THE IN VIVO AND IN VITRO TRANSPORT OF OXYGEN AND CARBON DIOXIDE IN BLOOD

by

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ABSTRACT

The relationship between the concentration of 2,3 diphosphoglycerate (2,3 DPG) in the red cells and the oxygen tension of whole blood at an oxygen saturation of 50% (P_{50}) has been studied in the blood of patients with various acid base disturbances, but particularly, in chronic hypoxaemia and hypercapnia resulting from chronic bronchitis and emphysema. P_{50} was determined by the mixing technique with whole blood samples, and corrected to a pH of 7.4 by a directly measured Bohr effect. 2,3 diphosphoglycerate concentrations were determined by a modification of Krimsky's enzymatic assay.

Values for both $P_{50(7.4)}$ and 2,3 DPG varied widely in the bronchitic patients from low, normal to high values, as compared to values in normal subjects, in contradistinction to the results observed by others in patients with hypoxaemia which is not associated with CO_2 retention, where 2,3 DPG levels are described as consistently raised.

A significant positive correlation was established between 2,3 DPG concentrations and $P_{50(7.4)}$ ($r = 0.597$, $p =< 0.001$), but there was considerable variance about this regression relationship.

The role of factors known to affect this relationship was studied; these included whole blood pH (and PCO_2), intraerythrocytic pH (measured by the freeze-thaw technique), haematocrit, Mean

Corpuscular Haemoglobin Concentration, red cell mass, and inorganic phosphate concentrations. The concept of conflicting regulatory actions on 2,3 DPG concentrations was considered, and it was concluded that chronic arterial hypoxaemia might directly affect the P_{50}/2,3 DPG relationship in these patients.

The physiological role of any increase in 2,3 DPG concentrations in these patients appears to be trivial, in so far as facilitation of oxygen transport is concerned, and the reasons for this result are discussed.

CHAPTER I

INTRODUCTION

In 470 B.C. Empedocles, a Sicilian aristocrat, put forward the idea that 'Blood is the Life' and 'The Heart is the Seat of Understanding'.

William Harvey endorsed this idea stating that the blood is of prime importance and the heart the mere organ for its circulation. His monograph 'Motion of the Heart and Blood' in 1628 dispelled many spirits, humours and vapours!

Many have speculated upon the function of the lungs. Pre-aristotelians regarded the lungs as the inlet for vitalizing spirits, others regarded the lungs as a cooling system. It was only in 1669 that Richard Lower observed that venous blood turned red on exposure to air, and that this took place during passage through the lungs.

The Oxy-haemoglobin Dissociation Curve.

Gustav Magnus, in 1837, left no doubt that blood contained oxygen and carbon dioxide. He proved this experimentally using a mercurial blood pump, similar to the present day Van Slyke apparatus. Twenty years later, whilst studying the chemistry of haemoglobin, Felix Hoppe-Seyler established its ability to combine 'loosely' with oxygen. He found that oxygen could be displaced from haemoglobin by carbonmonoxide, an observation discovered independently by Claude Bernard. In 1872 Paul Bert (an assistant to Bernard) obtained

data for the first dissociation curve whereby he demonstrated both in vivo and in vitro the dissociation of oxygen from haemoglobin at low pressures. In 1886 Christian Bohr presented his thesis which contained the dissociation curve for purified haemoglobin (Hb.). He later took great pains to map out the curve point by point, characterizing the sigmoid shape, so disproving Hüfner's theory that the reaction was unimolecular. Barcroft, in 1907, launched a full attack on factors affecting the oxyhaemoglobin dissociation curve following the important discovery by Bohr, Hasselbalch, and Krogh in 1904 that addition of CO_2 to blood displaced oxygen. That CO_2 'may assist' in the liberation of oxygen from blood had occurred to Magnus 67 years previously but it was only experimentally shown in 1904, and is still known as the Bohr effect. Bohr attempted to prove that the relationship was reciprocal, i.e. that the state of oxygenation of the haemoglobin might affect CO_2 transport, but this was not proved until 10 years later, 2 years after his death. L.J. Henderson explains the delay in his book 'A Study in General Physiology', as due to the natural state of bewilderment which is the usual condition of the investigator! To quote:- 'So little are physiologists accustomed to mathematics, and such is the natural inertia of the mind, that it remained for Christiansen, Douglas and Haldane to discover by experiment that the CO_2 dissociation curves of oxygenated and reduced bloods are different.'

The fact that reduced haemoglobin always 'absorbed' a very much greater volume of CO_2 than oxygenated haemoglobin at the same CO_2

tension is known as the C.D.H. or Haldane effect. They also showed that the response varied with the degree of saturation. The fact that oxygen might drive out CO_2 was suggested by Ludwig, and taken up by Holmgren in 1865, but with little success. Werigo in 1892 concluded that oxygen expels CO_2, for when he distended one lung with oxygen and the other with hydrogen he found that the concentration of CO_2 was higher in the oxygenated lung by 2-3%. However, he did not verify these observations in vitro. Henderson stressed the usefulness of the mechanism whereby oxygenation of haemoglobin in the lungs assists CO_2 release, and oxygen delivery in the tissues assists CO_2 uptake in his book 'A Study in General Physiology' (1928).

Christiansen, Douglas and Haldane attempted to explain the increased affinity of CO_2 for reduced haemoglobin by the differences in H^+ ion concentration. Measurement of bound CO_2 in oxygenated and reduced haemoglobin allowed pH to be calculated indirectly from the Henderson-Hasselbalch equation, and the pH was constantly higher in the reduced sample. Direct pH measurements were not available in 1914.

Barcroft had initially great difficulty in obtaining a reproducible dissociation curve. He worked with haemoglobin solutions, but it was only when he added the salts of the red blood cells of the particular species whose haemoglobin he was working with, that he found he could reproduce curves identical to the curves using the whole blood of the species. The influence of temperature, salt and carbonic acid on the dissociation curve is

described best by Barcroft. He measured the 'capillary pressure-head', i.e. the pressure that maintains the diffusion current through the capillary wall by measuring the partial pressure of oxygen corresponding to 50% saturation (P_{50}) of dialyzed haemoglobin at 16°C, and found it to be 0.3 mm Hg! Addition of salts gave the curve the characteristic sigmoid shape enabling blood to give up oxygen at low pressures and combine with it at high pressures. Adjustment of temperature to 37°C increased the 'pressure-head' to 7.5 mm Hg, but addition of a PCO_2 of 40 mm Hg raised the final 'capillary pressure-head' to 25 mm Hg. Barcroft must have been looking to the future when he wrote, 'Indeed, in the blood of many persons the O_2 pressure corresponding to 50% saturation is as high as 30 mm Hg.' He concludes:- 'This marvellous thing has therefore happened as the result of temperature, salts and carbonic acid; oxygen diffuses into the tissues at approximately 100 times the speed.' (Barcroft, 1928).

The concept of a sigmoid curve was thus established, and was fully substantiated experimentally and theoretically by Douglas, Haldane and Haldane, 1912, and Adair, 1925. The physical variables affecting the dissociation curve were investigated at regular intervals for the next 35 years.

Much controversy arose over the mechanism of the Bohr effect. The reduced oxyhaemoglobin affinity with increasing CO_2 tensions was initially considered to be entirely due to the change in H^+ ion concentration, especially as Barcroft (1914) maintained that a

similar shift occurred with addition of acids other than CO_2. Colman and Longmuir (1963) and Astrup, Engel, Severinghaus and Munsen (1965) still maintain that the effect is due to a pH change. Margaria and Milla (1955) showed that a solution of horse haemoglobin without CO_2 exhibited a higher oxygen saturation value than that when CO_2 was present. Naeraa, Peterson, Boye and Severinghaus (1966) confirmed this observation in whole human blood, where they found a correlation between the oxygen saturation and CO_2 tension, at a fixed pH, providing the PO_2 was not greater than 90 mm Hg.

Dill and Forbes (1941) constructed dissociation curves from data of one person as did Bock, Field and Adair (1924), the blood belonging to A.V. Bock. They based their calculations for correction factors for changing temperature and pH on these results. It is this work that is referred to in the Handbook of Respiration, 1958, by Severinghaus, Bartels and Opitz. Since curves before 1930 involved calculation of pH rather than measurement, Astrup, Engel, Severinghaus and Munson (1965) reinvestigated the influence of temperature and pH on the oxyhaemoglobin curve of human blood, obtaining results on 52 subjects. They derived a standard curve for all the subjects at pH 7.4 and 38°C, and though it differed from that of A.V. Bock at the two extremes, the 'straight part of the curve' was remarkably similar.

With standardisation of temperature, ionic strength and pH, some authors still found individual variations in the position of

the curve. (Naeraa, 1964, Astrup, 1965). Part of the variation was in fact due to variable carbon monoxide concentrations. (Douglas, Haldane and Haldane, 1912).

Two, Three Diphosphoglycerate.

Physiological interest in the significance of alterations in the position of the curve diminished towards the start of the 1960's, whereas interest was increasing in the biochemical approach, following the elucidation of the molecular structure of haemoglobin by Perutz (1960, 1968). However, both biochemical and physiological research was intensified in 1967, when Benesch and Benesch, and Chanutin and Curnish independently discovered that certain intracellular organic phosphates such as ATP, but particularly 2,3 diphosphoglycerate (2,3 DPG) exerted a profound influence on the affinity of oxygen for haemoglobin.

The existence of 2,3 DPG in the erythrocytes of pigs was discovered by Greenwald in 1925. It was later shown to be present in the red blood cells of many animals with its equivalent, inositol hexose monophosphate being found in birds.

The enzymes responsible for its synthesis and decomposition were studied by Rapoport et al. in 1939-1942, the pathway being known as the Rapoport Luebering pathway. (Rapoport and Luebering, 1951, 1952).

In the red blood cell, energy is derived from the breakdown of glucose to lactate by the Embden Meyerhoff pathway. Lactate can also arise from the hexose monophosphate shunt, but under normal

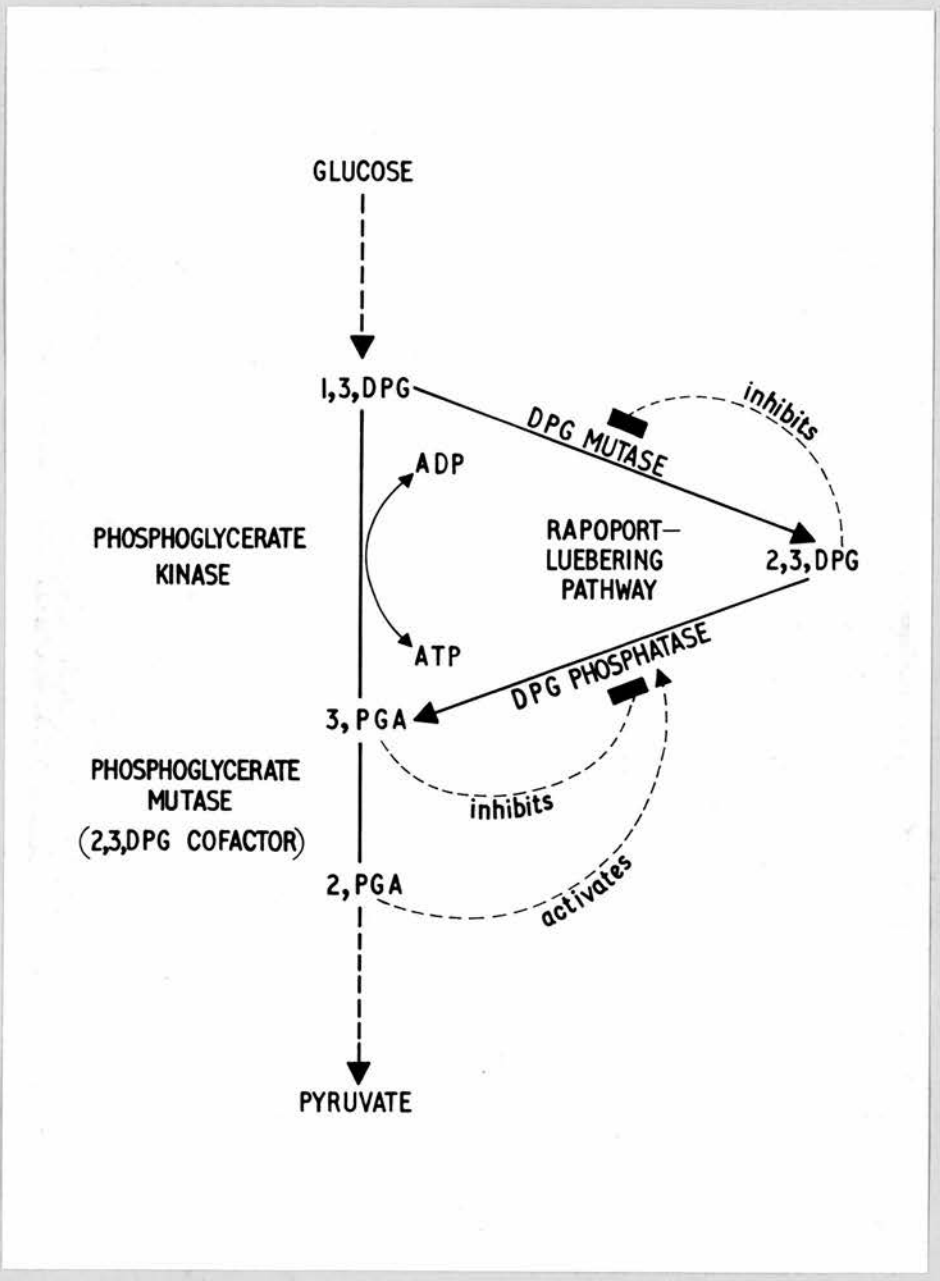


Figure 1.

The Rapoport-Luebering pathway together with the relevant intermediates of the main Embden-Meyerhof pathway.

conditions, this shunt is relatively unimportant. (Rapoport, 1968). At 37°C and pH 7.4, in one hour 0.5-2.5 mmoles glucose are consumed for each litre of cells, yielding 2 moles ATP, thus providing 90% of the energy required by the erythrocyte. Only 20-25% of the glycolytic intermediates enter the Rapoport-Luebering pathway, so that 0.6 mmoles 2,3 DPG are therefore formed/litre of cells/hour. As normal concentrations range from 4-6 mmoles/litre of cells, a relatively slow turnover will result, with a half-time of about seven hours. (Rapoport, 1968).

2,3 DPG is synthesised from 1,3 diphosphoglycerate and is catalyzed by diphosphoglycerate mutase. It is hydrolyzed to 3, phosphoglyceric acid (3 PGA), by diphosphoglycerate phosphatase, and thus back to the main pathway. (Fig. 1). 2,3 DPG is in turn a product inhibitor of the diphosphoglycerate mutase, so that the reaction is self-limiting. At the same time 2,3 DPG is a cofactor for phosphoglyceromutase, the enzyme that catalyzes the formation of 2, phosphoglyceric acid from 3, phosphoglyceric acid. 2,3 DPG is present in large concentrations in the erythrocyte, representing between 50 and 70% of all acid soluble organic phosphates. Its formation is irreversible, without production of energy, yet it is present in four times the concentration of ATP, and is in equimolar concentrations with haemoglobin. Apart from acting as a labile reserve of phosphate, no important function was attributed to it until 42 years after its discovery.

Benesch and Benesch, working with 'phosphate free' haemoglobin,

added increasing concentrations of 2,3 DPG in standard solutions, and found that the affinity of haemoglobin for oxygen decreased proportionally, without altering the shape of the curve. They determined the binding of DPG in dilute solutions of human haemoglobin in 0.1 M NaCl at pH 7.3 and found that DPG bound only to reduced haemoglobin, 1 mole DPG binding to 1 mole of deoxyhaemoglobin tetramer. Chanutin and Gurnish (1967) working with dilute haemoglobin solutions at low ionic strength at pH 6.5 and in the cold, demonstrated binding to mainly reduced haemoglobin, but also in small amounts to oxygenated haemoglobin.

Garby (1969) also found similar binding to oxyhaemoglobin but in very much smaller quantities. He worked with concentrated haemoglobin solutions at $4^{\circ}C$. According to Benesch and Benesch and Yu (1969) it is only in physiological salt concentrations that 1 mole of DPG will bind to 1 mole of reduced haemoglobin tetramer, and that at low salt concentrations, oxyhaemoglobin will begin to bind. DPG binding is relatively nonspecific. Very strong neutralised salt solutions such as NaCl, will negate the DPG binding, as will ATP or even pyrophosphates, if present in sufficient quantities. Similarly large concentrations of DPG will prevent other salts combining. DPG is present in such large quantities, with four negative charges at the physiological pH of the cell, approximately pH 7.3 that its effects are very much greater than NaCl. (Benesch and Benesch, 1969). Since DPG is a highly charged anion, it is bound to cationic groups on the haemoglobin protein.

An increase in pH will therefore decrease the affinity for DPG, and viceversa, a decrease in pH will favour binding. (Benesch, 1969, Garby and De Verdier, 1970).

The effect of 2,3 DPG in lowering the oxyhaemoglobin affinity decreases with increasing temperature. The binding of both 2,3 DPG and oxygen to haemoglobin are exothermic reactions. Since the binding of oxygen involves displacement of DPG, the temperature coefficient is lowered in the presence of DPG, so allowing unloading of oxygen over a wide range of temperature. Differences in pH, temperature and ionic strength has given rise to various association constants for reduced and oxyhaemoglobin and DPG.

The actual DPG binding sites on the haemoglobin molecule have not been entirely elucidated, though evidence has accumulated for three possible sites. Benesch and Benesch (1967) found that β rather than α chains bound DPG, and that it was preferentially bound to reduced haemoglobin. On deoxygenation, β chains move apart by 6 Angstroms (Muirhead, 1967) allowing entry of the DPG molecule to the central cavity, this binding to the inside of the β chains. On oxygenation, these chains move together and 2,3 DPG is expelled. An increase in 2,3 DPG will therefore resist the oxygenation of haemoglobin, so decreasing the overall oxygen affinity.

Site 1.

The main site is thought to be the 143rd aminoacid on the basically charged histidine residue on the β chain. (The

21st position in the H helix, B H21). (Garby, 1969). Evidence in favour of this site is found when comparing adult and foetal haemoglobin. Although equal concentrations of 2,3 DPG are present in foetal and adult red blood cells, the affinity of oxygen for foetal haemoglobin (HbF) is very much less than for adult haemoglobin (HbA). In the γ chain of HbF, histidine is replaced by an uncharged serine residue.

Site 2.

The N-terminal valine of the β chain has been suggested by Bunn and Briehl (1970). Blocking of this group prevents haemoglobin and DPG combining. Carbamate is formed at the uncharged amino group of N-terminal valine residues of each haemoglobin chain (Kilmartin, Rossi-Bernardi, 1962), whereas DPG is thought to bind to the charged amino group of the β chain only so that DPG and CO_2 binding will be competitive, the binding of DPG leading to a reduction in sites for CO_2. Normally, oxygenation of haemoglobin decreases carbonate affinity, CO_2 combining preferentially with reduced haemoglobin, but Bauer (1970), has shown that in the presence of a 6 mM solution of DPG, carbonate formation with oxyhaemoglobin is greater than with reduced haemoglobin, due to the antagonistic influence of DPG being stronger than oxygen.

Site 3.

Lysine- the 82nd amino acid on the β chain, evidence for which is only from stereochemical information.

The preferential binding of DPG to reduced haemoglobin allowed Benesch and Benesch to suggest the following sequence of events regulating DPG levels:-

Deoxygenation of haemoglobin results in a reduction of levels of 'free' DPG, due to increased binding, but as a result product inhibition of the diphosphoglycerate mutase is relieved, so that further DPG is synthesised. This response would occur in conditions of hypoxia, due to the increased ratio of reduced haemoglobin to oxygenated haemoglobin.

Factors Illustrating the Relationship Between DPG Levels and Hypoxia.

1. Asakura (1966) observed that the rate of red cell glycolysis and DPG synthesis was the same when haemoglobin combined with oxygen or CO, but increased significantly in haemoglobin incubated with nitrogen.
2. Oski (1970) observed a significant rise in DPG accompanying higher glucose consumption in anaerobic conditions, while ATP levels decreased. He found that if haemoglobin was absent, DPG synthesis was similar in incubation of CO or nitrogen, but when deoxygenated haemoglobin was added to the incubation system, 2,3 DPG only rose in the presence of nitrogen.
3. Further evidence for this regulatory mechanism came from high altitude studies, where the proportion of unsaturated haemoglobin is increased, due to the reduced barometric pressure. Barcroft (1910) carried out altitude studies in Teneriffe. He

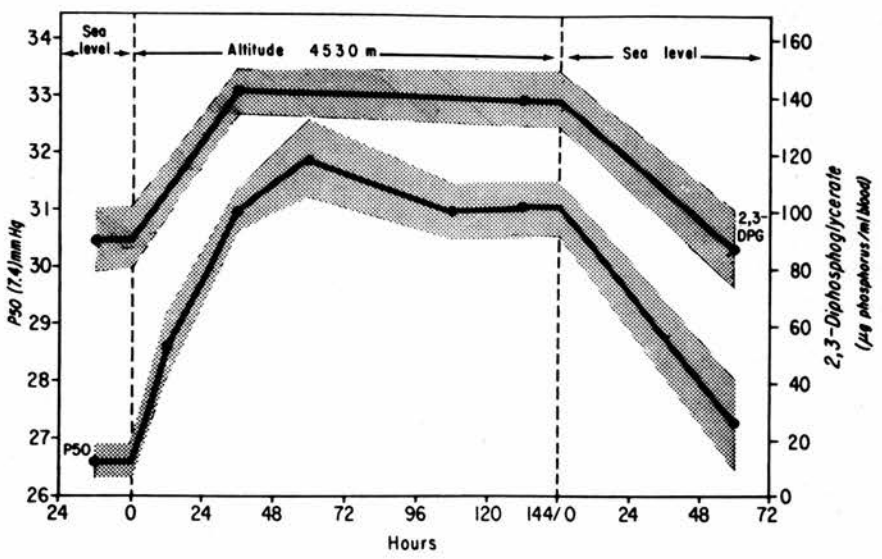


Figure 2.

Changes in $P_{50(7.4)}$ and 2,3 DPG of whole blood induced by high altitude. Parallel increases on exposure to high altitude and decreases on return to sea level were observed. (Lenfant, 1968).

found the dissociation curve shifted to the right, which he thought initially was due to increased lactic acid production. Improved methods of measurement of lactic acid proved that this was not the cause. The reduced affinity at altitude was verified by Aste Salazer and Hurtado (1944) but it was only in 1968 that Lenfant determined the relationship between oxygen dissociation and red cell organic phosphate concentration in subjects at sea level and altitude. (Fig.2).

Sea level natives increased both their P_{50} and DPG levels at altitude with a significant increase after 12 hours. The rise was complete after 36 hours, and was sustained for the 5 days. On return to sea level, it fell to normal levels. Natives living at high altitude had a high P_{50} and DPG level, which decreased to normal levels on descent to sea level. Circulating deoxygenated haemoglobin increased at altitude, stimulating production of DPG, whereas at sea level with the hypoxia removed, DPG levels remained normal.

4. In 1969 the effect of chronic hypoxia was reinvestigated by Lenfant in three groups of subjects; residents at altitude, patients with congenital heart disease, and patients with obstructive lung disease. (Fig.3).

The first two groups showed an adaptive shift in dissociation curve to the right, i.e. P_{50} was increased. There was a shift in dissociation curve in the third group to both the left and to the right. Lenfant correlated the shift with the haematocrit,

	P_{50} mm Hg	PaO_2 (Air) mm Hg	
Normal	26.8	90	Lenfant 1969
High Altitude			
(a) Residents	30.7	40	
HCT>70%	30.6	45	
HCT<70%			
(b) Sojourners	30.4		
Congenital Heart Disease	30.3	50	
Chronic Obstructive Lung Disease			
(a) Normal HCT	24.7	47	
(b) HCT>50%	29.2	48	
Cirrhosis	29.7	-	Mulhausen 1967
Cardiovascular (Coronary and Peripheral)	26.8	-	
Anaemia	30.7	-	

Figure 3.

Results of P_{50} and the corresponding PaO_2 level while breathing air in varying conditions of hypoxaemia. (Modified from Lenfant, 1969 and Mulhausen, 1967).

with a normal value correlating with the leftward shift and a high value correlating with the rightward shift, in these patients. Unfortunately, DPG levels were not measured.

5. Oski (1969) measured concentrations of 2,3 DPG in patients with congenital heart disease and found higher levels than in those patients with obstructive pulmonary disease although both were significantly higher than normal. Edwards (1968) had already found an adaptive increase in P_{50} in similar patients.
6. Woodson (1970) correlated increased P_{50} and DPG levels with cardiac disease in 39 patients. He found DPG levels and P_{50} values to be highly correlated with each other.
7. Anaemia, by lowering the oxygen capacity of the blood, reduces the supply of oxygen to the tissues despite increases in cardiac output. Decreased oxyhaemoglobin affinity has been found to correlate with the level of haemoglobin. (Rodman, 1960, Mulhausen, 1967, Edwards, 1968).

   Eaton and Brewer (1968) found a converse correlation between haemoglobin and DPG, and demonstrated that DPG levels increased with the severity of the anaemia. Torrance (1970), from the results of 171 subjects, found that for each gram of haemoglobin below normal, DPG rose by 0.23 mM, and P_{50} increased by 0.3 mm Hg.

   Gerlach, Dum and Deuticke (1969) demonstrated increased DPG levels in rats with anaemia, and decreased levels in rats with polycythemia.

8. Rats were made hypoxic by breathing mixtures of oxygen and nitrogen and significant increases of DPG were noted after 5 hours. Seven per cent oxygen, corresponding to an altitude of 7700 metres, was only tolerated for 24 hours, but a 60% rise in DPG was initiated. (Duhm and Gerlach, 1971).

The Effects of the Acidity of the Red Cell.

In 1924 Haldane correlated losses of inorganic phosphorus in urine with decreased organic phosphates in red blood cells, during NH_4Cl acidosis, an observation confirmed for diabetic acidosis in 1927 by Byrom. Ten years later, Rapoport identified the organic phosphate that decreased in red blood cells with acidosis to be 2,3 DPG. Rapoport and Guest (1939)[1] also observed that dogs with pyloric obstruction (with a plasma pH varying from 7.44 to 7.54), had an increased DPG level. They found a change of whole blood pH of 0.1 unit was followed by a 50% change in DPG in 24 hours.

A leftward shift of the dissociation curve in a patient in diabetic coma, and in a patient with uremia, was observed by Hasselbalch in 1917.

In 1969, Astrup, described by himself as 'an old medical acid-base chemist', confirmed Hasselbalch's findings, and demonstrated a low P_{50} and DPG level in 3 cases of metabolic acidosis. He also tried to confirm the previous reports of decreased oxygen affinity with anaemia, liver cirrhosis (Mulhausen, 1968), hypoxia due to congenital heart disease and obstructive pulmonary disease

(Edwards, 1968), hyperthyroidism (Snyder and Reddy, 1970), and high altitude studies (Lenfant, 1969). He found that the pattern of DPG and P_{50} was far less uniform than previously reported. He stressed that other factors, apart from haemoglobin concentrations and desaturation were of importance and although acid-base values were not measured in his study, Astrup stressed the importance of the pH-dependent change on 2,3 DPG concentration.

In this present study, the importance of the pH effect was shown when a patient in diabetic coma had an arterial pH of 6.75, a P_{50} of 15.36 mm Hg (normal levels 26-27) and an extremely low level of DPG. Five days later, when the pH had risen to 7.52, the P_{50} had risen to 28.5 and the DPG to normal levels.

Bellingham (1970) demonstrated low P_{50} and DPG levels in 6 patients with diabetic ketoacidosis, and stressed the danger of rapid correction of pH due to its contradictory role on oxyhaemoglobin affinity. pH affects the affinity of haemoglobin for oxygen directly and immediately via the Bohr effect, so that a fall in pH will decrease the affinity. The slower indirect effect through changes in DPG levels will act in the opposite way, a fall of pH decreasing DPG and thereby increasing oxyhaemoglobin affinity. These contradictory effects will tend to stabilize the P_{50} in vivo. Sudden correction of an acidosis will increase oxyhaemoglobin affinity initially, so impairing peripheral oxygen release, until DPG levels have time to increase sufficiently to counteract the change.

Both clinical and experimental metabolic acidosis and alkalosis have been shown to give rise to reduced and increased concentrations of 2,3 DPG respectively. In all cases, a rise in DPG correlated with whole blood pH. (Bellingham, 1971, Astrup, 1970, Rorth, 1970).

The influence of pH on DPG levels takes place through the enzymes involved in its synthesis and breakdown. (Hjelm and De-Verdier, 1970). Acidosis inhibits diphosphoglycerate mutase, and stimulates diphosphoglycerate phosphatase, whereas alkalosis stimulates the mutase and inhibits the phosphatase. It has also long been known that the rate of glycolysis is dependent on pH, increasing with alkalinity and decreasing with acidity, with a pH optimum of 8.1. (Chapman, 1962).

All glycolytic intermediates increase as pH rises due to stimulation of the rate-limiting enzymes, in particular, phosphofructokinase, the increase in 2,3 DPG being no exception. (Minakami and Yoshikawa, 1966). It must be remembered that increased pH will decrease the binding of DPG to cationic groups on the haemoglobin molecule, and therefore DPG levels may be affected by increased product inhibition of DPG to its own mutase.

The importance of the pH effect was stressed by Lenfant (1970) where acidosis was induced in 4 subjects before ascent to altitude by infusion of acetazolamide and oral NH_4Cl. The normal alkalosis due to hyperventilation was thus prevented as the pH did not rise above normal. The predicted changes in 2,3 DPG and P_{50} from altitude exposure was abolished, suggesting that the alkalosis

induced by hypoxia was the important stimulus. Similarly, Duhm and Gerlach (1971) abolished the rise in DPG levels in rats exposed to hypoxic mixtures by including 5% CO_2.

Anaemic patients with acidosis have low DPG levels, whereas anaemia without acid base disorders have immensely increased levels. Oski, Gottlieb, Miller and Delivoria-Papadopoulos (1970) demonstrated the importance of both hypoxia and pH in regulating DPG levels. Incubation of red blood cells in nitrogen and oxygen at pH 7.4, resulted in a rise of DPG in the deoxygenated sample. A further rise in DPG was demonstrated in both incubation systems when the pH was raised to 7.7, with the deoxygenated sample still retaining the higher levels.

As well as whole blood pH measurements, Astrup (1970)[2] measured the intraerythrocytic pH (pH_i) in his metabolic acidosis and alkalosis studies. He found that a change of 0.01 pH unit, either intraerythrocytic or in whole blood, gave a 4% increase in DPG, indicating that only a very small pH change influences DPG levels, and this led him to suggest that the mean red cell pH was a very important determinant of DPG levels.

Factors Determining Intraerythrocytic pH.

The four main factors affecting pH_i are:-

a) Degree of saturation of haemoglobin

b) CO_2 concentration

c) Dissociation of DPG

d) Extracellular pH.

All the factors must be considered as it will be the overall balance that will decide the pH_i.

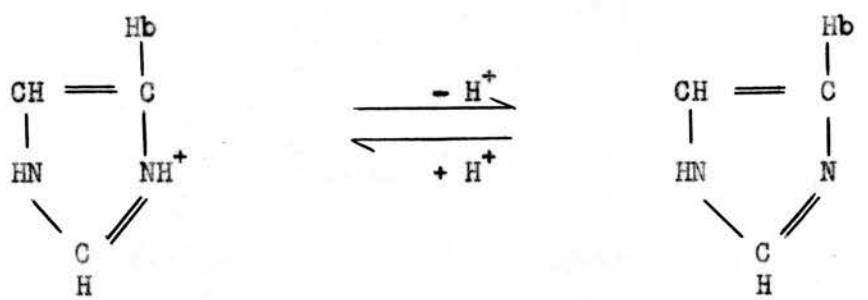
1. Degree of Saturation of Haemoglobin.

$$HHbO_2 \rightleftharpoons HbO_2^- + H^+$$

$$HHb \rightleftharpoons Hb^- + H^+$$

Reduced haemoglobin behaves as a weaker acid than oxygenated haemoglobin, therefore favouring H^+ acceptance by the reduced form.

By differential titration, Benesch and Benesch measured the change in H^+ when oxygenated haemoglobin was reduced. They found a pK of 7.93 for reduced haemoglobin and 6.68 for oxygenated haemoglobin.



reduced haemoglobin — Oxyhaemoglobin

Blockage of the nitrogen of the imidazole group as shown in Fig. above, prevented the release of H^+ (the Bohr proton) and the pK of the oxygenated haemoglobin increased to that of the reduced haemoglobin. An increase in circulating reduced haemoglobin will

raise the intraerythrocytic pH, so that the oxygenation/deoxygenation ratio, first supported as a direct regulatory mechanism for DPG, may also act indirectly via a pH change.

2. CO_2 Concentration.

a) Bicarbonate.

The well known reaction:-

$$CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons HCO_3^- + H^+$$

has a pK of 6.1 and takes place rapidly in the red blood cell due to the presence of carbonic anhydrase.

b) Carbaminohaemoglobin.

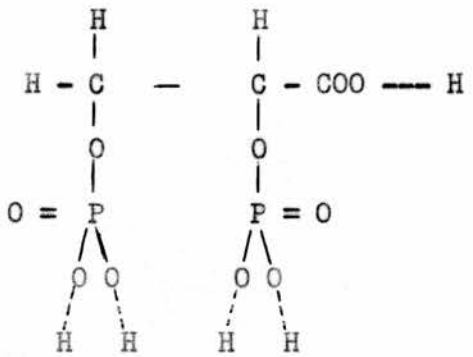
CO_2 combines with special amino groups of the deoxygenated haemoglobin, forming a carbamino compound.

$CO_2 + HbNH_2 \rightleftharpoons HbNHCOOH \rightleftharpoons HbNHCOO^- + H^+$. Carbamino haemoglobin is dissociated at body pH. However, it is a reversible reaction, so that if H^+ concentration rises, the carbamino reaction will be reversed and formation of 'carbamate' will decrease.

Although carbamino CO_2 only comprises 3.8% of the circulating CO_2 it accounts for 27% of the arterial-venous CO_2 difference. The H^+ produced by this reaction will depend on the concentrations of 2,3 DPG present, as DPG has been shown to compete with CO_2 for binding sites. (Bauer, 1970).

3. Dissociation of 2,3 DPG.

It was suggested by Garby (1971) that DPG exists in the cell in a dissociated form, with three or four of the five possible protons dissociated at the normal red cell pH.



2,3 Diphosphoglyceric acid.

This was confirmed by Siggaard Andersen (1971) who showed maximum buffering capacity of DPG at pH 7.1, indicating that this was the pK of some of the acid-base groups of the 2,3 DPG molecule.

Because H^+ and DPG^- are non-diffusable ions, there is an inequality of H^+ across the cell membrane, the degree of this inequality depending on the concentration of DPG. Verification of increased intraerythrocytic H^+ concentration with a rise in DPG levels came from experimental work on sheep by Battaglia (1970), He found that the organic phosphate concentrations, particularly DPG, were higher in the foetus than the lamb, and that the intracellular pH (pH_i) was correspondingly lower. This gives rise to an intraerythrocytic direct 'Bohr' regulatory mechanism, for

increased concentrations of 2,3 DPG will result in increased dissociation, the resultant rise in H^+ ion activity decreasing oxyhaemoglobin affinity. Bellingham, Detter and Lenfant (1971) attribute 35% of change in oxygen affinity to alteration of 2,3 DPG levels, in the range 9-19 μ moles/gm. haemoglobin, due to this direct H^+ effect. They induced acidosis and alkalosis in normal subjects and found a good correlation between DPG levels and the change in whole blood pH (pH_e) and intraerythrocytic pH (pH_i). The relationship pH_i to pH_e depended on the concentration of DPG.

Lenfant (1971) in his high altitude studies confirmed that at any pH, the pH_i is lower in samples with a greater concentration of DPG..

4. Extracellular pH (pH_e).

Astrup's studies in metabolic acid-base disturbances confirmed that DPG levels changed as pH changed, irrespective of whether pH_i or pH_e changed. Purcell (1961) found that changes in erythrocyte pH during clinical conditions of acute respiratory acidosis and alkalosis mirrored the extracellular pH change, indicating rapid diffusion of CO_2 across the cell membrane. This was confirmed by Battaglia (1965), and had been also found by Platts and Greaves (1957).

An observation of interest to this study was made by Platts and Greaves (1957) when they demonstrated that patients with chronic hypercapnia had a pH_i not significantly different from a control

group of similar age. Tushan (1970) verified these observations in 10 normal subjects and in 10 patients with chronic hypercapnia who were suffering from severe chronic obstructive lung disease. In the normal subjects pH_e was 7.42 and pH_i 7.12, not significantly different from pH_e of 7.38 and pH_i of 7.18 in the hypercapnic patients.

The reported increases in DPG level in obstructive pulmonary disease (Oski, 1969), along with a report of stable pH_i with hypercapnia (Tushan, 1970) is extremely relevant to this study, where chronic bronchitis and emphysema was accompanied in most patients by chronic hypercapnia.

The suggestion that the lowered pH_i regulated oxyhaemoglobin affinity at altitude (Lenfant, 1971) cannot be easily assumed to occur in the regulation of oxyhaemoglobin affinity in chronic lung disease, if the pH_i indeed remains constant in these patients, despite their high levels of arterial PCO_2.

5. Indirect Effect of pH via Mean Cell Corpuscular Volume (MCHC).

In addition to influencing oxyhaemoglobin affinity directly via the Bohr effect, and indirectly through 2,3 DPG levels, acidity affects the oxyhaemoglobin relationship in yet a third manner.

Bellingham (1971) found a delay of four hours before DPG levels changed sufficiently to affect oxyhaemoglobin affinity following experimental changes in pH_e. The oxyhaemoglobin affinity was, however, altered initially, and he correlated this change with the mean corpuscular haemoglobin concentration (MCHC). Onset of acidosis

decreased the MCHC, which returned to normal as acidosis continued. Alkalosis was accompanied by an increase in MCHC, which also later returned to normal. An increase in MCHC of 1.0 gm/100 mls. was accompanied by an increase of P_{50} (7.4) of about 0.5 mm Hg.

MCHC changes were found on exercise (Shappell, Murray, Bellingham, Woodson, Detter and Lenfant, 1971). After an eight week training period, DPG levels increased significantly yet P_{50} was unaltered. When the MCHC values were examined, a significant decrease was found, sufficient to counteract the DPG effect, so leaving P_{50} unaltered. Lenfant (1971) found MCHC changes both at sea level and at altitude and he correlated these changes with the acid-base status of blood.

6. Inorganic Phosphate Levels.

The effect of pH on oxyhaemoglobin dissociation is not always paramount. Astrup (1970)[1] demonstrated very high levels of DPG in a group of patients with uraemic acidosis, a result not expected from the pH values. Here the over-riding influence was high levels of inorganic serum phosphate.

It has been demonstrated in red cell glycolysis experiments, that the higher the inorganic phosphate concentration (Pi), the greater the rate of glycolysis, as a result of stimulation of phosphofructokinase and hexokinase activity. Between pH values of 7.0 and 8.0, all glycolytic intermediates, including 2,3 DPG have

been shown to increase. (Minakami and Yoshikawa, 1966).

P_i influx and efflux into the red cell are separate processes and are determined by differences in extracellular and intracellular concentrations, and by the permeability of the red cell membrane.

Large concentrations of P_i can be found in the plasma, with relatively small concentrations in the erythrocyte, due to rapid intracellular conversion to organic phosphates. Decreased organic phosphate concentrations have been correlated with phosphate deficiency (Lichtman, 1971), but smaller variations, within the normal range of inorganic phosphate, have not been shown to affect levels of DPG.

7. Relationship of 2,3 DPG with Other Glycolytic Intermediates.

Gerlach, Duhm and Deuticke (1969) demonstrated that dipyridamole (Persantin), in concentrations greater than 1×10^{-5}M, caused a diminished ADP level, and the greater the fall in ADP, the higher the rate of DPG synthesis. They experimentally excluded an effect on the mutase or phosphatase.

Assuming the phosphoglycerate kinase reaction is at equilibrium in the erythrocyte, then

$$1,3\ \text{DPG} \rightleftharpoons 3\ \text{PGA}$$

$$\text{ADP} \curvearrowright \text{ATP}$$

$$\text{therefore} \quad 1,3\ \text{DPG.}\ \text{ADP} = k.\ 3\text{PGA.}\ \text{ATP.}$$

$$\text{or} \quad \frac{1,3\ \text{DPG.}\ \text{ADP}}{3\ \text{PGA.}\ \text{ATP}} = k.$$

If 3 PGA and ATP levels remain constant, a decrease in ADP will be compensated by an increase in 1,3 DPG, resulting in increased synthesis of 2,3 DPG.

Hamasaki, Asakura and Minakami (1970) have suggested a possible role of DPG as a controlling factor in regulation of glycolysis under states of oxygenation and deoxygenation. Increased binding of DPG to reduced haemoglobin allows increased production of DPG and therefore a reduction in the phosphoglycerate kinase reaction. The ATP/ADP ratio will fall, and as ATP inhibits phosphofructokinase, a reduction in ATP will remove the inhibition, so stimulating glycolysis.

The interrelationship of DPG and glycolysis is demonstrable in clinical conditions with enzyme abnormalities. Hexokinase deficiency results in very low DPG levels with a correspondingly decreased P_{50}. Pyruvate kinase deficiency results in high DPG levels and a very much increased P_{50}. (Delivoria-Papadopoulas, 1969).

SUMMARY.

Although the regulation of DPG levels may be varied and complex, there is no doubt as to its importance. Providing pH is constant, and red cell metabolism is unimpaired, an increased level of DPG is a sure sign of an increased proportion of reduced haemoglobin in the circulation, and is therefore a sign of hypoxaemia. Two straightforward examples of a direct relationship between DPG and oxygen levels can be illustrated:-

a) Oski (1970) found that patients with cyanotic congenital heart disease, over 1 month of age, with an arterial PO_2 less than 60 mm Hg, had a consistently elevated red cell DPG level.

b) Woodson (1970) found a significant correlation between the mixed venous oxygen saturation and the increase in DPG levels in patients with non-congenital cardiac disease.

The concept of a mean intravascular PO_2 is important, for one might expect the slow turnover of DPG to respond to a mean value, rather than either the arterial or venous level alone. Hjelm (1969) suggests a DPG response to an increased 'time average concentration of deoxygenated haemoglobin'.

Some of the earlier studies demonstrated unpredictable DPG values, not significantly different from normal, yet these authors did not specify the acid-base status of the blood. Alkalosis will have an additive effect if present with hypoxia, whereas acidosis will

have a contrary effect. Both whole blood and intraerythrocytic pH will affect DPG levels, and the factors contributing to H^+ ion concentration have been discussed. The mean pH level will then affect DPG levels by:-

a) Influencing DPG and haemoglobin binding

b) Stimulation or inhibition of diphosphoglycerate mutase, and phosphatase

c) Overall glycolytic effect

d) Indirect effect via changes in mean cell haemoglobin concentrations.

Yet questions on the effects of acidity still remain. Why does the high altitude native have a relatively normal arterial pH, yet a compensatory rise in DPG? Following descent to sea level why does the DPG decrease to normal levels without an acidotic stimulus? Rorth (1970) in his in vitro studies, could not stimulate the rise in DPG by increasing the pH above 7.4, as seen in conditions of metabolic alkalosis in vivo. In most situations a rise in DPG is well correlated with shift in the oxyhaemoglobin dissociation curve, and Lenfant (1971) demonstrated clearly that where the DPG increase was prevented, there was no shift in P_{50}. Shappell (1970) demonstrated an increased DPG level after exercise training, with a constant P_{50}, explained by him to arise from the contradictory effect of MCHC changes on P_{50}. He has, as yet, not explained his findings in patients with Angina Pectoris, where he found an increased P_{50} in coronary sinus blood samples, yet with no change in DPG level,

and with constant arterial pH and MCHC.

P_{50} and DPG values therefore do not always correlate, many inter-dependent mechanisms influencing the relationship. The influence of three mechanisms on haemoglobin-DPG binding were illustrated clearly by Garby (1971)[1], maximum binding taking place at pH 7.0, with the smallest deoxygenated haemoglobin concentration.

It follows that all these factors discussed above must therefore be taken into account when studying the DPG/P_{50} relationship, i.e. mean intravascular PO_2, mean pH, MCHC, haemoglobin-concentration red cell mass, inorganic phosphate, and red cell enzyme deficiencies.

C H A P T E R II

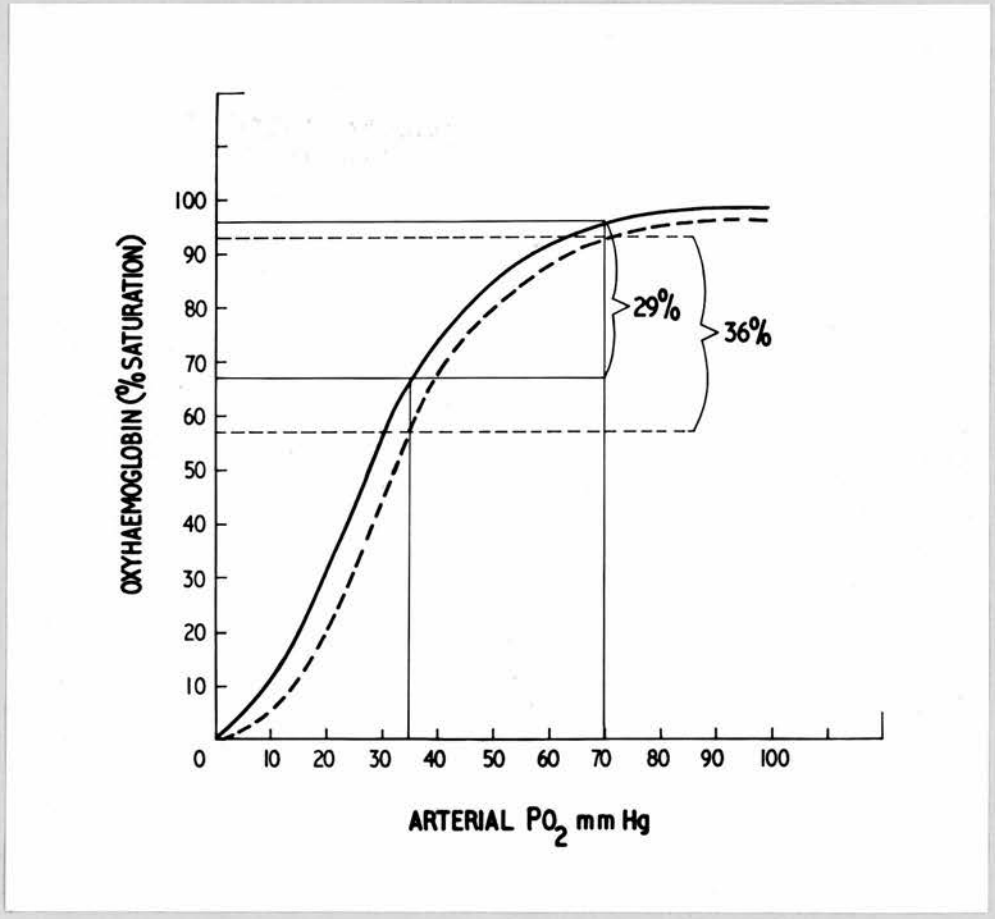


Figure 4.

An adaptive rightward shift of the oxygen dissociation curve in patients with hypoxaemia allows greater desaturation, and therefore greater oxygen release to the tissues.

THE AIM OF THE PRESENT STUDY.

In a number of conditions associated with hypoxaemia, DPG levels have been shown to increase, with a corresponding increase in P_{50}, i.e. there is a lowered oxygen affinity compensating for the oxygen deficit. Fig. 4 demonstrates the increased unloading of oxygen resulting from such an adaptive rightward shift.

Chronic hypoxaemia is a frequent complication of chronic bronchitis and emphysema, and it was hoped to correlate changes of P_{50} and 2,3 DPG levels in these patients in this study. Interest was first stimulated by the high altitude studies of Lenfant. The hypoxia of altitude is associated with a reduced arterial PCO_2 due to hyperventilation, whereas in chronic bronchitis, many patients develop ventilatory failure, with chronic elevation of arterial PCO_2. Could PCO_2 levels be related to any differences in 2,3 DPG levels and position of the oxygen dissociation curve? The extraordinary finding of Lenfant, whereby the oxygen dissociation curves of obstructive pulmonary disease correlated with haematocrit also stimulated interest. Many patients with chronic bronchitis and cor pulmonale develop increased haemoglobin concentrations and secondary polycythemia - an obvious adaptive mechanism. Could the degree of polycythemia be correlated with the position of the dissociation curve?

Increased haemoglobin levels have been shown to correlate with reduced DPG concentrations. Would the hypoxic stimulus of increased

circulating reduced haemoglobin counteract the contradictory increased haemoglobin concentration stimulus?

A small group of patients suffering from severe chronic hypoxaemia while breathing air are receiving long term oxygen therapy in the home. By measuring DPG levels and P_{50} in these patients, whilst breathing both air and oxygen, it was hoped to elucidate further the stimulus for changes in DPG and P_{50}.

The purpose of this study, therefore, initially was to answer the following questions:-

1. Are DPG levels raised in all patients with chronic obstructive lung disease?
2. Is there a corresponding shift in the dissociation curve and is there a strong correlation between DPG and P_{50}?
3. Is there a specific effect of chronic hypercapnia on DPG levels, in contrast to altitude studies where arterial PCO_2 levels are low?
4. Do abnormal levels of DPG alter the Bohr effect?
5. How important is the influence of haematocrit? Is it similar to Lenfant's findings, where a normal haematocrit is associated with an increased P_{50}, and a high haematocrit with a decreased P_{50}?

The question of intraerythrocytic pH control arose during the study and posed the following additional questions:-

6. Is the intraerythrocytic pH more stable in chronic hypercapnia, as suggested by Tushan (1970)?
7. Could all known factors discussed above, documented in the literature, explain the relationship between DPG concentrations and P_{50} which we found in this study in patients with clinical hypoxaemia, due to asthma, chronic bronchitis and emphysema?

C H A P T E R III

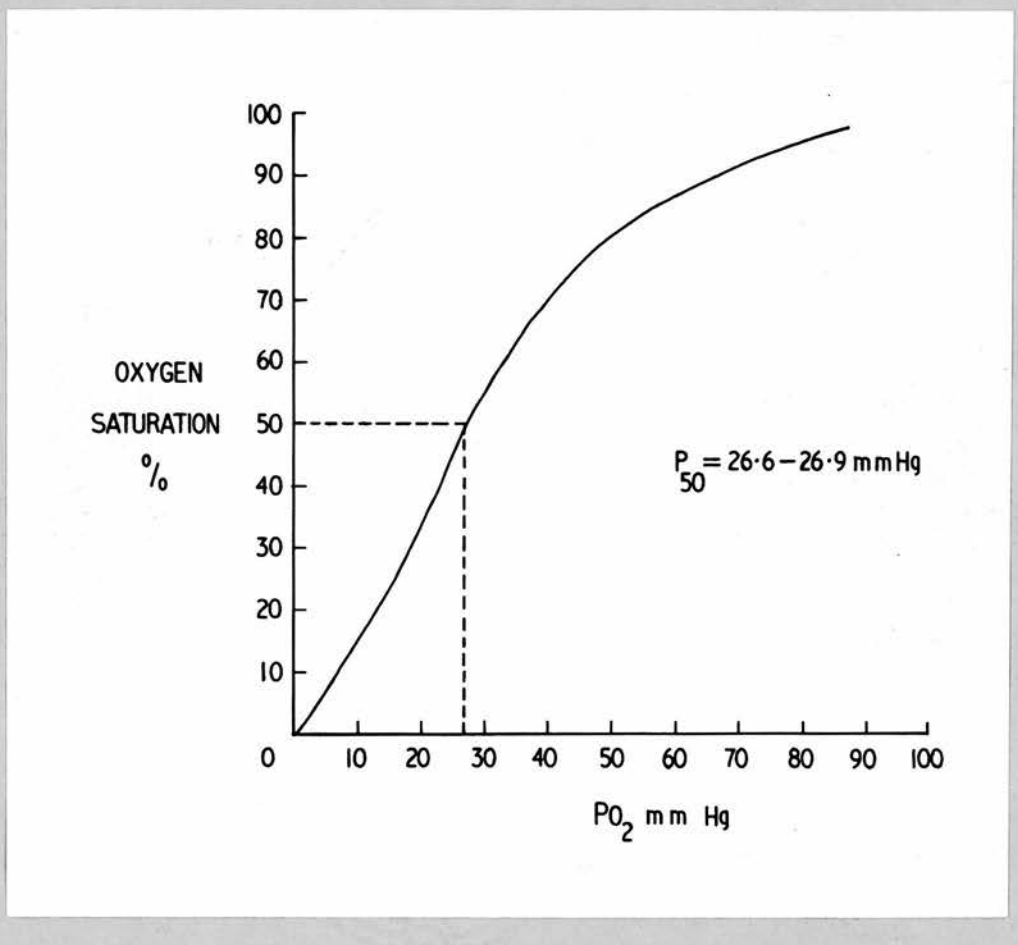


Figure 5.

P_{50} is the PO_2 at 50% saturation, when the pH is 7.40 and the temperature 37°C. Normal values by the 'mixing technique' range from 26.6 to 26.9 mm Hg.

METHOD OF P_{50} MEASUREMENT.

There are two basic methods for determining the oxygen dissociation curve of whole blood:-

(a) The technique whereby samples of fixed oxygen tension are prepared, and the percentage saturation calculated. (Bartels and Harms, 1959).

(b) The converse technique whereby samples of known saturation are prepared and the partial pressure of oxygen measured polarographically.

The main disadvantage of the first method, (a), is the length of time involved as equilibration of blood samples with several gas mixtures must be used with separate equilibration times for each. With method (b) a 'mixing' technique can be used, requiring only two gas mixtures, one to give a fully saturated sample, and the other to give a fully reduced sample. By taking varying proportions of such samples, at a fixed PCO_2, and measuring the oxygen tension, many points on the curve can be obtained. (Lenfant and Johansen, 1965).

The most frequently used point on the oxygen dissociation curve is the oxygen tension at 50% saturation (P_{50}), measured at 37°C and pH 7.4, i.e. the point where the ratio of fully reduced and fully oxygenated haemoglobin is equal. (Fig. 5).

The technique for measurement of P_{50} in this study was adapted from Edwards and Martin (1966) who developed the 'mixing' technique so that P_{50} could be determined with a single measurement.

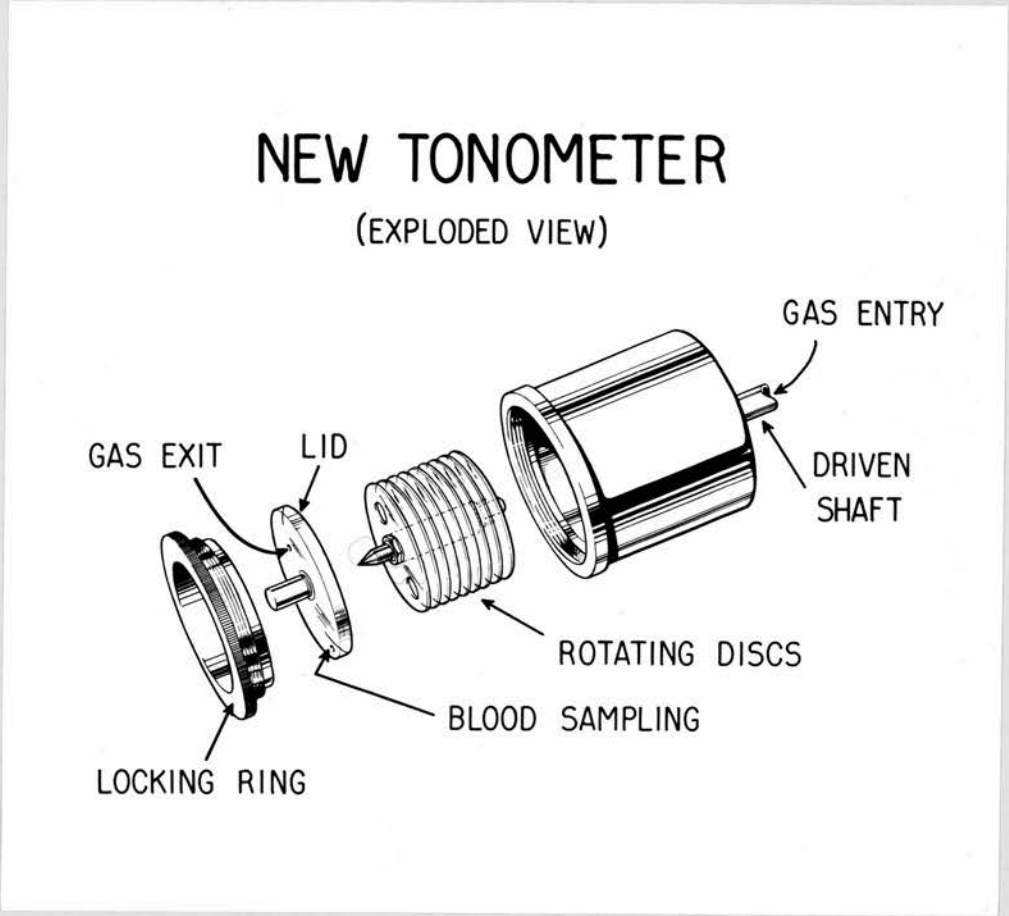


Figure 6.

The 'exploded' view of one of the two identical tonometers used in this study. It is placed horizontally in a thermostatted water bath.

Preparation of Blood Samples.

Fifteen mls. of heparinized venous blood were placed in two rotating disc tonometers (Flenley and Millar, 1967), situated side by side in a water bath maintained at 37°C. Each tonometer contains eight perspex discs, mounted on an axle. Humidified gas enters along the axle and escapes through apertures placed between each disc, so that on rotation, a film of blood is picked up by the discs and evenly equilibrated with the gas. (Fig. 6).

Gas Mixtures.

A mixture of nitrogen and carbon dioxide, and carbon dioxide and air, was required for the reduced and oxygenated samples respectively. Gas mixtures from large cylinders of CO_2 and 'white spot' nitrogen (oxygen-free grade, purity 99.9%) were passed along copper tubing to Wösthoff gas mixing pumps. Two overflow vessels and two safety traps were included in the circuit so that a slow continual overflow ensured a sufficient flow rate to each gas mixing pump. (Fig. 7a).

For the fully saturated sample, a Wösthoff SA 27/3F gas mixing pump was employed. This produces mixing ratios from 1.01 to 1.99 in 12 steps. An accurate outflow of 1% CO_2 and 99% air was humidified and led to Tonometer I via a water trap.

For the fully desaturated sample, the SA 18/2F pump was used, giving mixing ratios from 1.1 to 1.9 in 9 steps. The gears were adjusted to give an accurate outflow of 90% nitrogen and 10% CO_2. This was humidified and led to Tonometer II via a water trap. A

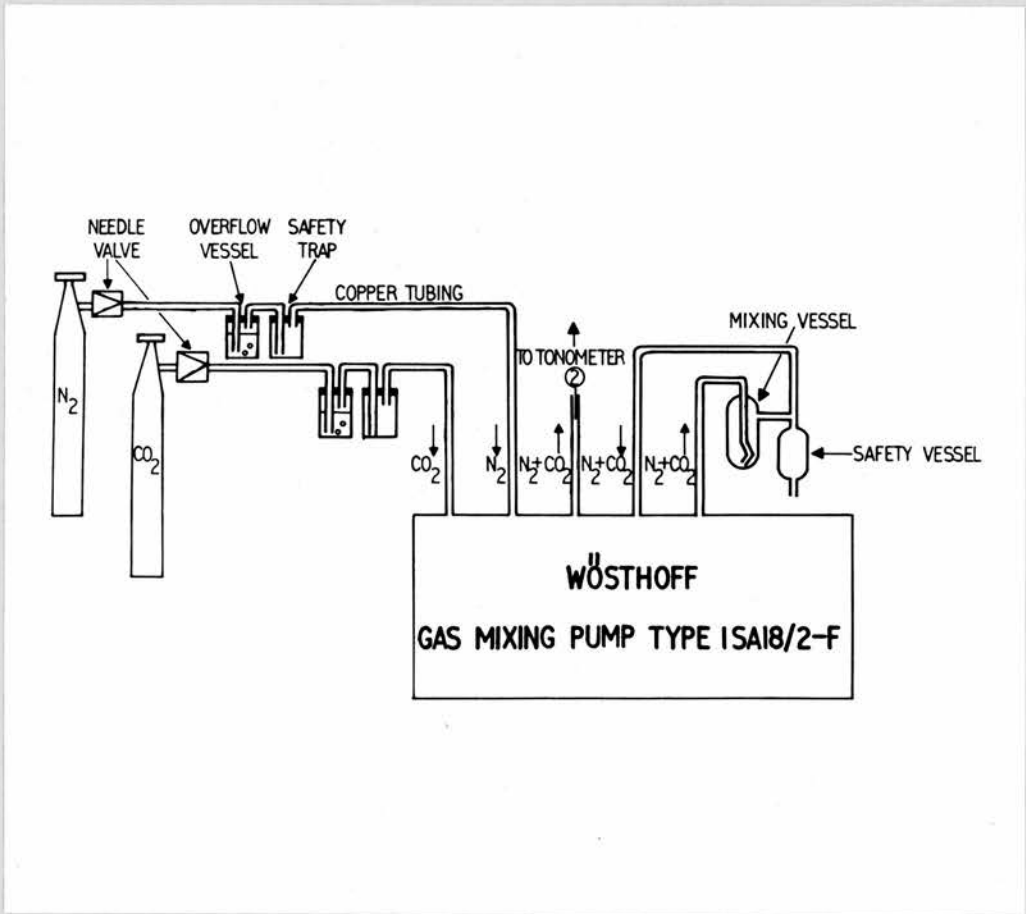


Figure 7a.

System of gas tranport whereby two pure gases are accurately mixed in a Wösthoff gas mixing pump. (H. Wösthoff o.H.G., Bochum, Germany).

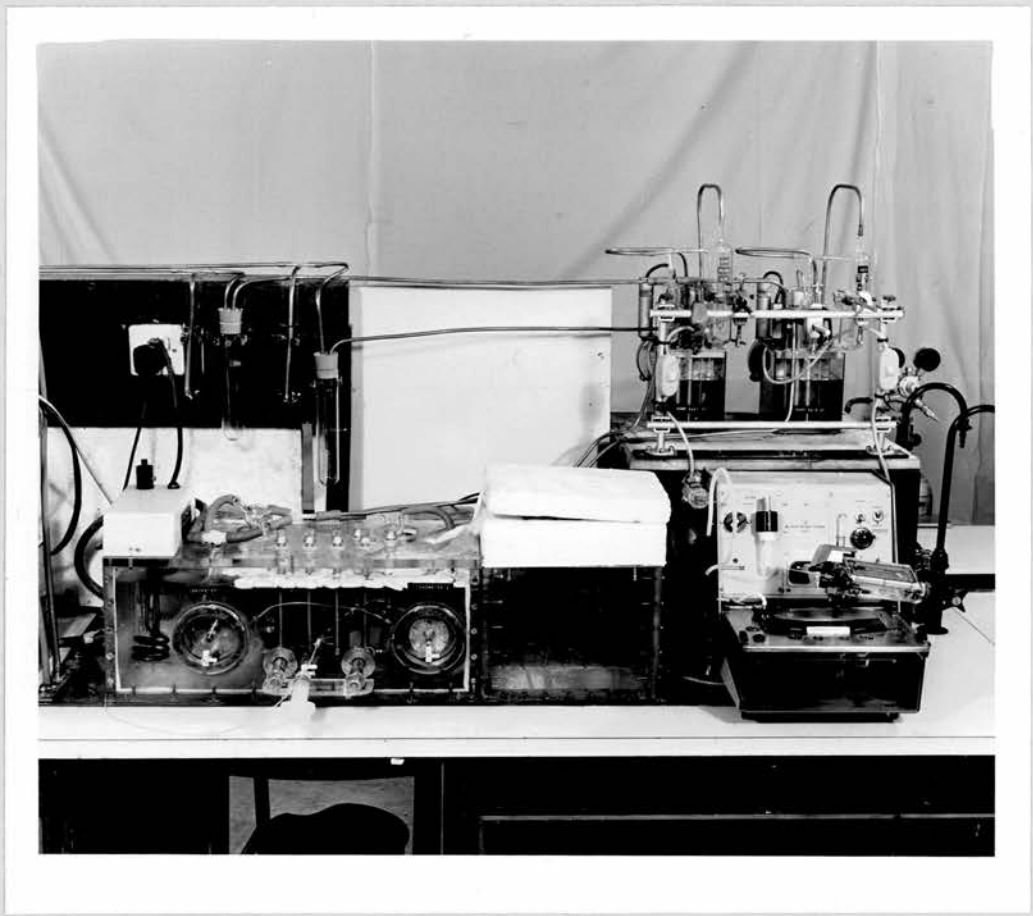


Figure 7b.

The two Wösthoff gas mixing pumps are placed in the upper right hand corner of the photograph. Gas outflow leads to two tonometers situated side by side in the main water bath.

photograph of this equipment is shown in Fig. 7b.

For complete reduction, one hour of tonometry was required, at the end of which the following values were normally obtained at the standard barometric pressure minus water vapour pressure.

Tonometer I	Tonometer II
PO_2 = 130-140	PO_2 = 0-4
PCO_2 = 7-9	PCO_2 = 70-80

P_{50} Measurement.

Equal quantities from fully reduced and oxygenated blood theoretically result in 50% saturation, therefore 2 mls. from Tonometer I and 2 mls. from Tonometer II were anaerobically drawn into a specially designed syringe, and mixed. To reduce the dead space to a minimum, a gas tight Hamilton syringe with needle (Model. No. 1010N) was used. The barrel was 'inset' so that a stainless steel mixing washer was flush with the top of the barrel, thus avoiding dead space at the end of the syringe. The needle (volume approximately 0.05 ml.) was filled with heparin before sampling.

Adjustable back-stops allowed withdrawal of the barrel to two fixed positions. For the P_{50} measurement, the 2 ml. and 4 ml. marks were calibrated accurately, after which they remained fixed. (Fig. 8).

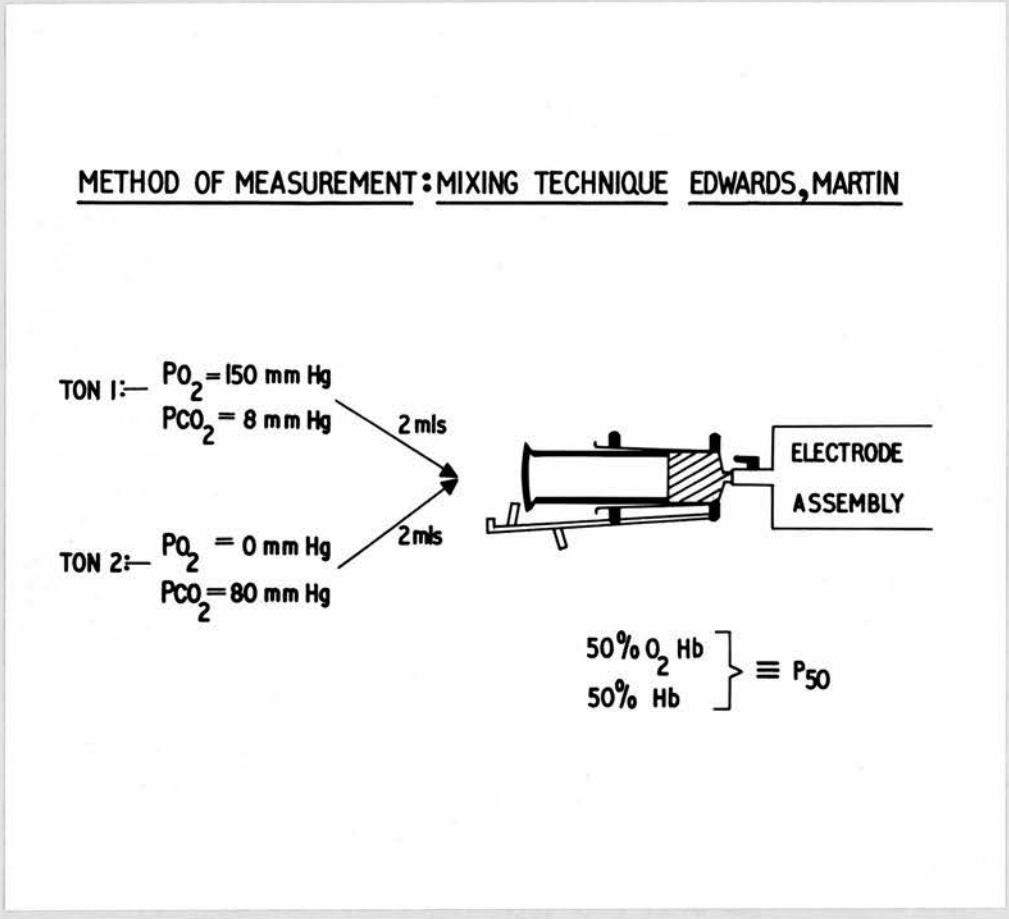


Figure 8.

The specially calibrated Hamilton syringe allows mixing of equal volumes of fully saturated and fully reduced blood.

Accuracy of P_{50} Mixture.

An attempt to verify the accuracy of the 50% saturation mixture was made using radioactive sodium chromate (Cr^{51}) to label red cells.

Tonometer I contained sodium chromate labelled blood, Tonometer II normal unlabelled blood. The count rate of the P_{50} sample should equal the count rate for

$$\frac{\text{Ton. I} + \text{Ton. II}}{2}$$

the arithmetic mean of the count rates from samples from the two Tonometers. However, the background count and the reproducibility were too variable for such accuracy. Instead samples from Tonometer I, Tonometer II and P_{50} were taken for manometric analysis of oxygen and carbon dioxide content. (Van Slyke and Neill, 1924).

A new method of measuring oxygen content, adapted from Laver (1965) was correlated with Van Slyke measurements. This involved mixing a small sample of blood anaerobically with ferricyanide solution, and measuring the increase in oxygen tension polarographically. The two methods correlated well, with $r = 0.991$, ($n = 39$), but when a similar technique was employed for CO_2 content, using lactic acid, the results were not good. As both oxygen and CO_2 content were required, the Van Slyke method was adopted. Much experience (essential for any gasometric technique), was gained while comparing the two methods for oxygen and CO_2 content measurements, and the very small error of $\pm 1.5\%$ in Van Slyke analysis considered acceptable.

Measurement of oxygen carrying capacity was made by spectrophotometric determination of the oxyhaemoglobin content of the blood (King and Wootton, 1959), after which a correction factor, determined by comparison with the Van Slyke manometric technique, was applied. (Staunton, 1966). ~~(Fig.)~~. Measurements were made in triplicate at 540 m using the Unicam Instruments Ltd. SP 600, and together with the oxygen content results, enabled the percentage oxygen saturation of each sample to be measured. This verified the complete reduction of samples in Tonometer I, the complete oxygenation of Tonometer II and the 50% saturation of the P_{50} sample.

Electrode System.

Having investigated the reliability of a system in obtaining samples of fixed and reproducible saturation levels it was necessary to ensure that PO_2, PCO_2 and pH measurements were equally dependable.

The electrodes were manufactured by the Radiometer Company, the PO_2 electrode type E5044, PCO_2 electrode type E5036, and micro pH electrode type BMS1.

The electrodes were set up in the normal way and maintained in good working order with the help of 'What the instructions didn't say', a small manual on blood gas electrodes by John W. Severinghaus and A. Freeman Bradley (1969).

White spot nitrogen and air were used as reference points when setting up the oxygen electrode. Ten alternate measurements giving a mean and SD of 0.44 ± 0.13 mm Hg for nitrogen, and 147·34 ± 0.467 mm Hg for air.

The PCO_2 electrode was set up with 3% and 11% CO_2 gas mixtures, the actual concentration being determined by the Lloyd Haldane apparatus, duplicate analysis of samples agreeing to within 0.03%. After 3 minutes, the electrode readings on gas mixtures were stable, eight alternate measurements giving PCO_2 25.78 ± 0.71 mm Hg and 76.45 ± 2.44 mm Hg respectively.

The pH electrode was calibrated with two Radiometer standard buffer solutions (pH 6.840 and pH 7.381). Ten alternate measurements of each gave a mean and standard deviation of 6.845 ± 0.001 and 7.384 ± 0.004 respectively. A potassium chloride valve established a new liquid junction for every 30μl sample of blood drawn into the capillary, so improving accuracy.

In this study, the sensitivity and stability of the signals from the PO_2, PCO_2 and pH electrode was increased by using specially designed preamplifier circuits within a fully automated print-out system, most of the equipment being designed and built in The Department of Medicine, University of Edinburgh.

Processing of oxygen, pH, and CO_2 electrode signals took place in three preamplifiers mounted in a watertight copper box, within the same water bath as the PO_2 and PCO_2 electrodes, and the two Tonometers, and was therefore maintained at 37°C. This arrangement reduced the problem of temperature drift and provided electrical screening for the sensitive preamplifiers.

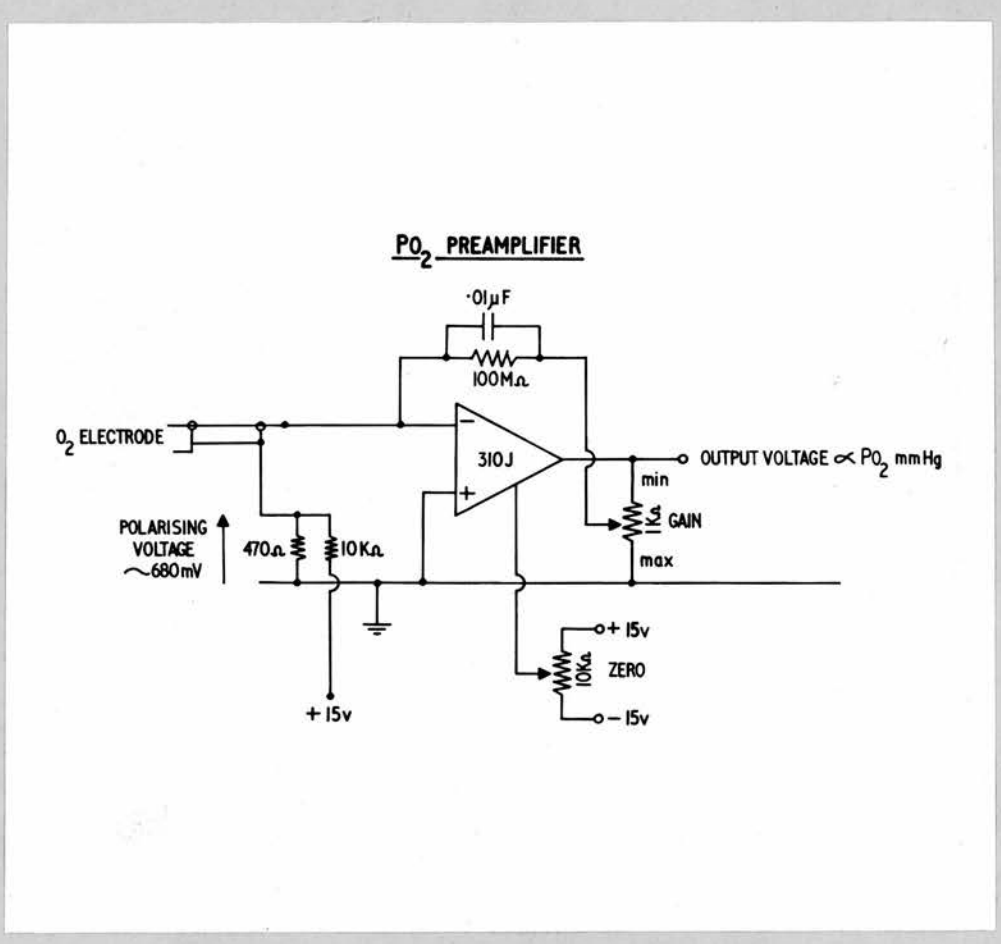


Figure 9.

Circuit used to measure PO_2 output voltage. (Electrometer amplifier, No.310J, manufactured by Analog Devices).

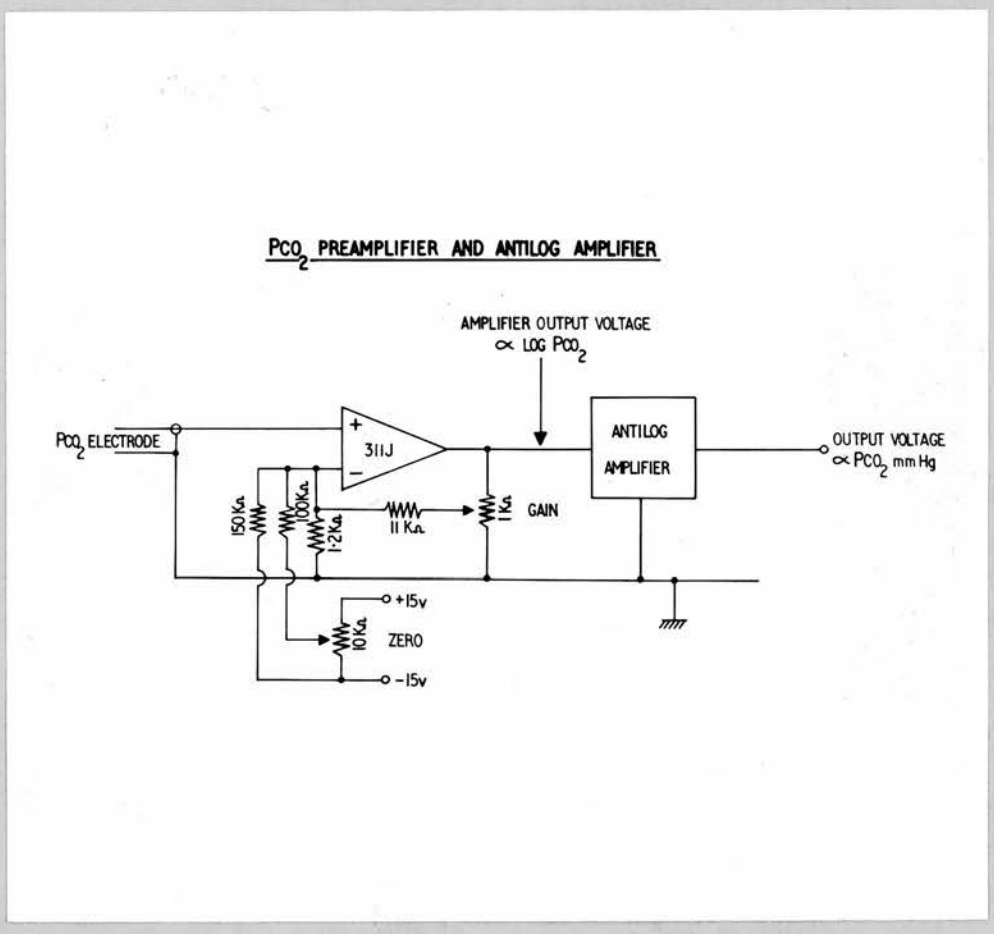


Figure 10.

Circuit used to measure PCO_2 output voltage. (Electrometer amplifier, No.311J, manufactured by Analog Devices).

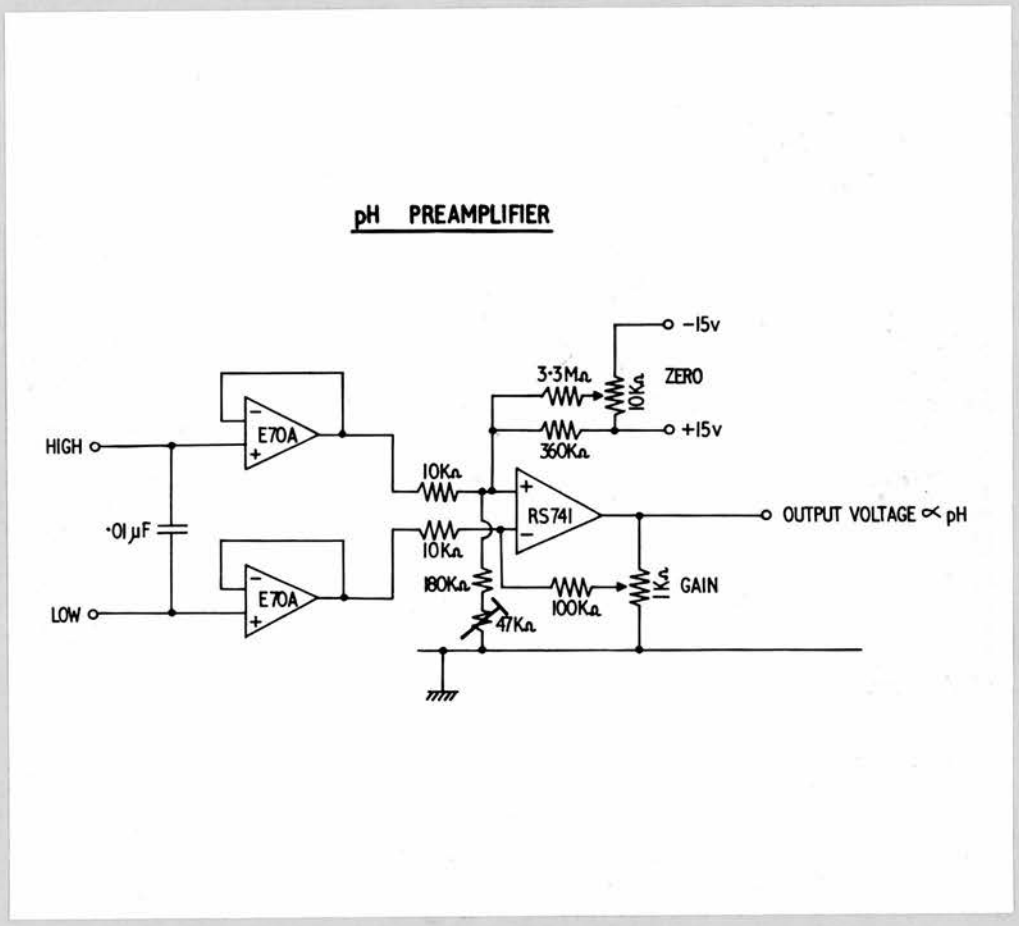


Figure 11.

Circuit used to measure pH output voltage. (Amplifier No. E70A manufactured by Computing Techniques and No.741, by R.S. Components).

PO_2 Preamplifier.

With the polarising voltage on the oxygen electrode maintained at a constant value in the range 600 to 700 mv, the current through the electrode is directly proportional to the PO_2 of the liquid in contact with the membrane. Fig. shows the circuit used to measure the elctrode current while maintaining a constant polarising voltage.

PCO_2 Preamplifier.

The PCO_2 preamplifier (Fig. 10) has a very high input impedance ($\simeq 10^{14}\Omega$) to enable it to measure the true voltage of the high impedance PCO_2 electrode. The output voltage of the preamplifier is proportional to log PCO_2 and therefore to obtain a linear output, this voltage is fed into an 'antilog' amplifier, the output of which is then proportional to PCO_2.

pH Preamplifier.

The Radiometer microelectrode is contained in a separate unit and therefore requires a differential input amplifier to reduce the effects of 'hum pick-up' from its own water bath. The circuit is shown in Fig. 11.

Readout System.

A block diagram of the overall system is shown in Fig. 12a and a photograph in Fig. 12b. The signals from the three preamplifiers are fed into the data logger, which, on command, measures each input

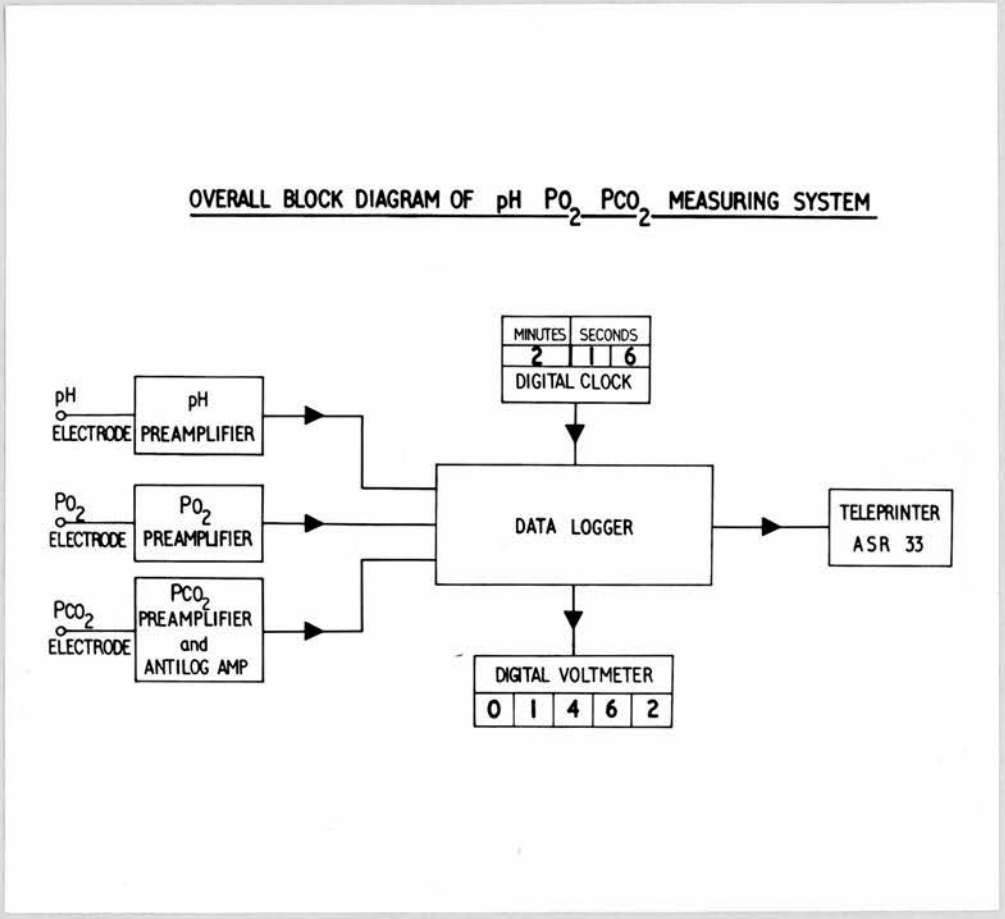


Figure 12a.

Block diagram of complete system. The electrode signals (left of diagram) are displayed on the digital voltmeter and automatically recorded by the teleprinter.

Figure 12b.

Photograph of all the equipment, including the automatic print-out system.

in turn on the digital voltmeter and prints out these values on the teletype along with the time from the digital clock.

During one sampling procedure, an automatic printout occurs at one minute and a half, 2 minutes, two minutes and a half, and 3 minutes, and can be initiated manually at any time by pressing the 'PRINT' button. When the preamplifiers are correctly adjusted the digital voltmeter reads PCO_2 and PO_2 to one decimal place, and pH to three decimal places. Accuracy was shown to increase by establishing a fixed reference point before each blood sample reading. Saline and air were passed through the PO_2 and PCO_2 electrode prior to each blood measurement. PO_2, PCO_2 and pH readings on samples from Tonometer I and Tonometer II were recorded so that the accuracy of the electrodes could be predetermined before sampling the P_{50} mixture. Readings from at least two separate P_{50} samples were determined and if the PO_2 difference was greater than 0.5 mm Hg, a third or fourth mixture was taken. Haemolysis after two hours of tonometry, as measured by the concentration of plasma haemoglobin (Shinowara, 1954) did not exceed 50 mg/100 mls. blood.

Measurement of the Bohr effect.

Fully oxygenated and fully reduced blood samples at different CO_2 levels were prepared. The Wösthoff M300/a-F gas mixing pump gave an accurate mixture of air and CO_2, and the Wösthoff 1SA 27/3F gave an accurate mixture of nitrogen and CO_2.

Three, six and nine per cent CO_2 concentrations were chosen, and

at each CO_2 level, samples of approximately 45% and 55% saturation were obtained by varying the volumes of blood drawn from each tonometer into the Hamilton Syringe. On each sample, the PO_2, PCO_2 and pH, oxygen and carbon dioxide contents (Van Slyke), and the oxygen capacity were measured, so that the accurate saturation of each mixture could be calculated. A plot of the data, using Hill's equation (Hill, 1910), $\log SO_2/100-SO_2 = n \log PO_2$ allows an indirect calculation of P_{50}. The term n, represents the slope and is indicative of the shape of the curve. A plot of P_{50} versus pH allows the determination of the Bohr effect, which is expressed as the ratio $\triangle \log P_{50} / \triangle pH$: This value was then used to correct the P_{50} measurement to a standard pH of 7.40.

From the PCO_2 and CO_2 content values measured in the P_{45} and P_{55} sample the in vitro CO_2 dissociation curves of whole blood were calculated.

SUMMARY.

P_{50} was prepared by the 'mixing' technique. Fully oxygenated and fully reduced blood samples were prepared by tonometry, with mixtures of air, nitrogen and CO_2, continuously produced in accurately known concentrations by the Wösthoff gas proportioning pumps. P_{50} was determined by measuring the PO_2 of equal quantities of blood from the two tonometers, after thorough mixing in a specially designed syringe. The PCO_2 and pH were also measured, and the P_{50} corrected to a pH of 7.4 by direct measurement of the Bohr effect. Radiometer electrodes with their preamplifier circuits were used, and an automated digital read-out recorded the results on an ASR 13 Teletype printer

MEASUREMENT OF 2,3 DIPHOSPHOGLYCERATE.

A search through the literature for a method of measurement of 2,3 DPG at the end of 1969, gave two alternatives:-

a) A chromatographic method

b) An enzymatic procedure.

The most popular chromatographic method was that of Bartlett (1959). A trial of the method did not give good results.

Extraction of phosphates from the red cell was a lengthy process, requiring separation and washing of cells, repeated centrifugation with Trichloracetic acid (TCA), removal of TCA with ether, followed by removal of ether with compressed air. The samples were then prepared by passage through Dowex 50 and subsequent neutralisation for column chromatography on Dowex 1-ion exchange resin. After passage of the appropriate eluent, the required phosphate fraction was obtained. The phosphorus content was analysed by a modification of the colourimetric Fiske and Subbarow reaction. (Bartlett, 1959).

For the quantitative analysis of glycerates, the chromotropic acid colourimetric assay was recommended. (Bartlett, 1959). This involved heating the glycerate sample with chromotropic acid in concentrated sulphuric acid at 100°C, not an ideal condition in any experiment. Recovery of phosphate from the column eluents was not good, and with the length of the process, and lack of initial success, thoughts were turned towards an enzymatic method, where fractionation of the extracted material was not required.

An enzymatic assay which depended on the catalytic effect of 2,3 DPG on phosphoglycerate mutase, the enzyme converting 3 phosphoglyceric acid (3 PGA) to 2 phosphoglyceric acid (2 PGA) was chosen due to its specificity. The method of Krimsky (1963) had the following advantages:-

1. Two reactions completed the assay.
2. It was a micro-technique with a final volume of one ml. instead of three, so economising in reagents.
3. No prior purification of substrates was required.
4. It was a suitable assay for a number of samples.

The reaction depends upon the decrease in optical density (O.D.) of phosphoenolpyruvate (PEP) recorded at 240 nm, the rate of decrease ($\triangle$OD/min) being proportional to the concentration of 2,3 DPG present. Four 2,3 DPG standards were run at the same time as the sample, so that the value of 2,3 DPG was obtained by reading from this calibration curve prepared for each batch, the value corresponding to $\triangle$O.D./min.

Initially, the Optica CF4 single beam spectrophotometer was used to record the decrease in O.D. manually, each minute, over a period of four minutes. The decrease was small and not reproducible, and therefore the following precautionary steps were taken to elicit the cause of the error.

a) The standard DPG samples were weighed by Dr. J.W. Minnis, B.Sc., Ph.D., Department of Biochemistry, whose accuracy in weighing is renowned.

b) The accuracy of two Oxford pipettes, calibrated to deliver .01 and .1 mls respectively, were found to be 0.0096 (SD .0007) mls and 0.0979 (SD .0014) mls respectively.

c) The substrate/enzyme ratio was confirmed to be at the accepted level of 10:1.

d) Increasing standard PEP concentrations correlated well with increased O.D. at 240 mμ, whereas 3 PGA solutions caused no increase in absorbance.

e) The pH within the reaction cuvette was measured and found to be 7.35 (the pH of the Tris buffer being 7.4). As the optimum pH of phosphoglyceromutase (PGAM) is 7.0, and enolase 6.2-6.9, enzyme inhibition was discounted.

f) A double beam recording ultraviolet spectrophotometer (Model No. Optica CF4DR) with scale expansion was used to increase the sensitivity.

g) Instead of the recommended 10^{-4} molar standard solution of 2,3 DPG, a wide range of 10^{-5} molar to 10^{-7} molar standard solutions was made up.

h) The assay was carried out at 37°C.

Following these modifications, eventually a reproducible decrease in O.D. was noted in the 10^{-6} molar fraction.

The sensitivity was finally adjusted by using the Unicam SP800A, ultraviolet recording spectrophotometer, with a potentiometric expansion recorder. Errors were only completely eliminated when the concentration of PGAM was doubled in strength from that recommended by Krimsky (1963). If the concentration of PGAM is insufficient, the decrease in optical density will be limited by the PGAM instead of the 2,3 DPG level. Lack of PGAM also explained the initial errors in recovery experiments.

All reagents were obtained from Boehringer-Mannheim.

Preparation of Solutions.

1. Tris Buffer (tris-hydroxymethyl-aminomethane).

   2.0M Tris buffer was made up, adjusted to pH 7.4 with 5 N HCl and diluted with distilled water to a final volume of 100 mls.

2. Magnesium Chloride.

   10.2 grams $MgCl_2$ .6 H_2O was dissolved in distilled water and made up to 100 mls.

3. Phosphoenolpyruvate (.025 M PEP)

   58.1 mg of the PEP tricyclohexylammonium salt C_3H_2O6P $(C_6H_{14}N)_3$ was dissolved in distilled water and made up to 5 mls. The solution is neutral and stable for several weeks in the frozen state.

4. Enolase.

The commercial preparation is contained in a crystalline suspension of 2.8 M Ammonium Sulphate solution, pH approximately 6.0. 1 mg protein/ml distilled water is required and was made up daily.

5. Phosphoglycerate mutase (PGAM)

The commercial preparation is contained in 2.6M ammonium sulphate solution of pH 6.0. An enzyme solution of 4 mg protein/ml distilled water was made up each day.

6. 2,3 Diphosphoglycerate (2,3 DPG)

4.167 mg of the crystalline pentacyclohexyl-ammonium salt was dissolved in distilled water and made up to 5 mls. The solution is neutral and stable for several weeks in the frozen state.

The final composition of the test and reference cuvettes was:-

Reagent	Test	Reference
Tris	0.02 ml	0.02 ml
$MgCl_2 6H_2O$	0.01	0.01
PEP	0.03	-
Enolase	0.01	0.01
Water	0.89	0.96
PGAM	0.03	-
2,3 DPG	0.01	-
Total	1.00 ml	1.00 ml

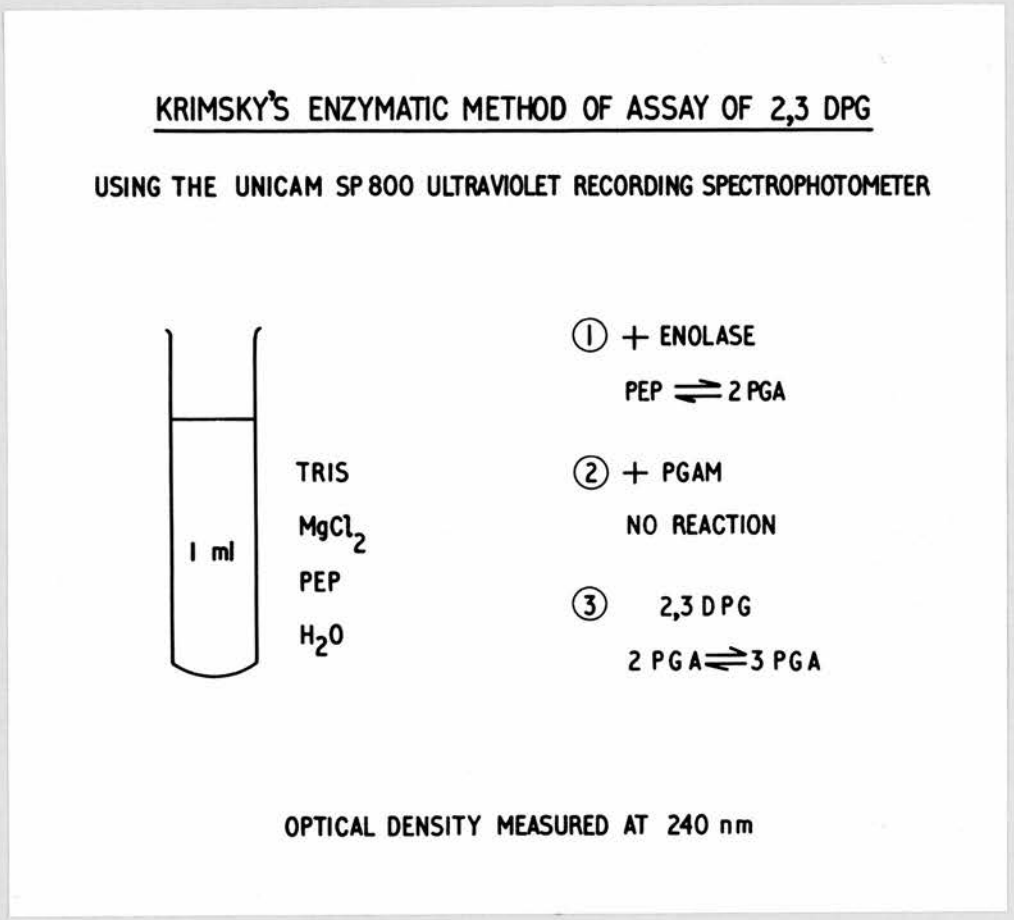


Figure 13.

The contents of the cuvette are shown on the left, before addition of enolase (reaction 1), phosphoglycerate mutase (reaction 2), and 2,3 diphosphoglycerate (reaction 3).

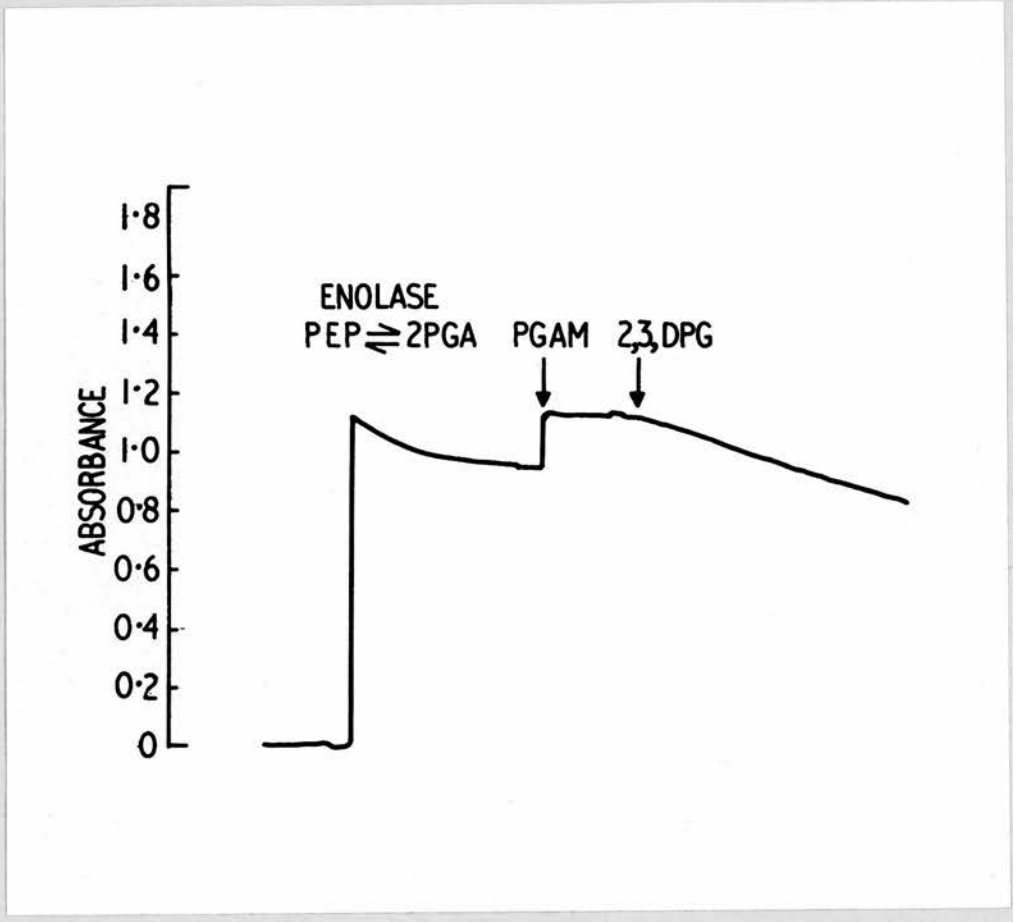


Figure 14.

The unexpanded trace of the 2,3 DPG assay from the Unicam SP 800 ultraviolet recording spectrophotometer.

Time scale:- 30 mms/minute.

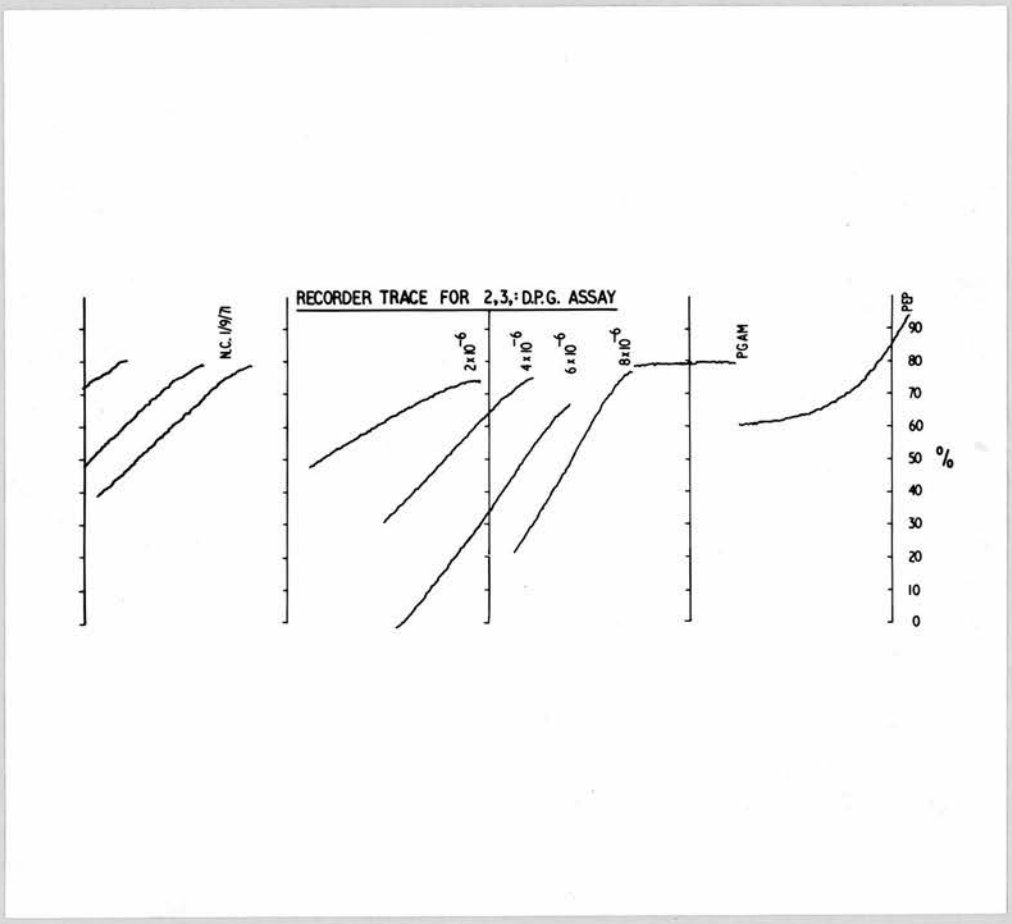


Figure 15.

The expanded trace of the 2,3 DPG assay from the expansion recorder of the Unicam SP 800 recording spectrophotometer. From right to left, the trace includes:- The equilibration of PEP, the lack of reaction with PGAM, the completed reaction with the four standard solutions, and a duplicate blood extract reaction.

2,3 DPG ASSAY.

The two reactions taking place are shown in Fig. 13. The addition of enolase to the test cuvette causes a decrease in PEP concentration, until equilibrium with 2 PGA is reached, after approximately 3 minutes. PGAM is added, and the trace allowed to run for 1 minute to make sure that the enzyme is free of contamination. Addition of standard DPG or extract then stimulates the PGAM activity and allows conversion of 2 PGA to 3 PGA. The concentration of PEP is therefore displaced from its equilibrium values, and the decrease is recorded at 240 m for 4 minutes. Fig.14 demonstrates the sequence of events without the scale expansion, and in Fig. 15 where sensitivy is increased by a factor of five, the change in O.D. of four standard DPG solutions, as well as of a sample extract, is shown.

Four standard DPG solutions were made up daily from a stock solution of 10^{-5} MDPG stored at -20°C. 2,4,6 and 8 x 10^{-6}M DPG standards were recorded, followed by the sample extracts of DPG, preparation of which is described below.

Extraction Procedure.

One ml of whole blood was added to 5 mls 0.6M perchloric acid (PCA). It was well mixed for one minute using a vortex stirrer, to ensure complete deproteinization. After centrifugation at 2000 (680g) revs/min for 20 minutes, the supernatant was removed and neutralised with 1M dipotassium hydrogen orthophosphate. It was recentrifuged for

3 minutes, and the supernatant either used immediately or stored frozen at -25°C. The extracts are stable up to four months. A dilution of 1:50 was necessary before analysis to bring the extract into the range of the standards. From the △ OD/min, the 2,3 DPG concentration was derived by interpolation from the graph of standard DPG solutions. However, this derived value must then be corrected for the various dilution stages in the preparation of the sample.

Calculation of Final Concentration:

If Y moles/litre of whole blood is the value from the standard curve, then the final concentration in μmoles/ml erythrocytes =

$$\frac{Y \times 6 \times 2 \times 51}{1000} \times \frac{100}{\text{Hct.}}$$

The factors are explained as follows:-

a) PCA : whole blood — 5 : 1

b) K_2HPO_4 : supernatant — 1 : 1

c) phosphate buffer : neutralised extract (to bring into range of standards) — 50 : 1

d) μmoles/l : μmoles/ml — $\frac{1}{1000}$

e) μmoles/ml blood : μmoles/ml RBC — $\frac{100}{\text{Hct.}}$

f) μmoles/ml RBC : μmoles/gm Hb. — $\frac{1}{\text{Hb.}}$

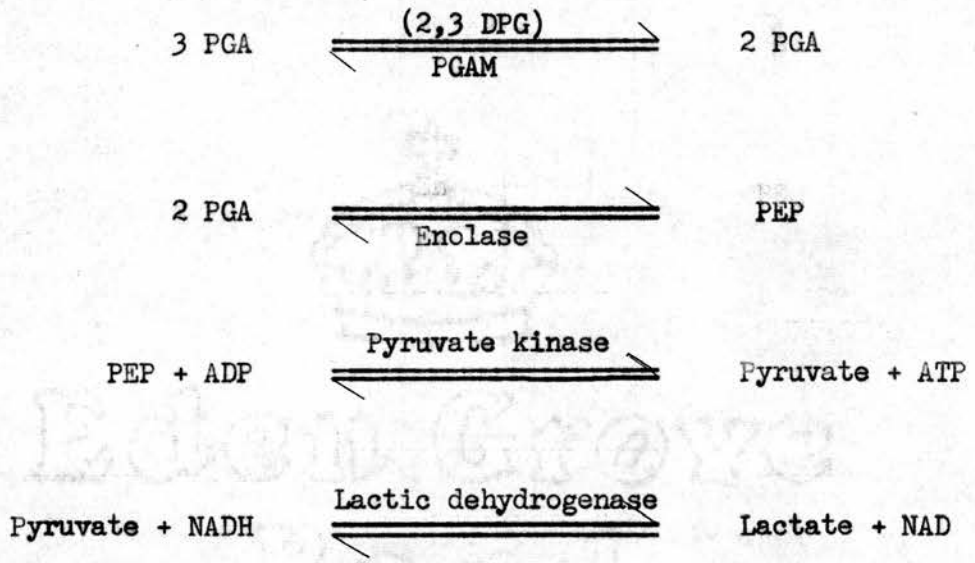


Figure 16.

Methods of enzymatic analysis of 2,3 DPG. a) Measurement of O.D. of PEP, or b) measurement of NADH/NAD reaction.

OTHER METHODS.

Several enzymatic methods are now in use, due to increased demand for measuring 2,3 DPG.

Production of enzymatic 'kits' by Sigma and Calbiochem are proof of the increasing demand for this assay. Boehringer, in the January, 1972 issue of 'Biochemica Service' gave a brief survey of 'promising' methods, including the catalytic effect of 2,3 DPG on the phosphoglycerate mutase, as developed for use in this study.

Towne, Rodwell and Grisolia (1957) also used this catalytic reaction of 2,3 DPG, but instead of measuring the decrease in O.D. of PEP, their initial substrate was 3 PGA, which was converted via 2 PGA to PEP, so that they measured the increase of PEP at 240 nm.

Schroter and Heyden (1965) continued the process just described with the conversion of PEP to pyruvate and lactate. The decrease in O.D. at 340 nm, due to the oxidation of NADH to NAD was measured. The full reaction is shown in Fig.16.

Further enzymatic procedures developed as a result of the discovery by Rose (1970)[1] that glycolate-2-phosphoric acid stimulates the phosphatase activity of PGAM, this property of PGAM having been described by Joyce and Grisolia in 1958.

Phosphatase activity on 2,3 DPG results in the formation of 3 PGA and an inorganic phosphate radical (P_i). P_i can be colourimetrically estimated by the Fiske and Subbarow reaction. Rose (1970)[2] converts the 3 PGA to lactate, measuring the NADH/NAD

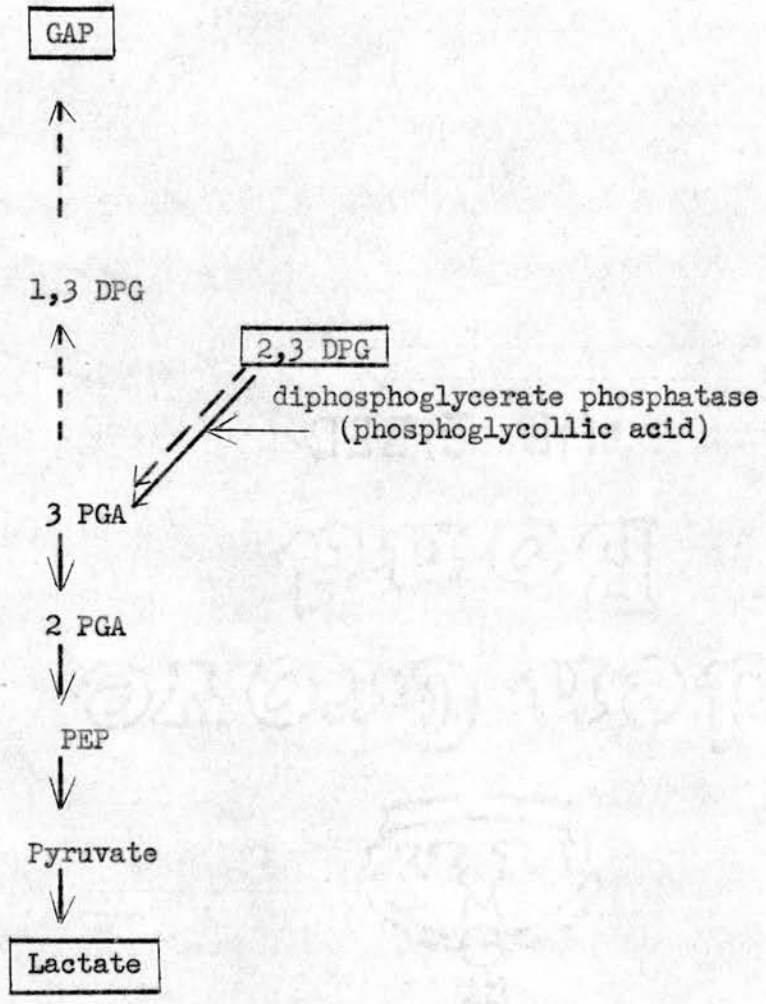


Figure 17.

Methods of enzymatic analysis of 2,3 DPG, using glycolate-2-phosphoric acid to stimulate the phosphatase activity of PGAM.

conversion as described previously.

Keitt (1971), also used the phosphoglycolic acid stimulant action of PGAM but instead of measuring P_i, or converting 3 PGA to lactate, he took the third alternative, and converted 3 PGA back to glyceraldehyde-3-phosphate (GAP) via 1,3 DPG, again measuring the decrease in O.D. at 340 nm due to oxidation of NADH. In all NADH/NAD reactions, the millimolar extinction coefficient for NADH at 340 nm was taken as 6.22.

Both reactions using 2,3 DPG as a substrate are shown in Fig.17.

The Sigman 'kit' assay uses the method of Keitt (1971) and on a few samples, 2,3 DPG was measured in duplicate, using both the modification of the Krimsky method described above, and the Sigma 'kit' method.

Standard haematological analysis of haemoglobin, haematocrit and mean corpuscular haemoglobin concentration were recorded on each sample in the routine laboratory. The Coulter Counter S1 was used.

SUMMARY.

2,3 Diphosphoglycerate concentrations were measured by a new modification of Krimsky's enzymatic method of assay, using the Unicam SP 800 recording spectrophotometer. Decrease in optical density of the substrate, phosphoenolpyruvate was measured at 240 nm., this decrease being proportional to the 2,3 DPG concentration, providing the compound is present in limiting amounts. The actual concentration is compared with 2,3 DPG standards.

METHOD OF INTRAERYTHROCYTIC pH MEASUREMENT (pH_i).

Methods of direct measurement of pH_i have given various results, depending on the method used. (Waddell and Butler, 1959). The two main techniques in use are:-

(a) The 'freeze-thaw' technique.

The pH of haemolyzed cells is directly measured by the glass electrode. Haemolysis is achieved by freezing on dry ice, and then thawing prior to measurement.

(b) Measurement of the distribution of a weak acid.

It is assumed that cell membranes are freely permeable to the undissociated form of the acid and impermeable to the dissociated form, the latter being distributed across the cell membrane according to the Donnan equilibrium. A weak acid will then be distributed between intracellular and extracellular space, according to the respective pH values and the pK of the weak acid used, so that if both the total amount of acid present and the pH of one compartment are known, the pH of the other compartment can be calculated. Measurement of bicarbonate ion concentration has previously proved unreliable, due to the presence of varying quantities of carbamino compounds. Chloride ion distribution is still measured by an electrometric silver titration method (Bromberg, 1966), but the main technique, developed by Waddell and Butler in 1959 is the

distribution of 5,5 dimethyl 2-4 oxazolidine-dione (DMO). This is a weak acid and therefore does not significantly alter the pH of the medium. DMO is not bound to plasma or protein, and is not metabolized.

Comparison of Methods.

Thomasen in 1963, compared the DMO and freeze-thaw methods of intraerythrocytic pH measurements and found a significant difference between them. Paymaster and Englesson in 1966 retested the accuracy and reproducibility of the DMO method, with a few minor modifications of Thomasen's technique, but still found higher values by the DMO technique compared with the direct freeze-thaw measurement. Bromberg in 1966 concluded that any of the three methods, freeze-thaw, chloride, or DMO, could be used for evaluating changes in intraerythrocytic pH but he could not judge one as being more accurate than another for determining absolute values.

In this study it was decided to measure the intraerythrocytic pH by the freeze-thaw technique, adapted from the method of Hilpert (1963).

Method.

Three mls. of heparinized arterial or venous blood was transferred anaerobically to a 5 ml. disposable syringe. The syringe was sealed, and the end of the barrel cut off. This ensured close contact between the base of the syringe and the centrifuge

holder and therefore did not allow air to enter during centrifugation due to movement of the barrel. The syringe was then centrifuged at 3,000 (1500 g) revolutions/min for ten minutes. The separated plasma, together with the top layer of red cells, was expelled by gently pushing the barrel of the syringe upwards. After sampling for plasma trapping the syringe was placed on dry ice (temperature -78°C) for 40 minutes. After thawing, haemolysis was complete and the pH of the red cells directly determined. Standard buffer solutions were sampled between each reading. Measurements of haematocrit, on red cell samples, was found to be constant at approximately 20%. Plasma trapping is recognised as an inherent error of the 'free-thaw' technique.

A review of intracellular pH was completed by Robson, Bone and Lambie in 1968, and one of these authors, Dr. J.M. Bone, having previously established the reproducibility of the DMO technique, confirmed the validity of this freeze-thaw method, by comparing the two methods on tonometered blood samples. Four samples of blood were tonometered with air and 12%, 8%, 5% and 2% CO_2 in air respectively, and the intraerythrocytic pH subsequently measured by both the freeze-thaw, DMO and 'nitrogen' techniques.

It was only possible to measure the intraerythrocytic pH on arterial and venous samples from six bronchitic patients, and one arterial and venous sample from a normal subject, due to the inclusion of this measurement at the end of the study.

CHAPTER IV

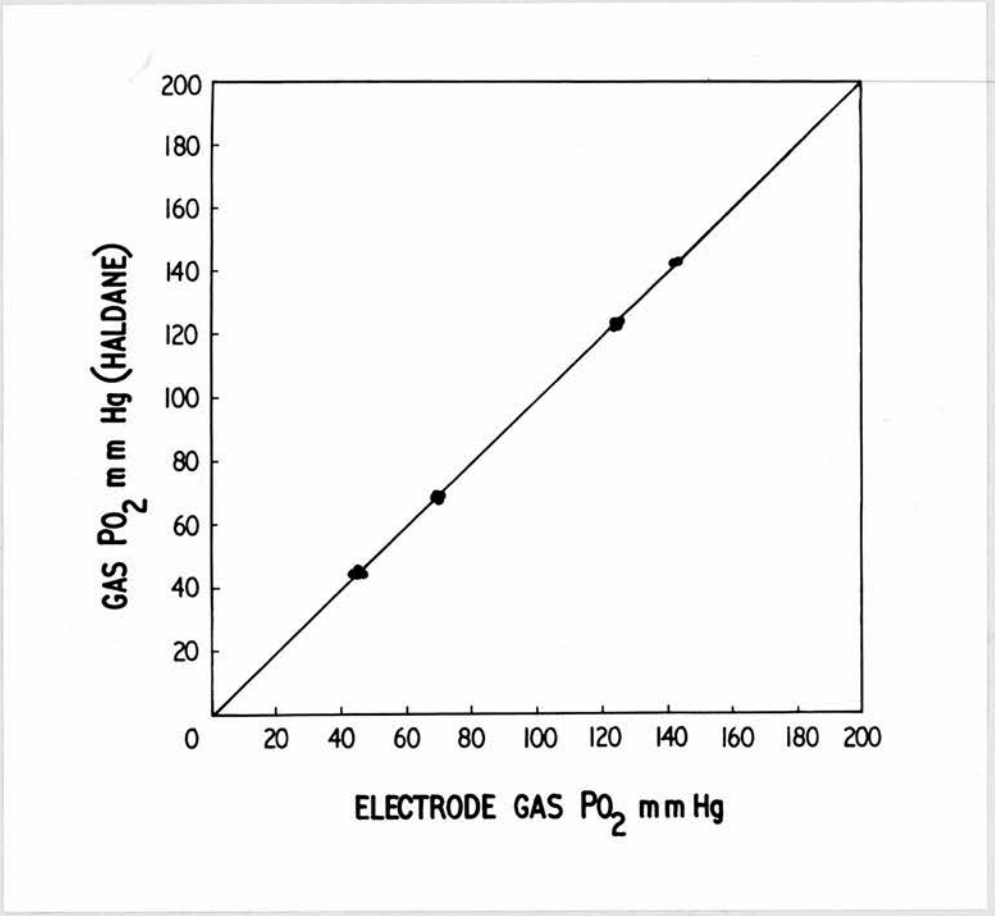


Figure 18.

Linearity of the PO_2 electrode : Gas Samples.

The electrode was set up with nitrogen and air, and the PO_2 of four other gas mixtures recorded by the electrode. The electrode reading was plotted against the PO_2 as determined by Haldane analysis. Four electrode readings were recorded for each gas mixture.

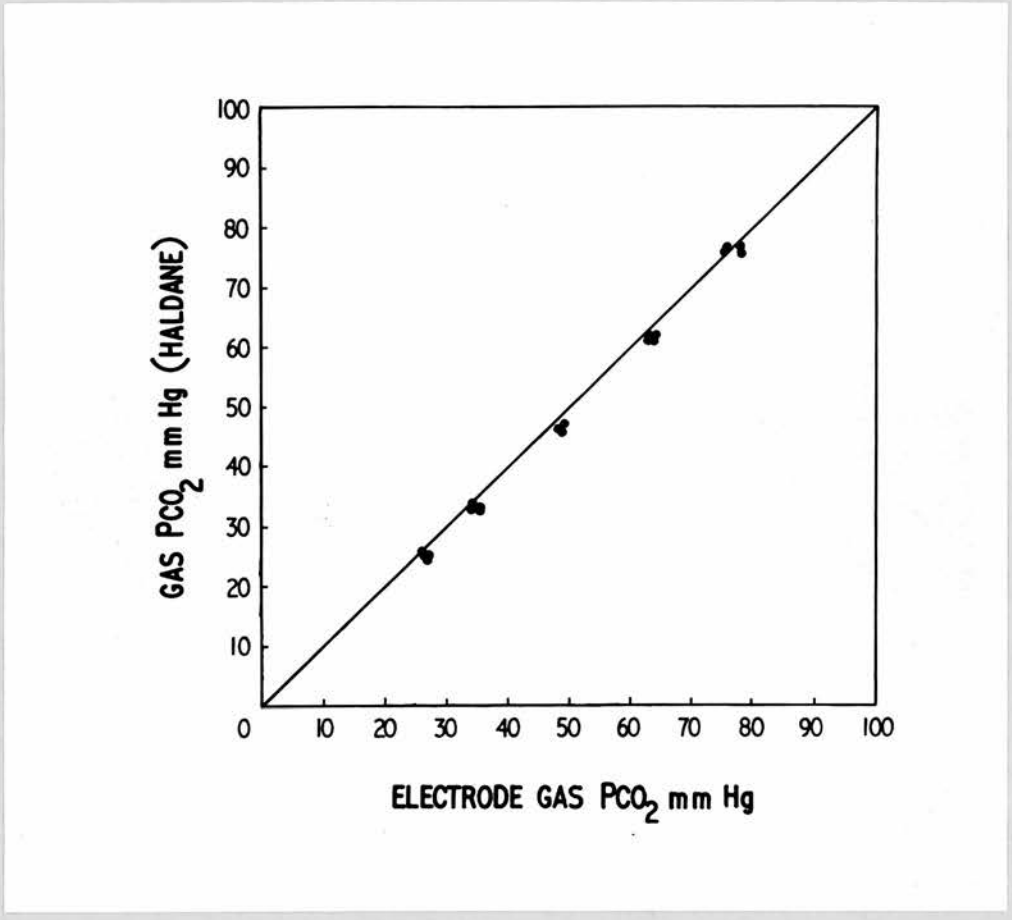


Figure 19.

Linearity of the PCO_2 electrode : Gas Samples.

The electrode was set up with two gas mixtures and the PCO_2 of five other gas mixtures recorded. The results were plotted against the PCO_2 of the mixture as determined by Haldane analysis. Four readings at each PCO_2 concentration were recorded.

RESULTS APPERTAINING TO THE METHOD OF MEASURING P_{50}.

The linearity of the oxygen and carbon dioxide electrodes were tested after the preamplifier circuits and automatic print out system had been set up.

Gas Samples.

Different concentrations of oxygen, nitrogen, and CO_2 were prepared in clyinders, and after overnight mixing their accurate composition was determined with the Lloyd-Haldane apparatus, duplicate analysis agreeing to 0.03%. After setting up the electrodes with two gas mixtures, the PO_2 and PCO_2 of five other gas mixtures were determined. This was repeated four times and the electrode readings compared with the accurate gas tensions calculated from the Haldane analysis and the barometric pressure. Figure 18 demonstrates the results for the oxygen electrode and Fig.19 the results for the CO_2 electrode. In each case the line of identity passes through the majority of points and the regression line does not differ significantly from this line of identity.

Blood Samples.

Having established the linearity of the electrode for gas samples, blood was tonometered with various O_2, CO_2 and N_2 mixtures, the accurate composition of these mixtures again being determined by

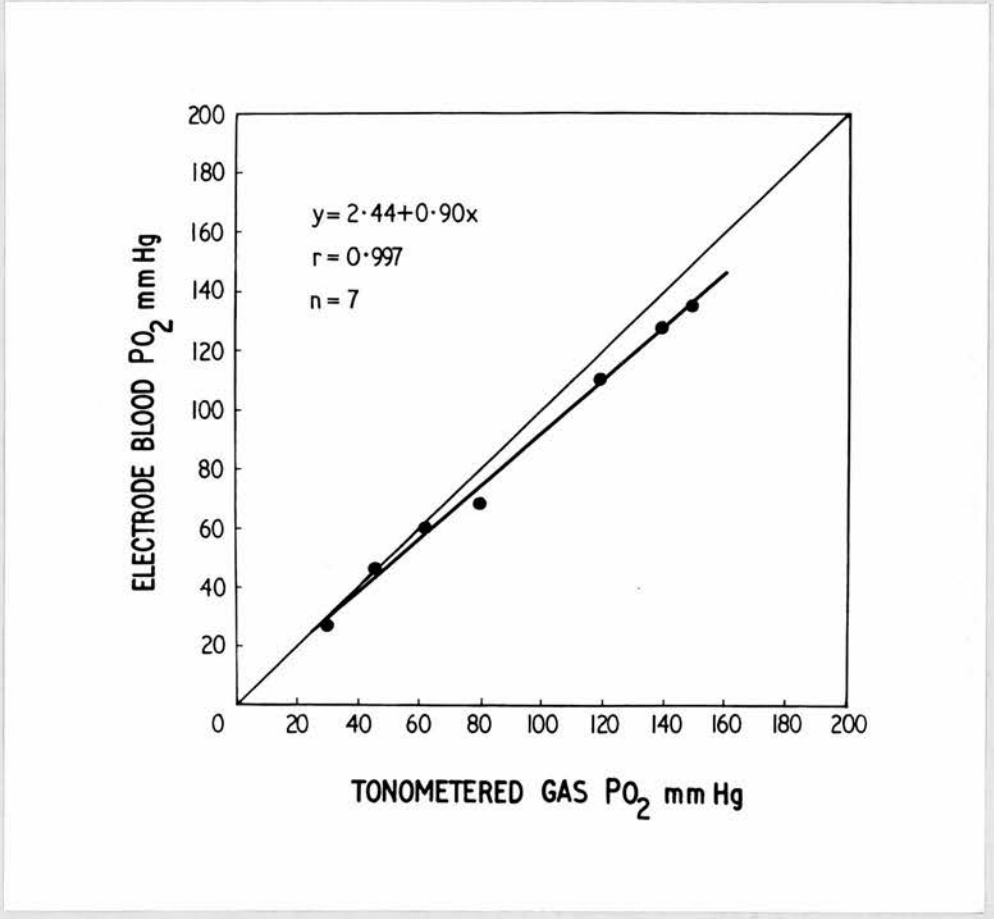


Figure 20.

Linearity of the PO_2 electrode : Blood Samples.

The electrode, set up on gas samples, was used to determine the PO_2 of blood samples (Electrode blood PO_2) prepared by tonometry with known gas mixtures where PO_2 was calculated from Haldane analysis. (Tonometered gas PO_2). The deviation from identity (but not linearity) is due to the well known blood/gas difference of these unstirred micro cathode PO_2 electrodes.

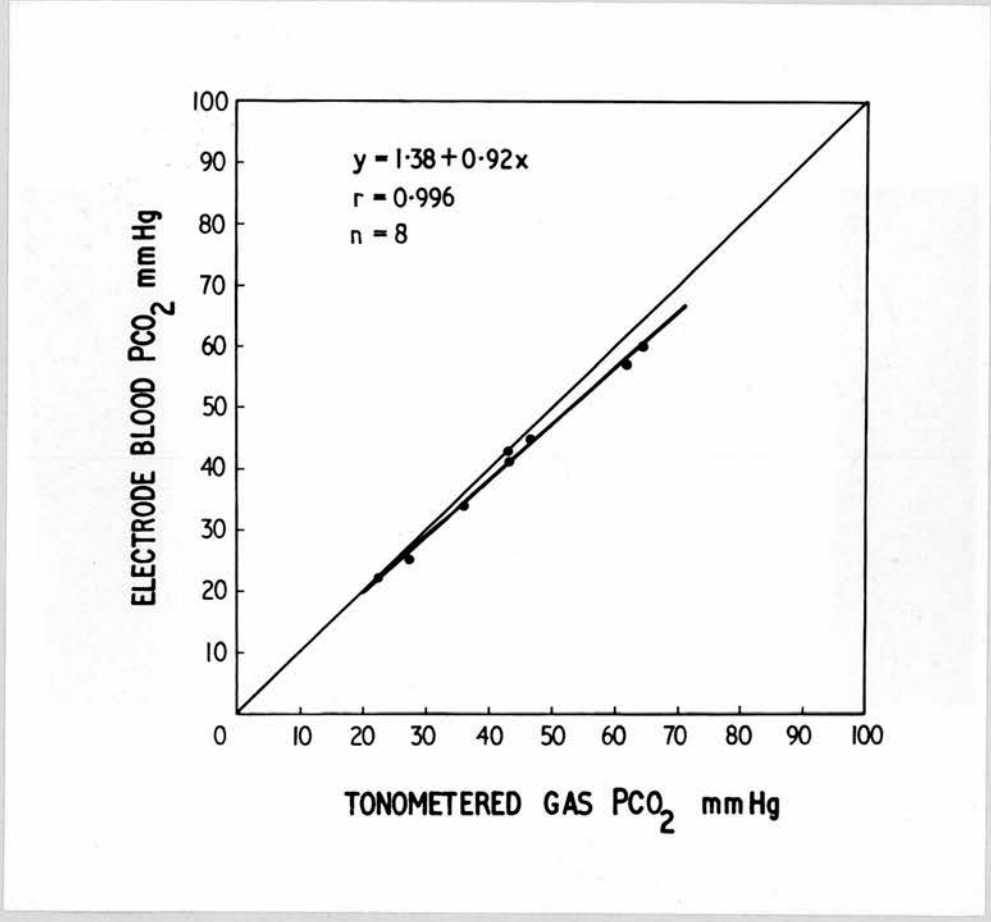


Figure 21.

Linearity of the PCO_2 electrode : Blood Samples.

The electrode, set up on gas samples, was used to determine the PCO_2 of blood samples, (Electrode blood PCO_2) prepared by tonometry with known gas mixtures where PCO_2 was calculated from Haldane analysis. (Tonometered gas PCO_2).

Lloyd-Haldane analysis, and the blood gas tension of the tonometered sample, as determined by the electrode, plotted against the gas tension of the tonometering gas, as calculated from Haldane analysis.

The deviation from the line of identity in the oxygen electrode values, Fig.20, is due to the blood/gas difference, characteristic of these unstirred microcathode PO_2 electrodes. The results for the CO_2 electrode were also lower than the gas readings, but at no time was the difference greater than 2 mm Hg. (Fig.21).

Accuracy of P_{50} Mixture.

PO_2, PCO_2 and pH measurements were made on Tonometer I and Tonometer II blood samples, prior to withdrawing the P_{50} mixture (Tables 1 and 2), to ascertain that the required degree of oxygenation and reduction had taken place at this time.

Oxygen capacity and oxygen content measurements were made on both the reduced, oxygenated and P_{50} samples. The close correlation (Fig.22) between capacity and content values for Tonometer I sample demonstrated that the oxygenated blood was indeed fully saturated.

The oxygen content in samples from Tonometer II ranged from 0 to 1.0 mls/100 mls blood. The relationship between oxygen content and oxygen capacity, when directly measured in the P_{50} mixture by Van Slyke analysis is shown in Fig.23. By taking the mean of the 100% and 0% samples for content and capacity, the relationship between these values, at a theoretical 50% saturation is calculated, and is also shown in Fig.23. Although the two regression lines are not

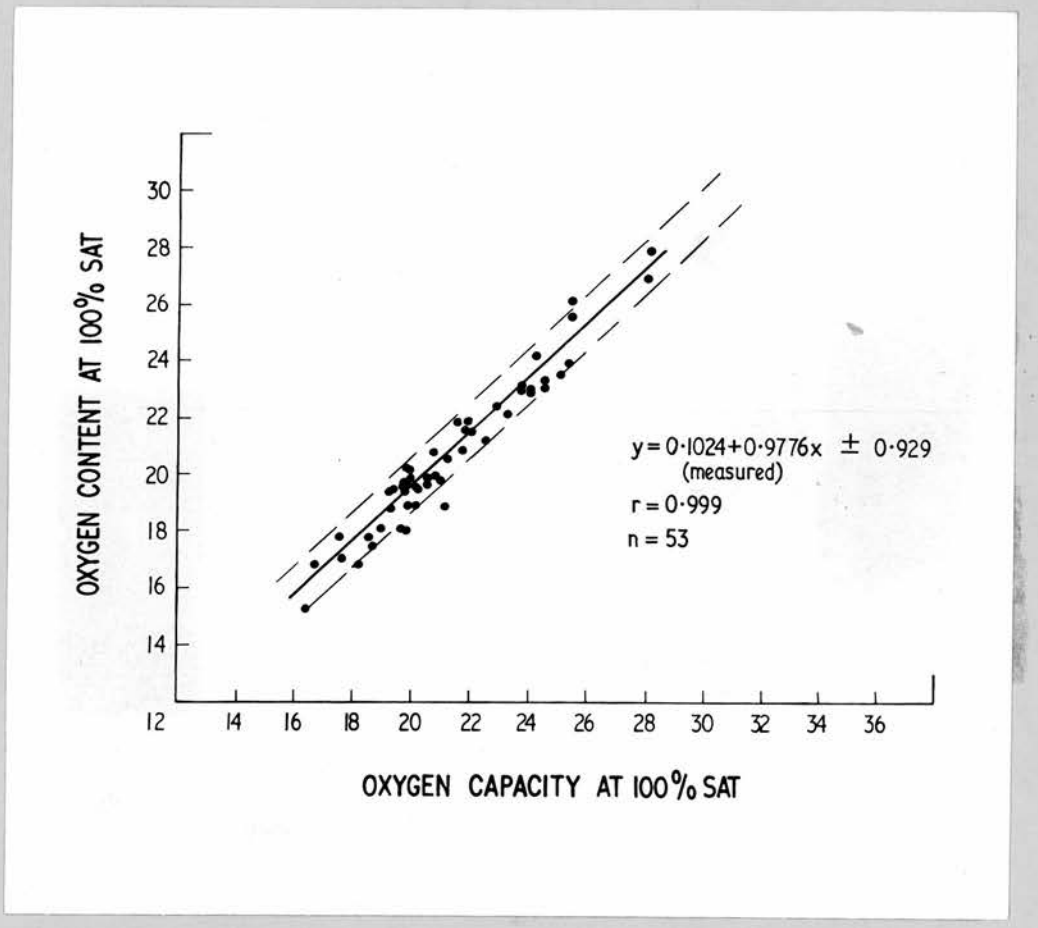


Figure 22.

The relationship between oxygen content and oxygen capacity at 100% saturation. The close correlation indicates the complete saturation of the oxygenated sample. — — — — indicates the 95% confidence limits of the least squares regression.

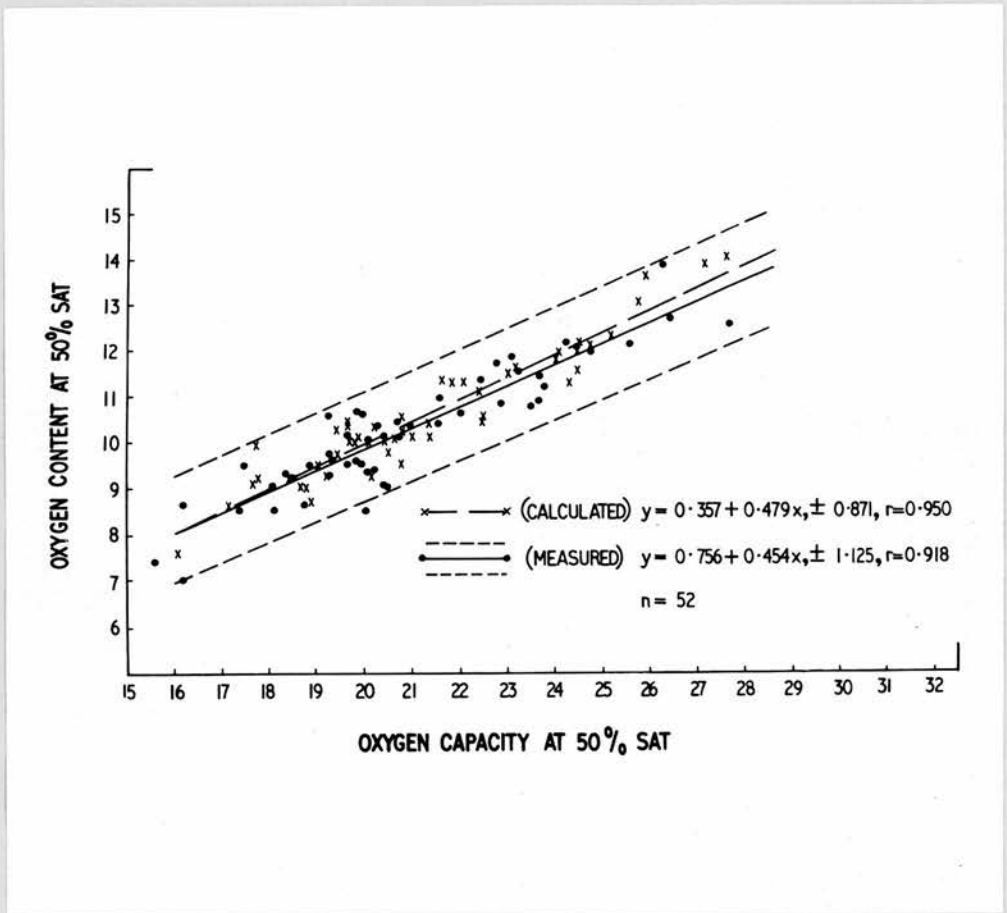


Figure 23.

The relationship between oxygen content and oxygen capacity at 50% saturation: a) when measured directly from the P_{50} sample (●) and b) when calculated from the mean of 100% and 0% saturated samples (x) — — — — indicates the 95% confidence limits of the least squares regression ●———●.

identical, the difference is insignificant and the 95% confidence limits of the least squares regression relationship between the measured and calculated values includes all the results.

The Bohr Effect.

To compare the affinity of haemoglobin for oxygen between subjects, P_{50} is standardized by correcting to a pH of 7.4 ($P_{50(7.4)}$), by using a Bohr factor. This was determined graphically as follows:- The data (Table 9a and 9b) was plotted in terms of Hill's (1910) equation:-

$$\log {}^{S}/_{100-S} = n \log PO_2.$$

Figure 24a shows this relationship in a normal subject and Fig.24b in a patient with chronic bronchitis. The value n is the slope of the line in these figures. It depends upon interaction between haem molecules and therefore is a measure of the shape of the oxyhaemoglobin curve. The normal and bronchitic subject did not differ in their value of n. Indirect estimation of P_{50} from these graphs at 3%, 6% and 9% CO_2 concentrations allows us to plot P_{50} as a function of the measured pH values, and thus determine the Bohr effect, expressed by the ratio $\frac{\triangle \log P_{50}}{\triangle \text{ pH}}$

Figure 25a demonstrates the calculation of the Bohr effect for the normal subject whose preliminary results were given in Fig.24a.

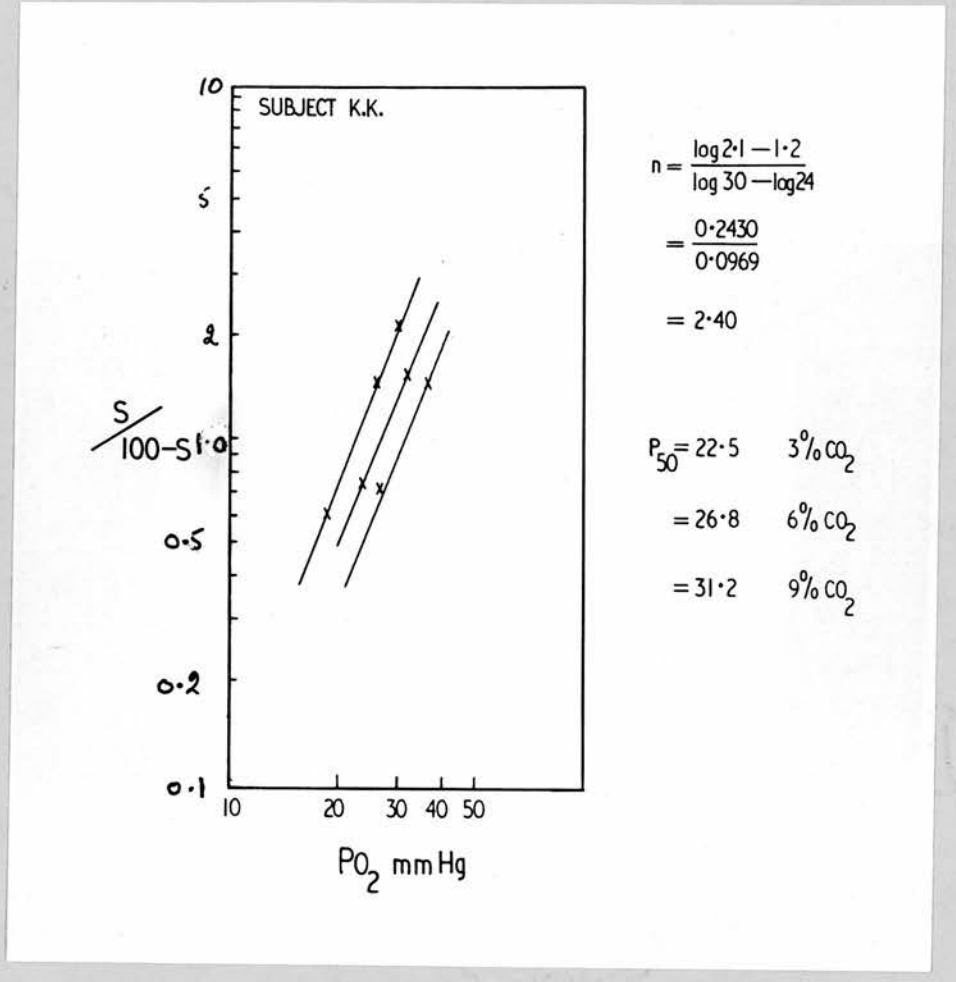


Figure 24a.

Measurement of two known levels of oxygen saturation (s), which were approximately 45% and 55% saturation, allows P_{50} to be estimated indirectly when the dissociation curve is plotted as Hills (1910) equation:-

$$\text{Log} \quad \frac{S}{100-S} \quad = \quad n \log PO_2$$

K.K. is a normal subject.

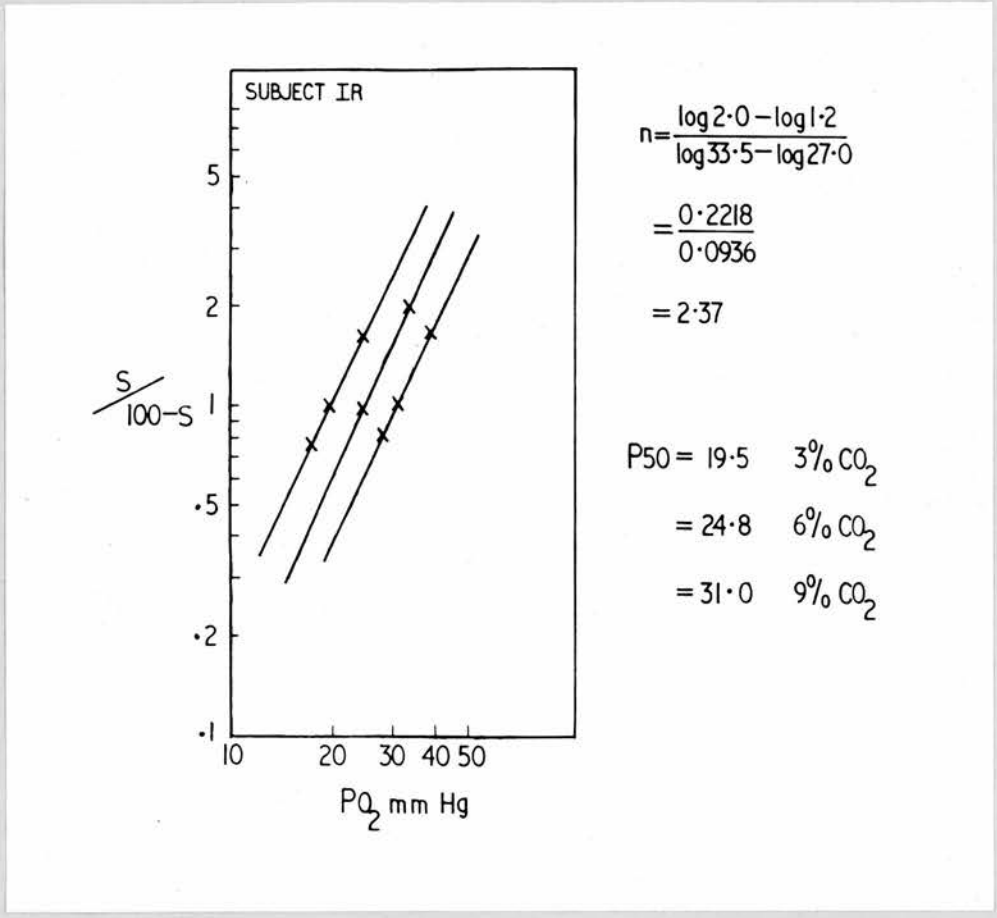


Figure 24b.

Measurement of two known levels of oxygen saturation (s) which were approximately 45% and 55% saturation, allows P_{50} to be estimated indirectly when the dissociation curve is plotted as Hills (1910) equation:-

$$\text{Log} \quad \frac{S}{100-S} \quad = \quad n \log PO_2$$

I.R. is a patient with chronic bronchitis.

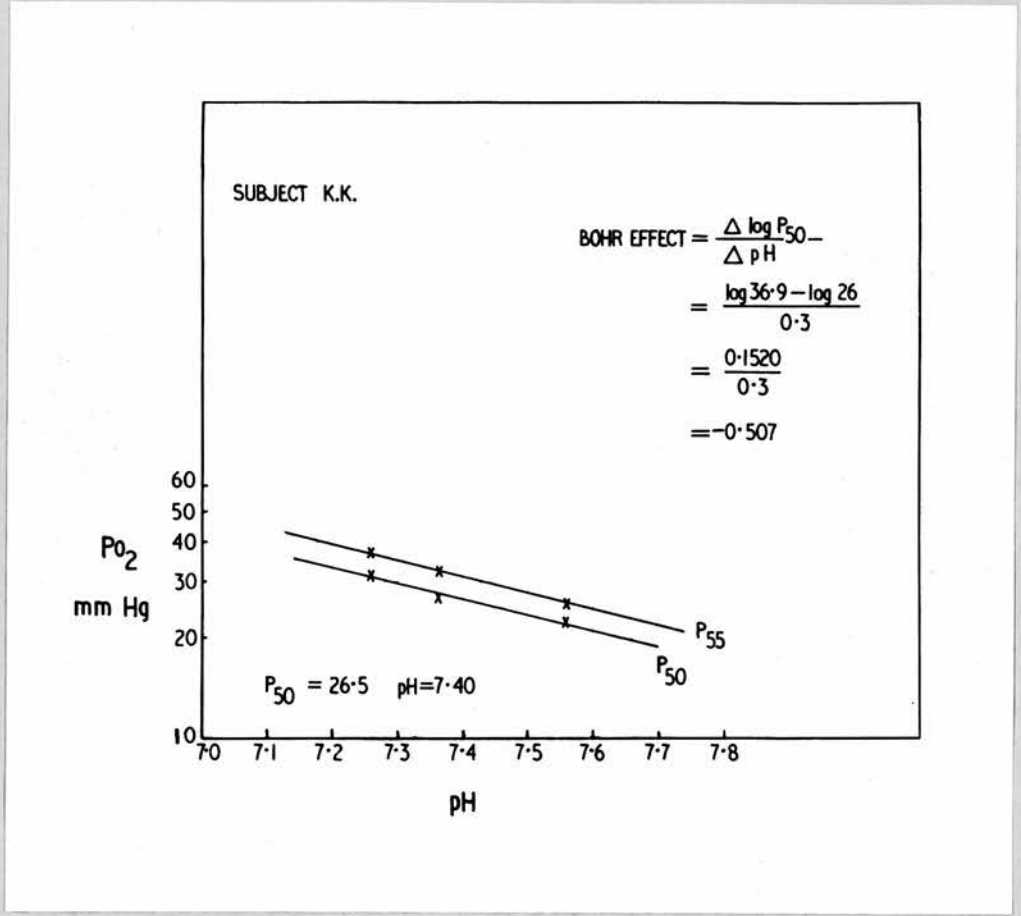


Figure 25a.

The indirect P_{50} measurements, calculated from figure 24a, when plotted against pH allows measurement of the Bohr effect.

K.K. is a normal subject.

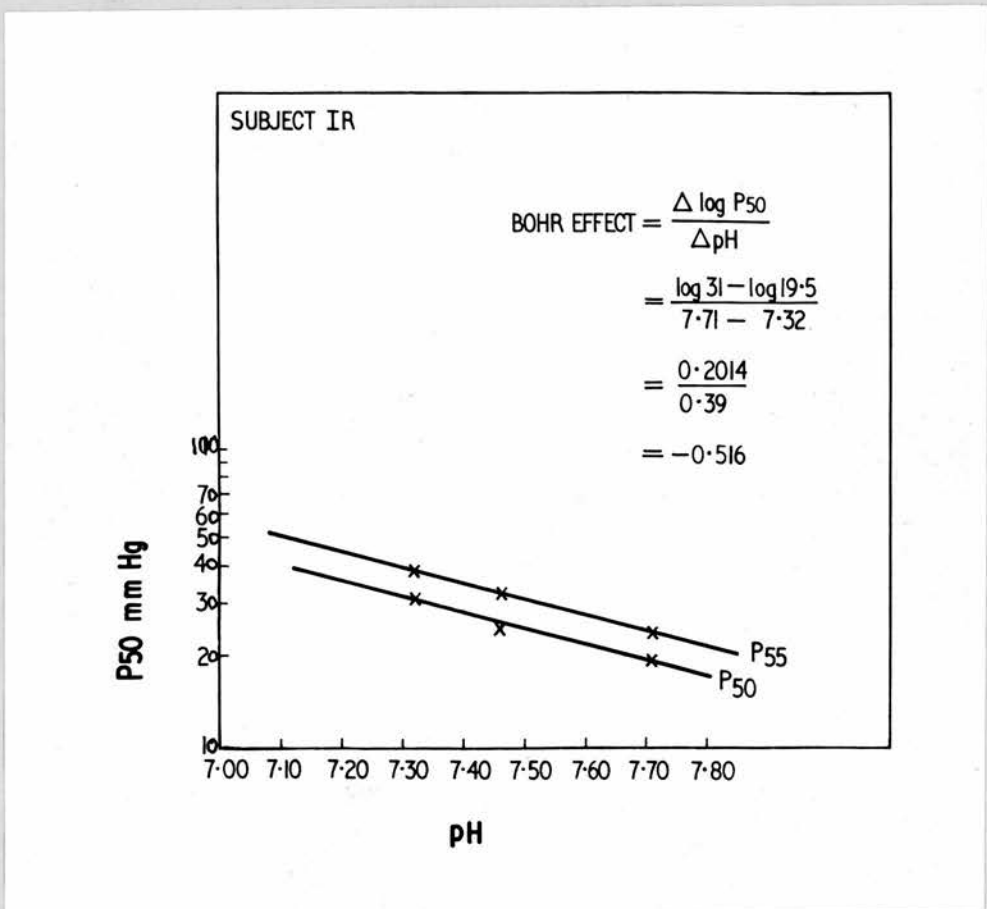


Figure 25b.

The indirect P_{50} measurements calculated from Figure 24b, when plotted against pH allows measurement of the Bohr effect.

I.R. is a patient with chronic bronchitis.

A similar Bohr factor (Fig.25b), was found for the patient with chronic bronchitis whose preliminary results were given in Fig.24b. The Bohr effect was measured on six patients with chronic CO_2 retention, the results of which are shown below.

Subject No.	Bohr Factor.
14	-0.537
15	-0.516
28	-0.521
31	-0.501
33	-0.535
45	-0.534
Mean (± SD)	-0.524 ± 0.014

CO_2 Transport.

The CO_2 content of whole blood samples tonometered in vitro to have an oxygen saturation of 45% and 55% are shown in Table 7b. This difference in oxygen saturation was too small to demonstrate an appreciable Christiansen-Douglas-Haldane (CDH) effect, although the majority of measurements indicated a higher CO_2 content in the sample at 45% oxygen saturation. The mean CO_2 content (at approximately 50% oxygen saturation) was plotted against the corresponding PCO_2, giving three points on an in vitro CO_2 dissociation curve (Fig.26). Little correlation was found between the slope or position of these curves and the 2,3 DPG levels, in either the six patients or the one normal subject studied.

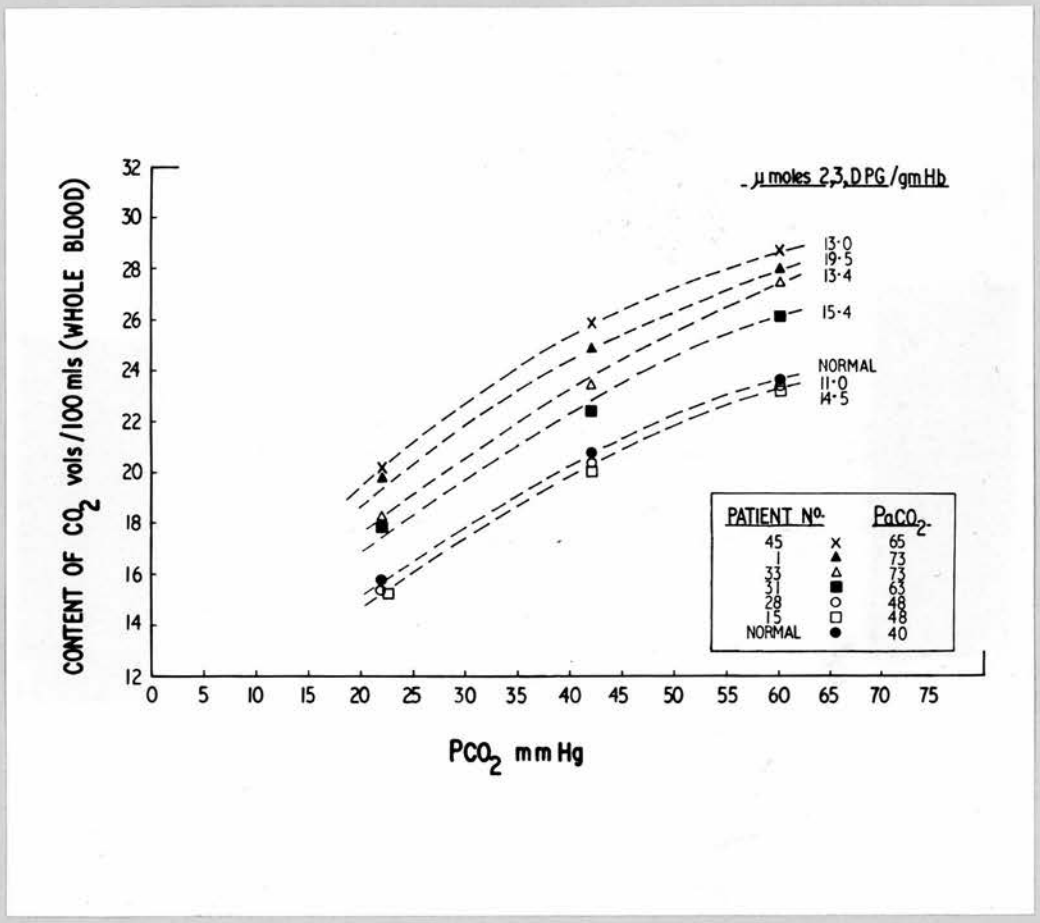


Figure 26.

The in vitro CO_2 dissociation curve of whole blood, at oxygen saturations of 45-55%, show little correlation with 2,3 DPG concentration and shape or position of the curve. The higher curves are from patients with chronic CO_2 retention.

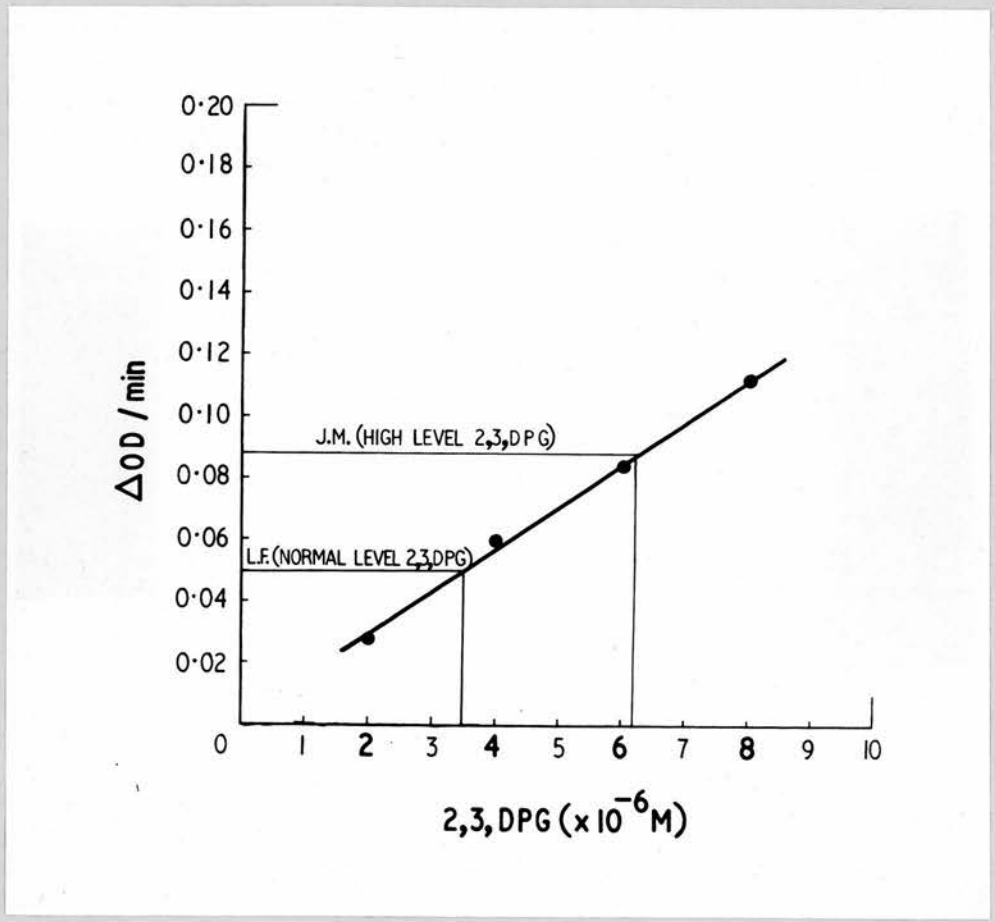


Figure 27.

Linearity of 2,3 DPG assay.

Demonstration of four standard solutions, 2,4,6 and 8 x 10^{-6}M, with the corresponding readings of a normal and a high level of 2,3 DPG from blood extracts.

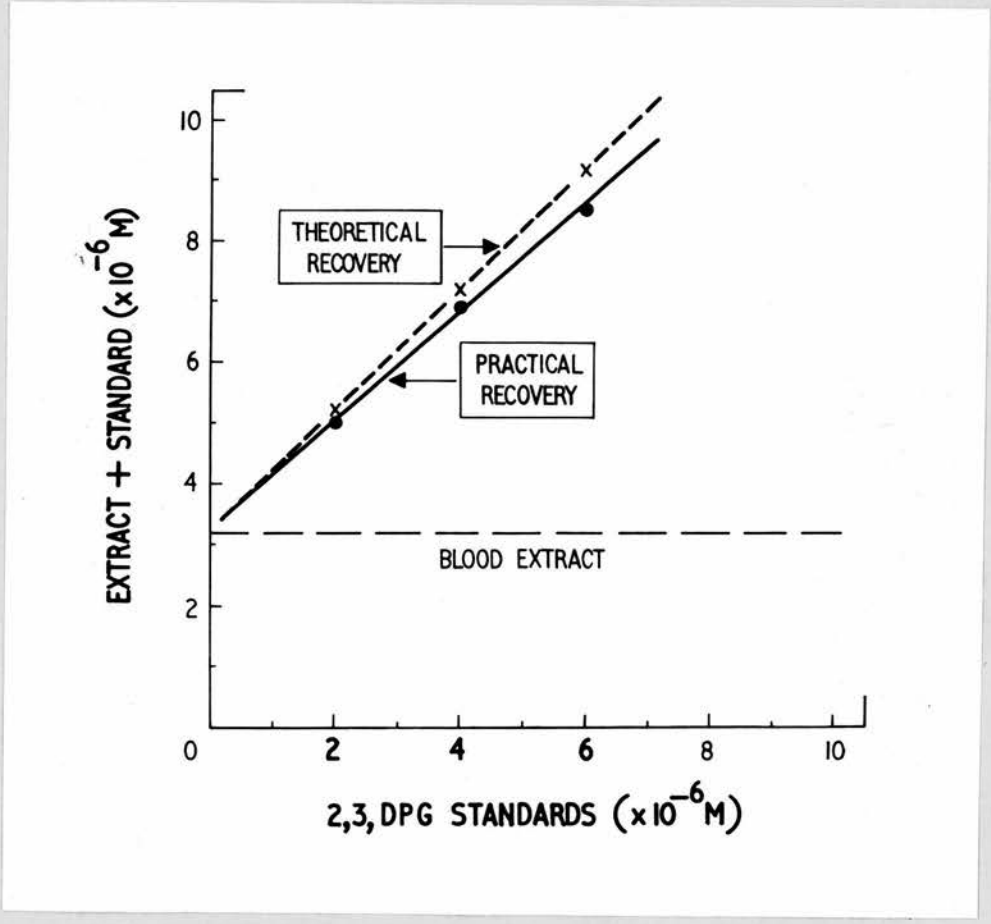


Figure 28.

The recovery of 2,3 DPG standards, which have been added to the blood extract prior to assay.

RESULTS APPERTAINING TO THE METHOD OF MEASURING THE CONCENTRATION OF 2,3 DIPHOSPHOGLYCERATE

Twenty-one different extracts were taken for 2,3 DPG assay from one normal sample of blood. The mean, in moles per ml of erythrocytes was 4.48 ± 0.15 representing an acceptable precision of 3%.

Figure 27 demonstrates the change in optical density with four standard solutions, 2,4,6 and 8 x 10^{-6} molar, and the readings from two extracts, one giving a normal result, the other a high result for 2,3 DPG.

A recovery experiment where standard DPG solutions were added to the extract prior to assay and the combined change in optical density measured is shown in Fig.28. The practical recovery is lower than the theoretical recovery for the combined results, but the 96%, 95% and 92% recovery for the extract plus 2,4 and 6 x 10^{-6}M standards respectively, is satisfactory.

According to Krimsky (1963), solutions of PEP and 2,3 DPG keep for several weeks in the frozen state, but no time limit was given for storage of the deproteinised neutralised blood extracts. Samples were always taken in duplicate for measurement of 2,3 DPG, but in certain subjects, an extra sample was taken and stored at -20°C. After a set period, measurement of 2,3 DPG was repeated, and compared with the previous value. The results for four subjects are shown below.

Subject No.	2,3 DPG Values in μmoles/gm. Hb.		
	Result at time of Study	Result after Storage at -20°C.	Storage Period
31	14.74	14.42	3 weeks
38	14.90	15.45	6 weeks
11	10.24	10.24	9 weeks
1	18.33	18.15	12 weeks

The largest error, shown in the samples from subject No.38 is 3.5%, equivalent to the precision of the method.

Levels of 2,3 DPG which were within these limits of precision were found in extracts taken directly from a heparinized venous sample prior to P_{50} measurement, from the P_{50} mixture itself, or from an arterial sample from the same patient taken simultaneously for blood gas analysis.

There was a significant correlation between the modification of Krimsky method, and the Sigman 'kit' method, when duplicate measurements of 2,3 DPG were made on extracts from 9 subjects. ($r = 0.881$, $p = < 0.01$).

The least squares regression differed significantly from the line of identity. (Fig.29).

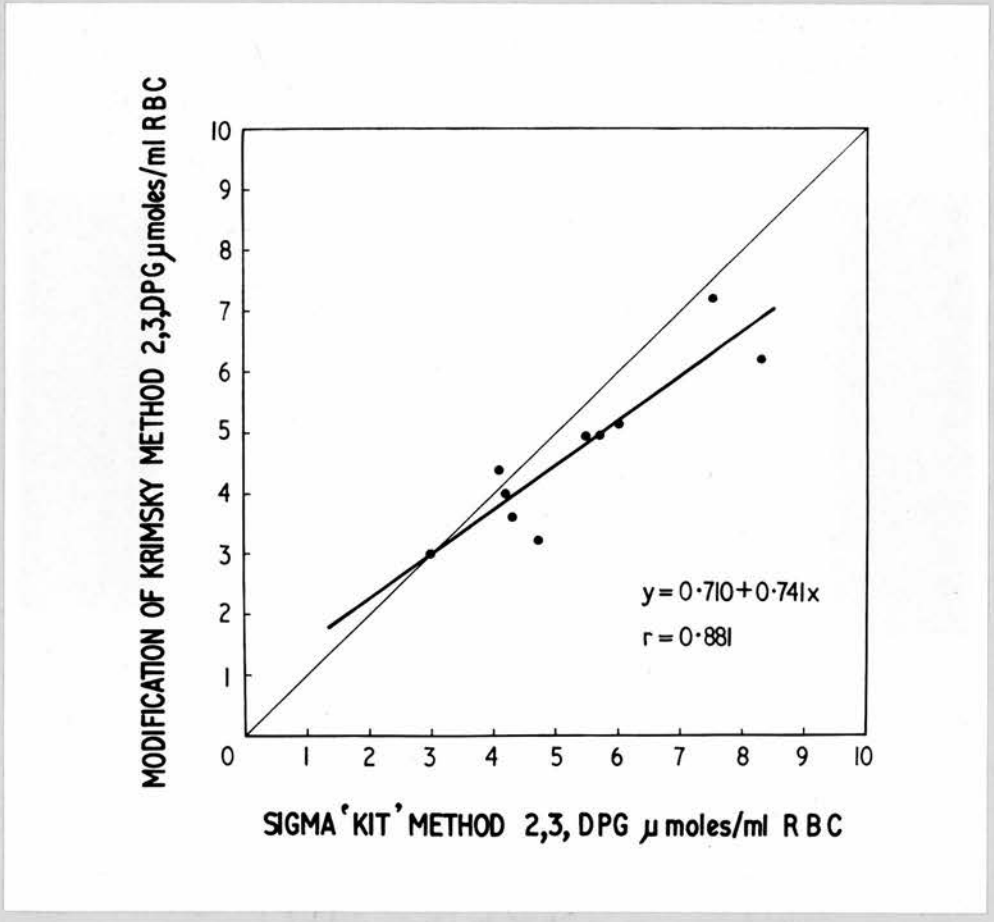


Figure 29.

Comparison of the modification of Krimsky method and the Sigma 'kit' method. The least squares regression is not significantly different from the line of identity.

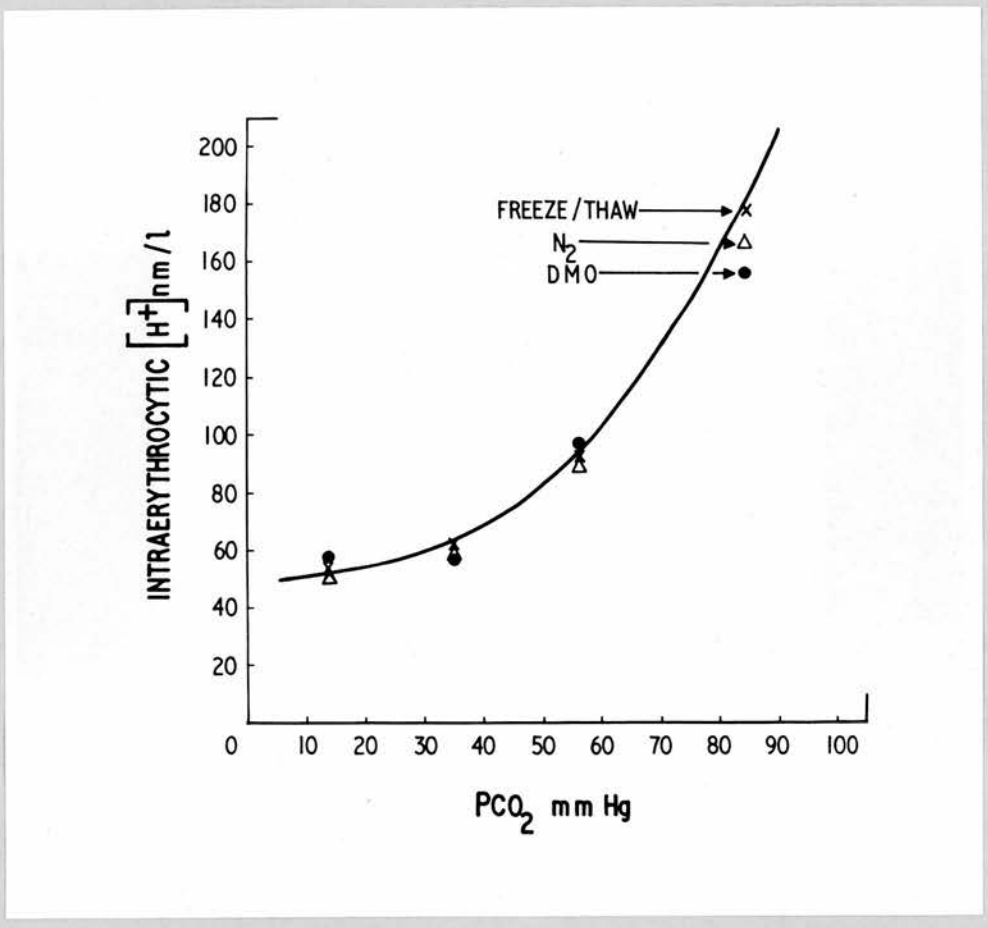


Figure 30.

The freeze-thaw method of pH_i measurement is compared with the DMO and N_2 techniques after tonometry at 4 different levels of CO_2 concentration. The line is drawn by eye.

RESULTS APPERTAINING TO THE MEASUREMENT OF INTRAERYTHROCYTIC pH.

The freeze-thaw, DMO, and nitrogen methods gave similar intraerythrocytic measurements on the four blood samples tonometered at different levels of PCO_2. The pH values were expressed as the activity of hydrogen ions and plotted against PCO_2. (Fig.30). The line joining the four sets of triplicate results was drawn by eye, and its only significant lies in demonstrating the close agreement between all three methods. Simultaneously drawn arterial and venous samples were used for intraerythrocytic pH measurements by the freeze-thaw technique in six bronchitic patients and one normal subject. PO_2, PCO_2, and whole blood pH measurements were determined at the same time. (Table 10).

Intraerythrocytic pH measurements were determined in six patients with chronic bronchitis. With the exception of subject number 12, all patients had low arterial PO_2 levels with persistent stable CO_2 retention. The mean and standard deviations of arterial and venous whole blood pH were 7.37 ± 0.04 and 7.28 ± 0.04 respectively. The corresponding mean and standard deviations of arterial and venous intraerythrocytic pH were 7.17 ± 0.02 and 7.10 ± 0.03 respectively. The correlation between whole blood and intraerythrocytic pH is shown in Fig.31. The intraerythrocytic arterial pH measurement of 7.16 for the normal subject was very similar to the mean value for the patients.

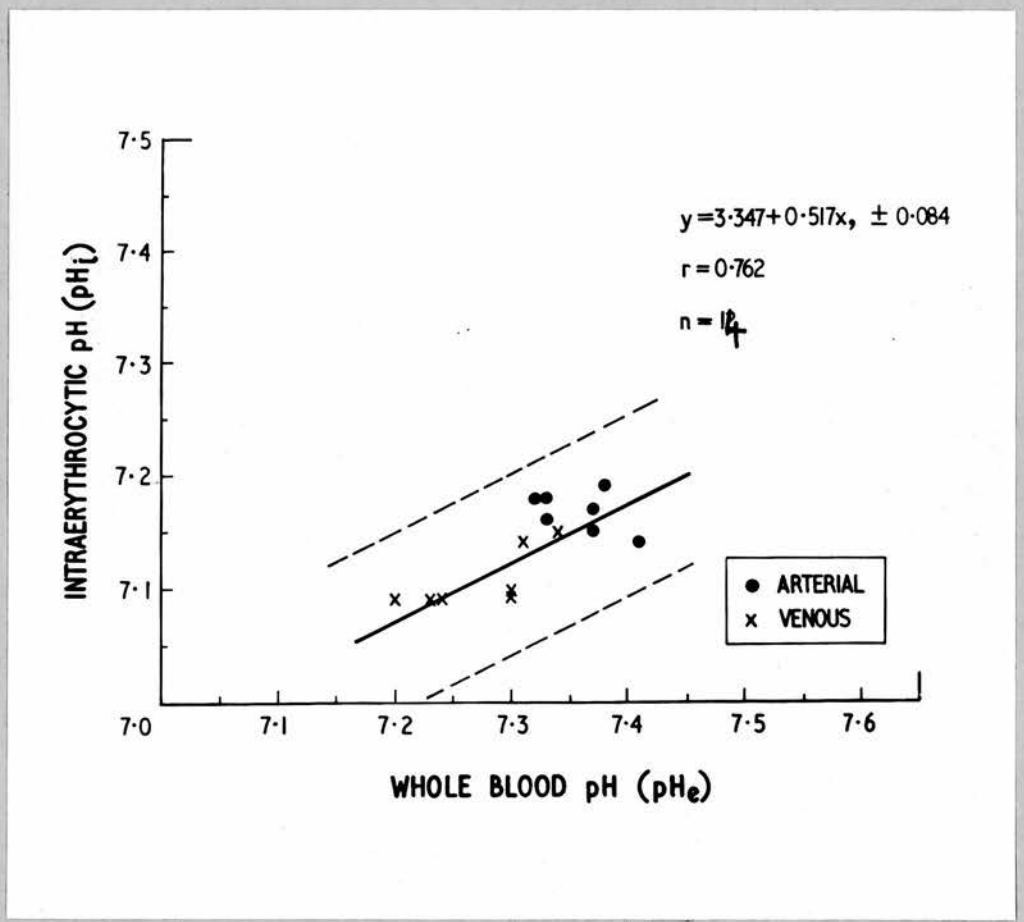


Figure 31.

Intraerythrocytic pH (pH_i) as a function of whole blood (pH_e).

RESULTS.

The mean and standard deviation of the $P_{50(7.4)}$ measurements found in this study for twelve normal subjects was 26.7 ± 0.65. This value does not differ significantly from other normal values quoted in the literature, and is identical to that of Astrup (1965), who studied 58 normal subjects. (Table 3).

The mean and standard deviation of the 2,3 DPG concentrations found in the same normal subjects, was 4.32 ± 0.69 μmoles per ml. of erythrocytes, or 12.8 ± 2.2 moles per gram of haemoglobin. Although normal values differ according to the method used, these values compare favourably with those quoted by different authors in the literature (Table 4).

Mean values for intraerythrocytic arterial pH, as measured by the freeze-thaw technique for normal subjects, are quoted by Purcell et al. (1961) as 7.19, and by Bromberg (1965) as 7.15. The mean of 7.17 found in this study for arterial intraerythrocytic pH in six patients with chronic CO_2 retention does not differ significantly from those values in normal subjects.

A Bohr effect ($\frac{\triangle \log P_{50}}{\triangle pH}$) of -0.500 was used throughout the study to correct the direct measurement of P_{50} to a standard pH of 7.40. Results of $P_{50(7.4)}$ together with the various parameters known to affect the oxyhaemoglobin relationship are shown for normal subjects in Table 5, and for all patients in Table 6. The twelve

normal subjects (8 males and 4 females) are all without cardiac or pulmonary disorders, and are non-smokers.

The results of the five patients, receiving long term oxygen therapy (Table 6) who were studied whilst breathing air, did not differ appreciably from the results obtained when they were breathing 30% oxygen. Taking the values obtained whilst breathing oxygen as their normal values, P_{50} was found to increase (Patients 44 and 48), decrease (patients 45 and 47) or remain constant (patient 46) whilst breathing air, with 2,3 DPG either increasing marginally or remaining constant, so that no consistent change was observed.

Data giving clinical information on 35 patients who had suffered from chronic bronchitis and emphysema for between 5 and 25 years, is presented in Table 5. Predicted values of forced expiratory volume in one second ($FEV_{1.0}$) and forced vital capacity (FVC) have been calculated from their age, height and weight from the data of Cotes (1968). With the exception of patient 17, all the $FEV_{1.0}$ and FVC values are at least 50% lower than the predicted values, indicating that they suffered from severe airways obstruction.

The mean and standard deviation of the arterial PO_2 in these patients when they were breathing air was 49.4 ± 9.9 indicating their severe hypoxaemia. The acid base relationships of their arterial blood is shown in Fig.32, where the results are plotted on an $[H^+]/PCO_2$ diagram. (Flenley, 1971). In most of these patients, these relationships lie within the 95% confidence limits of chronic respiratory acidosis. This arises from their longstanding CO_2

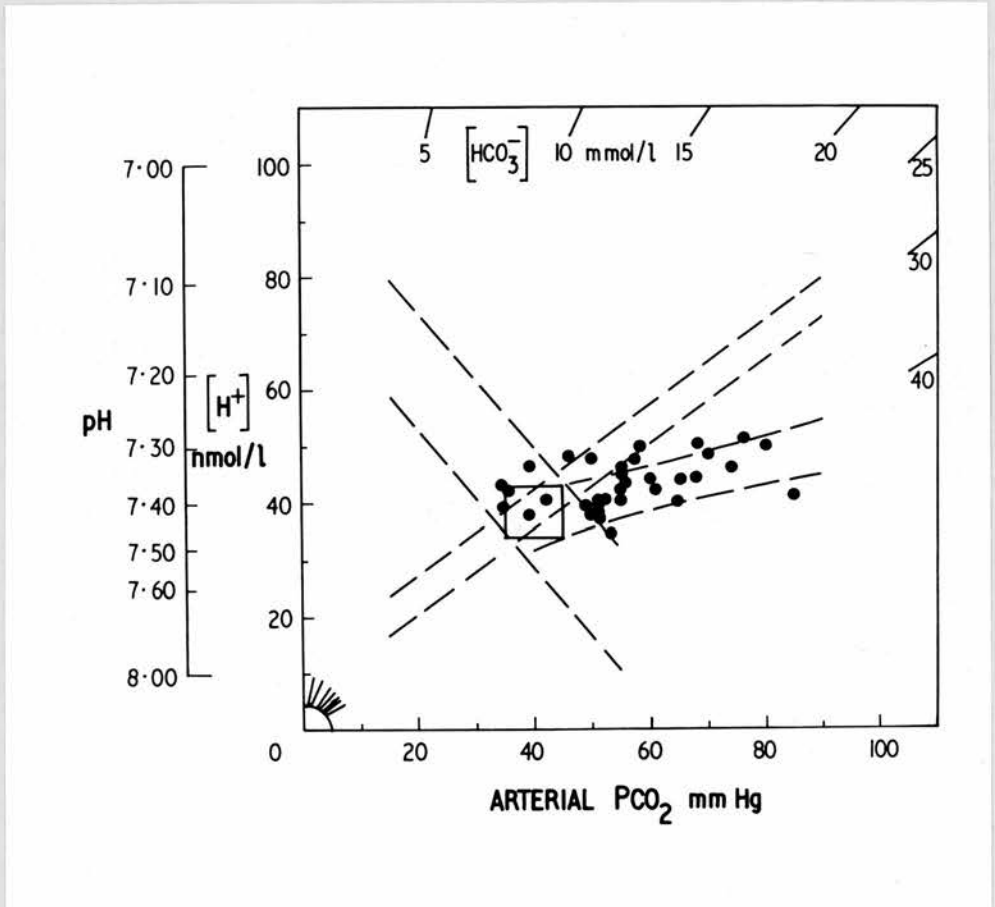


Figure 32.

Acid Base Relationship in Arterial Blood.

All patients with chronic bronchitis and emphysema are plotted on an $[H^+]//PCO_2$ diagram (Flenley, 1971), demonstrating the chronicity of their CO_2 retention and its renal compensation by tubular bicarbonate reabsorption.

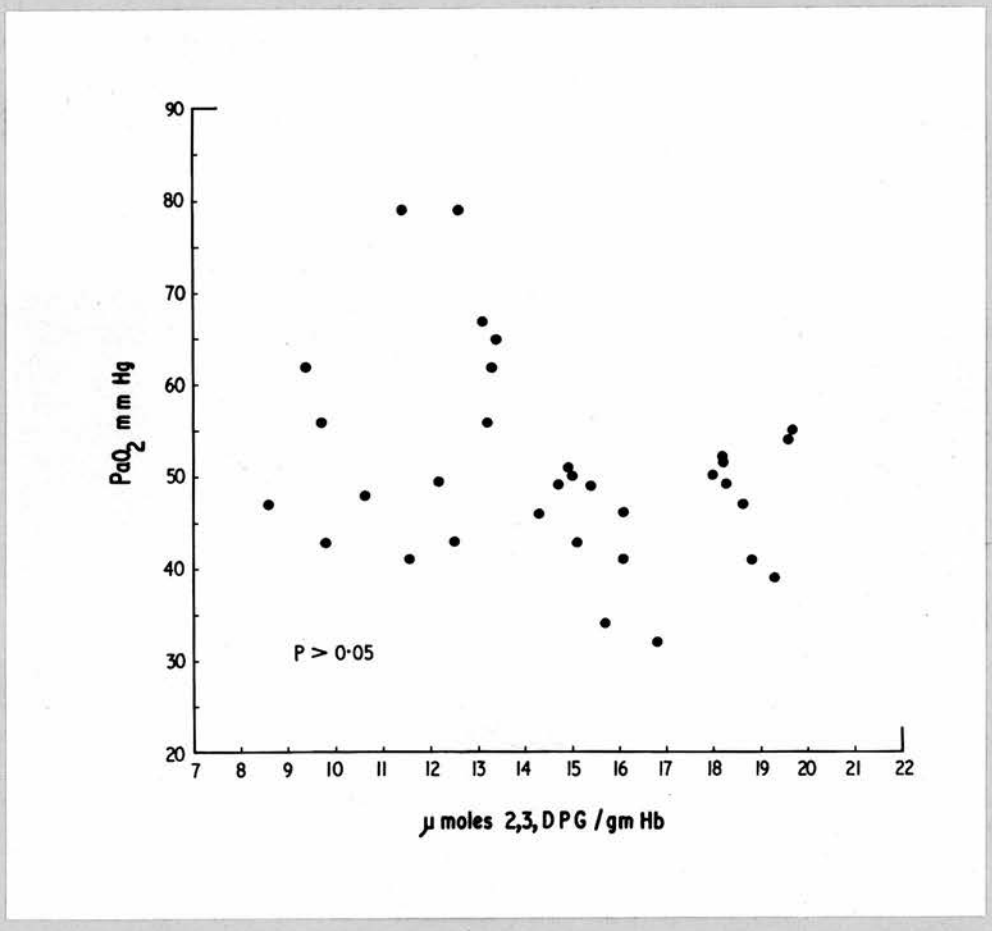


Figure 33.

The low arterial PO_2 of patients with chronic hypoxaemia did not correlate significantly with the levels of 2,3 diphosphoglycerate.

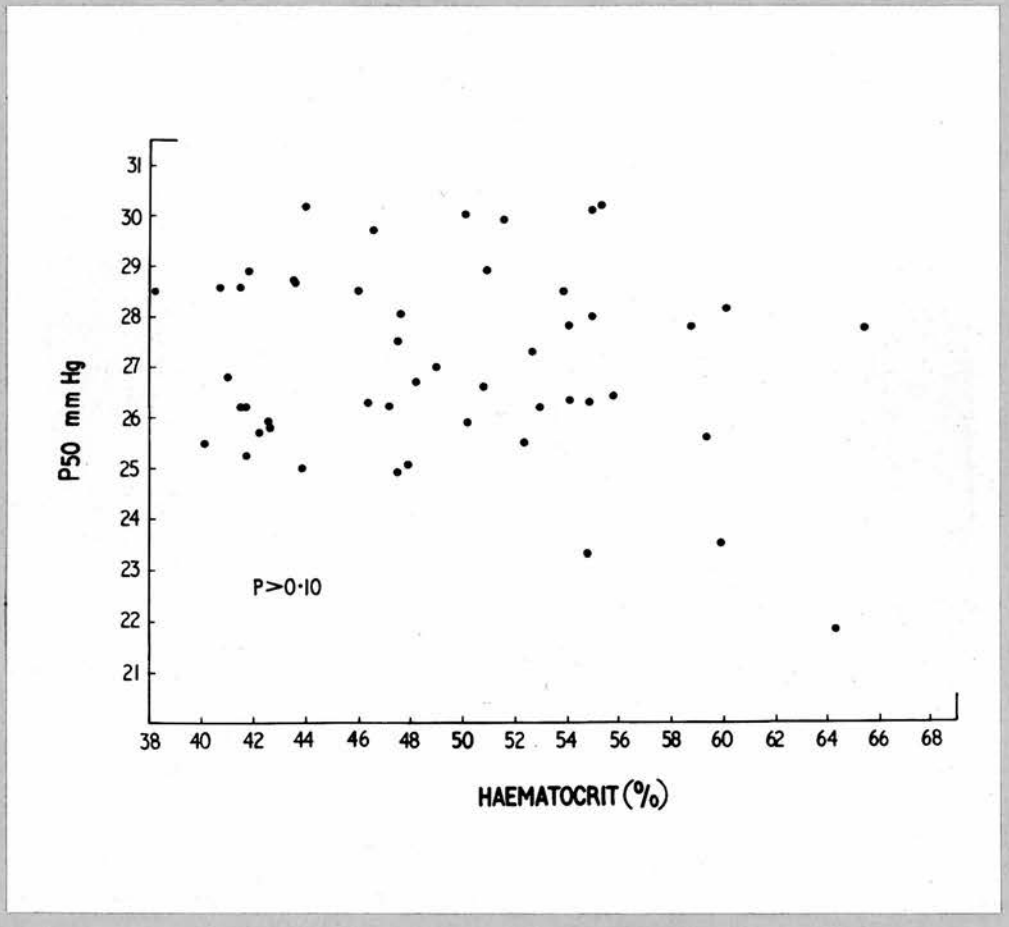


Figure 34a.

No correlation was found between $P_{50(7.4)}$ and the haematocrit.

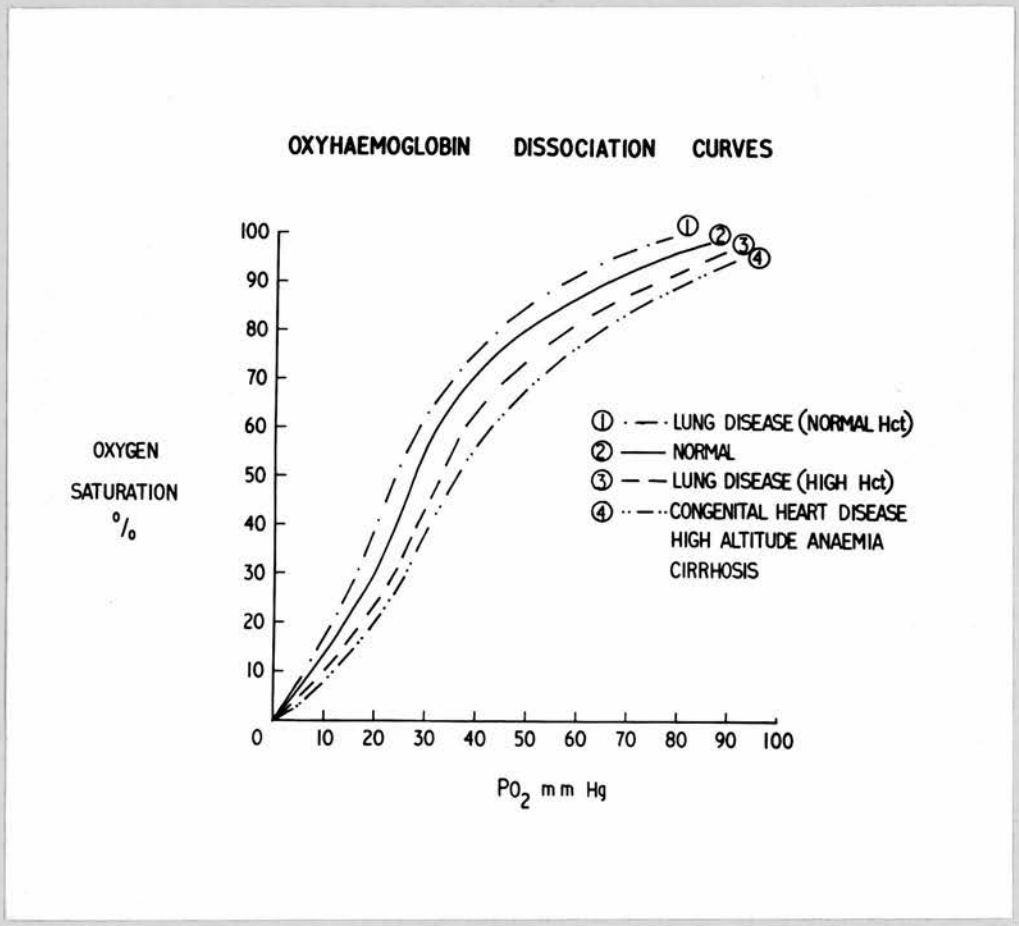


Figure 34b.

Shift in dissociation curve with different diseases. Adapted from Lenfant, 1969.

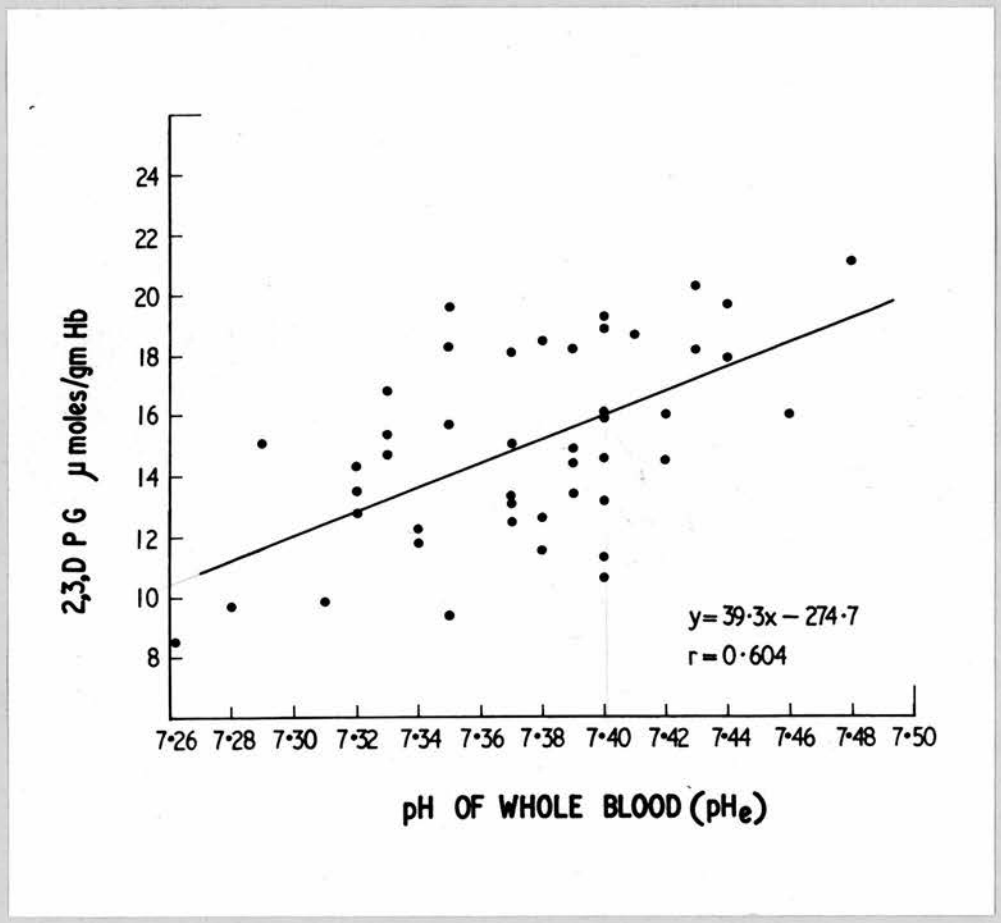


Figure 35.

2,3 diphosphoglycerate is plotted against the patient's arterial pH, taken at the time of study. ($r = 0.604$, $p = < 0.001$).

retention, with resultant renal compensation by increased bicarbonate reabsorption in their renal tubules. Data describing the condition of the remaining patients who did not suffer from chronic bronchitis is presented in Table 8.

The arterial PO_2 in all the patients, was plotted against the corresponding 2,3 DPG level, expressed as moles per gram of haemoglobin (Fig.33), no significant correlation being found.

The $P_{50(7.4)}$ value did not correlate with haematocrit (Fig.34a), contrary to the findings of Lenfant, who, in 1969, found a decreased P_{50} with a low or normal haematocrit and an increased P_{50} with a high haematocrit in patients with chronic obstructive lung disease. (Fig.34b).

A significant correlation was found between 2,3 DPG levels and arterial pH ($r = 0.604$, $p = < 0.001$) for all patients studied (Fig.35), but when the arterial pH was plotted against 2,3 DPG levels in these patients with chronic bronchitis alone, the correlation was not significant ($r = 0.215$, $p = > 0.1$).

P_{50}, as determined by the 'mixing' technique and corrected to pH 7.4, was found to be significantly correlated with 2,3 DPG levels, when data from all the patients and normal subjects was pooled (Fig.36) ($r = 0.597$, $p = < 0.001$). The scatter, however, was large, suggesting that other factors participated in this relationship. It was hoped to reduce this scatter by correcting for changes in Mean Corpuscular Haemoglobin Concentration (MCHC). According to Bellingham, Detter and Lenfant (1971), a rise in MCHC of

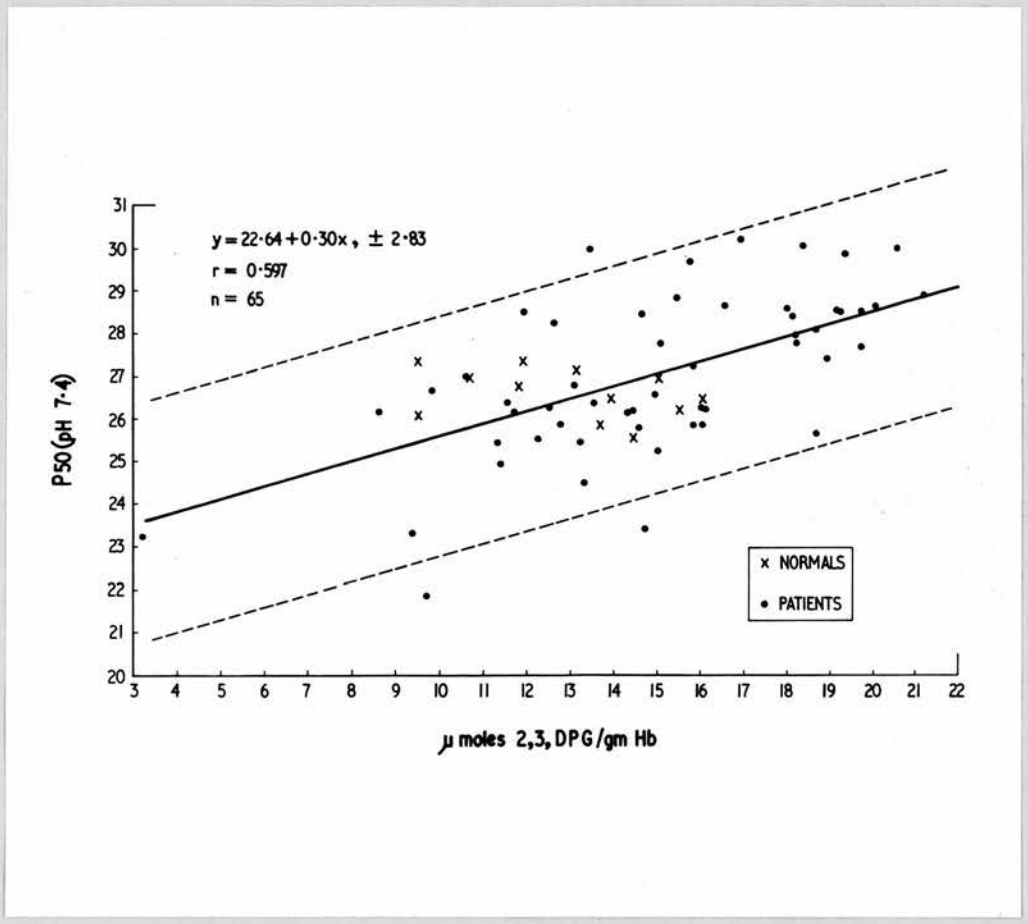


Figure 36.

$P_{50(7.4)}$ as measured by the 'mixing' technique, is correlated with levels of 2,3 diphosphoglycerate for all patients (●) and normal subjects (X) in this study. ($r = 0.597$, $p = < .001$.)

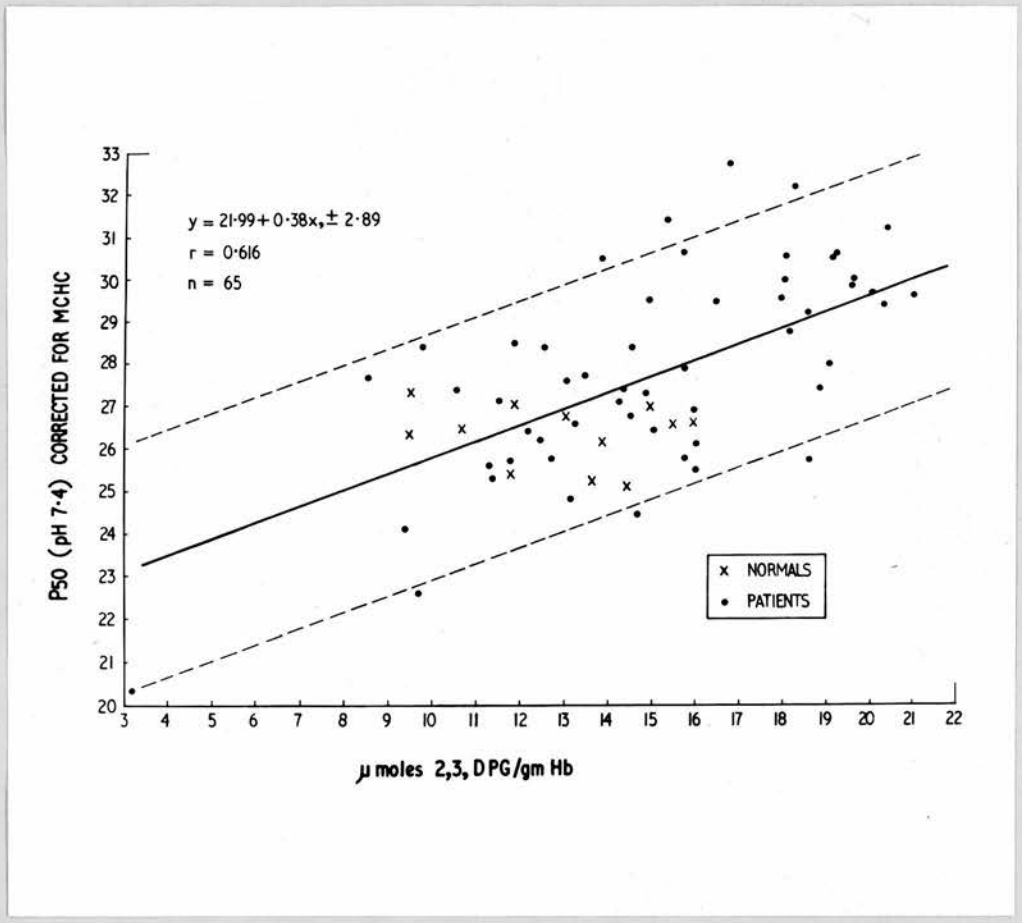


Figure 37.

$P_{50}(7.4)$, after correction for MCHC changes (Bellingham, 1971), is plotted against 2,3 DPG levels, in all patients (●), and normal subjects (X). ($r = 0.616$, $p = < .001$).

1.0 gm/100 ml, results in an increase of 0.5 mm Hg in $P_{50(7.4)}$.

Using this factor, all P_{50} results were corrected to a standard MCHC of 33 gm/100 ml (the normal value found in this study), and the values for the corrected $P_{50(7.4)}$ replotted against the 2,3 DPG concentrations, again expressed as μmoles/gm of haemoglobin. (Fig.37). There was a slight improvement in correlation ($r = 0.611$, $p = 0.001$) and an increase in slope of the relationship, but the scatter persisted.

Finally, $P_{50(7.4)}$ was calculated from the equation of Bellingham, Detter and Lenfant (1971), who included factors for 2,3 DPG and MCHC in addition to the normal correction factors of pH, temperature and base excess. The normal values for 2,3 DPG, P_{50}, MCHC and the Bohr effect of 12.8 μmoles/gm.Hb., 26.7 mm Hg, 33 gm/100 ml and -0.500 respectively, found in this study, were substituted in the equation.

As all measurements were taken at 37°C, the correction factor for temperature was therefore omitted, so that the final equation read as follows:-

$$\text{Log } P_{50(iv)} = \log \big/ 26.7 + 0.5\ (\text{MCHC}-33) + 0.69\ (\text{DPG}-12.8) \big/ + 0.0013\ \text{BE} + 0.5\ (7.4-\text{pH}).$$

$P_{50(7.4)}$ was calculated in this way for all patients with chronic hypoxia and CO_2 retention, after application of the experimentally determined Bohr effect to correct the in vivo P_{50} value to the

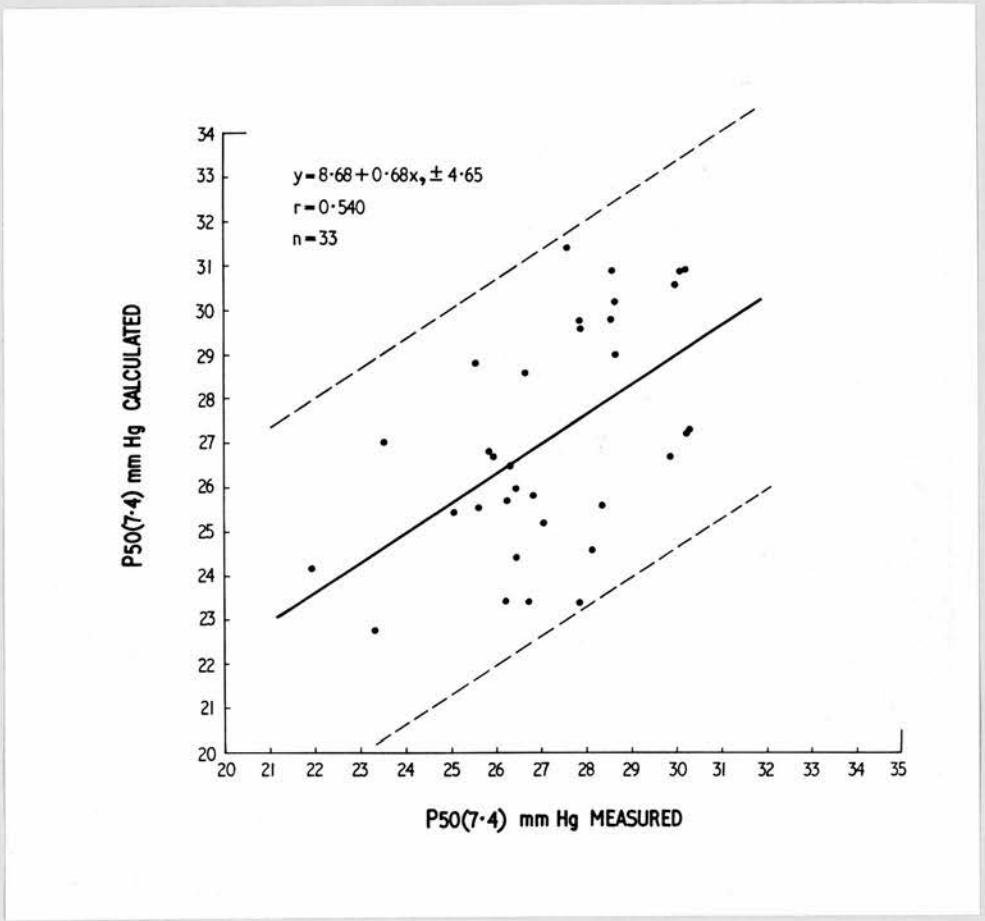


Figure 38.

$P_{50(7.4)}$ as measured by the 'mixing' technique is plotted against $P_{50(7.4)}$, as calculated from the equation of Bellingham, 1971, for all patients with chronic CO_2 retention and chronic hypoxia. A Bohr factor of -0.50, and the normal values for $P_{50(7.4)}$, 2,3 DPG and MCHC found in this study, were substituted in the equation.

Equation:-

$$\text{Log } P_{50(iv)} = \log \left[26.7 + 0.5\ (MCHC - 33) + 0.69\ (DPG - 12.8) \right] + 0.0013\ BE + 0.5\ (7.4 - pH) + 0.024\ (T - 37)$$

($r = 0.540$, $p = < .01$).

standard pH of 7.40. These values were compared to $P_{50(7.4)}$ measurements obtained from the 'mixing' technique (Fig. 38), and although the correlation was significant ($r = 0.540$, $p = < 0.01$), there was an appreciable scatter around the line of regression, suggesting that yet other factors might be determining the relationship between $P_{50(7.4)}$ and 2,3 DPG levels in these chronically hypoxic, hypercapnic patients.

C H A P T E R V

DISCUSSION OF METHODS.

a) The Determination of P_{50}.

As demonstrated in the 'Results' section, the 'mixing' technique gave rise to a $P_{50(7.4)}$ value on normal subjects which was within the accepted range of 26.6 to 26.9 for this method. Having shown that the P_{50} mixture in this study was indeed 50% saturated, future work using the same mixing technique would not require the oxygen content and the capacity to be measred on all the samples prior to sampling the P_{50} mixture. This would reduce the need for such a large blood sample, enabling duplicate P_{50} measurements to be made on 10 mls heparinized blood instead of 30 mls as used in this study. This mixing technique can be used if more than one point on the dissociation curve is required, merely by taking different proportions of the oxygenated and reduced samples and measuring the PO_2. In contrast, where the oxygen saturation is determined directly, many gas mixtures are required to establish different points on the oxygen dissociation curve. (Torrance and Lenfant, 1969/70).

Where the shape of the curve is of prime importance, the 'mixing' technique is no longer the most efficient. The rapid scanning method of Duvelleroy (1969) gives a complete picture of the whole curve in only 20 minutes on one 10 ml sample of blood. The method involves the rapid exposure of deoxygenated blood to pure oxygen while rapidly stirring. The increase in PO_2 of the deoxygenated sample is recorded on the X axis of an XY plotter by the PO_2

electrode, whilst simultaneously the decrease in oxygen (proportional to oxygen content) is measured on the Y axis of the XY plotter, again by a PO_2 electrode. Numerous technical difficulties are involved with this technique (Robert and Zaar, 1971), and there is a need for specially designed equipment. Bellingham and Lenfant in 1971 compared this technique with the 'mixing' technique and did not demonstrate any further advantages other than the ability to look at changes in shape of the curve.

Having established the accuracy with which the 50% saturated sample was obtained, equilibration at two CO_2 levels is then required to measure the Bohr effect. In this study the PO_2 at two known saturations was measured at three different pH values which enabled the derivation of P_{50} at more than one pH value, and thus calculation of the Bohr effect and also the n of Hill's equation, a measure of the interaction of haem molecules.

Recommended Bohr effect factors range from -0.45 to -0.58 (Torrance and Lenfant, 1969/70). The value -0.48 was originally given by Dill (1937) and later used by several authors including Severinghaus in 1958.

By keeping PCO_2 constant, and adding fixed acid or base to the blood, Astrup, Engel, Severinghaus and Munson (1965) recommended a factor of -0.50. The Bohr effect of -0.50 found in this study was calculated by varying the PCO_2, and values did not vary greatly from this figure in 6 patients with chronic CO_2 retention due to bronchitis and emphysema.

T The method of measurement of oxyhaemoglobin curves by Duc and Engel (1969/70) does not require correction for the Bohr effect, as any deviation of pH during measurement is automatically compensated by addition of 0.1N NaOH.

In this study, the Bohr effect did not vary greatly, and did not correlate with P_{50} or DPG which was also confirmed by Hilpert (1963). The experiments of Bauer (1969) demonstrated a reduction in the Bohr effect when 2,3 DPG was totally absent, with a PCO_2 of 40 mm Hg, but a normal Bohr effect when both 2,3 DPG and PCO_2 were at normal values, but he did not determine the effect of experimentally increasing the concentration of 2,3 DPG.

b) <u>The Assay of 2,3 DPG</u>.

Values for 2,3 DPG differ according to the method used, but the majority of whole blood measurements in normal subjects are included within the range 4-6μmoles per ml of erythrocytes (Garby, 1971). Normal 2,3 DPG values in this study have been quoted inμmoles/ml erythrocytes for comparative purposes, but the recent observation that the Mean Corpuscular Haemoglobin Concentration (MCHC) affects P_{50} (Bellingham, Detter and Lenfant, 1971), led to the presentation of results in terms ofμmoles per gram of haemoglobin. This is especially important in cases of chronic pulmonary insufficiency where the red cell water content is increased. (Grant, McDonald and Edwards, 1958, Tushan, 1970). The MCHC was reduced in the majority of patients in this study, so that expression of results as DPG/gm Hb.

prevents variation in the cell size from affecting the result.

Determination of 2,3 DPG by ion exchange chromatography of a trichloracetic extract, followed by phosphorus analysis on the individual eluents (Bartlett, 1959), has been modified by Robinson, Loder and de Gruchy in 1961, by Torrance, Jacobs, Restrepo, Lenfant and Finch in 1970, and by de Verdier and Killander in 1962. This method is superior where other glycolytic intermediates, such as ATP, are to be estimated, in addition to 2,3 DPG concentrations, but is less specific and more time-consuming than the enzymatic assays. The chromotropic acid assay of Bartlett (1959) is used to measure the concentration of 2,3 DPG in the appropriate eluent after passage through the ion exchange column.

The specificity of this determination by chromotropic acid has been questioned. Acid citrate dextrose (ACD) used as an anti-coagulant, adds to the colour of chromotropic acid (Eaton and Brewer, 1969), and substances appear in the plasma after exercise which also contribute to the development of colour after addition of chromotropic acid. (Faulkner, Brewer and Eaton, 1969). In 1968, Eaton and Brewer used 'the more specific enzymatic method' of Krimsky, to check the measurement of 2,3 DPG by the chromotropic acid method of Bartlett.

Enzymatic assays have increased rapidly in recent years culminating in the commercial production of enzymatic 'kits' for 2,3 DPG assay by two companies, Sigma and Calbiochem.

The cofactor reaction of 2,3 DPG on the phosphoglycerate mutase, with the subsequent measurement of an enzymatically linked reaction (Fig.16) has the advantage of specificity, for other substances present in the extract do not react in this way. Providing that the phosphoglycerate mutase (PGAM) is free from contamination, and that 2,3 DPG is present in limiting amounts, the initial rate of the reaction is proportional to the concentration of 2,3 DPG. The recent enzymatic assays where the phosphatase activity of the phosphoglycerate mutase is stimulated by phosphoglycolic acid (Fig.17), thus converting 2,3 DPG to a monophosphoglycerate, may result in contamination:- a) of 2 PGA, if the reaction involved is the conversion of pyruvate to lactate; or b) 3 PGA if the reaction involved is the conversion of 2 PGA to G-3-P; or c) ATP, if the inorganic phosphate from the hydrolysed 2,3 DPG is measured colorimetrically. This latter may be prevented by conversion of 2 PGA or 3 PGA to the assay end point prior to 2,3 DPG assay. Conversion of ATP to inorganic phosphate can be suppressed by addition of disodium ethylenediamine tetra-acetate (EDTA) (Maeda, Chang, Benesch and Benesch, 1971), although EDTA is known to increase erythrocyte glycolysis (Garby, 1970). Maeda et al. (1971) adapted the technique of Rose and Liebowitz (1969), where the inorganic phosphate liberated from 2,3 DPG hydrolysis is measured, to determine the 2,3 DPG concentration of a hemolysate. Only 0.2 mls of blood were required so that a finger prick sample sufficed. However, they used glycylglycine as a buffer, which according to Tsuboi and

Fukunaga (1970) increased red cell glycolysis.

There are disadvantages in using the 2,3 DPG cofactor action on phosphoglycerate mutase as an assay:-

1. There is considerable dilution of the extract before the concentration is within the range of standards.
2. The range of sensitivity, where the rate is proportional to the concentration of added cofactor is small.
3. The changes in optical density are small.
4. The reaction rate is very sensitive to minor variations in assay condition, in contrast to an end point assay.

In this study, accuracy in dilution was improved by use of Oxford Sampling pipettes (supplied by Boehringer), which are precalibrated to deliver an accurate amount (SD = $\pm$ 0.001). Although the sensitive range of the assay is small, an extract exceeding these limits can either be further diluted or concentrated to bring it into the range of standard solutions. The change in optical density, although small, can be magnified by using a potentiometric recorder, with a scale expansion device. The use of a recording spectrophotometer enables any deviation from linearity to be immediately identified. The ability to record each stage of the reaction enables impurities or contamination to be observed before addition of the extract.

The 2,3 DPG cofactor action on PGAM, used in this study was only preceded by one reaction, in contrast to the method of Nygaard and Rorth (1969) where the 2,3 DPG cofactor action on PGAM is coupled to four other reactions. This minimises the possibility of minor variations affecting the assay.

The Extraction Procedure.

Deproteinisation of the blood sample may be accomplished by addition of trichloracetic acid (TCA), perchloric acid (PCA) or by heat treatment.

TCA was avoided, as unless very small quantities of extract are taken, TCA interferes with optical density readings at 240 nm. (Krimsky, 1963).

Perchloric acid, in varying proportions is frequently used. Prior to enzymatic assay by the Krimsky method, Baumann, Bauer and Bartels (1971) used 9 volumes of PCA to 1 volume of extract, Oski, Gottlieb, Delivoria-Papadopoulos and Millar (1969), used 2 volumes of PCA to 1 volume of extract, and Hjelm (1969) used 5 volumes of PCA to 1 volume of extract. To ensure complete protein precipitation without excessive dilution a 5 to 1 ratio was taken in this study.

Diederich, Diederich, Luque and Grisolia (1969) claim that 2,3 DPG continues to bind to oxyhaemoglobin, even after desaturation of the blood by perchloric acid or heat treatment. They found the ratio of bound 2,3 DPG to oxyhaemoglobin as 1 : 20, so that assuming

an intraerythrocytic oxyhaemoglobin concentration of 5μmoles/ml of red blood cells, an error of 0.25μmoles/ml would occur if the haemoglobin was saturated. As venous samples were taken for 2,3 DPG analysis the error would approximate to 0.12μmoles/ml. This error is within the precision of the assay and would be consistent throughout the study.

DISCUSSION OF RESULTS.

The concentration of 2,3 DPG varied in the patients studied. The group with chronic bronchitis, hypercapnia and hypoxaemia, had low, normal, and high values. The impression from the literature suggests hypoxia in any form is compensated by an increase in 2,3 DPG despite the varying regulatory processes involved, but in this group of patients, with chronic lung disease, an adaptive increase in 2,3 DPG was not always apparent. Low, normal and high values were also found for $P_{50(7.4)}$ measurements, and these were found to correlate significantly with the 2,3 DPG concentrations ($r = 0.597$, $p = < 0.001$) (Fig.36). However, the large scatter around the regression relationship indicated that other factors contributed. The correction of P_{50} to a standard MCHC of 33 gm/100 ml marginally improved the correlation, but considerable scatter remained.

The lack of correlation between arterial PO_2 and 2,3 DPG in patients with chronic lung disease ($p > 0.1$) distinguishes this group of patients from other hypoxaemic conditions where levels of 2,3 DPG are increased despite the variation in the mechanism causing such a rise. This includes observations in non-congenital cardiac disease (Woodson, 1970), congenital heart disease (Oski, 1970), anaemia (Bonn and Jandl, 1970), high altitude studies (Lenfant, Torrance et al. 1968), and red cell mass deficits (Valeri and Fortier, 1969).

Out of the three groups of subjects studied by Lenfant, Ways, Aucutt and Eruz (1969), two groups increased their $P_{50(7.4)}$ values,

while the third group, suffering from chronic obstructive lung disease had a variable $P_{50(7.4)}$ response, some having values below normal, and some greater than normal. Unfortunately, 2,3 DPG levels were not measured in that study, and although $P_{50(7.4)}$ correlated significantly with the haematocrit, this observation has not been verified in further studies. Haematocrit and $P_{50(7.4)}$ were not significantly correlated in this study ($p > 0.1$) (Fig.34a).

A significant negative correlation between red cell 2,3 DPG concentration and whole blood haemoglobin concentration has been found in normal subjects (Eaton and Brewer, 1971), in clinical conditions of anaemia (Hjelm, 1969), and experimental conditions of anaemia and polycythaemia in rats (Duhm and Gehrlach, 1969), but not in patients with acidosis. (Astrup, 1969). In this study a negative correlation was found in the patients with chronic bronchitis between haemoglobin levels and 2,3 DPG but only at the 2% level ($r = 0.398$, $p = <0.02$). Secondary polycythemia, with an increase in haemoglobin may compensate for the failure of 2,3 DPG to be raised in several of the patients with chronic bronchitis. Gallagher in 1971, studied 16 adults with longstanding asthma and found that these subjects had no compensatory increase in 2,3 DPG, as measured by the method of Krimsky. He found a significantly higher haemoglobin concentration of 16.4 ± 1.4 gm/100 ml compared to a control value of 14.8 ± 1.3 gm/100 ml., an increase similar to that found in this study in the bronchitic group where the mean haemoglobin concentration of 15.7 ± 1.9 gm/100 ml exceeded the normal of 14.0 ± 0.95 gm/100 ml.

Gallagher took 6 arterial samples for blood gas analysis, finding a mean arterial PO_2 of 61 ± 16 mm Hg and arterial PCO_2 of 48 ± 9 mm Hg and an arterial pH of 7.39 ± 0.02. Although these values deviate less from normal than those found in the group of chronic bronchitics studied here, viz:- PaO_2 = 49 ± 10 mm Hg, $PaCO_2$ = 56 ± 13 mm Hg, pH = 7.36 ± 0.04, they illustrate similar conditions of hypoxia, hypercapnia and a compensated pH.

The acidity of the blood has been shown repeatedly to strongly influence the concentration of 2,3 DPG, decreasing with acidosis and increasing with alkalosis. It appears that in many hypoxic conditions raised levels of 2,3 DPG may largely result from concomitant increases in pH. In this study, taking the results from all patients studied, a significant correlation was found between whole blood pH and 2,3 DPG concentrations ($r = 0.604$, $p \leq 0.001$) (Fig.35). However, when those patients with metabolic alkalosis and ketoacidosis were excluded, and only the group with chronic bronchitis and hypercapnia included, the correlation was not significant ($p > 0.1$). The majority of pH values in these patients ranged from 7.30 to 7.40, as they had long standing CO_2 retention, with consequent renal compensation. A mild metabolic alkalosis may have contributed to these pH values due to the therapeutic administration of the diuretic, Frusemide, in these patients. PCO_2 and serum bicarbonate are known to increase in normal subjects after 4 days of oral Frusemide. (Iff and Flenley, 1972).

Author	Condition	Number(n)	Plasma pH	DMO^-	Cl^-
Tushan et al.	Normal	14	7.42 ± 0.03	7.12 ± 0.03	7.22 ± 0.04
	Hypercapnia	10	7.38 ± 0.06	7.18 ± 0.08	7.19 ± 0.06
Platts & Greaves	Normal	7			7.24
	Hypercapnia	13			7.21

Figure 39.

Results from Platts and Greaves (1957) and Tushan et al. (1970), indicating a normal pH_i in hypercapnia.

A very small change in pH can influence 2,3 DPG levels (Astrup, 1969), and therefore changes in intraerythrocytic pH were studied. The red cell pH has been shown to closely reflect changes in plasma pH during acute respiratory or metabolic acidosis and alkalosis. (Bromberg, 1965, Manfredi, 1967, Battaglia, Behrman, Hallegers and Battaglia, 1965). The correlation between intraerythrocytic and plasma pH depended on the concentration of 2,3 DPG in these acute studies. (Lenfant, Torrance and Reynafarje, 1971, Bellingham, Detter and Lenfant, 1971, and Duhm, 1971). Intraerythrocytic pH (pH_i) measurements have been determined in chronic hypercapnia by Platts and Greaves (1957), and Tushan, Bromberg, Shively and Robin (1970). They both found stable pH_i results, which were not significantly different from pH_i results in normal subjects. (Fig.39).

Studies of in vitro intracellular acid-base changes during chronic hypercapnia in isolated muscle cells indicated that the hydrogen ion concentration remains in the normal range even though the PCO_2 was as high as 70 mm Hg. (Relman, Adler and Roy, 1963).

The mean arterial pH_i of 6 hypercapnic patients, was 7.17 $\pm$ 0.02 in this present study. This together with the value of 7.16 found in one normal subject is very similar to the mean values of 7.15 reported by Pucell (1966), and of 7.19 reported by Bromberg (1970). pH_i correlated significantly with pH_e (the pH of whole blood) as shown in Fig. 31. The DMO technique for measurement of pH_i gave similar results to the freeze-thaw technique in tonometered blood

samples (Fig.30), so that although these results are insufficient to draw any firm conclusions, there is an indication that pH_i was within the normal range, as was pH_e, in these patients with chronic stable hypercapnia.

Long term residents at high altitude have a pH_e within the normal range for those at sea level, yet 2,3 DPG levels are increased in proportion to the concentration of deoxygenated haemoglobin, although this is of less degree than that found in short term sojourners at high altitude, where a raised pH_e value, (due to hyperventilation of altitude hypoxia) stimulates synthesis of 2,3 DPG. (Torrance et al. 1970/71).

The resident at high altitude may have similar hypoxaemia to these bronchitic patients, but contrasts in having a low PCO_2, unlike the bronchitic with chronic CO_2 retention. Any stimulus to synthesis of 2,3 DPG from increase in the proportion of reduced haemoglobin at altitude may well be counteracted by an increase in the formation of carbamino haemoglobin in the bronchitic for CO_2 combines preferentially with haemoglobin in the reduced form, and this will increase the acidity of the red cell cytoplasm. Thus the decreased dissociation of hydrogen ions from reduced haemoglobin which would stimulate diphosphoglycerate mutase and thus increase 2,3 DPG (Fig.40, factor 1), is counteracted by an increase in production of hydrogen ions from formation of carbamino haemoglobin which would inhibit diphosphoglycerate mutase, thus decreasing 2,3 DPG formation (Fig.40, factor 2). In addition, CO_2 and 2,3 DPG

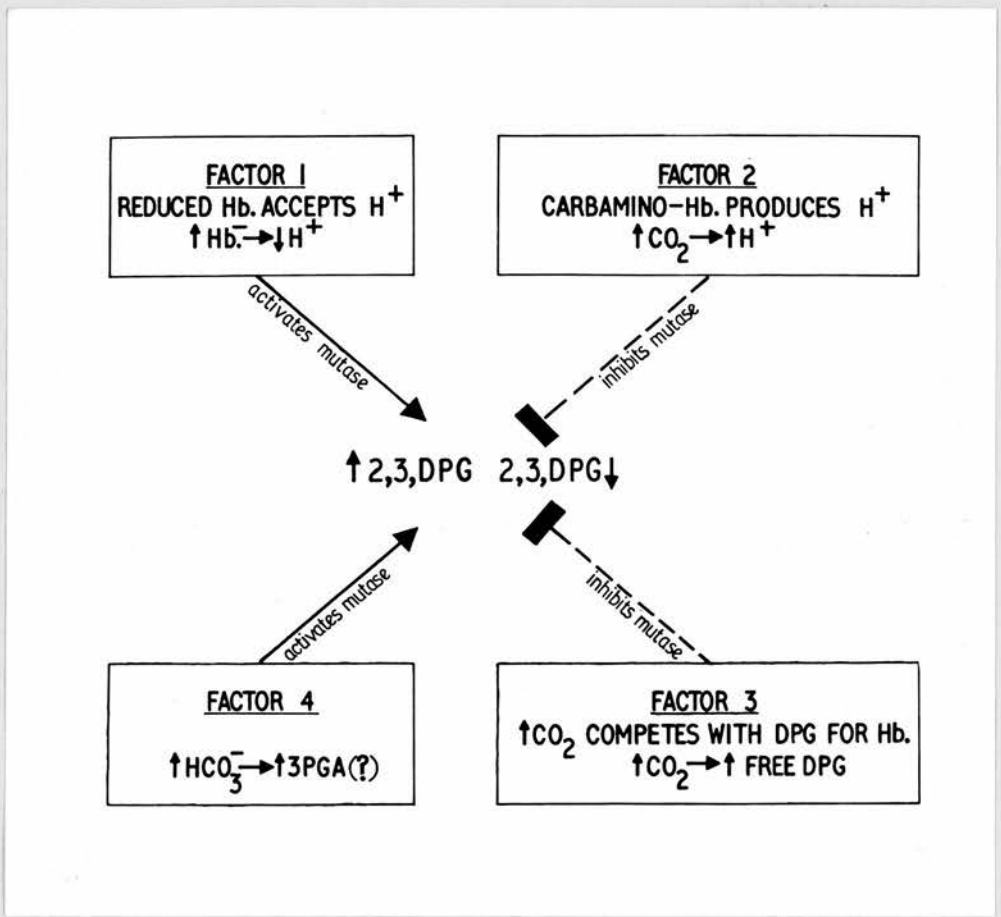


Figure 40.

Suggested factors participating in the regulation of 2,3 DPG levels by influencing DPG-mutase.

compete for the same binding sites on the haemoglobin molecule (Bauer, 1970), so that increased levels of CO_2 may result in decreased DPG binding, with a consequent increase in free 2,3 DPG, which in turn inhibits diphosphoglycerate mutase (Rose, 1968), so preventing an increase in formation of 2,3 DPG. (Fig.40, factor 3). However, this latter effect is probably trivial within the range of PCO_2 encountered in clinical conditions (Rorth, 1970).

In vitro studies by Forstner, Fortier and Lionetti (1968) showed that high concentrations of bicarbonate (40 mM) produced small but consistent increases in DPG synthesis. (Fig.40, factor 4). The mechanism was thought to be due to formation of 3 PGA, but this was not confirmed. 3 PGA can both stimulate 2, 3 DPG formation by acting as a cofactor for DPG-mutase, or decrease the synthesis of 2,3 DPG by inhibition of DPG-phosphatase. The activity of DPG mutase is four times as great as DPG-phosphatase, and Forstner et al. (1968) found that decline of DPG-mutase activity had a greater effect on 2,3 DPG than DPG-phosphatase stimulation. The overall effect on 2,3 DPG levels will depend upon a balance of all stimulatory and inhibitory effects.

It is now established that the level of inorganic phosphate also influences the level of 2,3 DPG, as for example in uraemic acidosis, retention of phosphate overcomes the influence of pH, so that 2,3 DPG is raised to very high levels. (Astrup, 1969). It is unlikely that inorganic phosphate (P_i) would change rapidly in these patients with chronic bronchitis, but measurements in two, both with

chronic bronchitis and CO_2 retention, one with 2,3 DPG levels above and one below normal, were made.

The inorganic phosphate was 3.75 mg/100 mls plasma in the first and 3.85 mg/100 mls plasma in the second, by the method of Gomorri (1942) (which gives a normal range of 2.5 - 4.5 mg/100 mls plasma), suggesting that inorganic phosphate levels were unlikely to vary enough in these patients to account for the differences in 2,3 DPG levels.

Valeri and Fortier in 1969 demonstrated an increase in 2,3 DPG levels in anaemia where the red cell mass was reduced, but they later found that 2,3 DPG levels were also increased in patients with heart and lung disease who were not anaemic. In the present study, red cell mass was measured in 9 patients. Three patients were within the normal range (30.3 mls/kg $\pm$ 4.5, Passmore and Robson, 1968), and 6 were greater, with 3 values of 58, 60 and 65 mls/kg. indicating very marked polycythaemia, secondary to their chronic hypoxaemia. However, no significant correlation was found between red cell mass and 2,3 DPG concentration in these patients ($r = 0.458$, $p => 0.1$).

Oski, Gottlieb and Papadopoulos in 1969 studied 2 groups of 5 patients with chronic hypoxia; one group with chronic lung disease and the other with cyanotic congenital heart disease. In their hands, normal levels of 2,3 DPG, as measured by Schröter and Heyden's (1965) modification of Krimsky gave a mean value of 4.1 $\pm$ 0.5μ moles/ml erythrocytes, with a range of 3.2 - 5.2μ moles/ml erythrocytes, not dissimilar to the normal values observed in the present study. The

5 patients with chronic lung disease were not defined as to their acid-base state, but some had a degree of hypercapnia, and they showed a significant increase in mean level of 2,3 DPG. (Mean = 5.3μmoles/ml cells, range = 4.7 - 5.7μmoles/ml cells). This, however, was much below the level found in patients with cyanotic congenital heart disease (Mean = 7.1μmoles/ ml erythrocytes, range = 5.4 - 8.9μmoles/ml erythrocytes). (It is quite likely that the arterial PCO_2 was below normal in this latter group, but no data was given on this point).

Edwards (1971) found low, normal and high values of $P_{50(7.4)}$ and also of 2,3 DPG concentrations in the red cells of 5 patients with chronic obstructive lung disease, where the arterial PO_2 was between 32 and 47 mm Hg and the arterial PCO_2 between 47 and 62 mm Hg, remarkably similar to the values observed in the present study. The arterial pH of 7.31-7.36 observed in their patients indicated a greater degree of acidosis than that found in the chronic bronchitic patients of the present study. Edwards concluded that the hypoxia induced a rise in 2,3 DPG, which was counteracted by the acidosis (inducing a reduction in 2,3 DPG), so that the in vitro $P_{50(7.4)}$ did not change. However, with an acidotic pH in vivo, the Bohr effect would result in a decrease in the affinity of oxygen for haemoglobin, thus counteracting the effects of a change in 2,3 DPG levels. Correction of $P_{50(7.4)}$ to the arterial pH obtained in vivo in the chronic bronchitic patients of the present study in fact resulted in an increase of P_{50} in vivo in many patients, but in some patients,

where the in vitro P_{50} was already low, and the arterial pH near to normal due to renal tubular reabsorption of bicarbonate, no such rise in P_{50} in vivo was observed.

It is probable that another, as yet undetermined factor affects the relationship between 2,3 DPG concentrations in the red cell and the $P_{50(7.4)}$ in these patients with chronic bronchitis and emphysema who have long standing persistent hypoxaemia and hypercapnia. In 1971, Bellingham, Detter and Lenfant proposed an equation which would yield the in vivo P_{50} based on measurements of 2,3 DPG and MCHC, in addition to the conventional determinants of temperature, pH and base excess, which are incorporated in the convenient slide rule described by Severinghaus (1966). Derivation of P_{50} in vivo from this equation (using normal values for MCHC, 2,3 DPG and $P_{50(7.4)}$ derived from the present studies), was possible in our bronchitic patients, where all these factors were measured. The Bohr effect measurement allowed conversion to $P_{50(7.4)}$, enabling a comparison to be made with the $P_{50(7.4)}$ as directly measured by the mixing technique. The correlation was significant ($r = 0.540$, $p = <0.01$) (Fig.38), but an appreciable scatter of values about the regression line persisted. It seems possible that <u>chronic</u> hypoxaemia, a major characteristic of our patients, unlike those on which Bellingham et al. (1971) based their prediction equation, may yet prove to have a direct effect on the 2,3 DPG/P_{50} relationship.

A remaining question resulting from these studies, is whether these patients with similar degrees of hypoxia, hypercapnia and

compensated respiratory acidosis, yet with increased 2,3 DPG levels, have any physiological advantage over those with normal or decreased levels of 2,3 DPG.

CHAPTER VI

The Physiological Significance of 2,3 DPG in Chronic Hypoxaemia.

The physiological significance of a shift in dissociation curve has recently been questioned. The assumption that a right-ward adaptive shift of the curve does increase the amount of oxygen delivered to the tissues in vivo has been adopted by several authors, for example by Lenfant in his early work.

Eaton, Brewer, Schultz and Sing (1969) experimentally determined the rate of oxygen extraction from whole blood passing through a permeable silastic tube surrounded by 95% nitrogen and 5% CO_2. They found a significant correlation between the rate of loss of oxygen from a solution of oxygenated blood and the sum of the concentrations of ATP and 2,3 DPG in the blood of 20 female subjects, with a similar, but not significant relationship in the blood of 20 male subjects. They found the mean rate of oxygen extraction to be significantly greater in the blood of two groups of patients with anaemia and one group of patients with lung disease. However, there were considerable differences in the degree of elevation of 2,3 DPG levels in their different groups of subjects, and they concluded that some other factors than 2,3 DPG were contributing to the delivery of oxygen.

As a part of their assessment for long term oxygen therapy, right heart catheterisation was carried out (Dr. B.J. Kirby) in patients 14,31,32,42,44,45,47, and 48, $P_{50(7.4)}$ and 2,3 DPG measurements being made on the same day. Mixed venous blood was

sampled from the pulmonary artery, and arterial blood from the brachial or left radial artery, samples being analysed for PO_2, PCO_2 and pH. (Table 11).

Using these measurements, it is possible to assess the physiological significance of any shifts in the dissociation curve as a result of variation in 2,3 DPG levels. The content of oxygen of the mixed venous and arterial blood was calculated in two ways:-

a) By assuming a normal dissociation curve, therefore using the Severinghaus (1966) slide rule to apply the conventional corrections for temperature, pH and base excess.

b) By using the directly measured in vitro P_{50} of the patient and calculating the percentage oxygen saturation of haemoglobin from Hill's equation ($\log \frac{S}{1-S} = n \log PO_2$) after correction to the actual arterial pH using the previously determined Bohr effect of -0.500.

Direct measurement of oxygen capacity (the oxygen content of fully saturated blood, assessed spectrophotometrically by the method of King and Wootton, 1959), enabled the oxygen content of these samples to be determined, so that the arterio-venous difference in oxygen content, assuming that the P_{50} was normal, could be compared with the arterio-venous difference in oxygen content at the actual in vivo P_{50} in that patient. A significant correlation ($r = 0.927$,

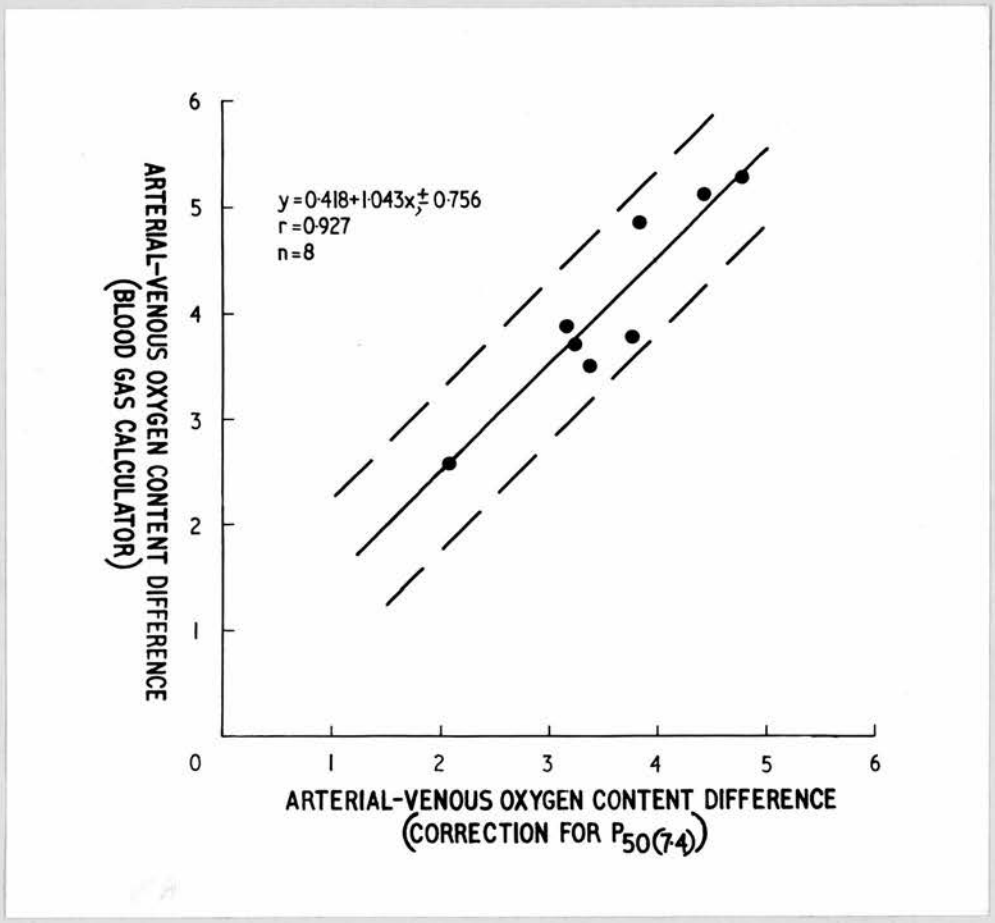


Figure 41.

The arterial-venous oxygen content difference as measured from a) saturation values corrected for P_{50} of patient (X axis) and b) saturation values as calculated from a standard P_{50} as used in a blood gas calculator (Y axis). ($r = 0.927$, $p = < 0.01$).

$p = < 0.01$) was found between the two estimates in these 8 patients, the arterio-venous oxygen content differences, as calculated by the two different methods, being very similar (Fig.41), the regression relationship not being significantly different from the line of identity. When the arterial PO_2 is sufficiently high to lie on the upper relatively horizontal portion of the dissociation curve, and the mixed venous blood lies on the steep part of the curve, a right-ward shift in the dissociation curve will increase the arterio-venous difference in oxygen content for a given level of mixed venous oxygen tension, as shown previously in Fig.4. The arterial PO_2 in these bronchitic patients was usually between 40-50 mm Hg, approaching the steep part of the dissociation curve, and was not far distant from the directly measured mixed venous PO_2 which averaged 34 mm Hg. A right-ward shift in the oxygen dissociation curve, however, in these patients would cause a very similar change in saturation of both the arterial and venous blood, with the result that the arterio-venous difference was little altered.

The oxygen content of the mixed venous sample was calculated from the directly measured PO_2, assuming that P_{50} was normal, and also by using the directly measured in vivo P_{50} as described previously. The difference in the oxygen content of the mixed venous blood thus obtained was related to the directly determined 2,3 DPG concentration in these same patients. (Fig.42). Although only 8 patients were available for study, the correlation was significant at the 5% level ($r = 0.781$, $p = < 0.05$).

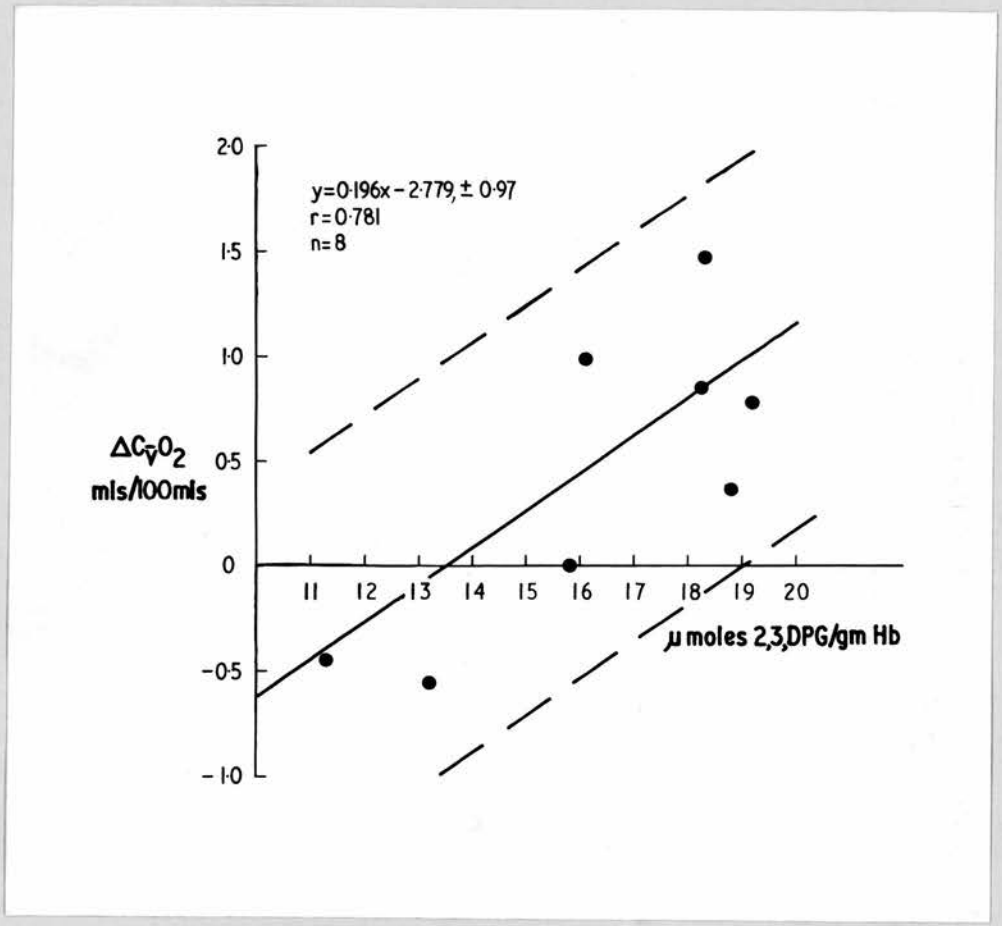


Figure 42.

The oxygen content difference in mixed venous blood measured from a saturation value using a standard $P_{50(7.4)}$ and a saturation value using the patients $P_{50(7.4)}$, is plotted against the concentration of 2,3 DPG in moles/gm. Hb. ($r = 0.781$, $p = < 0.05$).

These results indicate that any right-ward shift in the dissociation curve (expressed as an increase in $P_{50(7.4)}$) is associated with the increased concentration of 2,3 DPG found in the red cells of some of these chronically hypoxic hypercapnic patients. However, such a shift appears to play little, if any, part in allowing a reduction in cardiac output at any constant level of oxygen uptake in these patients, for as Fig.41 shows, the arterio-venous difference in oxygen content is unchanged as a result of this right-ward shift.

Let us consider the effects of a right-ward shift in dissociation curve, arising as a result of a change in $P_{50(7.4)}$ from the normal of 26.6 mm Hg to the highest level seen in these 8 patients, where direct measurements of mixed venous tensions were made, namely 30 mm Hg. If such a bronchitic patient had an arterial PO_2 of 50 mm Hg when breathing air, and if we assume that any respiratory acidosis was completely compensated so that the arterial pH was 7.40, a $P_{50(7.4)}$ of 26.6 mm Hg implies that his arterial oxygen saturation was 85% (working from the Severinghaus (1966) dissociation curve data).

If these patients were moderately polycythaemic as a result of this chronic hypoxaemia, with an oxygen capacity of 22 ml/100 ml of blood, then an arterio-venous oxygen content difference of 4 ml/100 ml of blood would mean an oxygen saturation arterio-venous difference of 18%. These assumptions result from a high normal cardiac output and oxygen consumption at rest. The mixed venous

oxygen saturation must therefore be 67%, and if the venous pH was 7.38, this means that the mixed venous oxygen tension was 36 mm Hg.

If this patient had in fact a $P_{50(7.4)}$ of 30 mm Hg, as did patient 14, and the same arterial PO_2 of 50 mm Hg (determined by the overall minute volume, and ventilation perfusion imbalance in his diseased lungs), it follows that his arterial oxygen saturation would be 77%, (directly measured from a plot of Hill's (1910) equation), assuming the arterial pH remained at 7.40.

Again, for the same cardiac output and oxygen uptake, the arterio-venous oxygen saturation difference remains at 18%, the mixed venous saturation would be 59% and the resultant mixed venous PO_2 is again 36 mm Hg.

It therefore appears that although the oxygen content of both mixed venous and arterial blood is affected by a right-ward shift in the curve at any given PO_2, the transfer of oxygen from the capillary to its site of usage as an electron acceptor in the mitochondria is unlikely to be assisted by any elevation in 2,3 DPG levels in these hypoxic patients. It appears that Lenfant, Torrance and Reynafarje (1971) came to a similar conclusion as to the benefits of any right-ward shift in the dissociation curve following ascent to high altitude. This is in contradiction to the earlier suggestions of Aste-Salazer and Hurtado (1944) which require that the arterio-venous content difference be sufficient to allow the arterial point to lie on the relatively horizontal portion of the curve (PO_2 above 80 mm Hg) and the mixed venous levels to be around the 30-40 mm Hg

level. The very large arterio-venous oxygen content difference that this implies must mean that the cardiac output was severely impaired, as in the shock syndrome. However, in exercise, in normal man, the PO_2 of effluent blood from the exercising muscles can fall to very low levels of below 20 mm Hg. Such a right-ward shift may thus help oxygen transport to exercising muscles in the bronchitic patient, particularly when breathing oxygen-enriched air, when his arterial PO_2 may be around 70-80 mm Hg.

SPECULATION ON THE PHYSIOLOGICAL SIGNIFICANCE OF 2,3 DPG IN CHRONIC HYPOXAEMIA

1. Exercise.

Significant increases in concentrations of red cell 2,3 DPG have been observed in normal subjects following 10 minutes of intense exercise (Faulkner, Brewer and Eaton, 1970), but this was not confirmed by Shappel et al. (1971) and further studies are needed to resolve the role of 2,3 DPG in exercise.

Patients suffering from severe chronic bronchitis and emphysema are only capable of mild exercise, such as level treadmill walking at a speed less than 1 m.p.h. King, Cooke, Leitch and Flenly (In Press) have shown that the resting hypoxaemia of these patients is aggravated by even this level of exercise, yet their hypoxaemia can be corrected by administration of 30% oxygen. They found that the arterial carbon dioxide tension, although raised at rest, did not rise further during exercise while breathing either air of 30% oxygen, and that arterial pH measurements did not fall significantly on exercise, as in normal subjects at such modest work loads.

This constancy of arterial PCO_2 and pH in these patients during exercise, might suggest that 2,3 DPG levels would also remain unchanged in exercise as a result of the poor correlation between arterial PO_2 and 2,3 DPG levels found in this study (Fig.33). The blood flow to the exercising muscles increases in exercise due to the

rise in cardiac output, and therefore an increased volume of blood perfuses the muscle fibres. Effluent venous blood from the muscles will have a very low PO_2 and pH, figures as low as 7.00 for pH having been recorded by Pirnay et al. (1972) in blood samples taken from the deep femoral vein during treadmill exercise. This difference between arterial and muscle effluent pH leads to the idea that the mean systemic pH, similar to the mean systemic PO_2 discussed earlier, may well be considerably lower than the arterial pH on exercise.

If it is agreed that whole blood pH is related to intra-erythrocytic pH (pH_i), and that pH_i determines 2,3 DPG synthesis and the interaction with haemoglobin, it follows that red cell 2,3 DPG levels during exercise may well depend upon this mean systemic pH, and not upon the arterial pH alone. It is therefore suggested the effects of 2,3 DPG on oxygen transport may not be mirrored in arterial PO_2, PCO_2 and pH measurements alone, and that regional PO_2, PCO_2 and pH_i measurements, as well as regional 2,3 DPG concentrations would be worthy of study. However, such studies would raise technical and ethical problems of considerable dimensions.

2. Cardiac Output.

Cardiac output can be measured by using either an Indicator Dilution method, where intravenous injection and subsequent measurement of the time concentration in arterial blood of a dye such as indocyanine green is involved, or by the direct Fick method. The

Fick method requires the accurate measurement of oxygen uptake, and also of the oxygen content of arterial and mixed venous blood. These measurements of oxygen content may be derived from a direct estimate of oxygen saturation, as obtained by the Kipp haemoreflector (Zijlstra, 1957) together with a spectrophotometric recording of oxygen capacity (King and Wootton, 1959), or may be derived from the partial pressures of oxygen in arterial and mixed venous blood, using values for saturation obtained from the standard dissociation curve (as in the Severinghaus Blood-Gas Slide rule). As discussed previously, the dissociation curve on which the slide rule is based depends upon a normal P_{50}. Thus an error may be incurred in calculation of cardiac output by using the Fick principle, when oxygen content values are derived from oxygen partial pressures and this slide rule.

The cardiac catheterisation data allowed arterial-venous oxygen content differences to be calculated both from the Severinghaus slide rule and the actual P_{50} of the patient. The A-V oxygen content differences were found to be closely correlated (Fig.41). Cardiac output values can be calculated from these values if one assumed a value for oxygen uptake. In practice, oxygen uptake varies, but if a constant figure of 250 mls/minute is assumed, cardiac output values calculated from the P_{50} of the patient and a standard P_{50} are closely correlated ($r = 0.83$) but yet with a significant difference between the two sets of results ($T = 3.86$, $P = 0.001$).

Any error incurred in calculation of cardiac output by use of a normal P_{50} instead of the P_{50} of the patient will only apply if the direct Fick method is used with the oxygen contents derived from partial pressure readings.

3. Methaemoglobin (MetHb)

Red blood cells consume very little oxygen themselves, mainly in slow oxidation of haemoglobin to Methaemoglobin (MetHb), the latter containing ferric iron rather than ferrous iron possibly by interfering with the MetHb reducing systems. The MetHb formed in 24 hours only amounts to approximately 0.5% of the total Hb, due to the efficient MetHb reducing systems present in the normal erythrocyte. Exposure of haemoglobin to amyl nitrate, aniline, nitrobenzene, etc., and clinical administration of drugs such as sulfonamides, acetanilid, phenacetin, salicylates, etc. induce the formation of MetHb possibly by interfering with the MetHb reducing systems. The patients suffering from chronic bronchitis and emphysema (Table7) did not receive any such drugs. Levels of MetHb were not measured in this study but concentrations of 2,3 DPG and P_{50} were recorded in one patient (No.3) where a high percentage of Met and Sulph-haemoglobin were present.

A shift to the left of the dissociation curve has been reported in the presence of methaemoglobin, both in vitro and in vivo. The conversion of ferrous to ferric iron in haemoglobin deprives the

haem iron of the physiological function of reversible combination with oxygen. The oxygen affinity of the remaining ferrous iron in the haemoglobin molecule is much increased, so that an increase in P_{50} is seen. In patient No.3 of this study, the P_{50} of 28.6 mm Hg could be either explained by the high concentration of 2,3 DPG (17,95 u moles/gm.Hb) or the high level of Sulph-haemoglobin which is known to decrease the affinity of haemoglobin for oxygen.

Schaefer et al. (1971) showed that exposure of guinea pigs to 15% CO_2 for 7 days results in a leftward shift of the oxygen-dissociation curve, which later returns to normal. The shift corresponds closely to the change in red cell pH decreasing initially and returning to normal later, due to bicarbonate reabsorption by the kidney tubules. MetHb. was found to increase after 24 hours of chronic hypercapnia, and although the level decreased after 7 days, the value was still significantly greater than normal. These authors suggested that the decrease in pH, as a result of increased CO_2, caused phosphofructokinase inhibition, which would limit the reduction of MetHb by decreasing NADH formation. 2,3 DPG measurements were not made in this study, so that the increased oxygen affinity may result directly from MetHb formation, or indirectly via a reduction in 2,3 DPG levels. Binding of 2,3 DPG to MetHb requires further study as Chanutin and Curnish (1967) found that 2,3 DPG binds to MetHb, whereas Benesch and Benesch's (1967) findings indicated lack of binding between 2,3 DPG and MetHb.

If increased levels of CO_2 can contribute to MetHb formation, and therefore increased oxygen affinity, the lack of increase in $P_{50(7.4)}$ found in some of these patients with chronic hypercapnia may be explained by the presence of MetHb antagonising the effects of 2,3 DPG. Spectrophotometric determination of MetHb may, therefore, be useful in a further study.

4. Other Phosphates in the Erythrocyte.

Haemoglobin phosphate complexes have been demonstrated after addition of 2,3 DPG, ATP and to a lesser extent, after addition of ADP and 3 PGA to haemoglobin solutions. Inorganic phosphates such as tripolyphosphates, tetra and hexametaphosphate, also resulted in increased haemoglobin-phosphate complexes (Chanutin and Curnish, 1965). Although organic and inorganic phosphates are capable of interacting with haemoglobin at 3-5 sites, these reactions need not necessarily induce the conformational changes responsible for causing a shift in the oxyhaemoglobin curve. Most of them will, however, alter the oxygen affinity via the Bohr effect of haemoglobin, due to accumulation of non-penetrating phosphate anions and consecutive changes of intracellular pH. Duhm (1971) found that when red cells were incubated with inosine, pyruvate and phosphates (IPP), the total concentration of organic phosphate greatly increased. Such phosphates included ATP, F-1-6-P, and various triose phosphates, but the 2,3 DPG concentration was particularly high, and the

subsequent increase in P_{50} exceeded 40 mm Hg.

It is generally agreed that ATP and 2,3 DPG have maximum effect on oxygen affinity and these two phosphates have been shown to behave identically at 37°C, so that under physiological conditions, oxygen affinity is determined by the sum of 2,3 DPG and ATP. ATP was not measured in this study as the concentration of 2,3 DPG in the intact cell is about 4 times that of ATP and, therefore, has the greater effect on P_{50}. Variations of 2,3 DPG in physiological and pathological states are much larger than those of ATP and it is now believed that 2,3 DPG is much more effective in moderating the oxygen affinity of haemoglobin than is ATP. 2,3 DPG is found to decrease the binding of ATP to deoxygenated haemoglobin, while inorganic phosphate up to a 10 m Molar solution does not affect the binding of either 2,3 DPG or ATP, so that in this study, 2,3 DPG concentrations alone were measured.

However, for a further study, measurement of total phosphate concentration, in addition to 2,3 DPG, may help to explain some of the residual variance of the P_{50} and 2,3 DPG relationship.

C H A P T E R VII

CONCLUSION

In final answer to the questions posed in Chapter II therefore:-

1. 2,3 DPG levels are not raised in all patients with chronic obstructive lung disease. Low, normal, and high levels are found in these patients with chronic bronchitis and CO_2 retention.

2. $P_{50(7.4)}$ correlates with levels of 2,3 DPG ($r = 0.597$, $p = 0.001$), but there is an appreciable scatter of results.

3. It is suggested that chronic hypercapnia affects 2,3 DPG levels, as the consistent rise in 2,3 DPG concentrations in residents at altitude was not seen in these patients, even although the degree of hypoxaemia and pH values were similar in the two groups.

4. 2,3 DPG levels do not alter the Bohr effect.

5. Haematocrit does not correlate with $P_{50(7.4)}$ in contrast to findings of Lenfant.

6. From initial studies, the intraerythrocytic pH appears to be within the normal range in chronic hypercapnia as suggested by Tushan et al. (1970).

7. It is suggested that factors, concerned with hypercapnia, but as yet undefined, affect the relationship between 2,3 DPG and $P_{50(7.4)}$, which we found in this study in patients with chronic bronchitis and emphysema.

Although a right-ward shift in the dissociation curve is associated with an increased concentration of 2,3 DPG, it is suggested that the shift is unlikely to be of physiological significance in these patients with chronic hypoxia and CO_2 retention.

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TABLES

Table 1.

Normal subjects breathing air.

Subject	Mixed Sample P_{50}					Oxygenated Sample Ton 1					Deoxygenated Sample Ton 2				
	PO_2	PCO_2	pH	TO_2	TCO_2	PO_2	PCO_2	pH	TO_2	TCO_2	PO_2	PCO_2	pH	TO_2	TCO_2
1.	28.0	29.7	7.37	9.76	15.94	129.6	7.8	7.64	20.02	6.18	4.7	71.5	7.16		25.82
2.	26.2	31.3	7.42	10.12	17.25	127	7.0	7.75	19.92	6.84	3.9	72.7	7.20	0.49	27.5
3.	23.9	30.0	7.48	8.5	18.7	131	7.8	7.84	17.5	9.5	2.0	71.7	7.22	0.97	27.0
4.	24.6	27.9	7.43	9.33	17.66	135	6.2	7.76	19.70	8.64	1.7	69.5	7.20	0.96	26.83
5.	24.6	30	7.44	10.37	18.74	130	8.8	7.78	21.93	8.18	2.7		7.21	0.72	28.86
6.	26.1	30	7.45	9.62	17.59	131	13.0	7.79	19.52	9.34	3.1		7.21	0.96	28.19
7.	27.4	26	7.42	10.41	15.73	131	8.0	7.72	21.85	7.67	4.1	60	7.19	0.73	25.89
8.	27.0	25	7.47	8.51	17.98	129	8.8	7.82	17.06	9.72	3.3	60	7.21	1.21	27.67
9.	26.3	25.4	7.38	10.58	15.03	133	7.9	7.76	20.19	8.04	1.0	67	7.20	0.73	27.45
10.	27.0	33.8	7.38	10.62	14.97	133	8.2	7.53	20.60	7.18	0.2	86	7.16	0.24	24.64
11.	27.1	31.6	7.38	9.38	14.67	134	8.1	7.59	16.84	6.87	0.8	83	7.12	0.6	27.37
12.	27.1	29.1	7.39	9.38	15.60	129	5.8	7.64	18.08	8.03	0.3	83	7.16		27.73
Mean	26.28	29.15	7.42	9.72	16.66	131.05	8.12	7.72	19.43	8.02	2.32	72.44	7.19	0.64	27.08
S.D.	±1.28	±2.65	±.04	±.74	±1.49	±2.33	±1.79	±.10	±1.72	±1.13	±1.54	±9.16	±.03	±.39	±1.15

PO_2 = partial pressure of oxygen
PCO_2 = partial pressure of carbon dioxide
TO_2 = content of oxygen
TCO_2 = content of carbon dioxide

Values of all patients involved in study.

Subject	Mixed Sample P_{50}					Oxygenated Sample Ton 1					Deoxygenated Sample Ton 2				
	PO_2	PCO_2	pH	TO_2	TCO_2	PO_2	PCO_2	pH	TO_2	TCO_2	PO_2	PCO_2	pH	TO_2	TCO_2
1.	23.5	28.5	7.53	10.63	19.08	135	7.3	7.84	21.20	10.15	2.2	73.1	7.27		
2.	24.1	29.5	7.54	10.28	20.8	131	7.2	7.85	19.84	10.54	3.0	74.1	7.28	0.24	31.03
3.	29.3	29.8	7.38	7.03	15.65	134	7.3	7.64	15.21	7.03	4.0	69.7	7.15	0.724	25.06
4.	27.1	32.1	7.37	11.87	16.83	123	7.3	7.63	23.42	6.91	4.5	73.7	7.19	0.48	27.87
5.	22.0	29.4	7.45	10.57	17.43	122	7.0	7.80	19.71	8.85	2.90	70.3	7.22	0.49	28.14
6.	28.8	50.6	7.40	9.06	14.85	122	9.1	7.66	18.79	7.49	2.7	101	7.19	0.24	26.46
7.	23.9	30.4	7.44	10.05	18.29	128	7.7	7.79	19.53	9.64	2.1	73.8	7.24	0.48	30.40
8.	28.6	28.6	7.39			130	5.9	7.62			2.8	72.4	7.13		
9a.	31.5	35.6	6.75	11.47	6.78	130	8.0	6.86			6.2	68.4	6.72		
b.	25.0	28.7	7.52	9.46	19.23	131	8.0	7.81	18.14	10.51	4.4	70.5	7.23	0.74	28.92
10.	24.0	26.1	7.45	13.86	19.40		7.0	7.69	26.21	8.38		68.3	7.24	0.97	32.06
11.	25.4	27.2	7.40	9.52	18.21	127	7.7	7.65	18.06	9.52	3.6	91.6	7.16	0.48	28.46
12.	24.1	26.2	7.58	9.05	23.39	132	6.9	7.95	19.78	15.76	4.2	68.9	7.33	0.49	36.36
13.	26.4	29.9	7.38	10.16	16.28	131	7.0	7.66	19.43	7.73	3.1	68.0	7.19	0.49	26.23
14.	26.0	26.8	7.50	11.35	20.11	130	7.0	7.84	22.17	10.52	3.9	70.0	7.34	0.98	32.2
15.	24.8	29.9	7.51	11.48	17.82	121	7.2	7.87	22.95	9.42	2.8	72.7	7.30	0.494	30.74
16.	24.5	30.1	7.59	10.12	20.89	128	7.8	7.96	19.99	12.80	2.9	74.7	7.32	0.25	34.26
17.	28.1	31.2	7.43	9.38	16.55	129	7.6	7.72	19.48	7.71	2.5	72.9	7.22	0.48	26.78
18.	24.4		7.54	10.37	21.98				20.20	12.4				0.49	34.79
19.	26.1	33.7	7.42	8.76	17.21	132	8.3	7.78	17.83	8.41	1.3	75.4	7.20	0.24	26.43
20.	24.1	32.4	7.52	11.96	21.18	130	8.6	7.93	24.01	11.06	1.9	76.5	7.33	0.61	32.08
21.	24.2	29.1	7.47	9.59	19.59	132	8.1	7.82	18.86	10.88	1.2	74.7	7.24	0.24	29.98
22.	29.6	35.9	7.41	12.45	15.83	138	8.6	7.73	22.46	7.49	2.6	82.6	7.22	0.48	27.48
23.	28.3	31.3	7.44	11.18	19.16	131	7.5	7.76	23.23	8.10	0.2	69.7	7.24	0.73	29.84
24.	25.6	33.6	7.42	10.78	16.05	130	7.7	7.69	23.64	6.94	1.0	71.6		0.37	26.48
25.	26.4	34.6	7.35	9.64	18.03	128	7.6	7.64	19.61	8.12	1.2	83	7.14	0.48	26.97
26a.	27	28	7.32	8.48	15.54	142	6.7	7.68	13.76	8.08	2.2	69.8	7.14	0.60	25.35
b.	28.2	31.5	7.42	7.46	16.66	132	7.2	7.80	14.32	9.38	2.3	78.2	7.16	0.72	25.29
27.	27.3	30.3	7.33	9.52	13.8	134	7.4	7.63	18.97	7.57	3.1	68.8	7.06	0.48	24.13
28.	22.6	28.8	7.43	12.67	17.19	135	6.3	7.73	27.04	8.08	2.8	74.9	7.22	0.61	28.19
29.	21.5	27.0	7.58	8.69	18.85	132	6.8	7.98	19.43	13.82	1.2	81.2	7.21	0.48	29.28
30.	28.5	27.8	7.41	7.48	14.48	126	7.1	7.78	16.84	9.52	3.8	70.0	7.16	0.48	25.84
31.	21.6	28.7	7.49	12.1	19.18	126	7.2	7.82	25.63	9.28	1.0	74.0	7.27	0.48	31.82
32.	23.4	26.0	7.59	10.78	23.08	136	6.8	8.02	22.09	11.84	3.5	76.6	7.35	0.48	33.89
33.	22.1	26	7.53	9.41	21.66	127	6.9	7.92	19.67	11.94	4.6	68.8	7.30	0.48	31.37
34.	34.7	30.8	7.23	8.54	11.25	122	7.4	7.49	18.02	4.83	1.3	72.1	7.04	0.72	19.40
35.	30.0	29.4	7.45	9.71	14.00	135	7.7	7.85	19.60	9.27	3.6	74.4	7.13	0.61	22.54
36.	26.7	32.7	7.38	10.48	14.60	136	6.7	7.69	23.43	5.41	2.7	70.8	7.20	0.24	25.55
37.	24.6	32.9	7.51	11.85	17.15	135	6.6	7.77	26.76	7.11	1.9	76.0	7.26	0.48	27.67
38.	23.7	30.6	7.51	10.66	19.61	136	6.3	7.85	22.70	9.99	1.6	75.8	7.26	0.61	30.58
39.	21.9	33.3	7.52	10.46	20.11	135	7.6	7.89	21.61	10.85	1.4	78.1	7.27	0.96	30.92
40.	22.1	26.6	7.59	11.35	21.48	132	7.8	7.95	22.42	11.27	3.0	75.4	7.32	0.72	33.2
41.	26.0	28.6	7.53	10.99	18.21	129	9.2	7.86	21.56	9.69	1.8		7.26	0.6	30.28
42.	24.2	29.1	7.55	9.50	19.57	130	8.8	7.88	17.75	11.83	2.9	61	7.28	0.73	31.19
43.	29.4	33.0	7.35	12.54	12.74	137	4.2	7.54	28.03	4.8	2.8	78.6	7.13	0.20	23.56
Patients on Long Term Oxygen Therapy.															
44. O_2	27.2	31.0	7.37	11.43	16.8	120	6.2	7.55	21.16	7.80	0.9	84.8	7.38	0.2	26.77
air	29.1	28.2	7.36	13.0	13.00	107	6.1	7.65	23.12	3.85	1.1	81.6	7.26	0.2	26.30
45. O_2	22.7	27.2	7.53	10.8	20.55	130	7.0	7.74	20.84	12.95	0.6	77.7	7.28	0.24	32.87
air	22.1	27.3	7.53	10.39	22.42	135	5.8	7.98	18.59	14.17	1.6	73.7	7.27	0.24	33.49
46. O_2	24	27.3	7.53	8.3	24.4	135	4.7	7.86	23.03	11.24	3.3	65.3	7.27	0.37	33.37
air	24.1	27.1	7.53	12.54	13.74	134	4.9	7.83	24.03	11.80	1.9	74.4	7.28	0.2	33.56
47. O_2	26.5	27.4	7.49	10.8	20.07	136	4.1	7.92	20.87	9.77	5.0	71.7	7.28	0.20	31.09
air	26.0	26.9	7.47	10.65	18.65	136	5.0	7.85	20.83	9.81	3.7	72.1	7.27	0.20	31.14
48. O_2	23.3	27.8	7.49	12.12	17.54	133	5.8	7.82	23.99	8.10	2.6	75.8	7.29	0.20	29.46
air	23.3	27.0	7.48	12.20	18.68	138	7.1	7.79	24.3	8.49	1.8	77.3	7.26	0.20	30.20

PO_2 = partial pressure of oxygen
PCO_2 = partial pressure of carbon dioxide
TO_2 = content of oxygen
TCO_2 = content of carbon dioxide

Table 3.

Normal Values of P_{50}.

Author	Year	P_{50}(mmHg)	SD	No. of Subjects
Dill	1941	26.3		1
Bartels	1961	26.8	±2.6	14
Naeraa	1964	30.0	±2.0	24
Astrup	1965	26.7		58
Severinghaus	1966	26.6		
Mulhausen	1967	26.9	±1.0	
Duc	1969	29.0	±1.0	7
Lenfant	1969	26.8	±0.6	14
Oski	1970	26.4	±1.1	9
Department of Medicine	1970-71	26.7	±0.65	12

Table 4.

Normal Values of 2,3 Diphosphoglycerate.

Author	Year	2,3 DPG	
		moles/ml RBC	moles/gm Hb.
Rorth	1969	5.4 ± 0.3	
Oski	1969	4.08 ± 0.52	
Woodson	1970	5.06 ± 0.44	
Department of Medicine	1970-71	4.32 ± 0.69	
Eaton and Brewer	1969		12.80 ± 2.32 (Males)
Nygaard and Rorth	1969		13.9 ± 0.9 (Males)
Valeri and Fortier	1969		12.4 ± 1.0
Department of Medicine	1970-71		12.8 ± 2.2

Normal Subjects Breathing Air.

Subject No.	P_{50} (7.4) mm Hg	Hb. gm.	HCT. %	MCHC %	DPG moles/ml RBC	DPG moles/ml Hb.
1.	27.20	14.2	42.1	33.9	4.42	13.09
2.	26.14	14.2	43.4	32.6	3.11	9.50
3.	26.36	12.8	38.7	32.7	5.12	15.5
4.	25.61	13.7	40	34.0	4.95	14.45
5.	25.90	15.1	43.3	34.3	4.36	13.08
6.	27.49	13.8	40.8	33.3	3.23	9.55
7.	27.49	15.1	43.7	33.9	4.12	11.92
8.	27.22	12.3	35.3	34.3	3.74	10.73
9.	27.00	14.6	43.5	32.8	4.97	14.81
10.	26.39	15.0	45.3	39.6	5.56	16.09
11.	26.50	13.1	38.8	33.6	4.70	13.92
12.	26.79	14.9	42.1	35.6	4.19	11.84
Mean	26.67	14.07	49.63	33.63	4.32	12.81
S.D.	±0.622	±0.948	±2.01	±0.897	±0.688	±2.21

P_{50} (7.4) Partial pressure of oxygen at 50% saturation and pH 7.4

Hb. Haemoglobin

HCT. Haematocrit

MCHC Mean corpuscular haemoglobin concentration

DPG 2,3 diphosphoglycerate.

a) moles per ml. of red blood cells
b) moles per gram of haemoglobin.

Table 6.

All patients breathing air.

Subject	PO_2	PCO_2	pH	$P_{50(7.4)}$	Hb.	HCT	MCHC	DPG	DPG
1.	48	49	7.40	27.02	15.8	48.5	32.3	4.89	14.67
2.	47	86	7.38	28.13	14.7	47.6	30.9	5.74	18.59
3.	79	47	7.44	28.6	11.7	37.7	31.1	5.57	17.95
4.	41	51	7.38	26.4	17.4	55.8	31.6	3.60	11.54
5.				23.25	14.3	41.3	34.8	1.09	3.15
6.				28.7	13.7	43.5	31.6	6.41	20.35
7.	79	35	7.40	24.98	14.2	43.8	32.4	3.69	11.38
8.	64	36	7.48	28.96	13.2	41.9	31.6	6.66	21.14
9a.	113	38.8	6.90	15.36	16.6	51	32		
b.			7.34	28.54	13.2	38.9	34.1	4.01	11.82
10.	49	54	7.34	25.56	18.6	59.3	31.3	3.84	12.24
11.		39	7.42	25.8	13.2	42.6	31.1	4.51	14.55
12.	34	67	7.35	29.77	14.5	46.6	31.2	4.89	15.71
13.				25.9	14.1	42.6	33.3	4.2	12.75
14.	49	65	7.35	30.13	15.7	54.5	28.8	5.28	18.33
15.	52	52	7.39	27.8	17.0	54.1	31.1	5.71	18.17
16.	32	58	7.29	30.25	15.1	55.4	27.0	4.60	16.87
17.				28.74	13.8	43.6	31.5	5.23	16.52
18.	52	61	7.40	28.48	14	46	30	5.52	18.13
19.	67	35	7.37	26.83	12.9	41.0	31.5	4.11	13.06
20.	43	68	7.29	27.84	17.6	58.8	29.7	4.5	15.03
21.(O_2)	82	57	7.32	26.4	14.1	46.4	30.3	4.11	13.52
22.	39	43	7.32	29.9	16.4	51.6	31.7	6.14	19.32
23.	62	55	7.35	23.3	17.3	54.8	31.4	2.96	9.37
24.	46	46	7.32	26.17	16.5	52.5	31.2	4.49	14.28
25.	62	34	7.37	24.98	14.2	47.5	29.8	3.98	13.30
26a.	64	36	7.37	25.3	12.7	41	31.0	4.60	15.10
b.	55	40	7.39	26.2	12.7	40.7	30.7	4.38	14.38
27.	63	26	7.41	25.7	13.7	42.2	32.8	6.04	18.65
28.	56	76	7.28	21.86	20.1	64.3	31.4	3.02	9.70
29.	47	80	7.30	26.23	14.0	47.2	30.0	2.54	8.56
30.	54	34	7.36	28.57	12.2	40.7	30.4	5.89	19.65
31.	49	55	7.33	23.47	18.3	58.9	31.3	4.58	14.74
32.	50	50	7.43	28	16.7	55.1	29.9	5.5	18.14
33.	43	70	7.31	26.68	14.4	48.2	29.6	2.93	9.81
34.	49	38	7.33	28.9	14.4	51	28	4.38	15.41
35.	65	55	7.39	30.18	14.3	44	32.3	4.36	13.41
36.	80	37	7.42	26.27	17.8	54.9	31.7	5.19	16.00
37.	55	60	7.35	27.8	19.2	66	28.5	5.77	19.68
38.	51	51	7.41	26.6	16.5	50.8	31.6	4.84	14.90
39.	50	51	7.43	25.14	15.8	47.9	32.2	6.70	20.31
40.				27.27	17.1	52.7	31.7	5.12	15.78
41.	36	58	7.30	30.03	15.4	50.1	30.7	6.29	20.45
42.	48	41.8	7.38	28.56	13	41.3	31.4	6.03	19.16
43.	79	29	7.38	28.3	20.1	60.2	32.8	4.23	12.60
Patients on Long Term Oxygen Therapy.									
44. O_2	43	53	7.40	26.3	17.7	52.0	33.3	4.96	14.57
air	41	51	7.40	28.5	18.5	53.9	33.4	5.51	16.05
45. O_2	62	74	7.40	26.2	14.8	40	34	4.35	11.76
air	56	65	7.40	25.5	13.9	40.1	34.5	4.58	13.21
46. O_2	48	55	7.46	27.7	17.5	52.3	33.3	5.28	15.78
air	46	53	7.46	27.7	17.2	50.2	33.8	5.49	16.02
47. O_2	44	50	7.42	28.6	16.2	47.0	34.2	6.57	19.06
air	41	50	7.40	27.5	15.9	47.5	33.2	6.32	18.88
48. O_2	48	54	7.41	25.5	18.3	55.8	32.3	3.71	11.31
air	43	55	7.37	26.3	17.6	54.1	32.2	4.65	12.48

PO_2, PCO_2 & pH = arterial sample.

Table 7.

Patients with Chronic Bronchitis and Emphysema.

Subject	Sex	Age (years)	FEV	FVC	BP	Duration of Disease (years)	Drug Regime
1.	F	44	0.35	1.95	110/80	5	S,A,D,Dig.
2.	M	61	0.5	1.75	115/90	10	S.
4.	M	80	0.8	2.25	130/70	15	A.
7.	M	64	0.6	1.7	175/100	12	S,D,A.
10.	M	64	0.5	1.5	150/70	15	D,B,Dig,K.
11.	M	69	1.0	2.8	210/105	4	D,A,Dig,K.
12.	M	50	1.5	2.5	145/95	3	A,D.
13.	M	64	1.2	3.7	130/90	30	B,S,K.
14.	F	44	0.35	1.9	110/70	5	Dig,D,K.
15.	F	60	0.5	1.7	140/70	15	Dig,D,S,A.
16.	F	68	0.7	1.7	150/80	20	D,Dig.
17.	M	62	2.2	3.65	130/70	20	A,B,K.
18.	M	61	0.6	1.5	160/85	20	D,B.
19.	M	70	0.6	1.8	100/55	20	Dig,A.
20.	M	47	0.5	1.6	130/90	5	Dig,K,A,D.
21.	M	74	0.7	1.7	130/80	20	S,A,D.
23.	M	58	0.7	1.8	110/70	5	Dig,D,K,A.
24.	M	78	0.7	2.55	130/85	13	A,D,Dig.
28.	M	52	0.6	1.45	130/80	9	S,A,D.
29.	M	74			120/70	14	A,B,Dig.
30.	M	66			135/70	20	A.
31.	M	63	0.4	1.6	140/90	20	D,K,S,Dig.
33.	M	62	0.35	1.10	110/70	20	A.
34.	M	69	0.5	1.7	130/60	2	A,D,Dig.
35.	M	52	1.5	2.5	145/95	4	A,D.
37.	M	72	0.7	1.7	140/90	12	D,K,Dig.
38.	M	67	0.55	2.0	130/80	5	A,B.
40.	M	63	0.4	1.6	95/70	20	S.
41.	F	73	0.6	1.2	160/80	25	D,B,Dig.
42.	M	63	0.95	2.6		10	A,D,K,B,Dig.
Patients on Long Term Oxygen Therapy							
44.	M	65	0.6	1.8	100/60	20	D,Dig,K.
45.	M	60	0.35	1.2	113/65	25	A,B,K,D,Dig.
46.	F	56	1.0	1.9	120/70	5	B,Dig,K,D.
47.	F	58	0.7	1.9	110/80	10	S,D,K.
48.	M	65	0.6	1.85	120/70	13	Dig,D,A,K.

Severe airways obstruction is indicated by the forced expiratory volume (FEV) and forced vital capacity (FVC) values. With the exception of No.17, all are at least 50% below the predicted value.

S = Steroid, A = Antibiotic, D = Diuretic
Dig = Digoxin, B = Bronchodilator, K = Potassium.

Table 8.

Patients without chronic bronchitis observed in study.

Subject	Sex	Age	Diagnosis
3.	F	74	Sulph and Met haemoglobinaemia (69% Met.Hb., 4.8% Sulph.Hb.)
5.	M	60	Haemolytic anaemia
8.	M	64	Metabolic alkalosis
9.	M	28	Diabetic ketoacidosis
22.	M	62	Acute pulmonary oedema
25.	M	64	Hypertension
26.	M	45	Acute renal failure (on dialysis)
27.	M	38	Acute renal failure (on dialysis)
32.	M	49	Myocardial infarction
36.	M	50	Polycythemia Rubra Vera
39.	M	68	Mediastinal obstruction.

Table 9a.

Values from which the Bohr effect was calculated in 6 patients with chronic bronchitis and in 1 normal subject.

Subject	3% CO_2							6% CO_2							9% CO_2						
		P_{45}			P_{55}				P_{45}			P_{55}				P_{45}			P_{55}		
	pH	PO_2	PCO_2	CAP	PO_2	PCO_2	CAP	pH	PO_2	PCO_2	CAP	PO_2	PCO_2	CAP	pH	PO_2	PCO_2	CAP	PO_2	PCO_2	CAP
1.	7.74	15.2	20.6	21.62	21.2	20.8	21.77	7.52	19.2	39.1	21.77	27.2	40.1	22.06	7.40	22.5	57.1	21.79	31.7	56.7	22.09
15.	7.71	17.2	17.2	22.2	24.7	21.0	23.08	7.49	23.7	39.0	22.25	32.8	40.0	22.8	7.32	27.8	59.1	23.04	38.9	60	22.88
28.	7.58	17.5	22.5	26.91	22.2	23.0	27.26	7.39	20.6	42.0	26.83	29.6	41.5	26.63	7.28	24.7	59.6	26.31	32.1	59.3	26.79
31.	7.65	17.7	22	25.64	24.8	22.9	25.72	7.45	22.8	42	25.48	32.6	42.4	26.39	7.31	26.3	59	26.44	37	58	25.64
33.	7.76	17.7	22	19.84	24.2	22	19.57	7.50	24.8	40	19.84	33.3	41.4	20.00	7.38	29.6	60	19.84	38.9	60.1	20.04
45.	7.68	16.7	22.1	21.36	21.9	21.1	21.96	7.48	21.9	39.9	21.24	28.8	41.8	21.05	7.36	26.0	60.4	21.13	34.2	58	21.09
10. (Normal)	7.56	18.6	21.8	22.10	25.6	21.1	21.30	7.37	23.4	40.5	21.69	31.5	40.1	21.45	7.26	26.4	60.3	21.45	37.0	59.2	22.09

CAP = Capacity
P_{45} = 45% saturation (approximately)
P_{55} = 55% saturation (approximately)

Oxygen and carbon dioxide transport measurements in six patients with chronic bronchitis and in one normal subject.

Subject	3% CO_2				6% CO_2				9% CO_2			
	P_{45}		P_{55}		P_{45}		P_{55}		P_{45}		P_{55}	
	TO_2	TCO_2	TO_2	TCO_2	TO_2	TCO_2	TO_2	TCO_2	TO_2	TCO_2	TO_2	TCO_2
1.	8.30	20.11	13.01	19.60	8.14	25.25	13.06	24.57	8.6	28.37	13.18	27.73
15.	9.99	16.48	14.21	14.29	10.95	20.48	15.21	19.60	10.26	24.18	14.24	23.24
28.	11.23	15.29	15.69	15.32	10.90	20.36	15.87	20.18	10.52	23.95	16.09	23.3
31.	11.52	18.06	15.87	17.73	11.08	22.68	16.05	22.0	11.30	26.27	16.60	26.16
33.	9.71	18.87	13.13	17.51	9.45	23.79	13.33	23.21	9.41	27.99	13.40	27.22
45.	9.07	20.81	13.23	19.5	9.40	25.35	13.38	24.61	9.01	28.27	13.38	29.1
10. (Normal)	8.33	15.67	12.65	15.73	9.24	20.41	13.13	20.82	9.00	24.7	13.01	23.60

TO_2 = Content of oxygen

TCO_2 = Content of carbon dioxide

P_{45} = 45% saturation (approximately)

P_{55} = 55% saturation (approximately)

Table 10.

Arterial and venous intraerythrocytic pH measurements on six bronchitic patients and one normal subject together with PO_2, PCO_2 and pH results.

Subject No.	ARTERIAL				VENOUS			
	PO_2	PCO_2	pH(e)	pH(i)	PO_2	PCO_2	pH(e)	pH(i)
12.	84	48	7.37	7.17	31	78	7.23	7.08
15.	63	53	7.42	7.14	29	69	7.30	7.08
28.	52	57	7.33	7.18	26	83	7.26	7.08
44.	46	50	7.37	7.15	14	84	7.24	7.08
47.	41	50	7.38	7.19	21	60	7.34	7.15
48.	53	61	7.32	7.18				
41.					24	72	7.30	7.08
Normal	95	40	7.33	7.16	45	41	7.31	7.14
Mean ± S.D.			7.37 ± .04	7.17 ± .02			7.28 ± .04	7.10 ± .03

$pH_{(e)}$ = whole blood pH

$pH_{(i)}$ = intraerythrocytic pH (freeze-thaw).

Table 11.

Cardiac catheterisation data on 8 patients with chronic bronchitis.
The percentage saturation is calculated from a normal P_{50} and from the patients' P_{50}.

Subject No.	Arterial			Mixed Venous			Capacity	% Saturation (Normal P_{50})		% Saturation (P_{50} of Patient)	
	PO_2	PCO_2	pH	PO_2	PCO_2	pH		Arterial	Venous	Arterial	Venous
14	39	63	7.35	30	70	7.34	22.5	68.3	51.5	61.5	45.0
31	45	68	7.33	33	83	7.30	23.23	75.5	54.5	70.6	54.5
32	54	46	7.40	35	53	7.39	23.67	87.4	65.0	81.5	61.5
42	55	60	7.36	38	68	7.34	17.49	86.0	66	80.8	61.5
44	40	51	7.45	36	68	7.39	24.5	76.0	65.5	69.7	66.5
45	47	69	7.36	34	62	7.35	19.68	79	60.1	79.2	63
47	43	47	7.42	32	54	7.41	20.99	78.5	60.1	73	58.3
48	44	51	7.37	31	57	7.36	23.94	77	55.5	75.6	57.4