

Heat at 100°C for 5 minutes then cool

add 0.1 ml. PP.

Stand at 30°C for 30 minutes, then place in a boiling water-bath for 90 seconds.

Read purple colour in SP 500 or SP 1300, at 560 mμ.

Fig. 13. GENERAL SCHEME OF THE METHOD OF BARKER AND SUMMERSON.

and optical density (OD) could both be read off directly. However the %T was always read since this was a linear scale so the reading of the value of the %T should have been subject to a constant error. The OD was on a log. scale so the greater the OD the greater the error in reading its value. The %T was readily converted to OD by applying the equation

$$OD = 2 - \log_{10} T.$$

The method is summarised in Fig. 13.

THE STANDARD LACTIC ACID CURVE

1. The lactic acid standard

In order to estimate unknown samples of LA a standard curve was determined. The LA standard solution was made according to the method of Lundholm et al. (1963b) : 60 mls. of 50% potassium lactate were added to 30 gms. of zinc lactate and mixed well. On standing, a thick white precipitate formed which was filtered off and recrystallised twice from water. The resulting powder was dried for 24 hours at 100°C in an oven, and for 24 hours in a dessicator over phosphoric oxide. According to Lundholm et al this powder consisted of 99.6% zinc lactate. A melting point determination was not done because no information about its melting point could be found for this compound.

297.57 gm zinc lactate $3H_2O$[a] → 180.16 gm. LA.

0.1651 gm zinc lactate in 100 mls → a solution containing 1000 µg LA / ml.

This solution was used as the stock LA solution, various dilutions being made.

2. The standard curve

According to Barker and Summerson, the graph of OD v. µg LA is a straight line over the range 0-12 µg LA. Two experiments were done to check the linearity of the line. For

a Formula from Handbook of Chemistry and Physics, (1949). Chemical Rubber Publishing Co. Ltd., Cleveland, Ohio.

RELATION BETWEEN LACTIC ACID CONCENTRATION AND OPTICAL DENSITY

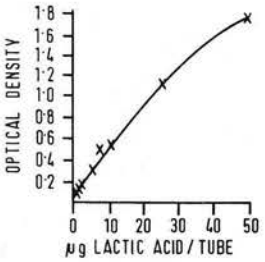


FIG. 14

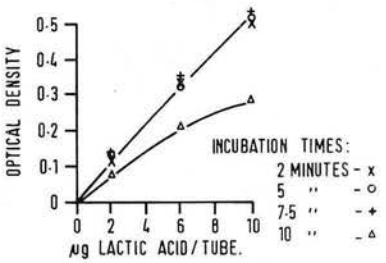


FIG. 15 EFFECT OF TIME ON THE LACTIC ACID → ACETALDEHYDE REACTION

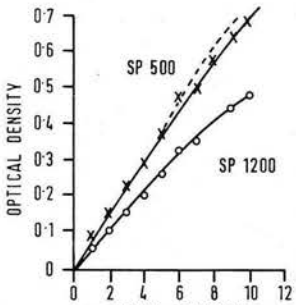


FIG. 16 EFFECT OF READING OPTICAL DENSITY IN SP 500 AND SP 1200 SPECTROPHOTOMETERS

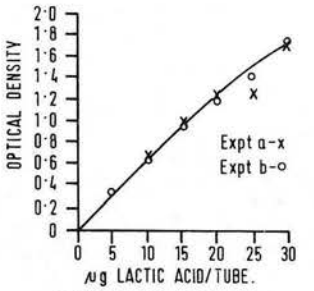


FIG. 17 OPTICAL DENSITY READ ON SP 500 SPECTROPHOTOMETER

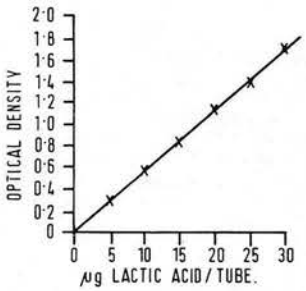


FIG. 18 EFFECT OF INCREASING P-HYDROXY-DIPHENYL CONCENTRATION

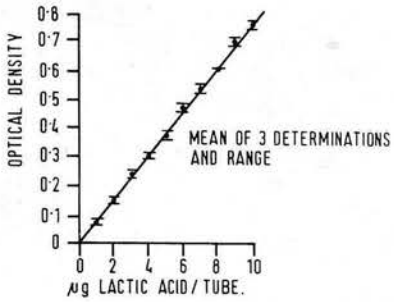


FIG. 19 THE STANDARD CURVE

Figures 14, 15, 16, 17, 18, 19.

TABLE 28. LACTIC ACID CONCENTRATION AND OPTICAL DENSITY

Lactic acid concentration per tube µg	Optical density		
	Test 1	Test 2	Mean
100	Colour too intense to measure.		
75			
50	1.7	1.7	1.7
25	1.08	1.10	1.09
10	0.535	0.523	0.529
7.5	0.458	0.475	0.4665
5.0	0.318	0.319	0.3185
2.5	0.192	0.177	0.1845
1.0	0.068	0.078	0.073
0.75	0.069	0.086	0.0775
0.50	0.055	0.031	0.043
0.25	0.047	0.040	0.0435

TABLE 29. LACTIC ACID CONCENTRATION AND OPTICAL DENSITY

Effect of time on the lactic acid → acetaldehyde reaction

Incubation period mins.	Lactic acid concentration per tube µg	%Transmission (=%T)			Log_{10} T	Optical density = 2 - $\log_{10}$
		Test 1	Test 2*	Mean		
2	2	76.0	-	76.0	1.8808	0.1192
	6	47.5	45.2	46.35	1.6661	0.3339
	10	32.0	30.5	31.25	1.4949	0.5051
5	2	74.2	-	74.2	1.8704	0.1296
	6	47.2	-	47.2	1.6739	0.3261
	10	32.0	30.0	31.0	1.4914	0.5086
7.5	2	72.0	76.0	74.0	1.8692	0.1308
	6	45.6	44.0	44.8	1.6513	0.3487
	10	30.5	-	30.5	1.4843	0.5157
10	2	82.5	84.5	83.5	1.9217	0.0783
	6	61.5	61.5	61.5	1.7889	0.2111
	10	51.5	-	51.5	1.7118	0.2882

* The $CuSo_4$ - $Ca(OH)_2$ treatment was done on a total volume of 10 mls. of lactic acid-containing solution. There was then insufficient volume for 12 x 1 ml. aliquots.

this purpose only the final colour reaction procedure was used (stages iii) and iv) of the method). The lactic acid concentrations used were 0-100 µg/tube (µg LA/ tube refers to the total amount of LA in the tube at stage iii) of the method onwards, the solution volume being 7.15 mls.) The results are shown in table 28 and fig. 14. It will be observed that the line is not linear over the ranges 0-10 and 0-50 µg LA/tube.

There were two obvious possibilities for the occurrence of this curve:

i) the incubation time during the reaction LA → acetaldehyde was incorrect, or

ii) the $CuSO_4$ - $Ca(OH)_2$ treatment is required.

3. <u>The effect of time on the LA → acetaldehyde reaction with the $CuSO_4$ - $Ca(OH)_2$ treatment included</u>

It was possible that the LA → acetaldehyde reaction was not continuing to completion particularly at the higher LA concentrations. An experiment was done to check this using three LA concentrations (2, 6, 10 µg LA /tube) with four incubation periods (2, 5, 7.5, 10 minutes). The $CuSO_4$ - $Ca(OH)_2$ treatment was also included. Table 29 shows the OD values at the various LA concentrations, while Fig. 15 shows the graph of OD v. µg LA/tube.

Table 30. LACTIC ACID CONCENTRATION AND OPTICAL DENSITY USING SP 500 AND SP 1300 SPECTROPHOTOMETERS.

SP 500				SP 1300			
Lactic acid concentration / tube µg	% Transmission (%T)	$Log_{10}T$	Optical density	Lactic acid concentration / tube µg	% Transmission (%T)	$Log_{10}T$	Optical density
1	83.6	1.9222	0.0778	1	90.0	1.9542	0.0458
2	71.1	1.8519	0.1481	2	79.3	1.8993	0.1007
3	60.0	1.7782	0.2218	3	70.7	1.8494	0.1506
4	51.9	1.7152	0.2848	4	63.6	1.8035	0.1965
5	42.4	1.6274	0.3726	5	55.2	1.7419	0.2581
6	33.4	1.5237	0.4763	6	46.8	1.6702	0.3298
7	31.5	1.4983	0.5017	7	45.0	1.6532	0.3468
8	26.5	1.4232	0.5768	8	-	-	-
9	22.8	1.3579	0.6421	9	36.0	1.5563	0.4437
10	20.5	1.3107	0.6893	10	33.1	1.5198	0.4802

Table 31. LACTIC ACID CONCENTRATION AND OPTICAL DENSITY-SP 500

Expt. a					Expt. b				
Lactic acid concentration / tube µg	% Transmission (%T)		$Log_{10}T$	Optical density	Lactic acid concentration / tube µg	% Transmission (%T)		$Log_{10}T$	Optical density
5	-		-	-	5	48.2) 46.2)	47.2	1.6739	0.3261
10	22.7) 22.7)	22.7	1.3560	0.6440	10	23.8) 22.3)	23.05	1.3626	0.6374
15	11.1) 11.3)	11.2	1.0492	0.9508	15	11.8) 11.4)	11.6	1.0645	0.9355
20	6.0) 6.4)	6.2	0.7924	1.2076	20	6.5) 6.1)	6.3	0.7993	1.2007
25	5.3) 5.7)	5.5	0.7404	1.2596	25	3.8) 4.2)	4.0	0.6021	1.3979
30	1.9) 2.1)	2.0	0.3010	1.6990	30	1.8) 2.0)	1.9	0.2788	1.7212

In all cases a curve was obtained, very similar curves being found for incubation times of 2, 5, and 7.5 minutes. It was therefore concluded that the LA → acetaldehyde reaction was continuing to completion, and that the $CuSO_4$ - $Ca(OH)_2$ treatment was not exerting a significant effect towards making the line linear.

4. Unicam SP 1200 or SP 500 for colour measurement?

Another possibility for the occurrence of the curve was that the SP 1300 spectrophotometer, which had been used up to this stage, was not sufficiently sensitive or that the band width was too wide (510 - 590 mμ). An experiment was therefore performed with 0-10 μg LA / tube with no $CuSO_4$ - $Ca(OH)_2$ treatment and measuring the %T on the SP 1200 and also on the SP 500. The results are shown in table 30 and Fig. 16. As usual the SP 1300 gave a curve, while the SP 500 gave a straight line over the range 0-8 μg LA / tube, the line then apparently falling off.

To determine whether this falling off of the line was experimental error or fact, two experiments were done over the range 0-30 μg LA / tube with no $CuSO_4$ - $Ca(OH)_2$ treatment, the colour being measured in the SP 500. The results are shown in table 31 and Fig. 17. Once again the graph shows a curve.

Table 32. LACTIC ACID CONCENTRATION AND OPICAL DENSITY - EFFECT OF INCREASING THE P-HYDROXYDIPHENYL CONCENTRATION

Lactic acid concentration µg/tube	% Transmission (%T)			$Log_{10}T$	Optical density
	Test 1	Test 2	Mean		
5	50.2	51.6	50.9	1.7067	0.2933
10	25.6	25.8	25.7	1.4099	0.5901
15	14.0	14.4	14.2	1.1523	0.8477
20	7.2	7.4	7.3	0.8633	L.1367
25	3.8	4.2	4.0	0.6021	1.3979
30	1.9	2.0	1.95	0.2900	1.7100

Table 33. LACTIC ACID CONCENTRATION AND OPTICAL DENSITY THE STANDARD CURVE

Lactic acid concentration µg/tube	Test 1		Test 2		Test 3		Mean	Mean
	%T*	OD+	%T*	OD+	%T*	OD*	%T*	OD+
1	83.1	0.0804	82.4	0.0841	85.8	0.0665	83.77	0.0769
2	69.4	0.1586	73.8	0.1319	72.0	0.1427	70.07	0.1506
3	56.0	0.2518	56.2	0.2503	57.6	0.2396	56.60	0.2472
4	47.5	0.3233	48.8	0.3116	50.7	0.2949	49.00	0.3098
5	42.4	0.3546	42.0	0.3969	42.2	0.3747	42.20	0.3750
6	33.2	0.4789	32.6	0.4868	35.0	0.4559	33.60	0.4737
7	28.4	0.5467	27.9	0.5544	29.6	0.5287	28.63	0.5431
8	24.9	0.6038	25.0	0.6021	24.6	0.6091	24.83	0.6050
9	21.5	0.6676	19.1	0.7190	-	-	20.30	0.6925
10	17.3	0.7610	17.3	0.7610	17.4	0.7595	17.38	0.7600

* %T = % transmission

\+ OD = optical density

5. The effect of increasing the p-hydroxydiphenyl concentration

Were there insufficient PP present to combine with the acetaldehyde, the colour would not develop properly at high LA concentrations. To test this, an experiment was done using 0.2 mls. PP (i.e. PP concentration doubled.) 0-30 µg LA / tube were used with no $CuSO_4$ - $Ca(OH)_2$ treatment. The colour was read in the SP 500. The results are shown in table 32 and Fig. 18. The graph suggests that under these conditions there is a direct relationship between the OD and the LA content of the tubes.

6. The accepted standard lactic acid curve

Since the expected range of LA concentration per tube was in the range of 0-10 µg, three experiments were done over this range with the $CuSO_4$ - $Ca(OH)_2$ treatment included, and using 0.2 mls. PP. The results are shown in table 33 and Fig. 19. The graph shows a good straight line which was calculated as:

$$y = 0.0764\ x + 0.00691$$

7. Conditions to be used in future estimations of lactic acid

The method used was as described previously with the exceptions that:

i) 0.2 mls. of PP were used;

ii) all colour measurements were made using the SP 500 spectrophotometer;

iii) on the few occasions that the %T was less than 10% (O.D. greater than 1.0) % dilution was made with H_2SO_4 such that the OD was less than 0.7.

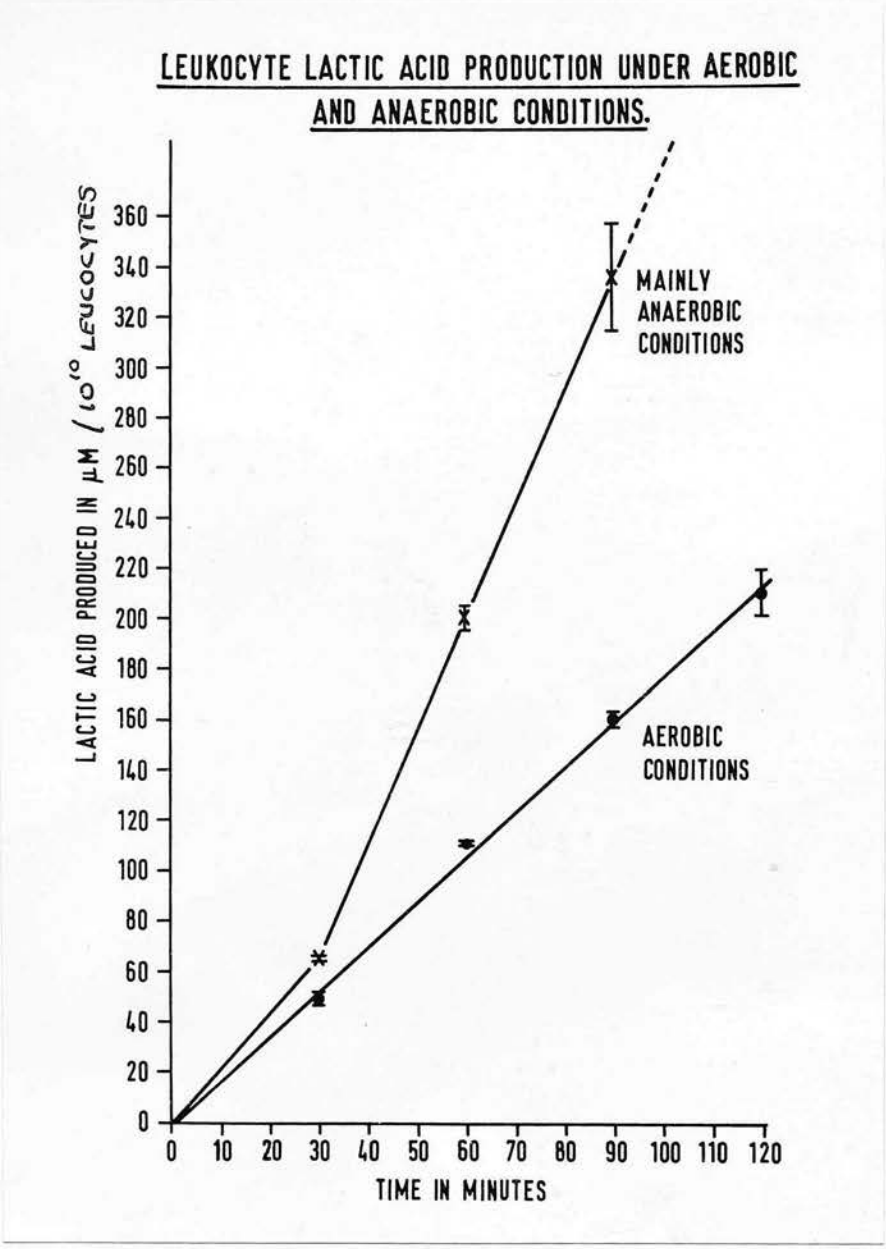

LEUKOCYTE LACTIC ACID PRODUCTION UNDER AEROBIC AND ANAEROBIC CONDITIONS.
LACTIC ACID PRODUCED IN µM / 10^10 LEUCOCYTES
0
20
40
60
80
100
120
140
160
180
200
220
240
260
280
300
320
340
360
0
10
20
30
40
50
60
70
80
90
100
110
120
TIME IN MINUTES
MAINLY ANAEROBIC CONDITIONS
AEROBIC CONDITIONS

Figure 20.

TABLE 34. AEROBIC LACTIC ACID PRODUCTION OF LEUCOCYTES

Incubation period - mins.	% Transmission	Lactic acid produced : $\mu M/10^{10}$ leucocytes
0	74.6	0.0
	74.8	0.0
30	68.9	46.2
	68.3	51.9
60	61.8	110.0
	61.6	110.6
90	56.4	164.1
	56.8	158.8
120	52.8	201.3
	51.2	219.0

TABLE 35. ANAEROBIC LACTIC ACID PRODUCTION OF LEUCOCYTES

Incubation period - mins.	% Transmission	Lactic acid produced : $\mu M/10^{10}$ leucocytes
0	61.6	0.0
	64.6	0.0
30	53.8	64.8
	53.2	65.1
60	38.1	194.8
	39.1	204.8
90	26.1	357.0
	28.4	316.0

LACTIC ACID PRODUCTION OF LEUCOCYTES -

PRELIMINARY EXPERIMENTS:

1. Aerobic lactic acid production of leucocytes

This experiment was done to make certain that the leucocyte LA production was measurable.

20 mls. of human peripheral blood were taken and anti-coagulated with 10 U / ml. heparin. The erythrocytes were sedimented with dextran. The erythrocyte-poor supernatant was centrifuged, the leucocytes being resuspended in 'phosphate' Hanks at pH 7.4. Two mls. of the leucocyte suspension + 2.0 ml. plasma were placed in Warburg flasks and incubated in the normal manner. One flask was removed every 30 minutes, the LA content being estimated in duplicate. The results are given in table 34 and Fig. 20. The LA production was found to be linear with time.

2. Anaerobic lactic acid production of leucocytes

The above experiment was repeated out of interest under approximately anaerobic conditions. The leucocyte suspension was placed in a stoppered tube at 37^{o}C and shaken frequently, aliquots being withdrawn at intervals for LA estimation. The results are shown in table 35 and Fig. 20. The LA production under anaerobic conditions was found to be greater than under aerobic conditions as would be expected. The explanation of the low

gradient over the time 0-30 minutes is possibly that the dissolved oxygen was being utilised.

3. Conclusions

It was concluded that the amount of LA produced during the oxygen uptake incubation period would be measurable.

THE EFFECT OF AMIDOPYRINE AND THIOURACIL ON LEUCOCYTE LACTIC ACID PRODUCTION

A number of experiments were performed to study the effects of amidopyrine and thiouracil on the oxygen uptake and LA production of separated leucocytes. These results will be given later. The important observation was that while the results obtained for the effects of these drugs on PMN LA production were acceptable, the lymphocyte results were quite unintelligible, being very erratic and with duplicate determinations being widely different. This was probably due to the lymphocyte LA production being lower than the PMN LA production, consequently experimental errors would have a more marked effect.

It was therefore considered necessary to use another method for the determination of the leucocyte LA production. The method chosen was the Biochemica[a] enzymatic method which was

a Biochemica Test Combination for Lactate determination. UV method. Boehringer Corp. (London) Ltd.

obtained in a kit form, this method not being known about at the beginning of this work.

LACTATE DETERMINATION - BIOCHEMICA METHOD

1. Principle of the method

This method depends upon the oxidation of L-lactate by nicotinamide adenine dinucleotide (NAD) to pyruvate, the reaction being catalysed by lactate dehydrogenase (LDH) (Hohorst 1965):

$$\text{L(+)-lactate} + NAD^+ \rightleftharpoons \text{pyruvate} + NADH + H^+$$

The equilibrium of this reaction lies to the left. In order to make the reaction proceed to the right, so giving a quantitative oxidation of the lactate, the protons are removed by use of an alkaline solution, while the pyruvate is trapped as the hydrazone:

$$\text{L(+)-lactate} + NAD^+ + \text{hydrazine} \xrightarrow[\text{pH 9.0}]{\text{LDH}} \text{pyruvate hydrazone} + NADH + H_3O^+$$

~~$+ H_3O^+$~~

The course of this reaction is measured spectophotometrically by the increased optical density due to the formation of NADH.

2. The method

Three solutions were provided:

solution 1 - 0.5M glycine buffer, pH 9.0, containing 0.4M hydrazine;

solution 2 - 2 mg/ml. LDH;

solution 3 - 0.027M NAD.

The sample to be estimated was deproteinised by addition of an equal volume of perchloric acid (6.5 ml. of 60% perchloric acid

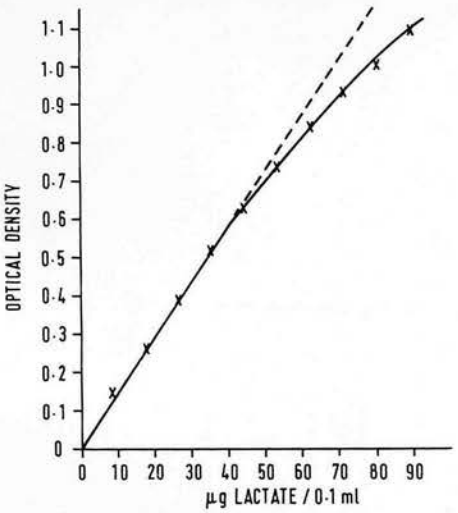


Fig.21. RELATION BETWEEN LACTATE CONCENTRATION AND OPTICAL DENSITY.

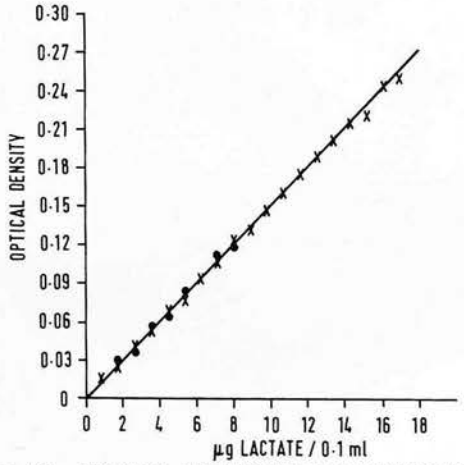


Fig.22. RELATION BETWEEN LACTATE CONCENTRATION AND OPTICAL DENSITY, THE STANDARD CURVE.

Figures 21, 22.

TABLE 36. LACTATE CONCENTRATION AND OPTICAL DENSITY THE STANDARD CURVE

Lactate concentration µg/0.1 ml.	Optical density	Lactate concentration µg/0.1 ml.	Optical density
0.9	0.0098	18	0.2583
1.8	0.0309	27	0.3871
2.7	0.0353	36	0.5139
3.6	0.0575	45	0.6239
4.5	0.0640	54	0.7323
5.4	0.0838	63	0.8368
6.3	0.0924	72	0.9289
7.2	0.1106	81	1.0065
8.1	0.1160	90	1.0895
9.0	0.1427		

TABLE 37. LACTATE CONCENTRATION AND OPTICAL DENSITY THE STANDARD CURVE

Lactate concentration µg/0.1 ml.	Optical density	Lactate concentration µg/0.1 ml.	Optical density
0.9	0.0116	9.9	0.1455
1.8	0.0236	10.8	0.1600
2.7	0.0400	11.7	0.1774
3.6	0.0522	12.6	0.1901
4.5	0.0690	13.5	0.1972
5.4	0.0761	14.4	0.2226
6.3	0.0929	15.3	0.2264
7.2	0.1044	16.2	0.2446
8.1	0.1230	17.1	0.2500
9.0	0.1315	18.0	0.2721

diluted to 100 mls. with distilled water). The solutions were pipetted into small tubes as follows:

deproteinised supernatant	0.10 mls.
solution 1	2.00 mls.
solution 2	0.03 mls.
solution 3	0.20 mls.

After mixing, the tubes were incubated for one hour at 25°C, the % transmissions then being measured at 366 mμ in 1 cm. light path glass cuvettes in a Unicam SP 500 spectrophotometer.

The % transmissions were measured against an air blank. A solution blank was made up using 0.1 ml. of trichloracetic acid in place of 0.1 ml. of the deproteinised supernatant.

A standard lactate solution was provided (1N L-lactate), a 1:1000 dilution giving a solution containing 9 μg L-lactate/0.1 ml.

3. The standard curve

To see over what range the relationship between OD and lactate concentration was linear, the OD was determined for lactate concentrations of 0-90 μg lactate/0.1 ml. sample. These results are given in table 36 and Fig. 21. The curve appears to be linear up to about 25 μg/0.1 ml.

The test was repeated over the range 0-18 μg/0.1 ml. which was the expected experimental range. These results are given in table 37. Fig. 22 shows the combined results from tables 36 and

37. It can be seen that the curve is linear over this range having as its best straight line the equation

$$y = 0.01495x - 0.000493$$

This line was taken as the standard lactate curve.

THE EFFECT OF AMIDOPYRINE ON LEUCOCYTE LACTIC ACID PRODUCTION ESTIMATED USING BOTH METHODS.

It was in the course of the experiments where the effects of differing concentrations of amidopyrine upon the leucocyte LA production were being investigated, that the defect of Barker and Summerson's method was first noticed. These LA determinations were then repeated using the Biochemica method of estimation. The results for these determinations are shown in table 38 and Fig. 23, the effects of the drug being expressed as the % inhibition, where

$$\% \text{ inhibition } (\%I) = \frac{\text{'no-drug' value - 'drug' value}}{\text{'no-drug' value}} \times 100$$

It can be seen from Fig. 23 that both methods give essentially similar results for the PMN, while there is no similarity between the results for the lymphocytes.

THE EFFECT OF THIOURACIL ON LEUCOCYTE LACTIC ACID PRODUCTION ESTIMATED USING BOTH METHODS

Following on from the experiments mentioned above, the

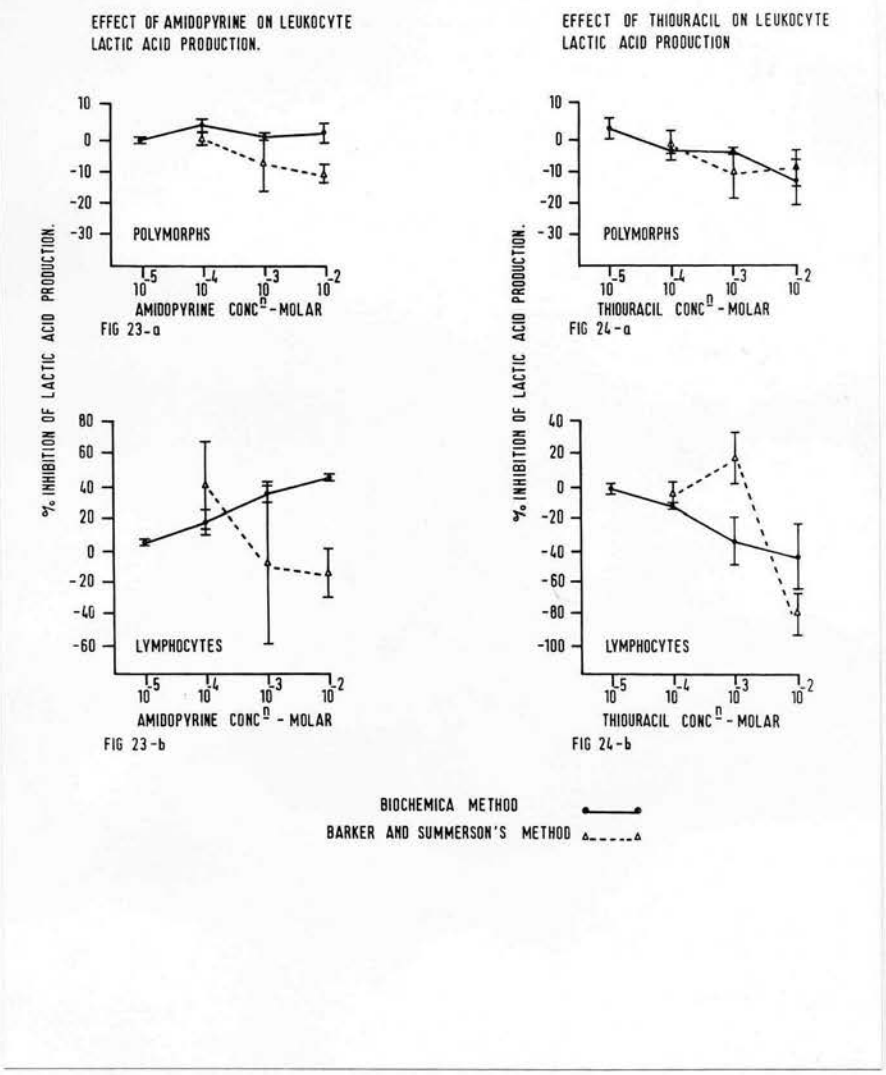


Figure 23a. Effect of amidopyrine on leucocyte respiration.
" 23b. Effect of amidopyrine on leucocyte lactic acid production.

Figure 24a. Effect of thiouracil on leucocyte respiration.
" 24b. Effect of thiouracil on leucocyte lactic acid production.

TABLE 38. EFFECT OF AMIDOPYRINE ON LEUCOCYTE LACTIC ACID PRODUCTION THE RESULTS BEING EXPRESSED AS % INHIBITIONS.

Amidopyrine concentration Molar.	Biochemica method of lactate determination		Barker and Summerson's method of lactic acid determination	
	PMN	Lymphocytes	PMN	Lymphocytes
1×10^{-5}	-1.6	3.1		
	-1.6	4.9		
1×10^{-4}	1.8	8.6	2.0	68.4
	6.0	26.1	2.2	12.3
1×10^{-3}	1.7	40.3	-17.2	42.0
	0.4	28.8	1.6	-60.0
1×10^{-2}	4.6	44.0	- 8.2	-29.0
	-1.9	45.4	-14.1	0.0

TABLE 39. EFFECT OF THIOURACIL ON LEUCOCYTE LACTIC ACID PRODUCTION THE RESULTS BEING EXPRESSED AS % INHIBITIONS

Thiouracil concentration Molar	Biochemica method of lactate determination		Barker and Summerson's method of lactic acid determination	
	PMN	Lymphocytes	PMN	Lymphocytes
1×10^{-5}	4.6	-0.1		
	-1.3	-5.4		
1×10^{-4}	-4.7	-13.1	0.4	1.9
	-6.3	-12.9	-7.0	-14.0
1×10^{-3}	-5.7	-49.9	-4.2	1.2
	-5.2	-19.2	-19.8	32.8
1×10^{-2}	-22.1	-23.0	-4.8	-64.2
	-8.3	-65.2	-16.0	-92.6

effects of thiouracil on leucocyte LA were determined using both methods for LA measurement. These results are shown in table 39 and Fig. 24. As with amidopyrine, the PMN results do not differ with the two methods. The effects of thiouracil would appear to vary depending upon which method is used to estimate the LA produced.

BIOCHEMICA METHOD Vs. BARKER AND SUMMERSON'S METHOD

1. Polymorphs

Either method would appear suitable for measuring PMN LA production, the Biochemica method giving less variation between duplicate experiments.

2. Lymphocytes

As can be seen from Figs. 23 and 24 the results obtained for the influence of amidopyrine and thiouracil depend entirely upon which method is used for the determination of the LA produced. The Biochemica method gives reasonably reproducible results in both cases showing a trend with increasing concentration of the drugs. Barker and Summerson's method, on the other hand, gives results which are not reproducible, the scatter of the results being so great that no trend with increasing drug concentration is observable. These latter results may be due to this method being insensitive at the low LA levels found, or possibly to the

presence of impurities which contribute a relatively large amount of "LA" relative to the amount of LA produced by the lymphocytes.

It was therefore decided that the Barker and Summerson method should be discontinued, the Biochemica method being adopted instead.

The significance of the effects of amidopyrine and thiouracil on leucocyte LA production will be considered later.

THE CORRECTION FOR ERYTHROCYTE CONTAMINATION OF THE TWO LEUCOCYTE SUSPENSIONS DURING THE DETERMINATION OF THE LACTIC ACID PRODUCTION

It was shown previously that the erythrocyte contamination of the two leucocyte suspensions contributed a negligible amount to their ΔO_2's. It was assumed that the erythrocyte LA production would be small compared to the leucocyte LA production. When almost all the drug experiments had been done it was apparent that a simple explanation for the curious lymphocyte results would not be forthcoming. It was decided to check that the above assumption was correct. Eight experiments were performed to determine the erythrocyte LA production, the results being shown in table 40, the LA production being expressed as $\Delta LA = \mu M/hour/10^{10}$ cells.

Table 40. The lactic acid production of different cell types

ΔLA : μM / hour / 10^{10} cells			
	Polymorphs	Lymphocytes	Erythrocytes
	818.1	340.5	5.4
	672.5	226.7	4.9
	947.3	117. 0	8.8
	817. 5	26.8	5.8
	930.5	127.6	6.0
	831.0	166.4	4.8
	823.0	130.5	4.4
	1061.0	99.6	9.6
Mean :	862.6	154.4	6.2
S.E.:	40.95	33.21	0.69

1. Correction for erythrocyte contamination of the PMN suspension

Assuming an average leucocyte : erythrocyte ratio of 4:1 as determined previously, the erythrocytes would account for about 6.2 μM in every 4 x 862.6 μM produced. This is equivalent to about 0.18% which is negligible.

2. Correction for erythrocyte contamination of the lymphocyte suspension

Assuming an average leucocyte : erythrocyte ratio of about 1 : 9 as determined previously, the erythrocytes would account for about 6.2 x 9 μM in every 154.4 μM LA produced. Thus the true

leucocyte LA production would be 154.4 - (6.2 x 9) = 98.6 μM LA. This is only 63.8% of the observed value. On attempting to correct past results by this percentage negative results were obtained in many cases for the lymphocyte Δ LA, while the PMN Δ LA was virtually unaffected.

3. Conclusion

This method for correcting for theerythrocyte contamination of the lymphocyte suspensions gave results which were extremely variable and quite uninterpretable. It was thus necessary to attempt another method for evaluating the erythrocyte contribution to the leucocyte LA. production.

4. The use of three leucocyte suspensions

Since the two leucocyte suspensions contained three cell types, (erythrocytes, lymphocytes, and PMN) it was thought that the LA production of the three cell types could be evaluated by using three leucocyte + erythrocyte suspensions and determining the LA production of each cell type by applying simultaneous equations in the manner detailed previously. The cell suspensions used were those following column separation and one obtained prior to the separation. This method for eliminating the erythrocyte contribution to the leucocyte LA production was not successful, some "no-drug" and "with-drug" values in the same experiment

being as different as +2000 and -2000 μM/hour/10^{10} lymphocytes. It was therefore concluded that this method for correcting for the erythrocyte contamination of the lymphocyte suspension was not going to be successful, though the reason for this was not clear. It was not possible, therefore, to obtain any results for the effects of drugs on lymphocyte LA production.

SUMMARY OF 'NO-DRUG' RESULTS

1. Polymorph lactic acid production

It was mentioned previously that when the effects of drugs upon leucocyte lactic acid production were being investigated, control experiments were done simultaneously, and that this resulted in a number of 'no-drug' (control) results. A similar situation existed for the leucocyte LA determination. Table 41 shows the PMN LA production in 36 'no-drug' determinations (column 4), the mean value being 918.5 μM/hour/10^{10} PMN. For comparison, Hedeskov and Esmann (1966) using column separated PMN found a value of 640 μM, while Rauch et al (1961) using a suspension consisting predominantly of PMN obtained a value of 403 μM.

2. Leucocyte crowding

It was shown previously that as the PMN concentration increased, the ΔO_2 decreased. A similar effect has been shown to exist for PMN lactic acid production in the past (Esmann, 1964). To ascertain if a crowding effect was present in these experiments a graph was drawn of the $\log_{10}$ (number of PMN per test) Vs. $\log_{10}$

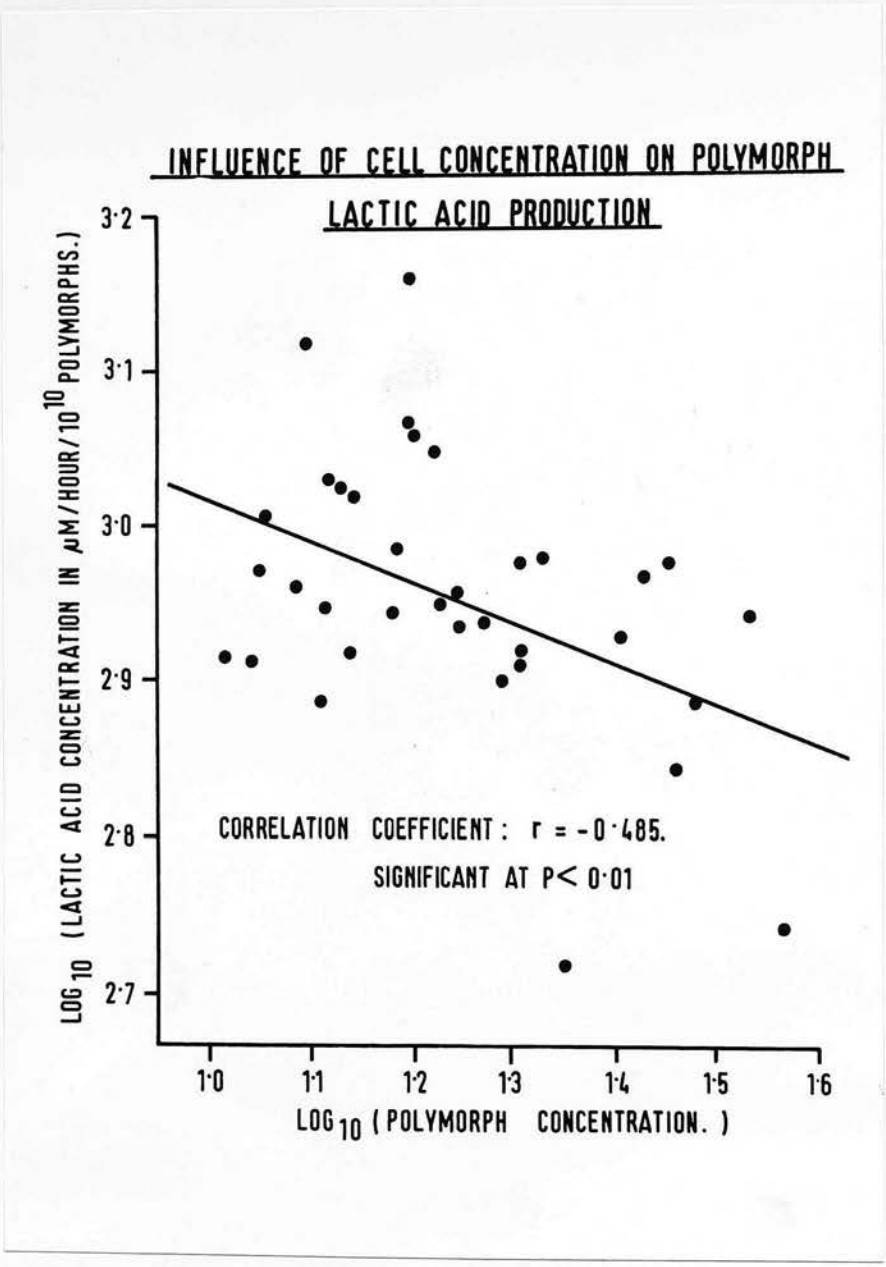

INFLUENCE OF CELL CONCENTRATION ON POLYMORPH LACTIC ACID PRODUCTION
LOG10 (LACTIC ACID CONCENTRATION IN μM/HOUR/10^10 POLYMORPHS.)
3·2
3·1
3·0
2·9
2·8
2·7
CORRELATION COEFFICIENT: r = -0·485.
SIGNIFICANT AT P< 0·01
1·0
1·1
1·2
1·3
1·4
1·5
1·6
LOG10 (POLYMORPH CONCENTRATION.)

Figure 25.

TABLE 41. INFLUENCE OF CELL CONCENTRATION ON POLYMORPH LACTIC ACID PRODUCTION

Experiment number	Number of * polymorphs x 10^6 / test = A	Log_{10} A	Lactic acid production in µM/hour/10^{10} polymorphs = B	Log_{10} B
95	17.7	1.2480	866	2.9370
96	12.9	1.1106	774	2.8882
98	36.9	1.5670	557	2.7461
99	30.2	1.4800	775	2.8891
100	17.6	1.2442	907	2.9572
101	12.1	1.0831	913	2.9607
102	13.8	1.1399	1042	3.0178
103	15.3	1.1853	969	2.9864
104	13.8	1.1386	828	2.9178
105a	17.0	1.2304	893	2.9509
106a	13.2	1.1189	1068	3.0286
107	15.8	1.1973	1158	3.0637
108	13.0	1.1127	884	2.9464
109	21.3	1.3284	957	2.9809
110	15.8	1.1973	1445	3.1599
112	33.9	1.5302	879	2.9439
113	16.7	1.2227	1116	3.0476
114	11.3	1.0546	1014	3.0059
115	19.6	1.2918	797	2.9016
116	22.7	1.3564	524	2.7916
117	11.2	1.0487	937	2.9717
119	20.5	1.3118	947	2.9762
120	20.4	1.3086	819	2.9133
122	28.3	1.4518	947	2.9764
123	12.7	1.1038	818	2.9125
124	27.0	1.4314	931	2.9687
125	20.4	1.3096	831	2.9196
126a	15.1	1.1793	877	2.9431
126b	15.1	1.1793	877	2.9431
127	10.4	1.0153	823	2.9155
128	13.0	1.1127	1061	3.0257
129	12.4	1.0934	1307	3.1161
130	25.5	1.4057	850	2.9296
131	18.7	1.2718	868	2.9384
132	15.9	1.2014	1150	3.0605
133	28.5	1.4553	701	2.8457
		Mean	918.5	
		S.E.	29.76	

* Number of PMN/test = 1/4 x number of PMN/Warburg flask.

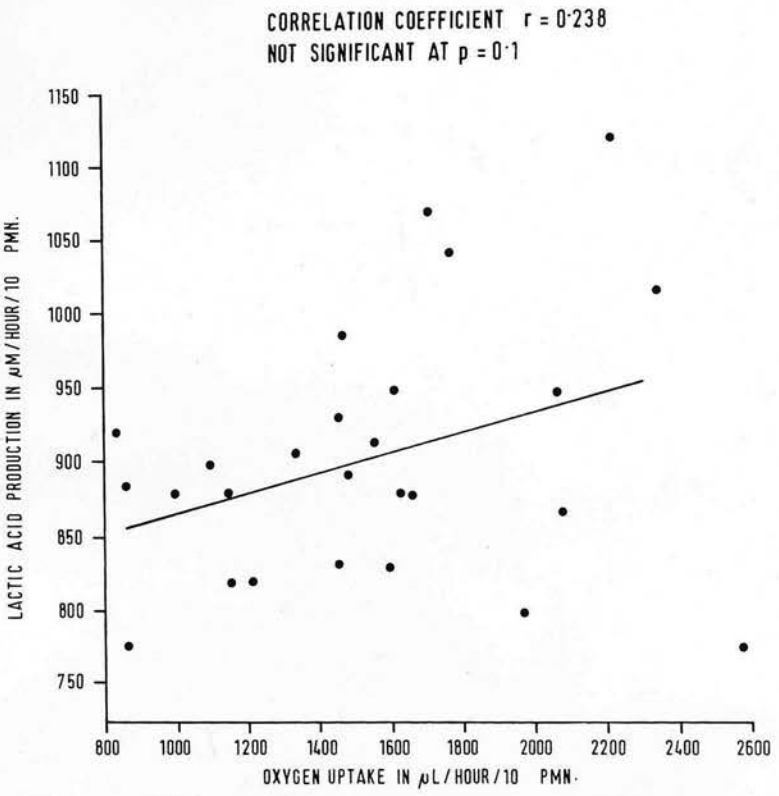


Figure 26.

Relation between oxygen uptake and lactic acid production of leucocytes.

TABLE 42. RELATION BETWEEN POLYMORPH OXYGEN UPTAKE AND LACTIC ACID PRODUCTION

Experiment number.	Oxygen uptake in $\mu L/hour/10^{10}$ polymorphs	Lactic acid production in $\mu M/hour/10^{10}$ polymorphs
86	1168	879
87	844	920
88	857	884
89	1477	897
95	2084	865
96	2566	773
99	860	775
100	1342	906
101	1568	914
102	1777	1042
104	1592	828
108	1716	1068
110	1484	884
112	1000	879
113	2229	1116
114	2357	1014
116	1091	896
117	1965	797
119	2072	947
120	1209	818
122	1613	948
123	1153	818
124	1457	931
125	1454	831
126	1643	877
127	1640	872

(LA production in $\mu M/hour/10^{10}$ PMN). This is shown in Fig. 25, the figures plotted being in table 41. The calculated best straight line through the points was

$$Y = - 0.268 X + 3.288$$

with a correlation coefficient (r) = -0.485, which is significant at $p < 0.01$ (at $p = 0.01$, $r = 0.4238$).

It was stated earlier that the cause of the crowding effect is not known.

3. The relation between leucocyte oxygen uptake and lactic acid production

It was of interest to see if there was any relation between PMN ΔO_2 and ΔLA.

Assuming that all PMN have the same ΔO_2 : ΔLA ratio, but that the metabolic rates of the leucocytes varies, then one would expect to find a linear relation with a positive gradient on the graph of ΔO_2 vs. ΔLA. If on the other hand there were no relation between ΔO_2 and ΔLA, i.e. these parameters varied independently from sample to sample, then one would not expect to find any correlation. To determine which was the case, a graph (Fig. 26) was plotted of the oxygen uptake in $\mu L/hour/10^{10}$ PMN against the lactic acid production in $\mu M/hour/10^{10}$ PMN, the appropriate values being given in table 42. The equation of the best straight line was calculated

as

$$Y = 0.0556\ X + 817.07$$

with the correlation coefficient (r) = 0.279 which is not significant at p = 0.1 (at p = 0.1, r = 0.3172). It would thus seem that there is no direct relation between these two parameters. There are a number of possibilities to explain this result:-

I) The PMN may have been damaged during the separation process. Since leucocytes exhibit the Pasteur effect, damage to the respiratory mechanism would result in an increased LA production, this leading to a degree of scatter between the results. Fig. 26 does show considerable scatter.

II) The crowding effect is more pronounced on the LA production (r = approx. -0.5) than on ΔO_2 (r = approx. -0.3). Thus as the PMN concentration increases there will be a greater decrease in ΔLA than in ΔO_2. In consequence there will be a lower gradient for the line than might otherwise be expected.

III) It is also possible that there really is no correlation between the parameters. It is believed by a number of workers that the production of LA by leucocytes "in vitro" is an artefact of separation, and that under normal "in vivo" conditions leucocytes produce no LA (Seelich, 1962). If this is the case, no correlation would be expected.

It is difficult to ascertain what is the normal ratio of ΔO_2 : ΔLA, due to different workers using a variety of methods for leucocyte separation, for $\Delta O_2/\Delta$LA estimations, and using different incubation media. For instance McKinney et al (1952) using unseparated leucocytes with a "great majority" of PMN found a ratio of 1.0 µM O_2 used for every 8.0 µM LA produced in a medium of 1 part plasma to 5 parts Hanks. Beck (1958) using homogenised unseparated leucocytes in a medium fortified by addition of ATP, DPN, and cytochromec found a ratio of 11.3 : 1.0, while Hedeskov and Esmann using column separated PMN and a Tris-phosphate-buffered plasma-free medium obtained a ratio of 5.5 : 1.0. In this work the ratio was 1.3 : 1.0.

From these figures it is apparent that there is no obvious relationship between oxygen uptake and lactic acid production.

SECTION 4. THE EFFECTS OF DRUGS ON LEUCOCYTE RESPIRATION AND LACTIC ACID PRODUCTION

PROCEDURE AND RESULTS

SECTION 4. THE EFFECTS OF DRUGS ON LEUCOCYTE RESPIRATION AND LACTIC ACID PRODUCTION

PROCEDURE AND RESULTS

THE USE OF DRUGS

The following drugs were investigated for their effects on leucocyte respiration and on PMN LA production:

chlorpromazine[a] (CPZ))
amidopyrine[b] (AP)) by these drugs is agranulocytosis
thiouracil[c] (TU))

phenylbutazone[d] (PB) which induces agranulocytosis and aplastic anaemia with equal frequency.

chloramphenicol[e] (CAP) which induces mainly aplastic anaemia

tetracycline[f] (TC))
sulphisoxazole[g] (SO)) these drugs are not generally associated with haematological disorders

All drug solutions were made up in 'phosphate' Hanks immediately prior to use after it had been found that a stock solution of CAP lost all its inhibitory activity with regard to PMN respiration in three days (see also Higuchi et al 1954). The resulting solutions were adjusted to pH 7.4 where necessary with 0.1N HCl or o.1N NaOH. All drugs were investigated at the same

a - chlorpromazine HCl (Largactil), May and Baker Ltd.,

b - amidopyrine was supplied by Geigy (U.K.) Ltd.

c.- thiouracil was supplied by Koch-Light Ltd.

d - phenylbutazone was supplied by Geigy (U.K.) Ltd.

e - chloramphenicol succinate (chloromycetin), Parke Davis and Co. Ltd.

f - tetracycline HCl (Achromycin), Lederle Laboratories.

g - sulphisoxazole (Gantrisin), Roche Products Ltd.

concentrations, these being 1.0×10^{-2}, 1.0×10^{-3}, 1.0×10^{-4}, and 1.0×10^{-5} molar. Because of the high potency of CPZ this drug was also used at 1.0×10^{-6}M. CPZ, PB, and TC were not soluble at 1.0×10^{-2}M, the highest concentrations of these drugs used being 1.0×10^{-3}M.

At all times when the effects of drugs on leucocyte respiration and LA production were being observed control determinations were carried out simultaneously. The procedure in each case was identical with the exception that the leucocytes were suspended in either drug-free or 'with-drug' medium. All determinations, including controls, were made in duplicate. Because of the large number of leucocytes required for the estimation of the oxygen uptake only one drug concentration and control could be investigated at any one time. Two experiments were conducted at each drug concentration.

Having determined the various ΔO_2 and ΔLA values as described previously, the effects of the drugs were expressed in terms of the percentage inhibition (%I), where

$$\%I = \frac{\text{'no-drug'}\ \Delta O_2/\Delta LA - \text{'with-drug'}\ \Delta O_2/\Delta LA}{\text{'no-drug'}\ \Delta O_2/\Delta LA}$$

The results were expressed in this form to eliminate the individual variation of the 'no-drug' ΔO_2 and ΔLA values. The

%I of the duplicate determinations carried out on different blood samples generally agreed to within 10%.

Log-dose v.s response curves were drawn for each drug, the %I being taken as the response.

RESULTS

The effect of the individual drugs on the leucocyte respiration and also on the PMN LA production are shown in tables 43a,b - 49a,b (the "a" tables showing the inhibition of leucocyte respiration, and the "b" tables the inhibition of PMN LA production). The results are also presented in graphical form in Figs. 27 - 31.

1. The effects of drugs on polymorph respiration

It is readily apparent that there are distinct differences between the drugs with regard to their actions on PMN respiration on a molar basis. CPZ is about ten times as potent as any other drug tested, the order of potencies of the other drugs, in descending order, being AP greater than PB, PB being very similar to TU, with CAP being the least potent. TC and SO were inactive.

2. The effects of drugs on lymphocyte respiration

None of the drugs investigated showed any marked effect on lymphocyte respiration.

3. The effect of drugs on polymorph lactic acid production

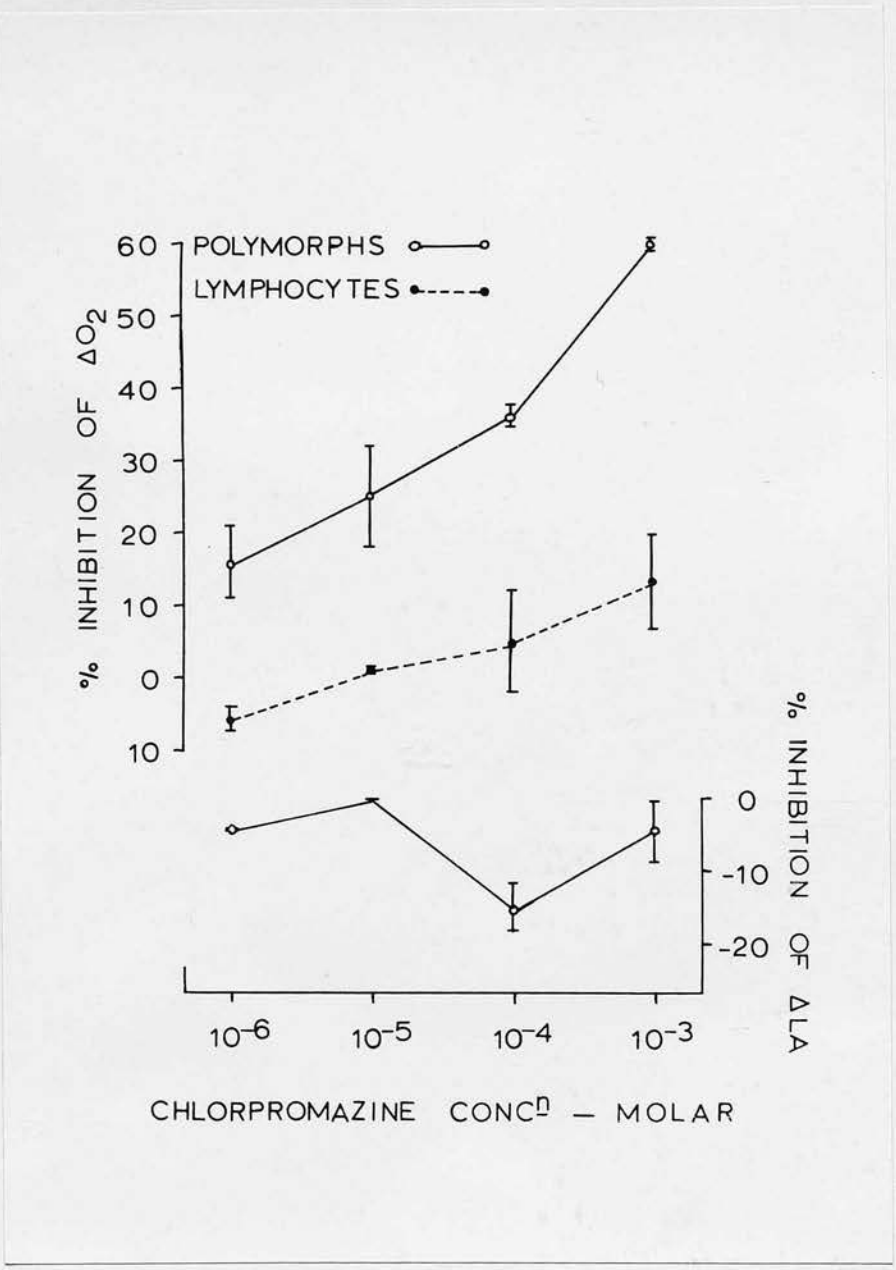


Figure 27.

Effect of chlorpromazine on leucocyte respiration (ΔO_2) and lactic acid production (ΔLA).

Table 43a EFFECT OF CHLORPROMAZINE ON LEUCOCYTE OXYGEN UPTAKE

Chlorpromazine concentration molar	Expt. no.	Polymorphs			Lymphocytes		
		No drug ΔO_2*	With drug ΔO_2*	%I +	No drug ΔO_2*	With drug ΔO_2*	%I +
-3	127	1747	690	61	2298	2149	6
1.0 x 10	128	2186	835	59	2834	2270	20
-4	126	2682	1744	35	2829	2482	12
1.0 x 10	133	1602	1000	38	2574	2625	-2
-5	129	1323	1084	18	2804	2755	2
1.0 x 10	130	2033	1546	32	3386	3366	1
-6	131	1932	1512	22	2982	3096	-4
1.0 x 10	132	1579	1406	11	2945	3163	-7

* ΔO_2 expressed as µl/hour/10^{10} leucocytes

+ % I = % inhibition

Table 43b. EFFECT OF CHLORPROMAZINE ON LEUCOCYTE LACTIC ACID PRODUCTION

Chlorpromazine concentration Molar	Expt. no.	Polymorphs		
		No drug ΔLA*	With drug ΔLA*	%I +
-3	127	823	896	-9
1.0 x 10	128	1061	1063	0
-4	126	1307	1548	-18
1.0 x 10	133	850	952	-12
-5	129	868	868	0
1.0 x 10	130	-	-	-
-6	131	1150	1203	-5
1.0 x 10	132	701	731	-4

* ΔLA expressed as µM/hour/10^{10} leucocytes

+ % I = % inhibition

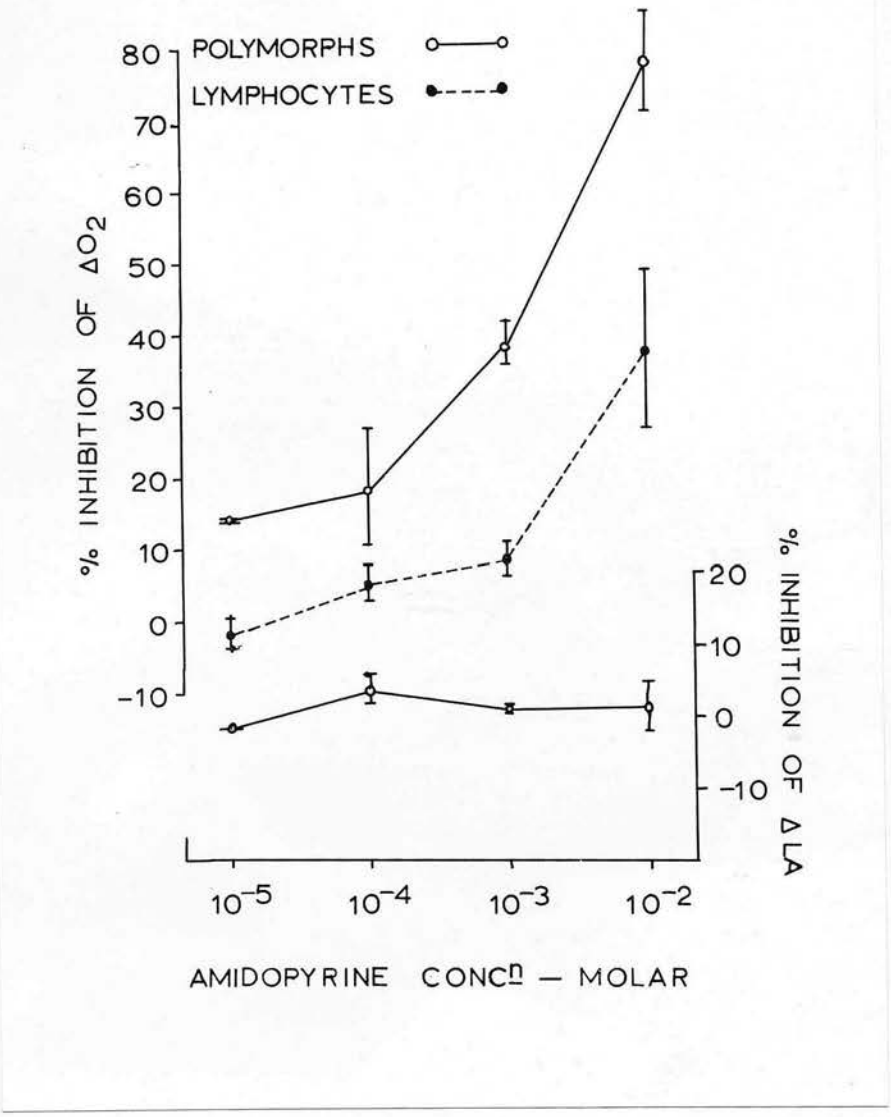


Figure 28.

Effect of amidopyrine on leucocyte respiration (ΔO_2) and lactic acid production (Δ LA).

Table 44a EFFECT OF AMIDOPYRINE ON LEUCOCYTE OXYGEN UPTAKE

Amidopyrine concentration Molar	Experiment no.	Polymorphs			Lymphocytes		
		No drug ΔO_2*	With drug ΔO_2*	%I +	No drug ΔO_2*	With drug ΔO_2*	%I +
1.0×10^{-2}	86	1163	336	71	2520	1311	49
	87	844	126	85	2485	1805	27
1.0×10^{-3}	88	857	549	36	2257	2111	6
	89	1477	859	42	3597	3182	12
1.0×10^{-4}	90	1018	746	27	3116	2858	8
	91	1644	1474	10	3175	3080	3
1.0×10^{-5}	92	705	605	14	3936	4096	-4
	93	1394	1201	14	2485	2466	1

* ΔO_2 expressed as μl/hour/10^{10} leucocytes

\+ %I = % inhibition

Table 44b EFFECT OF AMIDOPYRINE ON LEUCOCYTE LACTIC ACID PRODUCTION

One experiment. The 'no-drug' values were the same for all concentrations of amidopyrine.

'no-drug' PMN ΔLA's were 879 and 920, mean = 900 μM/hour/10^{10} PMN.

Amidopyrine concentration Molar	Polymorphs	
	ΔLA*	%I+
1.0×10^{-2}	858	5
	917	-2
1.0×10^{-3}	885	2
	896	0
1.0×10^{-4}	883	2
	846	6
1.0×10^{-5}	915	-2
	915	-2

* ΔLA expressed as μM/hour/10^{10} leucocytes

\+ %I = % inhibition

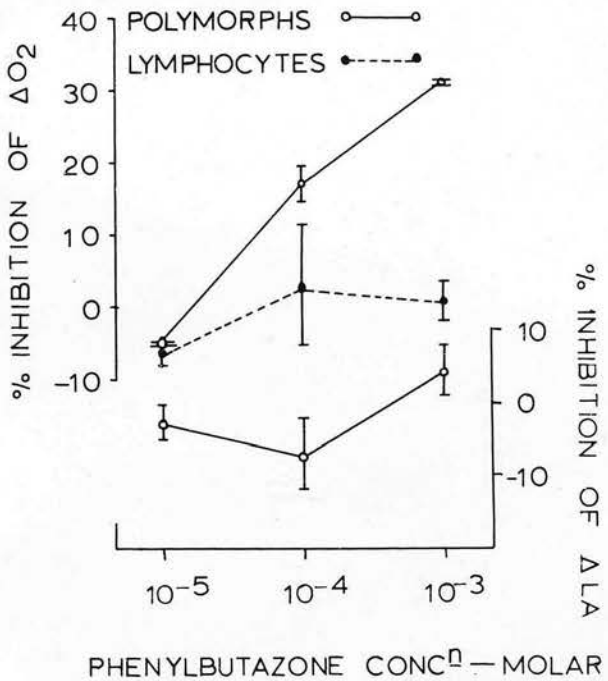


Figure 29.

Effect of phenylbutazone on leucocyte respiration (ΔO_2) and lactic acid production (ΔLA).

Table 45a EFFECT OF PHENYLBUTAZONE ON LEUCOCYTE OXYGEN UPTAKE

Phenylbutazone concentration Molar	Experiment no.	Polymorphs			Lymphocytes		
		No drug ΔO_2*	With drug ΔO_2*	%I +	No drug ΔO_2*	With drug ΔO_2*	%I +
1.0×10^{-2}		Maximum solubility in water at 22°C is 0.7 mg./ ml. = 2.3×10^{-3} molar.					
1.0×10^{-3}	112	1000	874	31	3098	2988	4
	113	2229	1538	31	2009	2052	-2
1.0×10^{-4}	114	2357	1894	20	2797	2470	12
	115	1839	1576	14	2453	2574	-5
1.0×10^{-5}	116	1091	1149	-5	3282	3460	-5
	117	1965	2065	-5	2331	2516	-8

* ΔO_2 expressed as µl/hour/10^{10} leucocytes

\+ %I = % inhibition

Table 45b EFFECT OF PHENYLBUTAZONE ON LEUCOCYTE LACTIC ACID PRODUCTION

Phenylbutazone concentration Molar	Experiment no.	Polymorphs		
		No drug ΔLA*	With drug ΔLA*	%I +
1.0×10^{-2}		See table 45a		
1.0×10^{-3}	112	879	873	1
	113	1116	1036	8
1.0×10^{-4}	114	1014	1033	-2
	115	797	891	-12
1.0×10^{-5}	116	524	524	0
	117	896	937	-5

* ΔLA expressed as µM/hour/10^{10} leucocytes

\+ %I = % inhibition

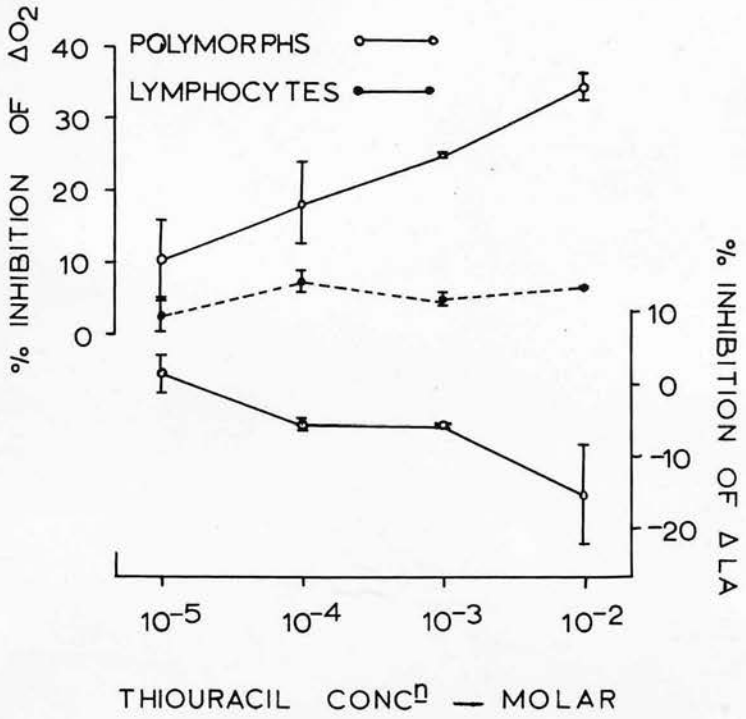


Figure 30.

Effect of thiouracil on leucocyte respiration (ΔO_2) and lactic acid production (ΔLA).

Table 46a EFFECT OF THIOURACIL ON LEUCOCYTE OXYGEN UPTAKE

Thiouracil concentration Molar	Experiment no.	Polymorphs			Lymphocytes		
		No drug ΔO_2*	With drug ΔO_2*	%I +	No drug ΔO_2*	With drug ΔO_2*	%I +
1.0×10^{-2}	95	2084	1406	33	2750	2572	7
	96	2566	1629	37	2613	2440	7
1.0×10^{-3}	98	1356	1015	25	2193	2062	6
	99	860	648	25	2606	2496	4
1.0×10^{-4}	100	1342	1170	13	2801	2548	9
	101	1568	1189	24	2204	2068	7
1.0×10^{-5}	102	1777	1482	17	2946	2790	5
	104	1592	1512	5	2481	2460	1

* ΔO_2 expressed as $\mu l/hour/10^{10}$ leucocytes

+ %I = % inhibition

Table 46b EFFECT OF THIOURACIL ON LEUCOCYTE LACTIC ACID PRODUCTION

Thiouracil concentration Molar	Experiment no.	Polymorphs		
		No drug ΔLA*	With drug ΔLA*	%I +
1.0×10^{-2}	95	865	1056	-22
	96	773	836	-8
1.0×10^{-3}	98	557	589	-6
	99	775	814	-5
1.0×10^{-4}	100	906	964	-6
	101	914	958	-5
1.0×10^{-5}	102	1042	1035	5
	104	828	839	-1

* ΔLA expressed as $\mu M/hour/10^{10}$ leucocytes

+ %I = % inhibition

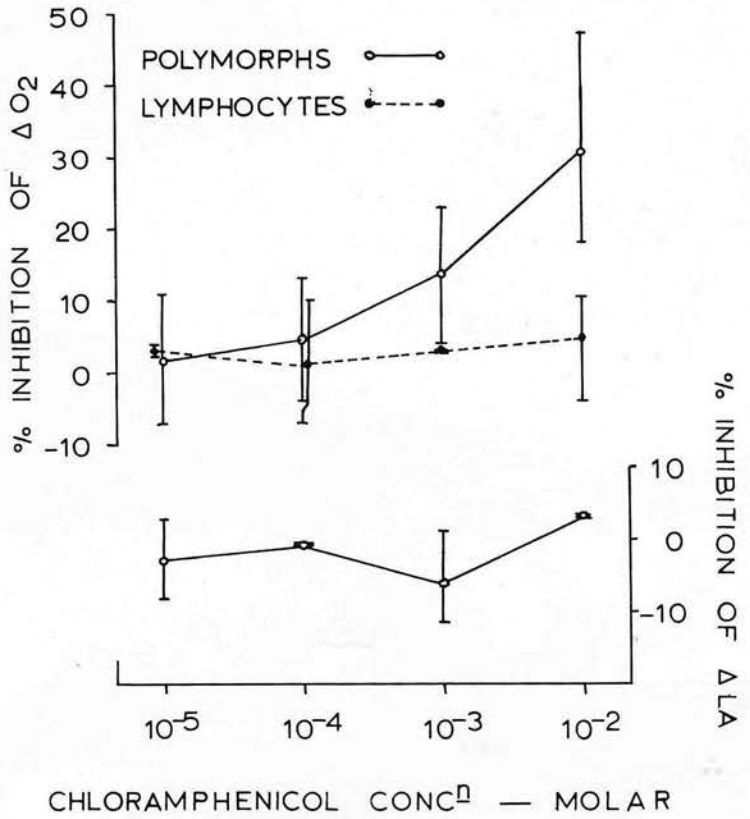


Figure 31.

Effect of chloramphenicol on leucocyte respiration (ΔO_2) and lactic acid production (ΔLA).

Table 47a EFFECT OF CHLORAMPHENICOL ON LEUCOCYTE OXYGEN UPTAKE

Chloramphenicol concentration Molar	Experiment no.	Polymorphs			Lymphocytes		
		No drug ΔO_2*	With drug ΔO_2*	%I +	No drug ΔO_2*	With drug ΔO_2*	%I +
1.0×10^{-2}	80	570	468	18	2710	2828	-4
	81	864	617	29	3480	3386	3
	82	893	534	34	2428	2171	11
	83	934	651	30	4035	3895	3
	84	1131	835	26	2987	2791	7
	85	949	500	47	2990	2726	9
			mean :	31		mean :	5
1.0×10^{-3}	105a	1202	1153	4	2492	2410	4
	105b	1438	1110	23	2989	2872	4
1.0×10^{-4}	106	1254	1091	13	2524	2280	10
	110	1484	1548	-4	3079	2738	-8
1.0×10^{-5}	108	1716	1530	11	2273	2184	4
	109	1490	1600	-7	2310	2240	3

* ΔO_2 expressed as μl/hour/10^{10} leucocytes

\+ %I = %inhibition

Table 47b EFFECT OF CHLORAMPHENICOL ON LEUCOCYTE LACTIC ACID PRODUCTION

Chloramphenicol concentration Molar	Experiment no.	Polymorphs		
		No drug ΔLA*	With drug ΔLA*	%I +
1.0×10^{-2}	110	1445	1393	4
	110	1445	1393	4
1.0×10^{-3}	110	1445	1417	2
	105b	893	995	-12
1.0×10^{-4}	106	1068	1069	0
	110	1445	1447	-1
1.0×10^{-5}	108	884	958	-8
	109	987	957	3

* ΔLA expressed as μM/hour/10^{10} leucocytes

\+ %I =%inhibition

Table 48a EFFECT OF TETRACYCLINE ON LEUCOCYTE OXYGEN UPTAKE

Tetracycline concentration Molar	Expt. no.	Polymorphs			Lymphocytes		
		No drug ΔO_2*	With drug ΔO_2*	%I +	No drug ΔO_2*	With drug ΔO_2*	%I +
1.0×10^{-3}	122	1613	1460	9	2777	2697	3
	123	1153	1403	-19	2822	2897	-3
	124	1457	1464	- 1	3375	3412	-1
	125	1454	1452	0	2778	2841	-2
1.0×10^{-4}	126a	1643	1605	2	1999	2062	-3
	126b	1643	1600	3	1999	1600	5

* ΔO_2 expressed as $\mu l/hour/10^{10}$ leucocytes

+ %I = % inhibition

Table 48b EFFECT OF TETRACYCLINE ON LEUCOCYTE LACTIC ACID PRODUCTION

Tetracycline concentration Molar	Expt. no.	Polymorphs		
		No drug ΔLA*	With drug ΔLA*	%I +
1.0×10^{-3}	122	948	887	6
	123	818	734	10
	124	931	912	2
	125	831	768	8
1.0×10^{-4}	126a	877	863	2
	126b	877	879	0

* ΔLA expressed as $\mu M/hour/10^{10}$ leucocytes

+ %I = % inhibition

Table 49a. EFFECT OF SULPHISOXAZOLE ON LEUCOCYTE OXYGEN UPTAKE

Sulphisoxazole concn. Molar.	Expt. no.	Polymorphs			Lymphocytes		
		No drug ΔO_2*	With drug ΔO_2*	%I +	No drug ΔO_2*	With drug ΔO_2*	%I +
1.0×10^{-2}	119	2072	2138	-3	3256	3312	-2
	120	1209	1165	4	3327	3307	1

* ΔO_2 expressed as $\mu l/hour/10^{10}$ leucocytes

+ %I = % inhibition.

Table 49b EFFECT OF SULPHISOXAZOLE ON LEUCOCYTE LACTIC ACID PRODUCTION

Sulphisoxazole concn. Molar	Expt. no	Polymorphs		
		No drug ΔLA*	With drug ΔLA*	%I +
1.0×10^{-2}	119	947	959	-1
	120	818	788	4

* ΔLA expressed as μM/hour/10^{10} leucocytes

\+ %I = % inhibition

Polymorph LA production was apparently unaffected by all of the drugs tested. It was mentioned previously (page 20) that leucocytes show a Pasteur effect, so it might be expected that a drug-induced inhibition of respiration would stimulate LA production. The fact that this was not observed suggests one of two possibilities:-

i) that the drugs inhibit PMN respiration in such a manner that the LA production is unaffected; this implies that no Pasteur effect is operating, or

ii) that the drugs exert a depressant action on both respiration and glycolysis, the depression of glycolysis not being observed due to the depressed respiration concurrently stimulating glycolysis, i.e. a Pasteur effect is operating.

SECTION 5. SUMMARY AND DISCUSSION

SUMMARY OF SECTIONS 1-4.

The purpose of this work as described in Section I was to investigate the actions of drugs known to induce agranulocytosis on the metabolism of human leucocytes. Some of the current theories as to the aetiology of agranulocytosis were discussed, and it was concluded that the underlying factors predisposing individuals to attacks of this dyscrasia were unknown. Since a number of conditions have been demonstrated to have hereditary connections it is possible that a similar process occurs in agranulocytosis. Thus a secondary investigation was to be a comparison of the effects of these drugs on normal leucocytes and on leucocytes obtained from drug-sensitive individuals and their siblings to determine whether any hereditary factor could be demonstrated with regard to the susceptibility of leucocytes to the "in vitro" actions of the drugs.

Section 2 dealt with the experimental procedure for the separation of lymphocytes and polymorphonuclear leucocytes (PMN) from whole blood and also for the measurement of the respiration of the separated cells. The bulk of the erythrocytes were removed by sedimentation with dextran, the resulting erythrocyte-poor leucocyte suspension being concentrated by centrifugation and resuspension of the leucocyte sediment in a smaller volume of the supernatant. The two leucocyte types were

separated by means of a column of small glass beads, the principle of this procedure being that in the presence of fresh plasma, and calcium and magnesium ions, PMN will adhere to siliconed glass surfaces while lymphocytes (and erythrocytes) will not. Thus, following addition of the leucocyte-rich suspension to the glass bead column and incubation at 37°C for 30 minutes, the PMN adhered to the beads; the lymphocytes could be recovered by washing the column with a 20% plasma - 80% Hanks solution. Subsequent washing of the column with a plasma-, calcium-, and magnesium-free solution containing 0.02% ethylene diamine tetraacetic acid allowed the PMN to be recovered.

The final yeild of leucocytes relative to the total initial number started with was not large. The dextran sedimentation of the erythrocytes led to a 38% loss of leucocytes, the differential count (%PMN : %lymphocytes) being altered slightly in favour of the PMN. On average about 30% of the PMN and 36% of the lymphocytes were recovered from the whole blood samples. The final lymphocyte suspension had a composition of 9 PMN : 91 lymphocytes : 890 erythrocytes, the corresponding figures for the PMN suspension being 93 : 7 : 24.

The oxygen uptake of the separated leucocytes was measured using a standard Warburg apparatus, it being necessary to use a minimum of 20 - 30 x 10^6 leucocytes per flask to produce

a measurable oxygen uptake over a two hour period. The incubation medium was 50% plasma : 50% Hanks solution with the phosphate buffer strength being increased to prevent the pH rising above its initial value of 7.4. The oxygen uptake was generally linear after the first thirty minutes, a somewhat higher rate being found over the 0 - 30 minute period. The erythrocyte contamination of the leucocyte suspensions was found to exert a negligible contribution to the oxygen uptake. The mean rate of oxygen uptake as determined over the 30 - 90 minute period (ΔO_2) was found to be

ΔO_2 PMN = about 1500 µl/hour/10^{10} PMN

ΔO_2 lymphocytes = about 2700 µl/hour/10^{10} lymphocytes.

These values for ΔO_2 were somewhat lower than those found by other workers, possible explanations being considered at the end of Section 2.

A crowding effect, where the ΔO_2 is depressed as the leucocyte concentration increases, was observed for both leucocyte types, it being significant at the 10% level for PMN, but not significant for lymphocytes.

Section 3 details the determination of the leucocyte lactic acid (LA) production. Chemical estimation was found to be unsatisfactory, an enzymatic method proving more accurate. For

reasons which were described it was not possible to estimate the lymphocyte LA production. The mean rate of LA production (ΔLA) for the PMN was found to be about 920 μM/hour/10^{10} PMN. A significant ($p < 0.01$) crowding effect was demonstrated.

In all experiments where the effects of the drugs on ΔO_2 and ΔLA were being determined, both 'no-drug' and 'with-drug' values were measured, the effect of the drug being expressed as the percentage inhibition (%I) = ('no-drug' $\Delta O_2/\Delta$LA - 'with-drug' $\Delta O_2/\Delta$LA) / 'no-drug' $\Delta O_2/\Delta$LA. This resulted in a number of 'no-drug' values for ΔO_2 and ΔLA from which the mean values given above were calculated.

The drugs investigated were chlorpromazine, amidopyrine, and thiouracil which all induce predominantly agranulocytosis, phenylbutazone which causes agranulocytosis and aplastic anaemia with equal frequency, and chloramphenical which induces mainly aplastic anaemia. Two drugs which are responsible for blood dyscrasias very infrequently - tetracycline and sulphisoxazole - were also used.

The results of these experiments are summarised in tables 50, 51, and 52, and in Figs. 32, 33, and 34. In all cases only the means of the two duplicate determinations at each drug concentration are shown. It is apparent that lymphocyte ΔO_2

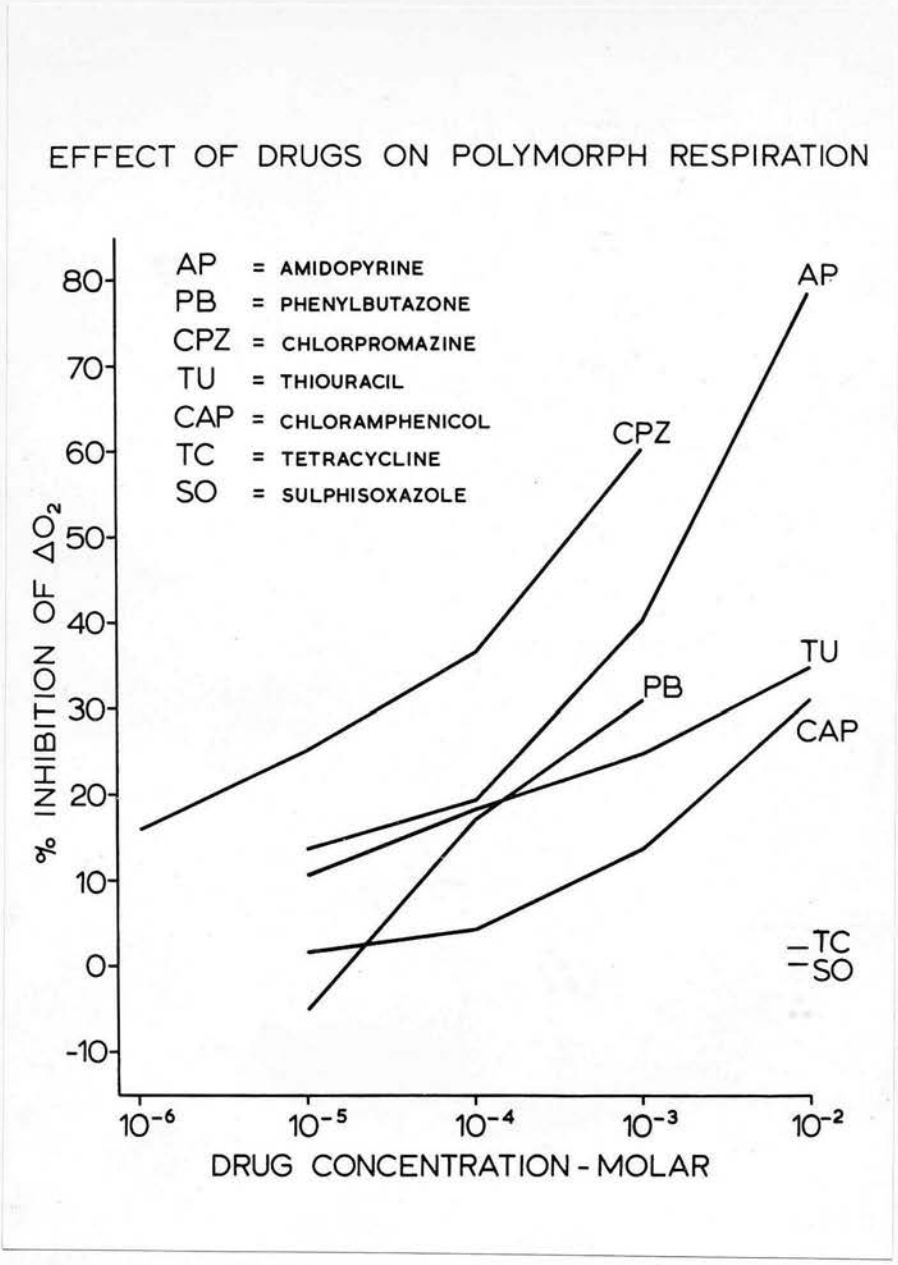

EFFECT OF DRUGS ON POLYMORPH RESPIRATION
AP = AMIDOPYRINE
PB = PHENYLBUTAZONE
CPZ = CHLORPROMAZINE
TU = THIOURACIL
CAP = CHLORAMPHENICOL
TC = TETRACYCLINE
SO = SULPHISOXAZOLE
% INHIBITION OF ΔO2
80
70
60
50
40
30
20
10
0
-10
AP
CPZ
TU
PB
CAP
TC
SO
10-6
10-5
10-4
10-3
10-2
DRUG CONCENTRATION - MOLAR

Figure 32.

Table 50. SUMMARY OF DRUG EFFECTS ON POLYMORPH RESPIRATION

Drug	1.0×10^{-2} M	1.0×10^{-3} M	1.0×10^{-4} M	1.0×10^{-5} M	1.0×10^{-6} M
Chlorpromazine	1S*	60.0	36.5	25.0	16.5
Amidopyrine	78.0	39.0	18.5	14.0	
Phenylbutazone	1S*	31.0	17.0	-5.0	
Thiouracil	35.0	25.0	18.5	11.0	
Chloramphenicol	31.0	13.5	4.5	2.0	
Tetracycline	1S*	2.5	2.5		
Sulphisoxazole	0.5				

* 1S = Insoluble

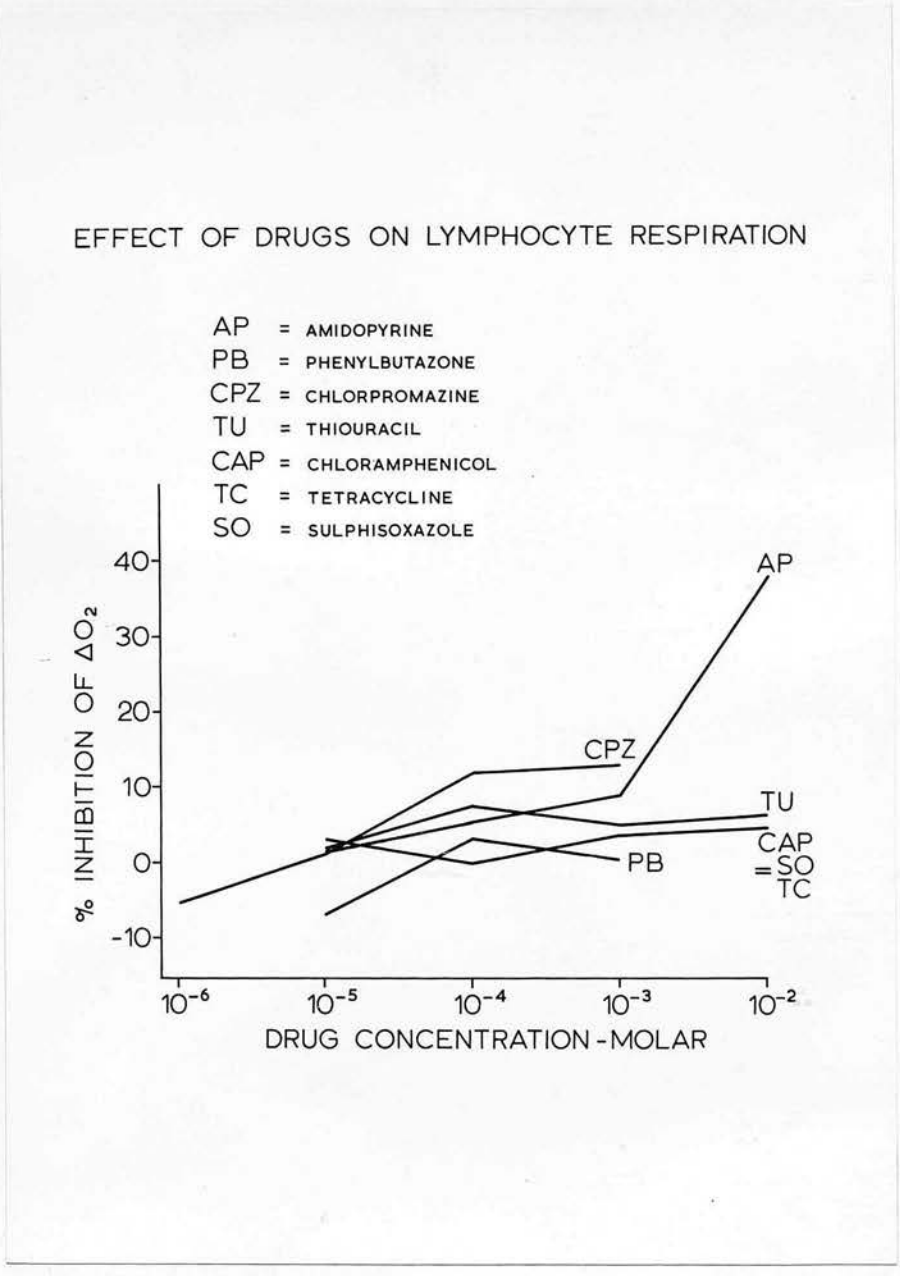

EFFECT OF DRUGS ON LYMPHOCYTE RESPIRATION
AP = AMIDOPYRINE
PB = PHENYLBUTAZONE
CPZ = CHLORPROMAZINE
TU = THIOURACIL
CAP = CHLORAMPHENICOL
TC = TETRACYCLINE
SO = SULPHISOXAZOLE
% INHIBITION OF ΔO2
40
30
20
10
0
-10
AP
CPZ
TU
CAP
=SO
TC
PB
10-6
10-5
10-4
10-3
10-2
DRUG CONCENTRATION - MOLAR

Figure 33.

Table 51. SUMMARY OF DRUG EFFECTS ON LYMPHOCYTE RESPIRATION

Drug	1.0×10^{-2} M	1.0×10^{-3} M	1.0×10^{-4} M	1.0×10^{-5} M	1.0×10^{-6} M
Chlorpromazine	1S*	13.5	5.0	1.5	-5.5
Amidopyrine	38.0	9.5	5.5	-1.5	
Phenylbutazone	1S*	1.0	3.5	-6.5	
Thiouracil	7.0	5.0	7.5	3.0	
Chloramphenicol	5.0	4.0	1.0	3.5	
Tetracycline	1S*	0.8	-1.0		
Sulphisoxazole	0.5				

* 1S = Insoluble

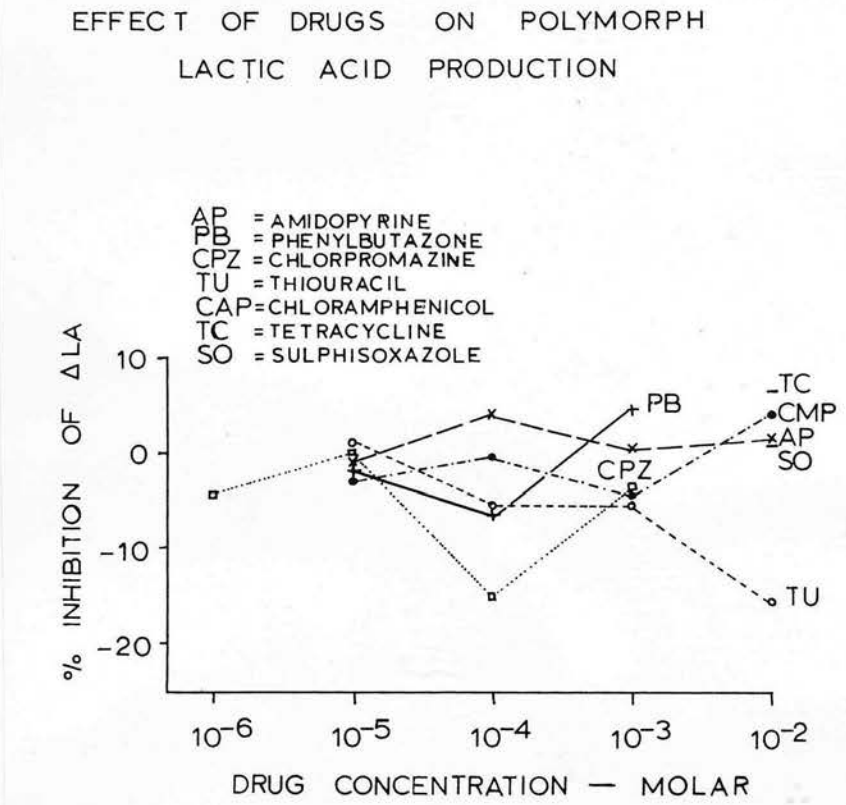

EFFECT OF DRUGS ON POLYMORPH
LACTIC ACID PRODUCTION
AP = AMIDOPYRINE
PB = PHENYLBUTAZONE
CPZ = CHLORPROMAZINE
TU = THIOURACIL
CAP = CHLORAMPHENICOL
TC = TETRACYCLINE
SO = SULPHISOXAZOLE
% INHIBITION OF ΔLA
10
0
-10
-20
PB
TC
CMP
AP
SO
CPZ
TU
10⁻⁶
10⁻⁵
10⁻⁴
10⁻³
10⁻²
DRUG CONCENTRATION — MOLAR

Figure 34.

Table 52. SUMMARY OF DRUG EFFECTS ON POLYMORPH LACTIC ACID PRODUCTION

Drug	1.0×10^{-2} M	1.0×10^{-3} M	1.0×10^{-4} M	1.0×10^{-5} M	1.0×10^{-6} M
Chlorpromazine	1S*	-4.5	-15.0	0.0	-4.5
Amidopyrine	1.5	1.0	4.0	0.0	
Phenylbutazone	1S*	4.5	- 7.0	-2.5	
Thiouracil	-15.0	-5.0	- 5.5	2.0	
Chloramphenicol	4.0	-5.0	- 0.5	-2.5	
Tetracycline	1S*	6.5	1.0		
Sulphisoxazole	1.5				

* 1S = Insoluble

and PMN ΔLA were unaffected by any of the drugs tested, while all the drugs which induce haematological disorders showed inhibitory tendencies to PMN ΔO_2.

DISCUSSION.

The literature indicates that the normal therapeutic plasma levels of these drugs are in the range 10^{-4} - 10^{-5} M. Thus it might appear that some of these drugs would have the potential to inhibit PMN respiration 'in vivo'. However, these plasma levels refer to the total amount of drug in the plasma and not to the amount of free drug present. The degree of plasma binding controls the amount of free drug present. The following tables show:

i) the inhibition of PMN respiration at the therapeutic plasma level assuming no plasma binding, and

ii) the inhibition of PMN respiration which might be expected to occur 'in vivo' taking into account the degree of plasma binding.

i) No plasma binding

Drug	Therapeutic plasma concentration Molar	%I of PMN respiration at this concentration
Chlorpromazine (CPZ)[9]	1.0×10^{-6} (1)	18
CPZ[9]	5.0×10^{-5} (1)	22
Amidopyrine (AP)	5.0×10^{-5} (2)	18
Phenylbutazone (PB)	2.4×10^{-5} (3)	22
Thiouracil (TU)	3.3×10^{-4} (4)	22
Chloramphenicol (CAP)	1.2×10^{-4} (5)	5

ii) With plasma binding

Drug	Degree of plasma binding - %	Therapeutic plasma concentration of free drug - Molar	% inhibition of PMN respiration at this concentration
CPZ(9)	very high, say 95. (6)	5.0×10^{-8}	-
CPZ(9)		2.5×10^{-6}	20
AP	15 (7)	4.2×10^{-5}	15
PB	95 (7)	1.2×10^{-6}	0
TU	0 (4)	3.3×10^{-4}	22
CAP	60 (8)	4.5×10^{-5}	0

(1) Huang and Kurland (1961)

(2) Brodie and Axelrod (1950)

(3) Burns et al (1953)

(4) Paschkis et al (1945)

(5) Glazko et al (1949)

(6) Salzman and Brodie (1956)

(7) Friend (1957)

(8) Goodman and Gilman (1965)

(9) The low dose is that administered for sedative purposes, the high dose being given to psychotic patients.

It will be observed that all the drugs which have a tendency to induce agranulocytosis, that is CPZ, AP, and TU all inhibit PMN respiration 'in vitro' at concentrations which would be expected to be present normally 'in vivo'.

If a comparison is made between these latter results and the incidence of agranulocytosis relative to aplastic anaemia induced by these drugs, the appropriate figures being shown in the table below, a correlation is apparent. Drugs which induce agranulocytosis but not aplastic anaemia, that is CPZ, AP, and TU, inhibit PMN respiration 'in vitro', while PB, which induces both these dyscrasias about equally, exerts a much lesser inhibitory effect on PMN respiration. The drug inducing predominantly aplastic anaemia, CAP, had no effect on PMN respiration. Drugs not generally inducing haematological disorders SO and TC, were also inactive as regards PMN respiration. These findings suggest that there may be some common factor between drugs which are liable to cause agranulocytosis.

Drug	Number of cases of the indicated dyscrasia due to the drug alone	
	Agranulocytosis	Aplastic anaemia
CPZ	83	3
AP	15	1
TU	23	0
PB	17	17
CAP	18	154

Figures from A.M.A. Registry on Adverse Reactions.

It was shown previously that the mechanism of agranulocytosis in AP-sensitive individuals is reasonably well understood though the reason why certain people are sensitive remains obscure. Although CPZ, AP, and TU all exhibit similar 'in vitro' effects, with variations in potency, they do not all seem to be identical with respect to their property of inducing agranulocytosis. For instance, although AP, when administered to a drug-sensitive patient as a single minute dose, produces a rapid and profound fall in the circulating PMN, CPZ under similar conditions must be administered as a number of quite large doses. A further difference is that although AP-induced agranulocytosis is associated with the presence of leukoagglutininis, which is suggestive of an immune response, there is only a single report of a CPZ-induced leukoagglutinin (Hoffman et al 1963), which suggests a direct toxic action for this drug. Alternatively, the methods used for the demonstration of leukoagglutinins may not be sufficiently sensitive, though this is improbable. In view of these differences in the manner in which agranulocytosis is apparently induced by these drugs, caution must be exercised when attempting to interpret the present results, particularly since the respiratory process involves a multitude of enzyme systems. Thus although CPZ, AP, and TU all show similar actions on PMN respiration, they may in fact all be acting in different ways.

One could list all the known actions of these drugs on specific enzymes in an endeavour to find common actions, but this approach is not likely to yield much information because of the wide variety of enzyme sources and methods used. For instance, TU inhibits cytochrome c of rat thyroid but not of rat liver and marrow (Paschkis et al 1945), while CPZ inhibits cytochrome c from rat liver mitochondria and inhibits the respiration of brain slices but not of kidney and liver slices Peruzzo and Forni (1953), PB does not act on rat liver mitochondria (Stenger 1956).

What is required, therefore, is a systematic study of the effects of these drugs on leucocyte metabolism, these studies being conducted using specific substrates and enzyme systems. Since this type of work would be carried out on cell-free systems, it would also be possible to determine whether the different results obtained in this work for the effects of the drugs on lymphocyte and PMN respiration were due to the two cell types having different respiratory systems, or to the drugs failing to penetrate the lymphocyte membrane. It may be that the drugs are acting on the membrane, by inhibiting transport mechanisms which are essential for PMN but not for lymphocytes. Since a method for separating marrow elements has recently been evolved

(Morrison 1967) similar useful work could be conducted using the immature cells of the myeloid and lymphoid series. Using unseparated human marrow it has been shown (Park, 1968) that AP exerts a similar inhibitory effect on the respiration to that found for peripheral leucocytes. The contamination of the marrow with peripheral leucocytes was considerable, so the apparent inhibition of respiration may be just a reflection of the action of the drug on the peripheral leucocytes.

It was found impossible to obtain any blood samples from drug-sensitive patients, so no investigations into the occurrence of any possible hereditary factors ~~was~~ were made. One supposedly normal female blood donor gave a remarkable response to tetracycline. As mentioned previously TC had no action on leucocyte respiration nor on PMN LA production in four experiments. On using this womans leucocytes, 1.0×10^{-3} M TC was found to cause complete inhibition of PMN respiration, PMN LA production and lymphocyte respiration being unaffected. According to her doctor she had no history of having taken any tetracycline drugs. On repeating this experiment on a fresh blood sample about six months later, and sacrificing duplicate determinations so that more drug concentrations could be used, ($1.0 \times 10^{-3}, ^{-4}, ^{-5}$ M) essentially identical results were obtained,

the PMN respiration being inhibited by 80% at 1.0×10^{-3}M but not at 1.0×10^{-4}M, the other two parameters being unaffected. Two requests to the woman's doctor for blood samples from her siblings produced no response. It is probable that these particular PMN were abnormally sensitive to TC; it would be a remarkable if both results were due to experimental error. Whether the donor had in fact taken tetracyclines at a much earlier date and had become sensitive to the drug, or whether her leucocytes had an abnormal respiratory system remains undetermined.

SUMMARY

This work was carried out in order to investigate the actions of drugs known to induce agranulocytosis on certain metabolic reactions of human leucocytes. Lymphocytes and polymorphonuclear leucocytes (PMN) were separated "in vitro" from samples of normal human blood, utilising the property of PMN whereby they will adhere to siliconed glass surfaces while lymphocytes do not adhere. The respiration and lactic acid production of the separated leucocytes was measured using manometric and enzymic methods respectively. It was found that drugs inducing predominately agranulocytosis - chlorpromazine, amidopyrine, and thiouracil - inhibited PMN respiration by 20% at concentrations which occur "in vivo" under normal therapeutic conditions. Drugs inducing both agranulocytosis and aplastic anaemia - phenylbutazone and chloramphenicol - showed little inhibitory activity on PMN respiration. No drugs exerted any apparent effect on lymphocyte respiration and on PMN lactic acid production. The significance of these results was discussed.

ACKNOWLEDGEMENTS

I should like to thank

Professor W. L. M. Perry for guidance and critical discussion in the course of this work.

Dr. R.A. Cumming and Dr. A.E. Robertson of the Royal Infirmary of Edinburgh Blood Transfusion Service for their generous supply of blood samples.

Miss Christine Cukrowski for the careful typing of the manuscript.

This work was carried out during the tenure of a Faculty of Medicine Post-Graduate Scholarship.

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