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CHARACTERISTIC pH

OF

HAEMOGLOBINS

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This is to certify that the work described in this thesis was carried out under our supervision by Oluyemi Samuel Adeosun in the Laboratories of the Department of Chemistry, University of Ibadan, between October 1968 and October 1971.

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ABSTRACT

The thermodynamics of the azide binding reactions of the hybrids of human and canine haemoglobins (i.e. $\alpha_2^A\beta_2^{Ca}$ and $\alpha_2^{Ca}\beta_2^A$) as well as the isolated α and β polypeptide chains of human and canine haemoglobins have been studied at various pH's and temperatures. Plots of $-\Delta H^\circ$ against pH, for all the species, show a distinct maximum. The pH at which the maximum value of $-\Delta H^\circ$ occurs is termed the 'characteristic pH' (pH_{ch}).

From the results it is concluded that the value of pH_{ch} for the haemoglobin tetramer is an average of the pH_{ch}'s of the separate α and β polypeptide chains although the form of the curve of $-\Delta H^\circ$ against pH is a function of the tetramer and cannot be obtained by averaging $-\Delta H^\circ$ values for the individual chains.

The Bohr effect of the two hybrids has also been measured and compared with that of the parent haemoglobins.

A study of the equilibrium reaction between oxyhaemoglobin and carbonmonoxide was carried out and the accompanying thermodynamic data assessed in terms of the mechanisms postulated for explaining ligand binding reactions of methaemoglobin.

CHAPTER I.

INTRODUCTION

Haemoglobin, the oxygen carrying component of blood cells has, without doubt, been studied more extensively than many other proteins. It is a readily available protein and this stimulated, at an early stage, the investigation of the function, properties and specificity of this substance. It has become a useful model for many different types of biochemical investigations. The enzyme chemist, for example, has been mainly concerned with the problem of elucidation of the mechanism of the enzyme at the 'active site'. While in recent years it has been realised that a large part of the protein may by means of configurational changes, be involved in the mechanism of an enzyme action, the general problem of how substituents on the enzyme can affect equilibria and kinetics at the 'active site' has not been fully rationalised.

Haemoglobin serves as an ideal model for this purpose for not only does it undergo a variety of simple reactions at the 'active site' (the central iron atom, in this case) but provides a class of closely related natural compounds with specific molecular differences. Thus we have available the abnormal human

haemoglobins S and C; which differ from one another and from normal human haemoglobin A by only two amino acid changes, thus providing the simplest substitutions. Reactivity studies on methaemoglobins S and C have unequivocally shown that changes in the amino acid composition of the protein even at positions remote from the iron atom, can affect the reactivity of the haem-iron of the haemoglobin (Anusiem, Beetlestone and Irvine, 1966).

In recent years, Beetlestone and his coworkers (Anusiem et. al. 1968b, 1968c, 1969; Bailey et. al. 1969; Bailey et. al. 1970a) have extended reactivity studies to several methaemoglobin species with a view to establish a model by which reactivity differences between different methaemoglobin species could be explained. It might be speculated that such a model might provide a basis for generalisation of mechanism of action of other proteins and enzymes.

The work described in this thesis is mainly concerned with developing this model. Furthermore, an attempt has been made to see if the model thus created to explain methaemoglobin reactions could lead to a useful model for interpreting oxyhaemoglobin reactions.

HAEMOGLOBIN.

Haemoglobin is a respiratory pigment which is present in the blood of most vertebrates and is responsible for the transport of oxygen in the body by virtue of its ability to combine reversibly with oxygen. Haemoglobin consists of two parts; the haem portion and the protein portion (globin). Haemoglobins from different species differ only in the protein portion while the haem groups are the same. Barcroft (1925) drew attention to this fact when he observed that different vertebrate haemoglobins have different relative affinities for oxygen and carbon monoxide and ascribed this to differences in the protein composition of the different haemoglobins. Hence differences in reactivity between one haemoglobin and another would be expected to be chiefly due to differences in their globin portion and how such differences affect reactivity at the iron atom of the haem.

Human haemoglobin consists of four polypeptide chains and four identical haems. Each haem contains an iron atom coordinated to four pyrrole nitrogens of protoporphyrin IX. When the iron atom is in the ferrous oxidation state, it can combine reversibly with oxygen without concommitant oxidation to the ferric oxidation state. The four polypeptide chains are identical in pairs (Ingram, 1956;

Rhinesmith et. al. 1957, 1958) and are designated α and β chains. Each α polypeptide chain contains 141 amino acid residues and the β chain 146 amino acid residues (Braunitzer et. al. 1961; Hill and Konigsberg, 1962). In terms of three dimensional gross structure, both the α and β polypeptide chains closely resemble the single chain of sperm whale myoglobin (Perutz et. al. 1960; Cullis et. al. 1962; Kendrew et. al. 1960).

In the first coordination position, according to the Werner system of numbering, the iron atom is bonded to the imidazole nitrogen of histidine F8 which is present in each of the polypeptide chains (Cullis et. al. 1962). This histidine residue is called the proximal histidine. On the other hand, not directly linked to the iron atom, lie histidine E7 and valine E11. The imidazole side chain of histidine E7 is called the distal imidazole. In all haemoglobin complexes the sixth coordination position is occupied by a ligand which lies between the iron atom and these two residues. In deoxygenated haemoglobin, this position is an empty hole (Nobbs et. al. 1966). Further removed from the iron atom, 60 atoms of the globin are in Van der Waals contact with the porphyrin ring (Perutz, 1970).

The four polypeptide chains are arranged tetrahedrally about

a two-fold axis of symmetry with haems lying in separate pockets on the surface of the molecule (Perutz et. al. 1960; Cullis et. al. 1962). The iron atom in each of the haem rings in the pocket is about 9Å inside the protein cavity and is formed by folds in the polypeptide chains. The iron atoms in neighbouring pockets between α and β chains are about 25Å apart. The distance between iron atoms in the β chains is 33.4Å and in the α chains 36Å.

The four chains are stabilised in the haemoglobin molecule by weak secondary forces; both hydrogen bonds and non-polar interactions. The interior of the individual subunits of haemoglobin molecule is made up of non-polar residues, almost everywhere in Van der Waals contact with their neighbours (Perutz, 1965). Many of the glycines and alanines being only weakly hydrophobic, lie on the surface of the molecule. Large non-polar side chains lie either in the interior of the individual subunits or in superficial crevices so designed as to minimise contact of these side chains with water or else at the points of contact between unlike subunits.

All side chains which are ionised at neutral pH lie on the surface of the subunits. The same is true of the other polar side chains, except for the haem-linked histidines and threonine C4

which is known from the structure of myoglobin, to be hydrogen bonded internally (Perutz, 1965). Thus the molecule conveys the general impression of being studded with polar groups all over its surface and most of them look as though they were designed to make contact with water rather than with other polar groups. Clearly the free energies of protein molecules are minimised if their exteriors are polar and their interiors non-polar as in soap micelles (Kauzmann, 1959), one reason being that groups carrying net charges or strong dipoles produce strong potential fields around them unless they are immersed in a medium of high dielectric constant like water.

The contacts between like subunits are few and limited to polar interactions near the α-amino and carboxyl termini. The contacts between unlike chains are of two different kinds i.e. $\alpha_1\beta_1$ and $\alpha_1\beta_2$. $\alpha_1\beta_1$ is the more extensive of the two contacts and is made up of 34 residues and about 110 atoms coming within a distance of 4Å of each other (Perutz et. al. 1968) and the majority of the interactions are non-polar. The contact $\alpha_1\beta_2$ is made up of nineteen residues contributing about eighty atoms within a distance of 4Å of each other. In the transition from oxyhaemoglobin to deoxyhaemoglobin, movement in the contact $\alpha_1\beta_1$

is slight. The relative displacement of atoms at this contact is only about 1Å. On the other hand, movement in the $\alpha_1\beta_2$ contact is large and relative displacement of atoms at this contact is as much as 5.7Å (Perutz et. al. 1968). The existence of a hole passing through the centre of the molecule along its dyad axis of symmetry was noted by Bragg and Perutz (1954). The hole is actually a wide cavity which extends all the way along the molecular dyad axis for a depth of 50Å. The internal cavity is lined with polar residues of all kinds. (Perutz et. al. 1968). Its width varies between about 5Å and 10Å. The dimensions of the central cavity and the concentration of polar groups which it contains leave no doubt that it must be filled with water (Perutz et. al. 1968).

METHAEMOGLOBIN (FERRIHAEMOGLOBIN)

The iron atom of the haem of haemoglobin can exist in two oxidation states. It can exist either in the ferrous or ferric state. In ferrohaemoglobin, the two pyrrole nitrogen atoms of the porphyrin ring neutralise the two positive charges of the ferrous iron such that the haem iron has a resultant neutral charge. The ferrous ion in ferrohaemoglobin can be oxidised to the ferric state with mild oxidising agents like potassium ferricyanide.

The oxidised haemoglobin is called ferrihaemoglobin or methaemoglobin. Strong oxidising agents like chlorate and permanganate will oxidise groups on the protein as well, hence they are not used for oxidising haemoglobin (Haurowitz and Hardin, 1954).

At pH 7.0 and below, methaemoglobin is chocolate brown in colour. In this compound, the sixth coordination position of the iron is occupied by a water molecule. At alkaline pH's, the colour of the methaemoglobin becomes wine red due to the ionisation of this water molecule. This position is now occupied by an hydroxyl group (Keilin and Hartree, 1952; Coryell et. al. 1937). In methaemoglobin, only two of the three positive charges on the iron are neutralised, hence it carries a resultant positive charge. Methaemoglobin, therefore, binds predominantly negative ligands such as azide (N_3^-), fluoride (F^-) and cyanide (CN^-). It can also bind some neutral ligands as well. These are usually nitrogen containing ligands such as ammonia, imidazole and methylamine.

ASSOCIATION-DISSOCIATION PHENOMENA IN HAEMOGLOBIN

The mammalian haemoglobin molecule is tetrameric and consists of two α and two β chains. Hence the tetrameric molecule can be

designated as $\alpha_2\beta_2$. The integrity of the tetramer is highly dependent on pH, ionic strength and nature of the solvent. Thus at both acid (Field et. al. 1955) and alkaline pH's, (Hasserrodt et. al. 1959) in strong salt solutions and in concentrated mercaptoethanol solutions (Gutter et. al. 1956), the human haemoglobin molecule has been demonstrated to undergo reversible dissociation-association equilibria although the reconstituted protein is native and maintains its oxygen capacity unchanged. Other mammalian haemoglobins also exhibit the same behaviour, although the degree of dissociation may vary (Steinhard, 1938; Gralen N., 1939). This dissociation may be demonstrated by a change in sedimentation behaviour (Field and O'Brien, 1955; Hasserodt and Vinograd, 1959; Kirschner and Tanford, 1964), by gel filtration (Ackers and Thompson, 1965; Huehns and Shooter, 1966), Electrophoresis (Gilbert, 1959; Gilbert and Jenkins, 1959), Chromatography (Cole, 1960; Thompson and O'Donnell, 1960) or by hybridisation between haemoglobins that differ in their component chains (Itano and Singer, 1958).

Itano and Singer (1958) were the first to be concerned with the mechanism of dissociation. They and Robinson and Itano (1960) concluded, on the basis of a series of recombination and

hybridisation experiments that the dissociation is asymmetrical both at low and high pH's. However, recent studies of the dissociation mechanism suggest that haemoglobin first dissociates into the symmetric $\alpha\beta$ dimer, which then splits to the monomer subunits i.e.

$$\alpha_2\beta_2 \rightleftarrows 2\alpha\beta \rightleftarrows 2\alpha + 2\beta$$

This mode of dissociation was first suggested by Vinograd and Hutchinson (1960) and was further supported by the membrane diffusion studies of Guidotti et. al. (1963). Guidotti and his coworkers specifically postulated that normal adult human haemoglobin is a solute in rapid association-dissociation equilibrium under all solvent conditions.

Recently, the dissociation of haemoglobin under special conditions has received much attention. Haemoglobin has been reported to undergo dissociation in concentrated urea solutions and solutions of other amides, in concentrated salt solutions, in strong mercaptoethanol solutions and in solutions at a pH below 6 and above 9.5. In this thesis, two different conditions have been employed in dissociating haemoglobins. In the preparation of the human and canine haemoglobin hybrids, the haemoglobins

(Human A and canine haemoglobins) were dissociated at acid pH (pH = 4.7) and then neutralised. For the preparation of the separate α and β chains of human A and Canine haemoglobins, the haemoglobins were dissociated with p-chloromercuribenzoate (PCMB). It is therefore relevant to discuss dissociation under these two conditions.

(a) DISSOCIATION OF HAEMOGLOBIN AT ACID PH

From the earlier work of Field and O'Brien (1955) and the more recent work of Wyman et. al. (1963) on the sedimentation and diffusion of human haemoglobin at pH values below 6.0, it is clear that in the pH range 6.0 to 4.5 haemoglobin undergoes reversible dissociation into subunits. Under this condition, the molecular weight of the protein approaches one half of its normal value. The dissociation is reversible and whole molecules are obtained upon neutralisation. Data obtained by light scattering (Wyman et. al. 1963) indicate that the effect of temperature on the dissociation is very small.

The concentration dependence of the dissociation at low pH values has been studied by several workers. The data reported by Field and O'Brien (1955) and the more detailed sedimentation and

light scattering values obtained by Wyman et. al. (1963) at different haemoglobin concentrations, show that the degree of dissociation increases with the dilution of the protein. When, however, experimental data were compared with those predicted by the mass law for a simple tetramer - dimer equilibrium, the dissociation appears to be less sensitive to protein concentration than expected; being greater than calculated at high concentration and smaller at low protein concentrations. This anomalous concentration dependence has been explained to mean that the dissociation is not a simple tetramer-dimer equilibrium which obeys the mass law equation as usually formulated. A careful analysis of the sedimentation behaviour of human haemoglobin in acid has recently been undertaken by Gilbert and Chiancone (1964). The results obtained indicate that even at pH values above 5, the dissociation equilibrium involves not only tetramers and dimers but also monomers. This is in agreement with the results obtained by Gwidotti and Craig (1963) on the rate of diffusion of haemoglobin through membranes.

The facts about the dissociation of haemoglobin below pH 6 suggest that the subunits are held together mainly by electrostatic forces linked to the ionisation of certain key groups on the surface

of the chains. Rossi Fanelli et. al. (1964) suggested that these key groups could be carboxyl groups. The very small effect of temperature on dissociation also suggests that the subunits obtained on dissociation are linked in the full molecule by weak electrostatic bonds.

One question that arises as regards the mechanism of dissociation at acid pH is whether the dissociation is symmetrical (i.e. $\alpha_2\beta_2 \rightleftarrows 2\alpha\beta$) or asymmetrical (i.e. $\alpha_2\beta_2 \rightleftarrows \alpha_2 + \beta_2$). It has been argued on the basis of hybridisation experiments that the dissociation in this case is asymmetrical (Itano and Robinson, 1960). However, this argument has been seriously criticised and it has been suggested that it is more probable that the dissociation is symmetrical (Rossi Fanelli et. al. 1964). The main argument for this comes from what can be deduced from the haemoglobin model of Perutz. (Cullis et. al. 1962). Thus the model shows greater and tighter contacts between the unlike than between like chains. Also the results on the oxygen equilibrium of haemoglobin (Rossi Fanelli et. al. 1961) and on denaturation experiments at acid pH (Bucci and Fronticelli, 1961) fail to show the heterogeneity which might be expected if α_2 and β_2 subunits were present in the solution.

(b) DISSOCIATION OF HAEMOGLOBIN BY P-CHLORO MERCURI BENZOATE (PCMB)

Bucci et. al. (1965) found that with carefully controlled conditions, human haemoglobin A (HbA) can be fully dissociated into its subunits by PCMB at pH 6.0. The isolated α and β chains obtained have been shown to be native since (a) they show full oxygen binding capacity (b) they combine in proper stoichiometry and (c) the recombination product has the same properties as the original haemoglobin (Bucci et. al. 1965; Geraci et. al. 1969).

Rosemeyer and Huehns (1967) studied the dissociation by PCMB of several human and animal haemoglobins with varying sulphydryl (-SH) content. It was shown by these authors that in the presence of excess PCMB, the dissociation of haemoglobin into its component chains varied with the concentration of sodium chloride present in the solvent. The production of subunits increases with salt concentration reaching a maximum at 0.1 to 0.2M NaCl. It was also observed that in the pH range 5.5 - 7.5, dissociation increased with decrease in pH and was complete at pH 6.0. At lower pH's, dissociation was complete but complete precipitation occured. For pH range 9-11, dissociation remained incomplete and decreased with increasing pH. The authors showed that optimum yield of the subunits were obtained when dissociation was carried out using

0.2M NaCl in a phosphate buffer pH 6.0 and allowing the mixture to stand overnight at 4°C. Gel filtration of the isolated α chain showed that this was a monomer. The isolated β chain was a tetramer in the absence of PCMB. However, when PCMB was added, 75% of the total haemoglobin were monomers.

While HbA dissociated under the conditions stated above, other haemoglobins dissociated, if at all, after prolonged exposure to PCMB except Canine haemoglobin which dissociates into subunits under similar conditions as haemoglobin A. Thus rabbit, horse, pig and fetal haemoglobins do not dissociate at pH 6.0. These results show that the degree of dissociation by PCMB depends on the nature of the haemoglobin which is reacted with this compound. It would be reasonable to expect that the extent of reaction would also depend on the quantity of reagent used. Thus when these authors performed further experiments with lower concentrations of the mercurial, they found that with haemoglobin A, electrophoretic analysis showed that small amounts of subunits appeared when the proportion of reagent to protein reached 4:1 and virtually complete separation was observed when this ratio was 6:1 or higher. A similar behaviour was observed for Canine haemoglobin in which appearance of subunits occured at ratio 6:1 and complete dissociatio at 8:1 level.

The results on the dissociation of haemoglobins by PCMB have led to a close study of the implications of particular -SH groups in the dissociation (Rosemeyer and Huehns, 1967). As the expected reaction with this reagent is with -SH groups, any difference in reaction should be related to the -SH content of these proteins. Rosemeyer and Huehns showed that complete dissociation into monomers occured only in those haemoglobins that have two -SH groups in the β chain. The β chain of Hb A has 146 residues with cysteine residues at positions 93 and 112. In Fetal haemoglobin, the cysteine residue at the position β 112 is replaced by threonine; hence fetal haemoglobin does not dissociate into monomers. This suggests that reaction at this site is a necessary requirement for production of monomers. Further support for this idea comes from results on dissociation of animal haemoglobins, canine haemoglobin, which has a second -SH group on the β chain (Cecil, 1963), does dissociate to form monomeric α and β chains while rabbit, horse and pig which do not have a second -SH group on the β chain, do not dissociate to form separate α and β subunits with PCMB.

From electrophoretic and sedimentation experiments, these authors showed that the dissociation of the tetramer to the dimer must be symmetrical. Hence the dissociation of the haemoglobin

tetramer to the dimer can be represented as $\alpha_2\beta_2 \rightleftharpoons 2\alpha\beta$. They further showed that dissociation into the dimer occured only in haemoglobins that have -SH groups at positions β 93 and α 104. It follows, therefore, that one of these sites must be responsible for the initial dissociation step. In haemoglobin A, the reactive -SH group has been identified as that at position β 93. (Goldstein, Guidotti, Konigsberg and Hill, 1961) and it is reasonable to expect the -SH group at position β 93 in other haemoglobins to be the reactive residue. For example, Schroeder (1963) suggested that the -SH group at position γ 93 in fetal haemoglobin is reactive while that at position α 104 is not. Furthermore, Rosemeyer and Huehns, proposed that before dissociation of the tetramer to the dimer is complete, the reaction at the other -SH group of the β chain (β 112 in Hb A) is occuring and that the reaction at this site is, in fact, competing with reaction at position β 93 so that dimer production from haemoglobin A is only complete when both β sulphydryls have reacted. A similar behaviour is expected for canine haemoglobin. Finally, as monomer production requires total substitution, the second dissociation step must depend on reaction at α-104. Since this residue does not appear to react in fetal haemoglobin, it would appear that its accessibility in adult haemoglobin depends on prior reaction at the position β 112.

(c) HYBRIDISATION IN HAEMOGLOBINS

Itano and Singer (1958) discovered that when mixtures of haemoglobins differing in some respect in the α or β or in both chains were exposed to acid or alkaline pH and then neutralised, new electrophoretic components that were not initially present, were obtained. This indicates the formation of "hybrid" haemoglobin molecules made up of parts exchanged between the molecules present at the beginning. Such hybrid haemoglobin species have been shown to form between human haemoglobin A and canine haemoglobin (Itano and Singer, 1958) and between Donkey and mouse haemoglobins (Riggs et. al. 1962). This phenomenon, which presupposes the dissociation of haemoglobin into monomers has found useful applications for the identification of the different chains in the abnormal haemoglobins (Itano and Robinson, 1960; Huehns et. al. 1962). Various and ingenious experiments made with different haemoglobin mixtures indicate that the hybrid molecules have exchanged the two α and the two β chains i.e. the two α and the two β chains in any of the components are the same while different α or different β chains in the same molecule are consistently absent. (Itano and Robinson, 1960; Vinograd and Hutchinson, 1960). Thus the hybridisation has arisen by a simple exchange of α_2- and β_2- subunits between the two

parent haemoglobins (X and Y) as indicated by the equation

$$\alpha_2^X\beta_2^X + \alpha_2^Y\beta_2^Y \rightarrow \alpha_2^X\beta_2^Y + \alpha_2^Y\beta_2^X$$

From these results, Itano and Robinson (1960) concluded that the dissociation of haemoglobin is asymmetrical both at high and low pH (i.e. $\alpha_2\beta_2 \rightleftarrows \alpha_2 + \beta_2$). However, reversible dissociation of haemoglobin as revealed directly by physico-chemical methods can take place under conditions in which hybridisation does not occur. Therefore, the reversible splitting into halves of haemoglobin is not by itself a sufficient condition for hybridisation. Indeed hybridisation occurs only when the haemoglobin mixture is exposed to a pH near 4.7 or a pH near 11. Thus no hybridisation was observed when canine and human haemoglobins were kept together at pH 5.2 for 24 hours and then neutralised (Antonini et. al. 1962). Yet it is known that these haemoglobins are largely dissociated at this pH. Furthermore, hybridisation takes place only when the haemoglobin mixtures are exposed to these very low or high pH's for a long time. (Itano and Robinson, 1960). In the case of human and canine haemoglobins, it was found that the amount of hybridisation was greatly reduced when the time of exposure to alkaline pH was lowered from 4 hrs to 20 minutes. Yet all the

evidence is that the dissociation involves a rapidly established equilibrium and that under the conditions of these experiments, it was always substantial (Hersserodt and Vinograd, 1959; Kurihara and Shibata, 1960). If so time should make no difference.

More recently, Vinograd and Hutchinson (1960) have shown that when haemoglobin A and labelled haemoglobin S which had been kept separately for 24 hours at pH 11 were suddenly brought together and neutralised within 10 seconds, only a very slight amount of hybridisation occured; although the molecules were certainly mainly dissociated at the time of neutralisation. Moreover, the formation of hybrid molecules is not observed in mixtures of haemoglobin dissociated in concentrated salt solutions. (Antonini et. al. 1962).

All these observations are difficult to reconcile with the hypothesis of an asymmetrical splitting of the molecules. On the other hand, they agree well with the idea of a symmetrical dissociation as postulated by Vinograd and Hutchinson (1960). Vinograd and Hutchinson pointed out that if the dissociation were symmetrical, all the experimental data and some of the inconsistencies mentioned above could be rationalised with the aid of two assumptions viz:-

(a) that the dimers, obtained on dissociation of the tetramer, are in equilibrium with monomers at both high and low extremes of pH and

(b) that only like dimers recombine to form tetramers i.e. a high degree of specificity is required in the recombination of the single chains so that only like α (or β) chains combine to form dimer subunits to the final exclusion of tetramer hybrids of the type $\alpha^X \alpha^Y \beta_2^Y$ etc.

Vinograd's postulates have been modified by Guidotti et. al. (1963) who maintains that the postulated equilibria between tetramers and dimers and between dimers and monomers are a function of pH and ionic strength. These equilibria can be represented as

$$\alpha_2\beta_2 \overset{k_1}{\rightleftharpoons} 2\alpha\beta \overset{k_2}{\rightleftharpoons} 2\alpha + 2\beta$$

where k_1 and k_2 are the equilibrium constants for the successive dissociation steps. Guidotti et al. (1963) have found that the value of k_1 is small but measurable at neutral pH and increases at both high and low pH's and that the value of k_2 is very small at neutral pH and increases at the extremes of pH. Also, the value of k_1 does not change while the value of k_2 becomes very small in strong sodium chloride solutions.

With this equilibrium model, Guidotti et.al. pointed out that all the hybridisation data are easily explained and the puzzling observations mentioned above could be resolved without necessarily making Vinograd's second assumption that only like dimers recombine to form tetramers. They explained some of these anomalies as follows:

(a) Hybridisation occurs only when haemoglobin mixtures are exposed to high or low pH's because the dissociation constant k_2 is not very pH dependent but increases appreciably only below pH 6 or at above pH 11, except at high ionic strength where it is diminished. (b) The rate of hybridisation is a function of the rate of monomer formation and therefore is not directly related to the rate of dimer formation. (c) Hydridisation does not take place in concentrated salt solutions because the value of the dissociation constant k_2 does not favour monomer formation. (d) There is no specificity in the association of the $\alpha\beta$ subunits and species with unlike α and β chains do form but are not detected by the usual analytical methods because of the existence at neutral pH of a rapid equilibrium between the $\alpha_2\beta_2$ tetramer and the $\alpha\beta$ subunits. One exception to the rule that haemoglobins with an unlike pair of chains cannot be detected is haemoglobin F_I, a

fetal haemoglobin which Schroeder et. al. (1962) have shown to have the composition $\alpha_2{}^{A}\gamma^{F}\gamma^{Acetyl-F}$. This haemoglobin is known to be stable when isolated. Guidotti et. al. could not give a satisfactory explanation for this. (e) A tetramer of haemoglobin containing only α chains is not produced because it is not as stable as $\alpha\beta$ subunits. Finally, it is to be expected that the equilibrium constants (k_1 and k_2) for the dissociation reactions will vary depending on the type of haemoglobin and the species and these variations are reflected in the relative rates of hybridisation in the different haemoglobin species.

A part of this thesis is concerned with physico-chemical studies on the hybrid haemoglobin species of human and canine haemoglobins. The dissociation (at pH 4.7) and recombination (pH 7.0) of human adult and canine haemoglobins produce two new species. The hybridisation reaction can be represented by the equation

$$\alpha_2{}^{A}\beta_2{}^{A} + \alpha_2{}^{Ca}\beta_2{}^{Ca} \rightarrow \alpha_2{}^{Ca}\beta_2{}^{A} + \alpha_2{}^{A}\beta_2{}^{A} + \alpha_2{}^{Ca}\beta_2{}^{Ca} + \alpha_2{}^{A}\beta_2{}^{Ca}.$$

The hybrid haemoglobins could be resolved from one another and the parent haemoglobins by electrophoresis at pH 8.5 (Huehns et. al. (1962). In the present study, they are resolved by Column Chromatography. Since only the more rapidly migrating species is

formed when canine haemoglobin is dissociated and recombined with haemoglobin H (i.e. β_4^A); its composition is $\alpha_2^{Ca}\beta_2^A$ and that of the slower hybrid species is $\alpha_2^A\beta_2^{Ca}$ (Huehns, Shooter and Beaven, 1962). The amounts of the two hybrid species in the dissociated and recombined mixture increases with time of exposure to acid pH, reaching a maximum after 24 hours. The equilibrium mixture attained after this time usually contains equal proportions of the two hybrids and of the two parent haemoglobins (Huehns et. al.1964).

LIGAND BINDING REACTIONS OF HAEMOGLOBIN

In all haemoglobin complexes the sixth coordination position is occupied by a ligand but in deoxygenated haemoglobin this position is an empty hole (Nobbs et. al. 1966). The structure of deoxygenated haemoglobin is thus unique and different from that of all other derivatives which have similar quarternary structures to one another (Perutz, 1970). Therefore, when deoxygenated haemoglobin reacts with a ligand there is usually a change in quarternary structure and some minor changes in the tertiary structure of the molecule (Perutz, 1970). This structural change is probably triggered off by movement of the iron atoms relative to the plane of the porphyrin, for transition from deoxyhaemoglobin

to oxyhaemoglobin involves a movement of the proximal histidine (F8) towards the plane of the porphyrin by 0.75 - 0.95Å. For transition from deoxyhaemoglobin to the high spin acid methaemoglobin (Coryell et. al, 1937), the movement is by 0.45 - 0.65Å in the same direction. On formation of low spin complexes from high spin methaemoglobin, there is a further movement of the iron atom from a position out of plane to the plane of the porphyrin ring (Hoard et. al, 1969). But this last movement does not lead to any significant change in quarternary structure of the molecule, for the quarternary structure of methaemoglobin is similar to that of its complexes (Perutz, 1970).

The iron atom in methaemoglobin carries a net positive charge hence it forms complexes mostly with negatively charged ligands like azide, fluoride, cyanide, hydroxyl and hydrosulphide ions. It can also form complexes with neutral ligands having lone pairs of electrons e.g. imidazole and methylamine (Scheeler, 1958; Ige, R.O. 1971). In acid methaemoglobin, the sixth coordination position of the iron atom is occupied by a water molecule and this water molecule is displaced by a ligand on complex formation. This can be represented as

$$Hb^{+} H_2O + L^{-} \rightleftarrows HbL + H_2O$$

where $Hb^{+}H_2O$ represents the methaemoglobin, L^{-} the ligand and HbL, the complex formed. The types of complexes formed can be grouped according to the spin state of the iron atom. Thus low spin complexes are formed with azide, hydrosulphide and cyanide ions (Coryell et. al, 1937) while fluoride ion forms a high spin complex and hydroxyl ion forms complexes whose magnetic susceptibilities are intermediate between ionic and covalent (Coryell et. al, 1937).

The thermodynamics of the reaction of several methaemoglobin species with azide, cyanide hydrosulphide, fluoride and hydroxyl ions have been adequately studied with a view to correlate differences in reactivity with differences in structure (Anusiem, Beetlestone and Irvine, 1966, 1968a, 1968b, 1968c; Bailey, Beetlestone and Irvine, 1969). The general features of these reactions as reported by these authors can be summarised as follows:

(i) The standard free energy change (ΔG°) of formation of these complexes vary by only a few hundred calories $mole^{-1}$ over the pH range 6 - 9 for all the ligands studied; while the variation in the enthalpy change (ΔH°) for the formation of the azide and cyanide complexes vary by as much as 10 kcal/mole between different

methaemoglobins and 5-7 kcal/mole over the pH range 6-9 for any one methaemoglobin. However, for the formation of low spin complexes with the hydrosulphide ion, ΔH° varies by only 2-4 kcal/mole over the pH range 6-9 for any one methaemoglobin and by as much as 7 kcal/mole between different haemoglobins while values of ΔH° for the formation of the fluoride complex are smaller than those observed for the other ligands (about 0-3 kcal. $mole^{-1}$ over the pH range 6-9).

(ii) For the azide methaemoglobin complex, the variation of $-\Delta H^{\circ}$ with pH for all methaemoglobins is a characteristic bell-shaped curve, the maximum value of $-\Delta H^{\circ}$ being different in each haemoglobin and occuring at different pH's; while for the complexes formed between methaemoglobin and hydrosulphide ion, the curve of $-\Delta H^{\circ}$ against pH shows a minimum value at a pH very close to that at which a maximum value of $-\Delta H^{\circ}$ is observed for the methaemoglobin-azide complex. Hydroxyl ion behaves similarly to the hydrosulphide ion. Beetlestone and Irvine designated this pH where the abrupt change in $d(\Delta H^{\circ})/d(pH)$ occurs, the "characteristic pH" (pH_{ch}) (Bailey et. al, 1970a).

(iii) For all the complexes, the relative magnitude of the variations in $T\Delta S^{\circ}$ and ΔH° are such that they compensate one another

with the result that they make little contribution to ΔG°.

(iv) A rough correlation was obtained between the characteristic pH and the isoionic point of the methaemoglobin and a linear correlation was observed between the characteristic pH and a function of the amino acid composition of the haemoglobin (Bailey et. al, 1970a).

(v) A correlation was also obtained between the characteristic pH and the magnitude of the acid Bohr effect for several haemoglobin species (Bailey et. al, 1970b).

Beetlestone and Irvine tried to explain the variation of changes in free energy and enthalpy of formation of methaemoglobin complexes with pH and between different methaemoglobins, in terms of the differences in electrostatic interactions in different haemoglobins by using the dielectric cavity model developed by Kirkwood (1934) for proteins. (Beetlestone and Irvine, 1964, 1965, 1968a). Using this model, these authors were able to account satisfactorily for the ΔG° variation with pH except for a narrow range of pH. This pH varies from one methaemoglobin to another and it is found to be the same as the pH where $d(\Delta H^{\circ})/d(pH)$ changes abruptly i.e. the characteristic pH. However, the dielectric cavity model could not explain the large and variable changes in

$-\Delta H^{\circ}$ with pH for the formation of methaemoglobin complexes and so it was necessary to look for an additional mechanism, besides electrostatic interactions, which would explain the variation of $-\Delta H^{\circ}$ with pH. This additional mechanism must not make a large contribution to the free energy since the dielectric cavity model successfully accounts for the free energy behaviour. Furthermore, such a mechanism must be specific to the iron atom since the dielectric cavity model is satisfactory for the interpretation of the thermodynamics of the ionisation of amino acid side chains (Anusiem, Beetlestone and Irvine, 1968a).

To account for the large ΔH° variation with pH, these authors postulated a mechanism involving changes in protein configuration and hydration. Large compensating enthalpy and entropy changes can arise in these systems as a result of either (a) configurational changes in the protein which will give rise to enthalpy and entropy changes of the same sign which would compensate to some extent or (b) hydration changes which would not give rise to any contribution to the free energy change since water in the hydration sheath is always in equilibrium with the solvent water, but which would be accompanied by compensating enthalpy and entropy changes. (Ives and Marsden, 1965). Clearly, changes in the configuration of

the protein will give rise to changes in hydration. Beetlestone and Irvine postulated three mechanisms by which such changes could arise:

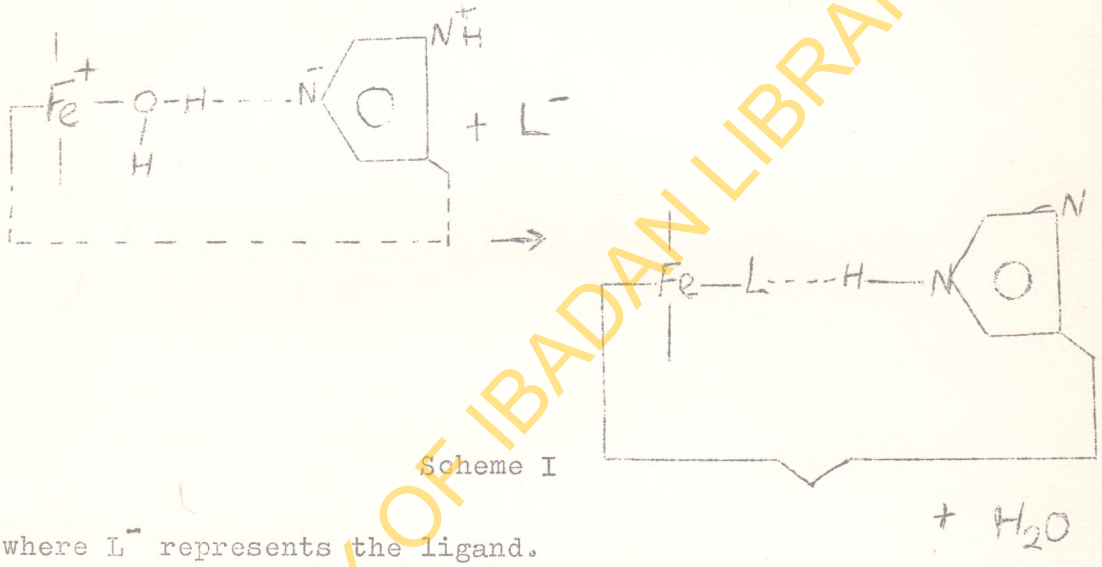
(1) The change of spin state on the iron atom which occurs when the ligand is hydroxyl, azide, cyanide or hydrosulphide ion is known to be accompanied by small changes in the protein structure (Watson and Chance, 1966; Ogawa and McConell, 1967).

(2) The replacement of the neutral water molecule by a charged ligand may remove polarisation on groups in the vicinity of the iron atom, particularly the distal imidazole.

(3) Hydration changes can arise by a "Proton shift" mechanism (Beetlestone and Irvine 1969). The proton-shift mechanism was based on the interpretation of Stryer et. al. (1964) for the changes in the electron density map observed when sperm whale myoglobin reacts with azide ion in the crystal. They suggested that the change involves the release of a sulphate ion (present as part of the mother liquor of the crystal) from a position adjacent to the tertiary nitrogen of the distal imidazole ring, which is exposed to the solvent. Beetlestone and Irvine argued that in dilute aqueous solution of low ionic strength it is unlikely that any anion would be specifically bound to the

imidazole nitrogen in methaemoglobin, but that Stryer's observations suggest a possible mechanism which could give rise to hydration changes. Specifically, Beetlestone and Irvine (1969) postulated that on complex formation, a hydrogen bond is formed between the ligand and the distal imidazole. The hydrogen atom involved may originate either from the ligand or by the transfer of a proton between the two nitrogen atoms of the distal imidazole. Hydration changes could arise if complex formation with some ligands is accompanied by a shift of a proton on the distal imidazole from the nitrogen atom on the surface of the molecule to the nitrogen atom which points towards the iron atom. Depending on the overall structure of the water of hydration in the region around the imidazole, this change could be a structure-forming or a structure-breaking process and hence could give rise to positive or negative entropy changes. The gross structure of water of hydration will vary with pH if only as a result of the changes in the net charge on the molecule and hence large variations with pH, of entropy and compensating changes in enthalpy would be expected. By contrast, when a ligand carries its own hydrogen atom into the complex there will be no "proton-shift" and hence the hydration changes which accompany proton-shift would be absent.

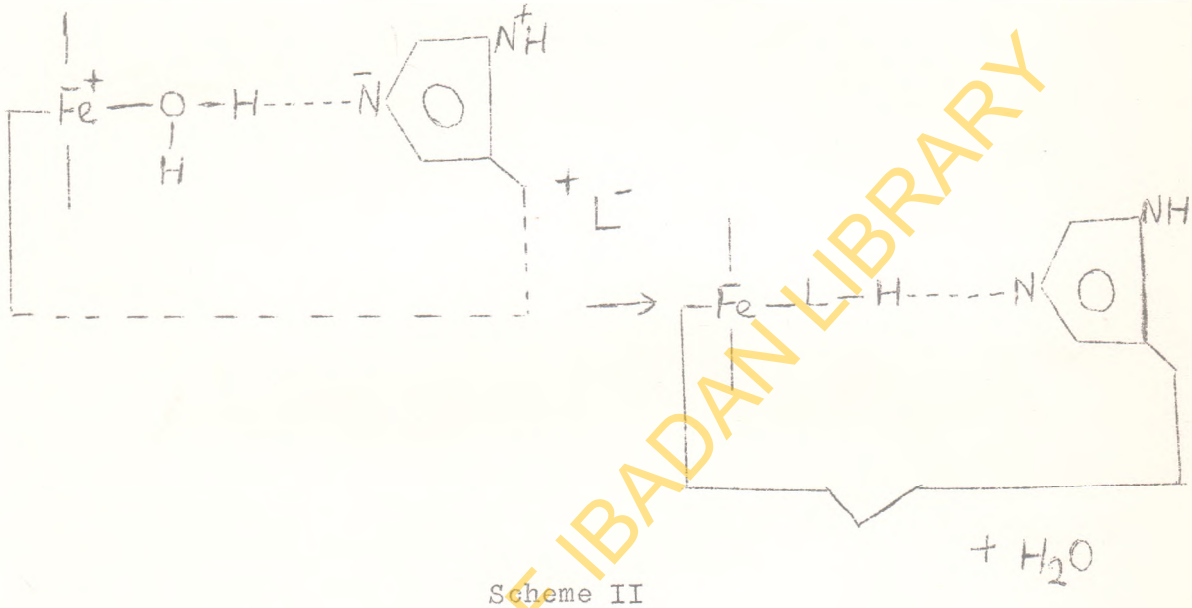
Thus the formation of the cyanide and azide complexes could be represented as in Scheme I below.



Scheme I

where L^- represents the ligand.

The loss of the hydrogen atom from the nitrogen of the distal imidazole which 'sees' the solvent will give rise to hydration changes whose precise nature will depend on the structure of the water of hydration in this region of the protein. On the other hand, hydrosulphide and hydroxyl ions both carry a hydrogen atom into the complex and we can represent the formation of these complexes as in Scheme II below.



Scheme II

Here, the hydrogen atom remains on the 'outside' of the distal imidazole and hence the hydration changes which accompany the removal of this hydrogen are absent. This hypothesis implies that any ligand which carries with it into the complex a hydrogen atom which can form a hydrogen bond with the distal imidazole will behave similarly to hydrosulphide ion rather than azide or cyanide ions. This has been tested by Ige (1971) who showed that methylamine which also forms a low-spin complex and carries its own hydrogen atom into the complex behaves like hydrosulphide ion rather than azide ion.

While the mechanisms discussed above can adequately account in

a qualitative way for the behaviour of different ligands, they in no way account for the particular form of the variation of $-\Delta H^{\circ}$ with pH for any methaemoglobin, that is, why the maximum or minimum value of $-\Delta H^{\circ}$ occurs at a particular pH for each methaemoglobin. This pH has been termed the characteristic pH (pH_{ch}). In order to account for this Beetlestone and Irvine postulated that over a narrow pH range around pH_{ch}, a structural pertubation of the protein occurs such that the charged groups on the surface of the molecule adopt different configurations above and below pH_{ch}. There is a correlation between pH_{ch} and the isoionic point of the haemoglobin (Anusiem, Beetlestone and Irvine 1968c) but the pH_{ch} varies very much more than the isoionic point, hence the pH_{ch} cannot be a function directly of the isoionic point. These authors therefore concluded that other factors in addition to the net charge determine the hydration structure and hence the variation of $-\Delta H^{\circ}$ with pH.

As stated above (page 28 of this thesis), the dielectric cavity model failed to explain the free energy behaviour only at a narrow pH range which was found to be the same as the characteristic pH. This suggests that at the characteristic pH, methaemoglobin undergoes a configurational change which dramatically changes the hydration sheath of the protein but which does not

significantly affect the affinity for ligands. Furthermore, Bailey, Beetlestone et. al. (1970a) have obtained a linear relationship between pH_{ch} and a function of the charged amino acid residues of the protein. By charged amino acid residues we refer only to those amino acid residues that remain in a completely charged state over the pH range of the experiments (5.8 - 9.0). The amino acid residues which come under this category are the basic lysine and arginine and acid glutamic and aspartic residues, the propionic side chains of the haem groups and the terminal amino and carboxylic acid end groups.

Beetlestone et. al. found that irrespective of the position in the molecule, a gain of a lysine residue or the loss of a glutamic acid residue leads to an increase of pH_{ch}. This suggests that pH_{ch} may be directly related to the difference between the numbers of negatively and positively charged groups irrespective of their positions in the molecule. This difference was given as (lys + Arg glu - Asp - 8) where the symbols respectively represent the number of lysine, arginine, glutamic acid and aspartic acid residues in the haemoglobin tetramer. The number 8 appears in order to take account of the two propionic side chains on each haem ring and it is assumed that the charges on the N- and C-terminal ends of the

polypeptide chains cancel one another. When pH_{ch} was plotted against (lys + Arg - Glu - Asp - 8) for several haemoglobin species a straight line was obtained (Bailey et. al. 1970a). Thus it appears that what actually determines pH_{ch} for different haemoglobin species is the number and type of charged amino acid residues in the molecule irrespective of their relative positions.

From the correlation between pH_{ch} and the charged amino acid composition of the haemoglobin tetramer, it appears as if the value of pH_{ch} is an average of the contributions from the α and β polypeptide chains. In other words, the pH_{ch} of the tetramer appears to be the average of the pH_{ch}'s of the separate α and β polypeptide chains. This has been shown to be the case even when one of the polypeptide chains is not reacting, by the recent work of M.M. Ogunlesi (1971) on the binding of azide ion by the valency hybrids of human haemoglobin A i.e. $\alpha_2^{II}(CO)\ \beta_2^{III}$ and $\alpha_2^{III}\beta_2^{II}(CO)$ where she obtained the same value of pH_{ch} for both hybrids as was obtained for normal human haemoglobin A. The shape of the curve of $-\Delta H^{o}$ vs pH is, however, a function of the tetramer and cannot be obtained by averaging $-\Delta H^{o}$ values of the separate α and β chains.

Further, it has been suggested above that pH_{ch} is a manifestation of a pH dependent configurational

change in the protein involving charged groups on the surface of the molecule. This postulated configurational change could be of two types:

(1) The configurational change may involve only charged group on flexible side chains on the surface of the molecule without affecting the tertiary structure of the molecule

or (2) The configurational change may also involve changes in tertiary structure. Since pH_{ch} is a function of the tetramer, this possibility implies that the configurational change involves a concerted pertubation in the tertiary structure of α and β polypeptide chains. This in turn would depend on the presence of crucial interchain interactions.

We can investigate the effect of changing the structure of one of the polypeptide chains while at the same time, as far as possible, retaining interchain interactions. This has been done in this thesis by studying the thermodynamics of the reaction of azide ion with the hybrids of canine and human haemoglobins which show normal functional properties (Antonini E. et. al, 1965).

If the correlation between pH_{ch} and the charged amino acid composition of the protein is an exact one, then it implies that the contributions of the α and β polypeptide chains to determining

pH_{ch} are additive since the correlation also holds for the α chain of haemoglobin A (Bailey et. al, 1970a). That is to say, for any methaemoglobins

$$pH_{ch}(\alpha_2\beta_2) = \tfrac{1}{2}\left[pH_{ch}(\alpha) + pH_{ch}(\beta)\right]$$

where $pH_{ch}(\alpha_2\beta_2)$, $pH_{ch}(\alpha)$ and $pH_{ch}(\beta)$ are respectively the characteristic pH's of the tetramer, the α chain and the β chains. If this equation is correct, we should be able to calculate pH_{ch} for canine human A and the two hybrid haemoglobins from a knowledge of pH_{ch} fo the isolated chains of human and canine haemoglobins. Hence in thi thesis, the thermodynamics of the reaction of azide ion with the α and β chains of canine and human methaemoglobins have also been studied. Since the met-derivatives of the separate chains are unstable under ordinary conditions, the equilibrium reactions on these chains were carried out in 1 molar glycine, in which they are known to be stable (Banerjee and Cassoly, 1969). As stated above, a correlation has been obtained between pH_{ch} and the magnitude of the acid Bohr effect of the haemoglobin. Hence, to further test this correlation, the Bohr effect of the hybrids of human and canine haemoglobins has been studied.

As mentioned above (page 29 of this thesis), the large variation of ΔH^{o} with pH observed for ligand binding reactions of

methaemoglobin has been interpreted in terms of hydration changes. Such hydration changes could arise if complex formation with some ligands is accompanied by a shift of a proton on the distal imidazole from the nitrogen atom on the surface of the molecule to the nitrogen atom which points towards the iron atom.

The question then arises whether one gets this 'hydrogen shift' in the reactions of haemoglobin itself. In other words, suppose we have the hydrogen atom 'out' in deoxyhaemoglobin; does binding of oxygen shift this hydrogen inside? If this is so, then we should observe a similar ΔH° variation with pH as in methaemoglobin reactions. However the ligand binding reactions of haemoglobin are complicated by the presence of haem-haem interaction. Hence we have to look at a much simpler reaction which is easier to study. A section of this thesis will therefore be devoted to a study of the reaction of carbon monoxide with oxyhaemoglobin. This is indeed a simple reaction since, structurally, there is no change in the quarternary structure of the protein and one ligand replaces another in the same coordination position rather than there being a change of coordination number as in the reaction of either carbon monoxide or oxygen with haemoglobin. Also, the formation curve of this reaction is hyperbolic unlike the reactions of oxygen or carbon

monoxide with haemoglobin which are sigmoidal.

Suppose we have the hydrogen atom 'out' in oxy-haemoglobin and the hydrogen atom 'in' in carbon monoxyhaemoglobin or vice versa then this reaction is accompanied by a proton shift and hence we should observe a variation of ΔH^{o} with pH similar to that observed for methaemoglobin reactions. This reaction can be represented as

$$HbO_2 + CO \rightleftarrows HbCO + O_2$$

and we can define the equilibrium constant k_m for the reaction as

$$K_m = \frac{[HbCO][O_2]}{[HbO_2][CO]}$$

where [HbCO], [HbO_2], [O_2] and [CO] are respectively the equilibrium concentrations of carbon monoxy haemoglobin, oxyhaemoglobin, oxygen and carbon monoxide.

The value of K_m has been determined spectrophotometrically and a simple tonometer has been devised for this purpose. With this method, accurate and reproducible values of K_m can be obtained. It is therefore possible to investigate enthalpy, entropy and free energy changes in this reaction as a function of pH for a number of haemoglobin species by determining K_m as a function of pH and temperature.

CHAPTER II

EXPERIMENTAL

I. Materials

Buffer Solutions: All buffer solutions were made with analytical grade reagents. Between pH 5.8 and 8.0 phosphate buffer solutions prepared according to Gomori (1955) were used. In the pH range 8.0 to 10.2, borate buffers prepared from boric acid and sodium hydroxide were used. Above pH 10.2, bicarbonate buffer solutions were used (Gomori, 1955). Unless otherwise stated, the ionic strength of all buffers were adjusted to 0.05 with analar sodium chloride.

Sodium Azide: Sodium azide used for azide equilibrium measurements was a B.D.H. product. It was further purified by recrystallisation from hot water.

Glycine: The glycine used for the stabilisation of the separate chains of human and canine haemoglobins was a B.D.H. product and was used without further purification.

Ion Exchange Celluloses: Carboxymethyl cellulose (CMC.32) and Diethylaminoethyl cellulose (D.E.A.E.32) used for chromatographic separations were Whatman products.

Other Reagents: Hydrolysed starch obtained from Connaught Medical Research Laboratories, Toronto, Canada, was used for starch gel electrophoresis. Sephadex G-25 medium, used in the separation experiments, was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). p-chloromercuribenzoate (P.C.M.B.) used for the dissociation of haemoglobin and mercapto ethanol used for demercuration were both B.D.H. products.

Blood Samples: Blood samples containing haemoglobin A were obtained from the blood bank of the University Hospital, Ibadan and had been characterised as such by paper electrophoresis in the routine screening of blood taken from donors. Blood of dog was obtained from a single animal. The anticoagulant used was either oxalate or citrate.

II. APPARATUS

Optical density measurements were made on a Unicam SP 500 Spectrophotometer with a constant temperature cell compartment, unless otherwise stated.

Measurement of pH was done using a Radiometer pH Meter 4d, unless otherwise stated.

III. PROCEDURE

Preparation of Haemoglobin:- The blood sample was centrifuged at a speed of about 18,000 r.p.m. for 20 mins at 5°C and the supernatant sclution sucked off at the water pump. The red cells were then washed three times with saline (9.5 gm/litre of NaCl) at 5°C, centrifuging between washings at 7,000 - 10,000 r.p.m. for 15 mins. The cells were then lysed by adding equal volumes of cold distilled water and cold purified ether equal to the volume of the packed cells. The mixture was well shaken and centrifuged at 7,000 - 10,000 r.p.m. for 20 mins. After the lysing, excess ether was removed by first sucking at the water pump and then by evaporating the remaining traces by gently blowing air into the centrifuge tube. The haemoglobin solution was then carefully decanted from beneath the case of cell debris.

Sodium chloride was added to 5% (weight/volume) to precipitate the non-heme proteins and the haemoglobin solution was left to stand at 5°C for 20 mins and centrifuged at a speed of 18,000 r.p.m. Any remaining ether was evaporated by gently blowing air into the centrifuge tube. The haemoglobin solution was then dialysed against 0.05M NaCl (pH 6.5 - 7.5), until the smell of ether was no longer detectable. The dialysis was usually done for about 12 hours with three changes of the NaCl solution.

Preparation of Methaemoglobin: The concentration of the haemoglobin solution was first determined by measuring the optical density at 540 nm of the cyanide complex of methaemoglobin, taking the value of the molar extinction coefficient at 540 nm as 10.9×10^3 (Cameron, 1964).

Approximately one molar solution of potassium ferricyanide (prepared by adding 1 ml of water to 0.33 gm of potassium ferricyanide) was prepared and just enough was added to the haemoglobin solution to provide a two-fold excess of ferricyanide. Excess ferricyanide was removed by slowly passing the methaemoglobin solution through a Dintzis column. (Dintzis, 1952).

Preparation of Dintzis Column for Deionising Proteins.

Hydrogen Form of Resin: Amberlite resin IR 120 was packed in a colum About 10 times its volume of 3M HCl was passed through it slowly. The resin was then washed with distilled water until the effluent was neutral to litmus.

Acetate form of Resin:

Amberlite resin IRA 400 was packed in a column and about 10 times its volume of 3M HCl was slowly passed through it. The resin was then washed with distilled water until the effluent was neutral

to litmus. About 10 times its volume of 3M sodium acetate solution was then slowly passed through the column. The resin was again washed with distilled water until the effluent gave no precipitate with silver nitrate solution.

Ammonium form of Resin:

Amberlite IR 120 was packed in a column and about 10 times its volume of 3M NaCl was slowly passed through it. Then the resin was thoroughly washed with distilled water until the effluent gave no precipitate with silver nitrate solution. Then about 10 times its volume of 3M NH_4Cl solution was slowly passed through it; and the resin was again washed thoroughly with distilled water until the effluent gave no precipitate with silver nitrate solution.

Packing of Dintzis Column:

A small quantity of the hydrogen form was first packed into the column to a length of about one inch. This was followed by about six inches length of mixed bed permitit "Biodeminrolit" resin. Then about three inches length of the acetate form was packed into the column, followed finally, by about three inches layer of the ammonium resin. The column was then washed with distilled water.

Chromatographic Separations.

In this thesis two different methods of chromatographic separation of haemoglobins were used. The hybrids of canine and human haemoglobins were separated from each other and from their parent haemoglobins by chromatography on carboxymethyl cellulose (C.M.C.32) using gradient elution technique. A similar method was used for the isolation of the α and β chains of canine haemoglobin. On the other hand, in the separation of the α and β chains of human haemoglobin, a two-column selective filtration procedure was used according to Geraci et.al, (1969). The chains obtained from the latter method were shown, by Geraci et. al. to be more stable than those obtained from a single C.M.C. column separation (Bucci and Fronticelli, 1965).

Pre-cycling and Equilibration of C.M.C. Resin.

A weighed amount of Whatman C.M.C.32 ion-exchanger was stirred into 15 volumes (volume of liquid/gm. dry resin) of 0.5N NaOH and left for at least 30 minutes. The supernatant was then decanted and the resin washed with distilled water (by stirring, allowing to settle and decanting), until the pH of the Resin was about 8. The resin was then stirred into 15 volumes of 0.5N HCl and left for at

least 30 minutes. The second treatment was repeated and the resin was washed until the pH of the resin was near neutrality. The C.M.C. resin was then equilibrated by stirring the resin into a small volume of 0.01 M phosphate buffer pH 6.0 (or phosphate buffer pH 6.6 for the resin used for the isolation of the β chains of human haemoglobin A) and titrated with 0.1M Na_2HPO_4 solution until the pH of the resin was about 6.0 (or 6.6 for the separation of the β chains of human haemoglobin). The supernatant was then decanted and the resin stirred into 0.01 M phosphate buffer of the same pH (pH 6.0 or 6.6 as the case may be). The resin was allowed to settle and the supernatant decanted. The equilibration was continued until the supernatant had exactly the appropriate pH. The resin was then stored in the cold at 5°C.

Pre-cycling and Equilibration of D.E.A.E. cellulose resin.

A weighed amount of Whatman D.E.A.E. 32 ion-exchanger was stirred into 15 volumes of 0.5N HCl and left for at least 30 minutes. The supernatant was then decanted and washed with distilled water until the pH of the resin was about 4. The resin was then stirred into 15 volumes of 0.5N NaOH and left for at least 30 minutes. The supernatant was decanted and the second treatment was repeated and followed by washing with distilled water until the pH of the resin

was near neutral. The resin was then equilibrated by stirring into a small volume of 0.01 M phosphate buffer pH 8.0 and titrated with 0.1M NaH_2PO_4 solution until the pH of the resin was about 8.0. The supernatant was decanted and the resin stirred into 0.01M phosphate buffer pH 8.0. Equilibration with 0.01M phosphate buffer pH 8.0 was continued until the pH of the resin was exactly 8.0. The resin was then stored in the cold at 5°C.

Packing of Column:

A 25 x 4 cm glass column plugged with glass wool at one end, was clamped vertically with the aid of a spirit level; and some phosphate buffer (of the same pH and ionic strength as the resin) was added. The stirred resin was then carefully poured into the column. The resin was first allowed to settle on its own and later the effluent was allowed to run to waste by opening the tap of the column. More resin was added until the packed resin had reached the desired level. The resin was then equilibrated overnight with phosphate buffer (of the same pH and ionic strength as the resin) at 5°C by allowing this buffer to run at a steady rate, through the resin.

Preparation and Purification of the hybrids of canine and human haemoglobins.

For the hybridisation experiment; equal amounts of human and canine carbon monoxy-haemoglobins were mixed together and dialysed at 5°C against two volume changes of 0.2M acetate buffer pH 4.6, for 24 hours (Itano and Robinson, 1959). The solution was then neutralised by dialysis at 5°C against two volume changes of 0.1M phosphate buffer pH 7.0 for 24 hours.

Previous workers on the hybrids of canine and human haemoglobins have always isolated the separate hybrid haemoglobins by preparative block-electrophoresis of the haemoglobin mixture on maize starch. However, in this thesis, a successful attempt has been made in the separation of these hybrids by column chromatography. The chromatographic procedure employed is described below.

The separation is carried out on carboxymethyl cellulose (C.M.C. 32) by gradient elution (both pH and ionic strength). 10 ml of about 10^{-3}M of the haemoglobin mixture, which had previously been dialysed against the first eluting buffer (phosphate buffer pH 6.0, I = 0.01) was applied carefully down the side of the glass column on to the C.M.C. resin bed with a syringe.

The haemoglobin mixture was allowed to drain into the resin

bed and some first elution buffer added to the top of the column. Gradient elution was started immediately. This was done by allowing phosphate buffer pH 8.0, I = 0.02 (the second elution buffer), to pass into an equal volume of phosphate buffer pH 6.0 I = 0.01 (first elution buffer) in a separate compartment which was continuously stirred by a magnetic stirrer. A buffer of continuously changing composition (ionic strength and pH) thus passed through the column. Elution was carried out at a steady rate of about 2 ml per minute and 10 ml fractions were collected.

After the separation, the optical density of each 10 ml portion was measured at 540 nm and a plot of optical density against tube number was obtained (fig. 1). Four peaks could be seen. Fractions around each peak in the elution profile were pooled together and concentrated. The different haemoglobin components were identified by starch gel electrophoresis, at pH 8.3, of each fraction from the column against a mixture of the hybrids and untreated canine and human haemoglobins. Huehns et. al. (1962) have shown by starch gel electrophoresis at alkaline pH that all four bands have anodic mobility. In sequence from the anode to the cathode these represent Hb $\alpha_2^{Ca}\beta_2^{A}$, HbA, Hb canine and Hb $\alpha_2^{A}\beta_2^{Ca}$. Thus from the relative mobilities of these fractions on electrophoresis at alkaline pH,

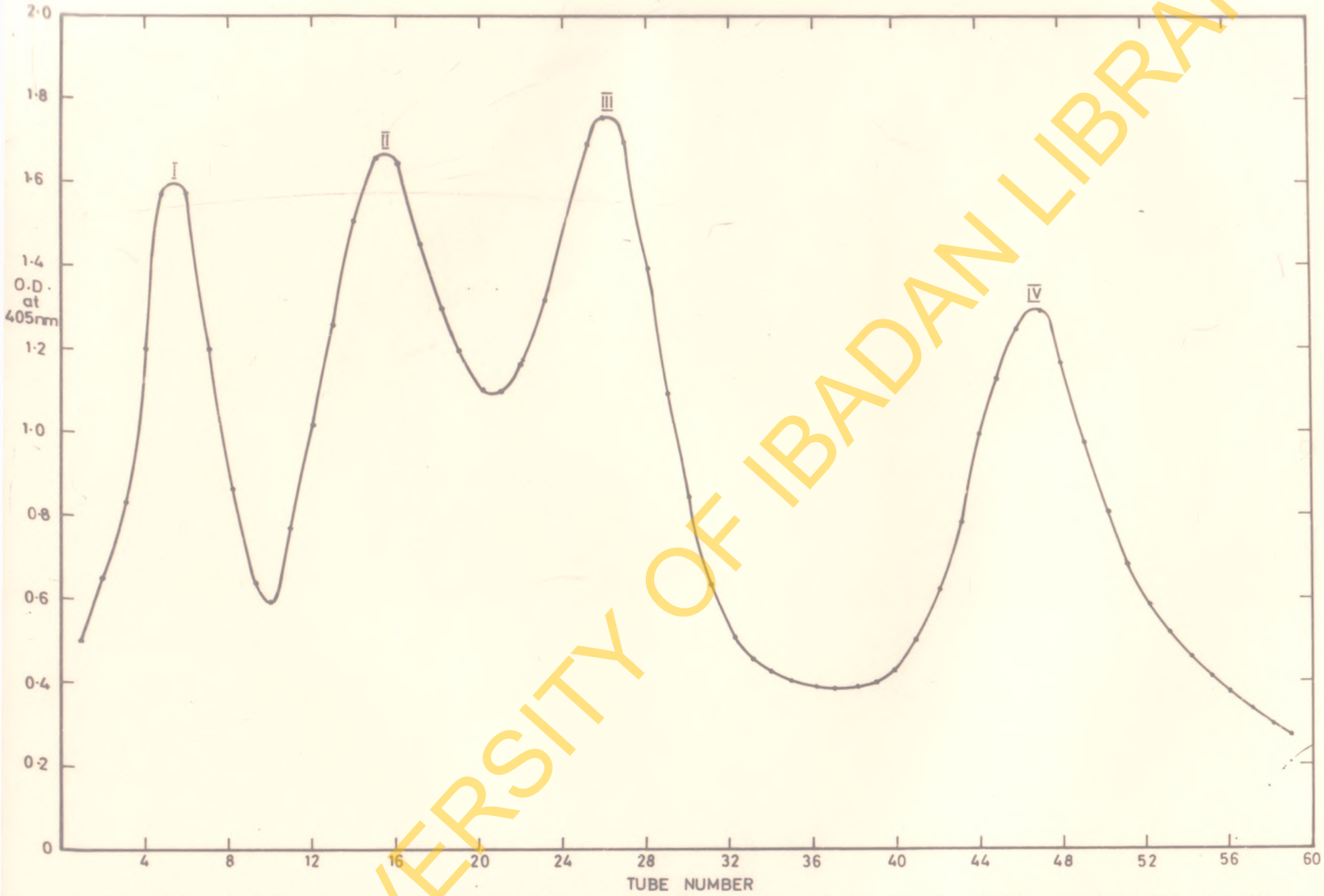


Fig. 1. ELUTION PATTERN OF THE CHROMATOGRAPHIC SEPARATION OF THE HUMAN—CANINE HYBRID HAEMOGLOBINS ON CMC 32 RESIN BY GRADIENT ELUTION (IONIC STRENGTH GRADIENT 0·01—0·02; PH GRADIENT 6·0—8·0.) RATE OF FLOW 2ml./min.

they could be identified. A table of the relative distances moved by each of these fractions after electrophoresis was carried out for 12 hours (at 300 volts and 16mA) is shown in Table 2. From this table, it can be seen that peak I in the elution curve is Hb $\alpha_2^{Ca}\beta_2^{A}$ while peak IV is Hb $\alpha_2^{A}\beta_2^{Ca}$ and peaks II and III correspond to HbA and canine haemoglobin respectively. Furthermore, the purity of the separated components was demonstrated using electrophoresis on starch gel.

Preparation and Purification of the α and β Chains of canine haemoglobin.

The method used for the dissociation of canine haemoglobin was essentially that described by Rosemeyer and Huehns (1967) for the dissociation of haemoglobins by p-chloromercuribenzoate (PCMB).

A 5% solution of PCMB was prepared by dissolving 0.25 gm of PCMB in 3 ml of 2N NaOH in a 5 ml standard flask and adding 1 ml of distilled water. The pH of the mixture was then adjusted with concentrated acetic acid solution until it gave a slight precipitate. The pH at this stage was between 10.5 and 11.0.

The haemoglobin solution was then dissociated by mixing 6 ml of carbon-monoxy haemoglobin ($\sim 10^{-3}$M) with 2.4 ml of 1M NaCl

solution and 7.0 ml of 0.2M phosphate buffer pH 6.0. 1.2 ml of 5% PCMB solution was then added drop by drop and the mixture left in the cold (5°C) for about 20 hours. The PCMB solution was added last to avoid too much precipitation of the chains. Complete dissociation of the haemoglobin into subunits as demonstrated on starch gel electrophoresis at pH 8.3, usually occurred after reaction with PCMB for about 20 hours but on some occasions, some undissociated haemoglobin remained.

The method used for the separation of the α and β chains from the mixture was similar to that described above for the separation of the hybrids of canine and human haemoglobins except that phosphate buffer pH 7.6, I = 0.02 was used as the second elution buffer instead of a phosphate buffer pH 8.0; as the former gave a better resolution of the components.

Elution profile of the separation gave three peaks (fig. 2). Upon concentration and electrophoresis at pH 8.3, peak I migrates faster towards the anode while peak III migrates slowest (Table 4). It has been shown that under electrophoresis at alkaline pH, the separated α chains of canine haemoglobins move faster than untreated canine haemoglobin and the β chains move more slowly than the untreated canine haemoglobin (Lehmann and Huntsman, 1966).

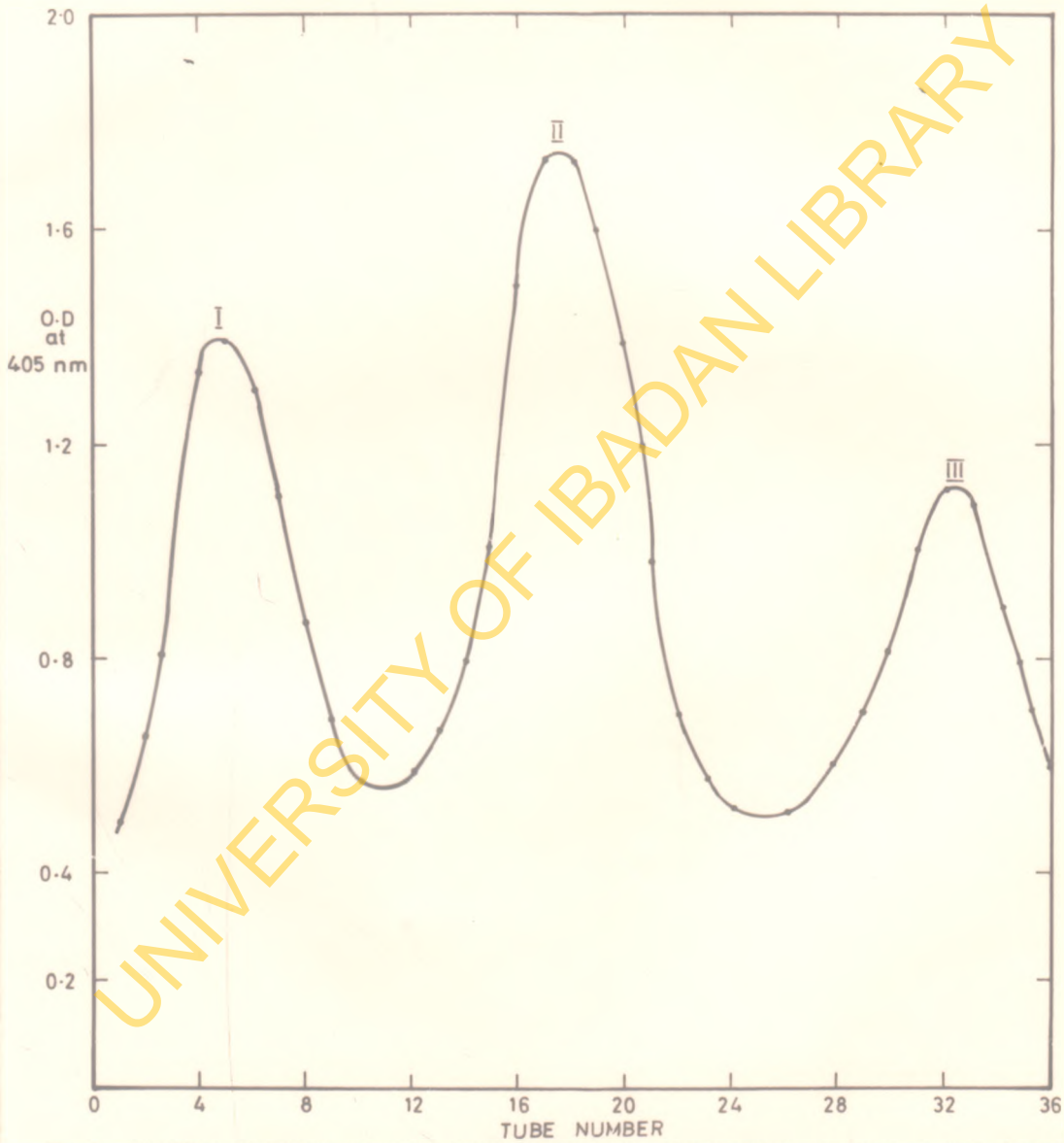


Fig. 2. ELUTION PATTERN OF THE CHROMATOGRAPHIC SEPARATION OF THE α AND β CHAINS OF CANINE HAEMOGLOBIN ON CMC 32 RESIN BY GRADIENT ELUTION. (IONIC STRENGTH GRADIENT 0·01 – 0·02; PH GRADIENT 6·0 – 7·6) RATE OF FLOW 2 ml./min.

Thus peak I corresponds to α chain of canine haemoglobin while peak III corresponds to the β chains. Peak II is the undissociated canine haemoglobin. The separate chains are unstable and they were stabilised by running directly into 4 molar phosphate buffer pH 7.0.

PCMB was removed from these chains with mercaptoethanol by the method of Geraci et. al, 1969. The chains were first dialysed against a phosphate buffer pH 8.0 and loaded on separate DEAE columns equilibrated to pH 8.0 (I = 0.01). The columns were then washed with 4 column volumes of 15mM mercaptoethanol in the same buffer for 4 hours. The columns were freed from mercapto ethanol by washing with phosphate buffer pH 8.0 until the smell of mercapto ethanol was no more detectable. The chains were then eluted with 0.1M phosphate buffer pH 7.0. SH titrations on the PCMB-free chains were carried out according to Boyer (1954). The α chain gave consistently between 1.57 and 1.60 SH groups per haem while the β chain gave between 1.59 and 1.62 SH groups per haem.

Preparation and Purification of the α and β chains of human haemoglobin (HbA).

The dissociation of human haemoglobin A was carried out as described above for canine haemoglobin except that complete

dissociation occured after 12 hours' reaction with PCMB. Hence the haemoglobin-PCMB mixture was left in the cold at 5°C for 12 hrs.

For the separation of the α and β chains, a two column selective filtration procedure was used according to Geraci et. al, 1969. In this method, haemoglobin A and one type of chain remain bound on the resin while the other chain was eluted. To obtain the α chains, 10 ml of the dissociated haemoglobin solution was adjusted to pH 8,0 by filtration through sephadex G 25 which had been equilibrated with 0.01M phosphate buffer pH 8.0. The haemoglobin mixture was then passed through a DEAE cellulose column equilibrated with 0.01M phosphate buffer pH 8.0 and α-PCMB chains were eluted with the same buffer. Under these conditions undissociated haemoglobin and β chains remain bound to the column.

β chains were obtained by adjusting 10 ml of the haemoglobin-PCMB mixture to pH 6.6 by filtration through sephadex G 25 equilibrated with 0.01M phosphate buffer pH 6.6. The mixture was then loaded on a C.M.C. 32 column which had been equilibrated with 0.01M phosphate buffer pH 6.6. The β-PCMB chains were eluted from the column with the same buffer. Under these conditions, haemoglobin A and α chains remain bound to the column.

PCMB was removed from both chains according to the method of Geraci et. al. (1969). PCMB was removed from the α chains by first

incubating for 10 minutes at 5°C with 15mM mercapto ethanol solution. The α-PCMB chains in 0.01M phosphate buffer pH 6.6 was applied to a C.M.C. 32 column (20 x 4 cm in dimension) which had been equilibrated with the same buffer. It was then washed over a period of about 30 minutes with 3 column volumes of 15mM mercaptoethanol in the pH 6.6 buffer. The column was then washed with pH 6.6 phosphate buffer until the smell of mercaptoethanol was no longer detectable. The α chains were then eluted with 20mM Tris-HCl buffer pH 8.0.

PCMB was removed from the β chains by loading the concentrated β chains in 0.01M phosphate buffer pH 8.0 on to a DEAE 32 cellulose column (40 x 4 cm) which had been equilibrated with the same buffer. (A longer column was used for the β chain as it tended to migrate down the column during washing). The column was washed with 4 column volumes of 15mM mercaptoethanol in the same buffer for 4 hours. The column was then freed from mercaptoethanol by washing with phosphate buffer pH 8.0 until the smell of mercaptoethanol was no longer detectable in the effluent. The β chains were then eluted with 0.1M phosphate buffer pH 7.0.

Complete removal of PCMB from both chains was checked by SH titrations (Boyer, 1954). SH titrations on the α chain gave between 0.88 and 0.93 reactive SH groups per chain while the β chains gave

between 1.77 and 1.81 reactive SH groups per chain. These results are in agreement with the values obtained by Geraci et. al. (1969). Purity of the chains was demonstrated by electrophoresis on starch gel at pH 8.3. The β chain of haemoglobin A moves much faster than haemoglobin A while the α chain moves much more slowly than haemoglobin A. (Table 3).

Concentration of haemoglobin samples:

The haemoglobin samples were concentrated by vacuum dialysis in the cold (5°C). The dilute haemoglobin solution was placed inside a visking tubing (grade 8/32) connected to a glass tubing which was fitted to a 5-litre flask with a rubber bung. The flask was then evacuated by sucking out air with a high-pressure pump. The level of the haemoglobin solution fell as the flask was evacuated and when the level of the haemoglobin solution was steady the rubber lead at the neck of the flask was tightly clipped thus creating a vacuum in the flask. The flask was then kept in the cold and solvent molecules exuded from the solution through the visking tube.

Starch gel Electrophoresis:

The procedure used was essentially the same as that of Fessas (1963). Discontinuous Tris-E.D.T.A.-Barbitone buffers were used. The vessel buffer pH 8.2 (I = 0.05) contained Diethyl Barbituric acid (Barbitone) (5.5gm) and sodium barbitone (10.3 gm) all made up to one litre with distilled water while the gel buffer pH 8.3 I = 0.05 contained 3.0 gm of Tris-hydroxymethylaminomethane; 0.390 gm Ethylene diamino tetra acetic acid (E.D.T.A.) dissolved in warm water and 2.5 gm boric acid all made up to 1 litre with distilled water.

The starch gel was made by adding 46.5 gm of hydrolysed starch into an Erlenmeyer flask containing 400 ml of the gel buffer. The flask was heated on a bunsen burner, the flask being continuously moved over the flame so that heat was evenly distributed. In a few minutes the starch is transformed into a viscous mass after which heating is continued for about another minute when the starch becomes less viscous. Prolonged heating was avoided as this would result in production of air bubbles. Any air bubbles in the gel were removed by connecting the flask to a vacuum pump until the solution finished boiling. The liquid was then carefully poured into a plastic tray (33 x 16 cm).

The warm starch solution in the tray was allowed to cool to room temperature. Meanwhile four sheets of Whatman 12 x 15 cm were dipped into the warm gel at each end of the tray. The gel was allowed to stand in the cold at 5°C.

A sheet of Whatman 3 mm. filter paper about 15 x 5 mm. in size was dipped in the haemoglobin solution. The excess haemoglobin was removed by touching the paper strip gently on a filter paper. Previously, by means of a razor blade inserted vertically in the gel, slots were cut transversely at about the middle of the tray at a distance of about 15 mm. from either side of the tray. The razor blade was inclined gently to one side in order to open the slit and the paper strip carrying the haemoglobin solution was inserted into the gel. The slits were covered with a few drops of warm starch solution and the gel was connected to the electrode vessels by means of paper bridges. The gel was covered with a sheet of plastic in order to prevent evaporation and a current of 16mA applied for 10 - 12 hours.

For electrophoresis of concentrated haemoglobin solutions, it was sufficient to inspect the coloured bands of the haemoglobin without staining, but with very dilute haemoglobin solutions, benzidine stain was used. The gel was stained by carefully

removing it from the tray and dipping it for 20 minutes in benzidine solution made up of 70 ml methanol to which were added 5 ml of 1.5M acetic acid, 5 ml of 1.5M sodium acetate, 20 ml of distilled water and 100 mg of benzidine (always freshly dissolved). 0.8 ml of 30% hydrogen peroxide was added to the solution just before use. The gel was then washed three times with a washing solution made up of 70 ml methanol, 5 ml of 1.5M acetic acid, 5 ml of 1.5M sodium acetate and 20 ml of distilled water.

Preparation of the met-derivatives of the chains.

The met-derivatives of both the α and β chains of canine and human haemoglobins are very unstable under ordinary conditions, hence they were stabilised in 1 molar glycine solution (Cassoly and Banerjee, 1969). Potassium ferricyanide was added to the chains in 1 molar glycine in a vessel equilibrated at about 10°C. Excess potassium ferricyanide was then removed by passing the met-derivative through a sephadex G-25 column which had been equilibrated with 1 molar glycine. The met-derivatives were always kept at low temperature (about 5°C) as precipitation of the chains occured at high temperatures.

Determination of the "active"-SH groups in haemoglobin by Titration with p-chloromercuribenzoate (PCMB)

The method used was that of Boyer (1954). PCMB solution approximately 2.1 x 10^{-2} molar was prepared by dissolving solid PCMB in 0.1 molar NaOH solution. It was then diluted in the ratio 1:25 with phosphate buffer pH 7.6, I = 0.05 and centrifuged. The optical density of the solution was determined using 1 cm silica cuvette at 250 nm.

The haemoglobin solution was diluted into phosphate buffer pH 7.6 (I = 0.05) so as to give an optical density of about 0.5 at 250 nm. 3.0 ml of the diluted haemoglobin solution was put into the 1 cm silica cuvette and its optical density measured at 250 nm. 0.01 ml increments of the PCMB solution were added using an Agla syringe and the solution was stirred with a small glass rod and the optical density at 250 nm measured after each addition. This was continued until the successive increases in optical density could be accounted for in terms of the absorbance of the PCMB solution itself. The readings were corrected for dilution and for the absorbance of the PCMB and a graph of change in optical density (ΔD) against volume of PCMB was plotted. The maximum change in optical density ($\Delta D_{max.}$) was taken as the point where

graph leveled off. The number of SH groups which have reacted per tetramer is then given by the equation

$$\text{Number of SH groups} = \frac{\Delta D_{max.}}{[Hb] \times 0.76 \times 10^{4}}$$

where [Hb] is the concentration of haemoglobin in gm. moles of haemoglobin tetramer and 0.76×10^{4} is the change in extinction coefficient on reacting one SH group with PCMB (Boyer, 1954).

Determination of the isoionic point of methaemoglobin

The methaemoglobin solution (about 4×10^{-4} molar) was passed through a freshly prepared Dintzis ion-exchange column very slowly into a thermostated vessel which was magnetically stirred and through which carbon dioxide-free air was passed. Sodium chloride was added to make the solution 0.05 molar. The pH of the solution was measured when its pH value became steady. This pH is the isoionic point of the methaemoglobin at the particular temperature of the experiment.

Determination of Equilibrium constants for the reaction of azide ion with methaemoglobin:

Dilute methaemoglobin solution having an optical density of 0.4 - 0.7 at 405nm was prepared in a buffer of known pH (I = 0.05).

5 ml portions of such a solution were pipetted into a series of 5 ml standard flasks into which various volumes (0.01 - 0.08 ml) of stock sodium azide solution of known concentration had been added from an Agla microsyringe. Also, some haemoglobin solution was put into a flask containing no azide, and another flask containing a few crystals of potassium cyanide. The reaction flasks were equilibrated in a thermostated water bath for about 8 - 12 hours at 13°C, 4 - 6 hours at 20°C and 3 - 5 hours at 27°C (The longer times being for alkaline pH's). The optical density of the contents of each flask was measured at 405 nm, except for the one containing the cyanide. The concentration of the methaemoglobin was then determined by measuring the optical density of the methaemoglobin cyanide complex at 420 nm. The molar extinction coefficient for the cyanide complex at 420 nm is 11.5×10^4 (Cameron, 1964). A similar procedure as described above was used for the separate α and β chains of canine and human haemoglobins except that all buffers used were made 1 molar in glycine so as to stabilise the met-forms of these chains. Also, measurements were taken at 6°C, 13°C and 20°C as higher temperatures tend to cause precipitation of these chains.

The complex formed between methaemoglobin and azide ion was first described by Smith and Wolf in 1904 and was characterised

spectrophotometrically by Keilin in 1936. At alkaline pH the reaction is in competition with the ionisation of methaemoglobin to its alkaline form. The two equilibria may be represented by the reaction scheme

$$H^+ + HbOH \overset{k_a}{\rightleftharpoons} Hb^+OH_2 + N_3^- \overset{k_L}{\rightleftharpoons} HbN_3$$

where Hb^+OH_2, HbOH and HbN_3 respectively represent methaemoglobin, alkaline methaemoglobin and methaemoglobin azide. Following Anusiem, Beetlestone and Irvine (1966), the reaction of azide ion with methaemoglobin can be described by the following equations and equilibrium constants:

$$k_a = [HbOH][H^+]/[Hb^+OH_2] \quad \ldots\ldots\ldots\ldots\ldots \quad (a)$$

$$K_L = [HbN_3]/[Hb^+OH_2][N_3^-] \quad \ldots\ldots\ldots\ldots\ldots \quad (b)$$

$$K_{obs} = [HbN_3]/[Hb^+OH_2 + HbOH][N_3^-] \quad \ldots\ldots\ldots \quad (c)$$

where $[Hb^+OH_2 + HbOH]$ is the total methaemoglobin concentration referred to a standard state of 1g. mole per litre of heme iron and $[N_3^-]$ is the unbound azide.

It follows from equations (a), (b) and (c) that

$$K_L = K_{obs}\left\{\frac{k_a + [H^+]}{[H^+]}\right\}$$

For methaemoglobin solution of a particular concentration and volume with varying concentrations of azide ion, D_o, D and D_∞ are defined respectively as the optical density of methaemoglobin solution alone; methaemoglobin solution with a finite concentration of azide and the methaemoglobin azide complex at 100% formation. The observed equilibrium constant is K_{obs} and is given by

$$K_{obs} = \frac{D_o - D}{(D - D_\infty)[N_3^-]}$$

where $[N_3^-]$ is the concentration of azide ion not bound to methaemoglobin. A typical calculation of K_{obs} is shown in Table 7. Thus the value of K_L can be calculated at a given pH from the value of K_{obs} if the value of K_a is known. The method for the determination of K_a for methaemoglobin is described below.

Determination of pK_a for the ionisation of methaemoglobin:

At about pH 8, the colour of methaemoglobin changes from brown to red. This spectral change was attributed to the ionisation of the water molecule in the sixth coordination position of the iron atom of methaemoglobin (Keilin and Hartree, 1952). A detailed study of these ionisations has been made by George and

Hanania (1953) for horse methaemoglobin. They proposed a number of equilibria represented by the following equations:

$$H_2Pr - Fe^+ OH_2 \rightleftarrows HPr^- - Fe^+OH_2 + H^+; pK_1' \quad \ldots\ldots\ldots (1)$$

$$H^-Pr - Fe^+ OH_2 \rightleftarrows Pr^{2-} - Fe^+OH_2 + H^+; pK_2' \quad \ldots\ldots\ldots (2)$$

$$Pr^{2-} Fe^+ OH_2 \rightleftarrows Pr^{2-} Fe^+OH^- + H^+; pK_3' \quad \ldots\ldots\ldots (3)$$

Pr stands for the globin moiety; $H_2PrFe^+(H_2O)$ and $HPr^-Fe^+(H_2O)$ being respectively the conjugate acid and base associated with pK_1'. pK_2' and pK_3' are defined in a similar manner.

The ionisation expressed in equation (3) is that responsible for the marked change in spectrum observed at about pH 8, and it is the one that will be considered here. A detailed study of this ionisation has been carried out for some mammalian methaemoglobins by Anusiem, Beetlestone and Irvine (1964, 1965). The ionisation constants were determined using the spectrophotometric method described by George and Hanania (1952, 1953) in which the pH and optical density of methaemoglobin solution are measured. pK_3' is given by the relation,

$$pK_3' = pH + \log_{10} \frac{[\text{Acid metHb}]}{[\text{Alkaline metHb}]}$$

where [Acid metHb] and [Alkaline metHb] are the concentrations of acid and alkaline methaemoglobins respectively i.e. of the species $Pr^{2-} Fe^{+} OH_2$ and $Pr^{2-}Fe^{+} OH^{-}$.

If D_o and D_∞ are the optical densities of acid and alkaline methaemoglobin respectively at any wavelength and D is the optical density at the same wavelength of a solution of the same total haemoglobin concentration but at such a pH that significant proportions of both forms are present, then

$$\frac{[\text{Acid metHb}]}{[\text{Alkaline metHb}]} = \frac{D_\infty - D}{D - D_o}$$

Optical density measurements were made at 405 nm. D_o was measured at pH 6.0 while D_∞ was measured at pH 10.50 and D was measured using solutions of pH ranging from 8.2 to 9.2. Table 5 shows a typical set of readings for the determination of pK_3' for $\alpha_2^{Ca}\beta_2^{A}$ methaemoglobins.

A similar procedure as described above was used for the α and β chains of canine and human haemoglobins except that phosphate or borate buffers made 1 molar in glycine were used in order to stabilise the met-derivatives of these chains.

Calculation of ΔH° for the formation of Azide-Methaemoglobin Complex

$$K_L = K_{obs} \left\{ \frac{K_a + [H^+]}{[H^+]} \right\}$$

Using the value of K_{obs} calculated as described above in this equation together with the experimentally determined value of K_a, K_L can be calculated at any particular pH.

The standard free energy change (ΔG°) is calculated from the relationship $\Delta G^{\circ} = -2.303\ RT \log_{10} K_L$. A graph of log K_L vs. pH is plotted (fig. 10) and by plotting the interpolated values of log K_L at fixed pH's against 1/T (fig. 11), ΔH° can be calculated. The slope of the plot of log K_L vs. 1/T has the value $-\Delta H^{\circ}/2.303\ R$ where R is the gas constant which is equal to 1.99 cals/mole/$^{\circ}$C. This procedure assumes that $\Delta C_p = (\partial(\Delta H)/\partial T)_p$ is zero or negligible over the temperature range under which the experiments were carried out and this would appear to be justified by the good linearity of the plots shown in fig. 11.

Measurement of the Bohr Effect for the hybrids of canine and human haemoglobins

The Bohr effect is observable either as a change in pH on binding of ligands by deoxygenated haemoglobin or as a change in

affinity of the haem iron for ligands as a function of pH. At a pH above 6.0 haemoglobin takes up protons on release of haem ligands; this is known as the alkaline Bohr effect. Below pH 6.0, the Bohr effect is reversed, protons being liberated on release of the haem ligands.

Wyman (1948, 1964) derived the relationship

$$\frac{d\log P_{½}O_2}{dpH} = -\Delta h^+$$

where $P_{½}O_2$ is the oxygen pressure at which haemoglobin is half saturated and Δh^+ is the number of moles of hydrogen ion produced when one mole of oxygen reacts with haemoglobin. Because of this relationship, two methods are available for the determination of the Bohr effect. One is the determination of $\Delta \log P_{½}O_2/\Delta pH$ and the other is the determination of the protons released when haemoglobin is oxygenated. The latter method is the one used in the present study. The experimental method is the same as that used by Bailey, Beetlestone and Irvine (1970b).

Essentially, it involves measuring the pH of deoxygenated haemoglobin, measuring the pH of haemoglobin after oxygenation and finally after titrating the oxyhaemoglobin back to approximately the

same pH as the deoxygenated haemoglobin using known volumes of acid or base of known concentration. The pH was measured using a Radiometer GK 2641C combined glass and calomel electrode. The Radiometer electrode was connected to a Cary 401 Vibrating Reed Electrometer. The Electrometer was used in conjuction with a Cary pH Switch and an external E.M.F. from a Pye Precision Vernier Potentiometer. By using the 3mv setting of the Electrometer, changes of pH of 5×10^{-4} could be detected. A continuous record of the pH was obtained by connecting the Electrometer to a Hartmann and Braun Potentiometric pen recorder. The entire electrode assembly was placed in a shielded air thermostat.

Before the determination of the Bohr effect, bound carbon monoxide was removed from the haemoglobin by shaking the solution (thermostated at 13°C to prevent denaturation) gently in an atmosphere of oxygen while exposed to a strong artificial light. (This was usually done with a 60 watt globe for 2 hours). The haemoglobin was then centrifuged and passed through a Dintzis ion exchange column to remove ionic contamination. The haemoglobin was diluted with carbon dioxide-free distilled water to give the required concentration and solid sodium chloride was added to make the solution 0.05M in sodium chloride.

This unbuffered haemoglobin solution was deoxygenated by stirring it magnetically for about 2 hours in a vessel through which nitrogen was passed. The small amount of oxygen usually present in the commercial nitrogen was removed by passing the nitrogen through an acidic solution of chromous sulphate in contact with amalgamated zinc. After deoxygenation, 4 ml of the reduced haemoglobin was transferred with a pipette into the thermostated vessel into which the Radiometer combined glass and calomel electrode was dipped. After addition of haemoglobin to the electrode vessel, the solution was magnetically stirred under nitrogen and titrated to the required pH using oxygen free 0.1 molar HCl or NaOH. When a constant pH was obtained, the e.m.f. was recorded and nitrogen was replaced by oxygen. When a new e.m.f. was obtained, a known volume, V ml, of 0.01 molar HCl or NaOH, was then added to return the e.m.f. to approximately its original value. The number of moles of hydrogen ion released or taken up per mole of oxygen reacting is then given by

$$\Delta h^{+} = \frac{10^{-2} . V . \Delta E_o}{4 . C . \Delta E_a}$$

where C is the concentration of haemoglobin in gm moles Fe/litre and ΔE_o and ΔE_a are, respectively, the changes in e.m.f. upon

adding oxygen and on adding V ml of 0.01M acid or alkali.

Since the haemoglobin solutions contain a small percentage of methaemoglobin which does not react with oxygen, the concentration required is that of oxyhaemoglobin. This was obtained by measuring the optical density at 500, 510, 542 and 577 nm of the solution after suitable dilution with pH 6.0 buffer (I = 0.05). By using the extinction coefficients of methaemoglobin and oxyhaemoglobin at these wavelengths, the concentrations of oxyhaemoglobin and methaemoglobin were calculated by solving sets of simultaneous equations (Cameron, 1964). In all the experiments on the hybrid haemoglobins, the samples contained between 5 and 10% methaemoglobin and the concentration of haemoglobin solution used was of the order of 10^{-4}M.

Bohr effect measurements were also made on human and canine haemoglobins obtained from the same chromatographic preparation as the hybrid haemoglobins.

Determination of the Hill Constant 'n'

The set up of the apparatus used for the determination of 'n' is the one described by Ige, R.O. (1971) and reproduced in fig. 3.

Bound carbon monoxide was removed from the haemoglobin sample

by shaking the solution gently in an atmosphere of oxygen while exposed to a strong artificial light. The haemoglobin solution was then deionised by passing it through a Dintzis column and diluted with the appropriate buffer to a concentration of about 4×10^{-4}M in iron. Solid 'Analar' sodium chloride was then added to make the haemoglobin solution 0.05M in NaCl.

About 50 ml of the haemoglobin solution was put into the degassing vessel [5]. (Numbers in square brackets refer to the diagram)(fig.3). The haemoglobin solution was then deoxygenated under nitrogen gas previously passed through acidic chromous sulphate in contact with amalgamated zinc to remove oxygen and also through 2M NaOH to remove carbon dioxide. The haemoglobin solution was stirred using paddle rotated by a motor in order to increase the efficiency of deoxygenation and to reduce possible denaturation by friction. While the haemoglobin was being oxygenated, the oxygen electrode was calibrated for use.

After the calibration of the oxygen electrode, nitrogen was passed through the tube [3] into the 2 cm cuvette [10] to displace all the air in the cuvette. The reading on the oxygen meter was then zero. The stirring of the haemoglobin solution was stopped and the end of the tube [3] was dipped into the deoxyhaemoglobin

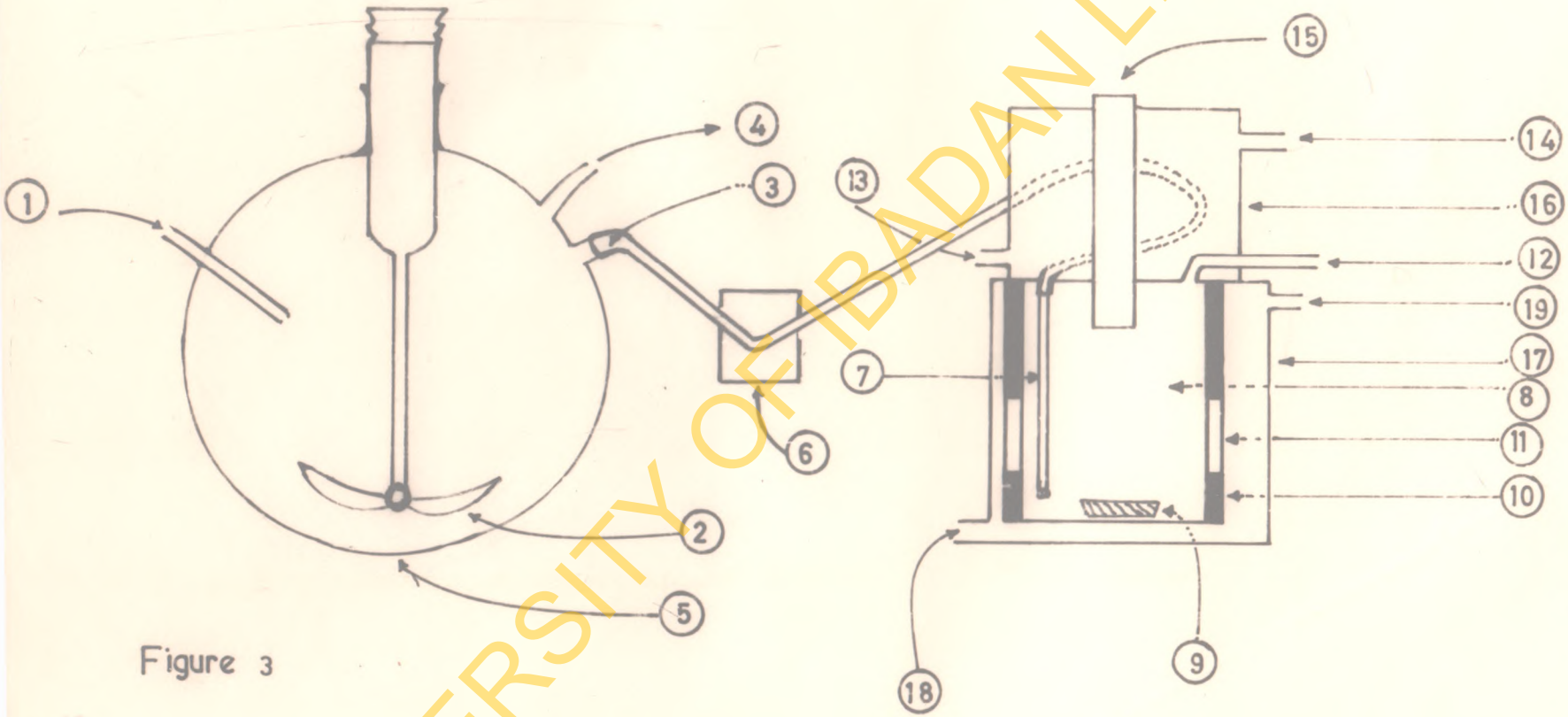


Figure 3

Apparatus for oxygenation studies

solution. The deoxy-haemoglobin was then pumped into the cuvette [10] with the sigma motor, [6] used to control the flow rate of solution, at the rate of about 7.5 ml per minute; the reading on the pump which gave this rate was about 80 units. The full scale reading of the meter was 100 units. During the time of filling the cuvette, nitrogen gas was still passed through the deoxygenating vessel. When the cuvette was filled, the sigma motor pump was switched off and the outlet from the oxygen electrode compartment was put into the degassing vessel, though not inside the haemoglobin solution. The sigma motor pump was switched on and the speed of the pump was changed to 30. The reading on the oxygen meter and the optical density of the haemoglobin at 650 nm were recorded. The nitrogen supply was then turned off. The haemoglobin in the degassing vessel became oxygenated and as this mixed with the deoxygenated haemoglobin in the cuvette (inside which was a magnetic stirring bar) the oxygen pressure and the optical density changed. These values were recorded simultaneously until a constant value of optical density was attained. 650 nm was chosen as the wavelength because of the wide separation between the extinction coefficients of oxy- and deoxy-haemoglobin at this wavelength.

After each experiment, a sample of the haemoglobin was taken for the determination of the pH and the percentage of methaemoglobin in the sample. A graph of log $\frac{Y}{1-Y}$ against log Po_2 was plotted where Y is the fractional saturation with oxygen.

If D is the optical density at a given oxygen pressure and D_o is the optical density at zero oxygen pressure and D_∞ is the optical density of the sample when saturated with oxygen, then Y is given by $D_o - D$, and 1-Y is given by $D - D_\infty$.

The value of 'n' was taken as the slope of the graph of log $\frac{Y}{1-Y}$ against log Po_2 between the points for 20% and 80% formation of oxyhaemoglobin. All determinations were carried out at 20^oC.

CHAPTER III

EXPERIMENTAL RESULTS

SPECTRA

Hybrid haemoglobins: Figs 4 and 5 show, respectively, the absorption spectra of $\alpha_2^{Ca}\beta_2^{A}$ and $\alpha_2^{A}\beta_2^{Ca}$ methaemoglobins at pH 6.0; the spectrum of methaemoglobin A under the same conditions is also included for comparison. It can be seen that the spectra of both hybrid methaemoglobins at acid pH are identical with that of methaemoglobin A. A check on the nativity of the haemoglobin sample was made by comparing the optical density at 570 nm with its value at 630 nm. For a native methaemoglobin sample, this ratio should be 0.84 ± 0.02 (Cameron, 1968) and only methaemoglobin samples which had a ratio within this range were used for the azide equilibrium measurements.

Met-α chain of haemoglobin A. Fig. 6 shows the visible spectrum of the met-α chains of human haemoglobin A (α_A^+) in 1 molar glycine at 5°C (pH = 6.0, I = 0.05) together with that of methaemoglobin A under the same conditions. The spectrum was shown to be reproducible in several independent preparations with minor differences in the regions 530-540 nm, 560-570 nm and 620-635 nm.

The spectrum of the met-α chains is very similar to that of methaemoglobin A. However, differences in extinction coefficient of about 11% in the region 525 - 540 nm and about 18% in the region 550 - 570 nm were observed. These were the maximum differences obtained from five different preparations. The spectrum of the met-α chains is identical with the one obtained by Banerjee and Cassoly (1969). The slight differences in the magnitude of the extinction coefficient reported in the two cases can be accounted for by the fact that Banerjee and Cassoly normalised the concentration of the α chains and methaemoglobin A by using the value of 11.5×10^3 at 540 nm as the extinction coefficient of the cyano-met derivative at this wavelength; while in the present study a value of 10.9×10^3 obtained by Cameron (1964) for methaemoglobin A has been used.

Magnetic susceptibility studies on the fresh met-α chains showed it to be 95% high spin (Kushimo, 1971). The chains were normally stored under carbon monoxide at $5^{\circ}C$ and were oxidised to the met-forms on the day they were used for azide equilibrium measurements. The spectrum of the met derivative was still reproducible three days after the preparation of the carbon-monoxy form. Beyond three days, the spectrum of the met derivative

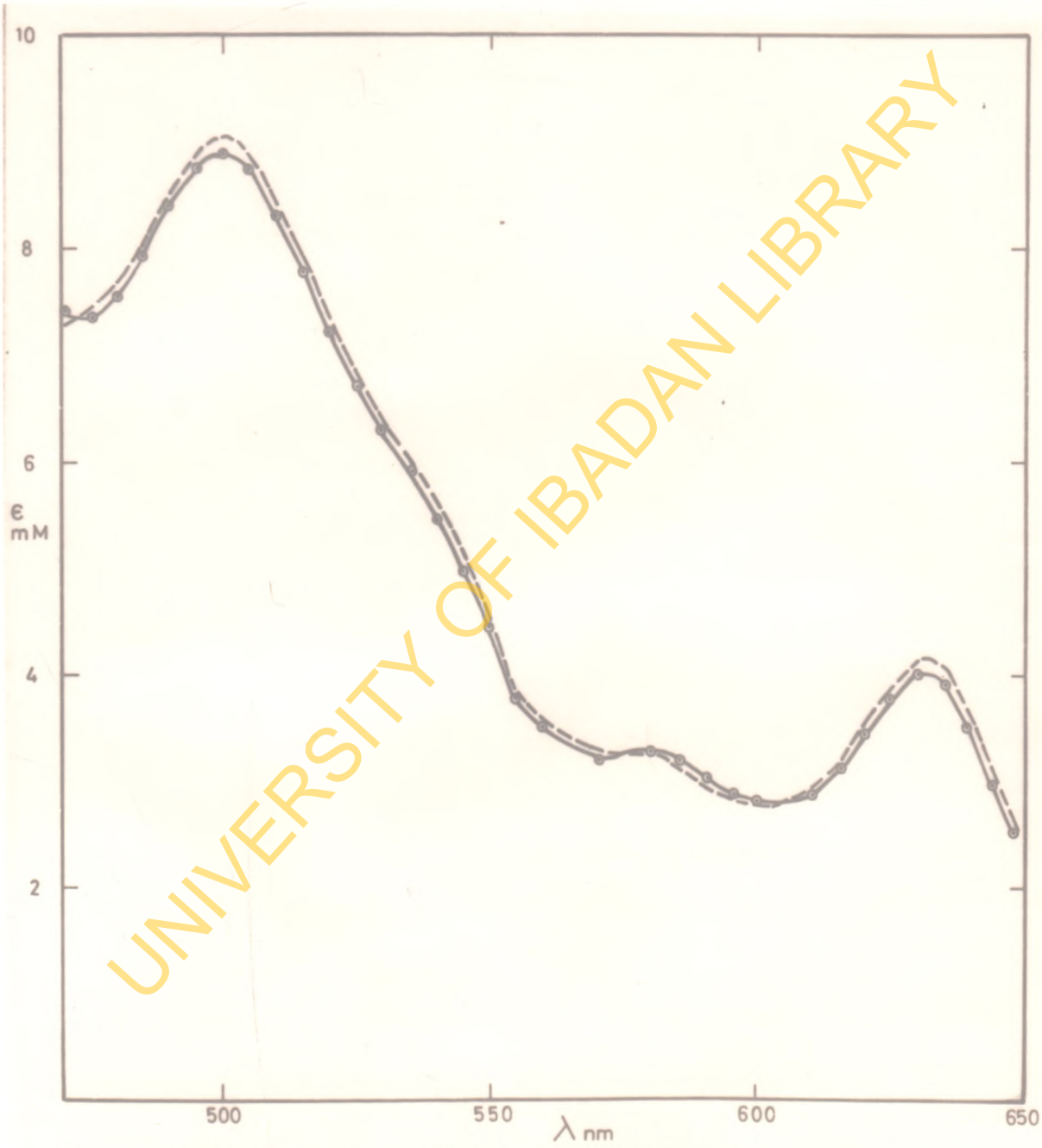


Fig. 4. SPECTRA OF $\alpha_2^{Ca}\beta_2^{A}$ METHAEMOGLOBIN ⊙—⊙—⊙ AND METHAEMOGLOBIN A ———; PH = 6·0, I = 0 05; t = 20 °C.

became significantly different from the normal spectrum and hence they were not used for azide binding studies. The acid met spectrum obtained after three days of preparation of the carbon-monoxy derivative was found to be a mixture of high and low spin forms.

Met-β chain of haemoglobin A: Fig. 7 shows a typical acid met spectrum of the β chain of normal human haemoglobin A (β_A^+) in 1 molar glycine at 5°C (pH = 6.0; I = 0.05). The acid met spectrum of haemoglobin A under similar conditions is also shown for comparison. It can be seen that the acid met spectrum of β^+_A is clearly different from that of haemoglobin A. The spectrum was reproducible in several independent preparations with minor variations in the magnitude of the extinction coefficients around the region 500 - 540 nm. The acid spectrum of β_A^+ is similar to the one obtained by Banerjee and Cassoly (1969) but there are slight differences in the magnitude of the extinction coefficients reported in the two cases. The reason for this is the same as the one given above for the α chains.

Banerjee et. al. (1969a) have shown from electron spin resonance studies that the abnormal spectrum of β_A^+ could be due to its spin state. They showed that while α_A^+ and methaemoglobin A are essentially high spin, β_A^+ shows a considerable amount of the low

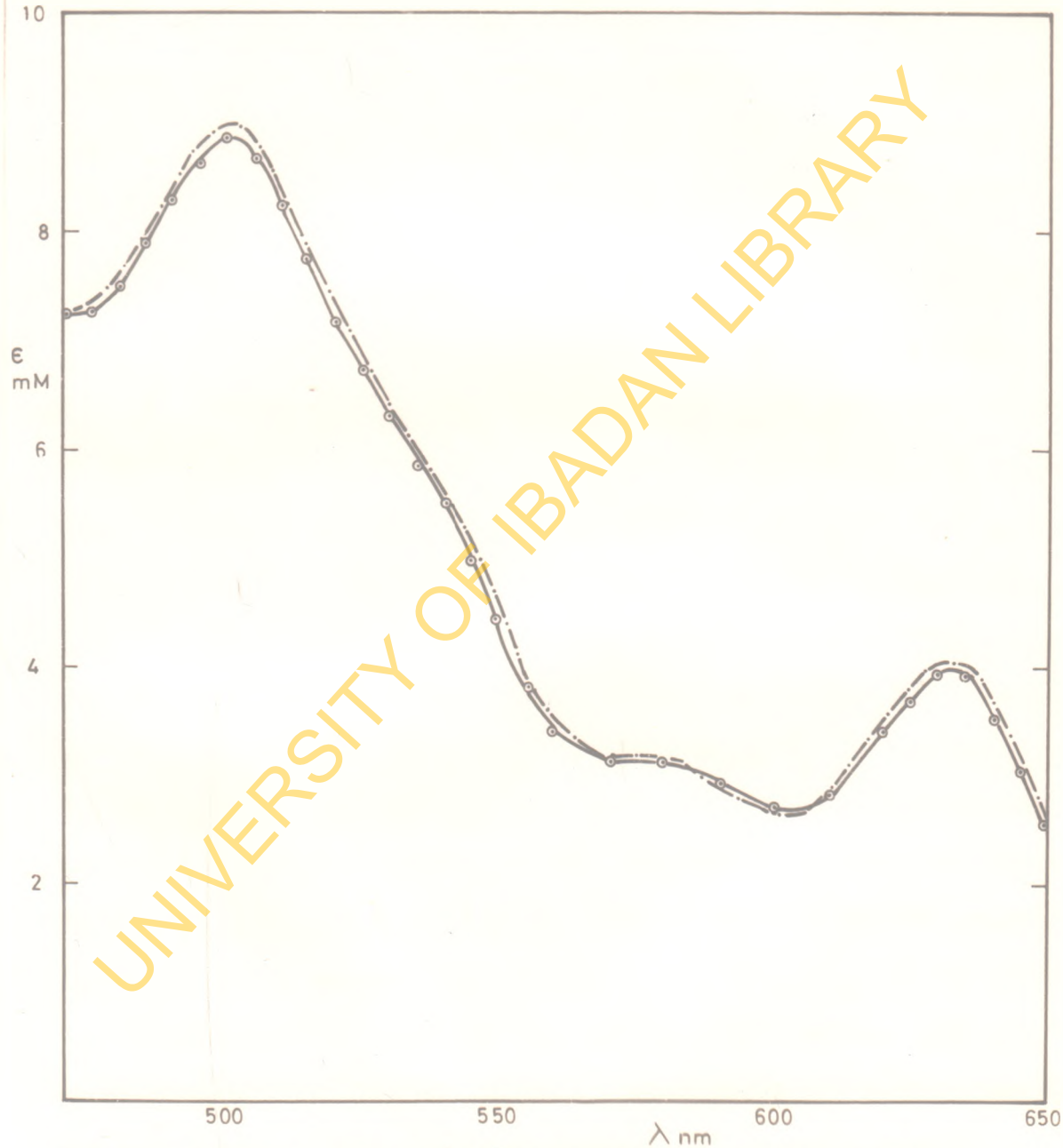


Fig. 5. SPECTRA OF $\alpha_2^A\beta_2^{Ca}$ METHAEMOGLOBIN ⊙-⊙-⊙ AND METHAEMOGLOBIN — — — PH = 6·0; I = 0·05; t = 20 °C.

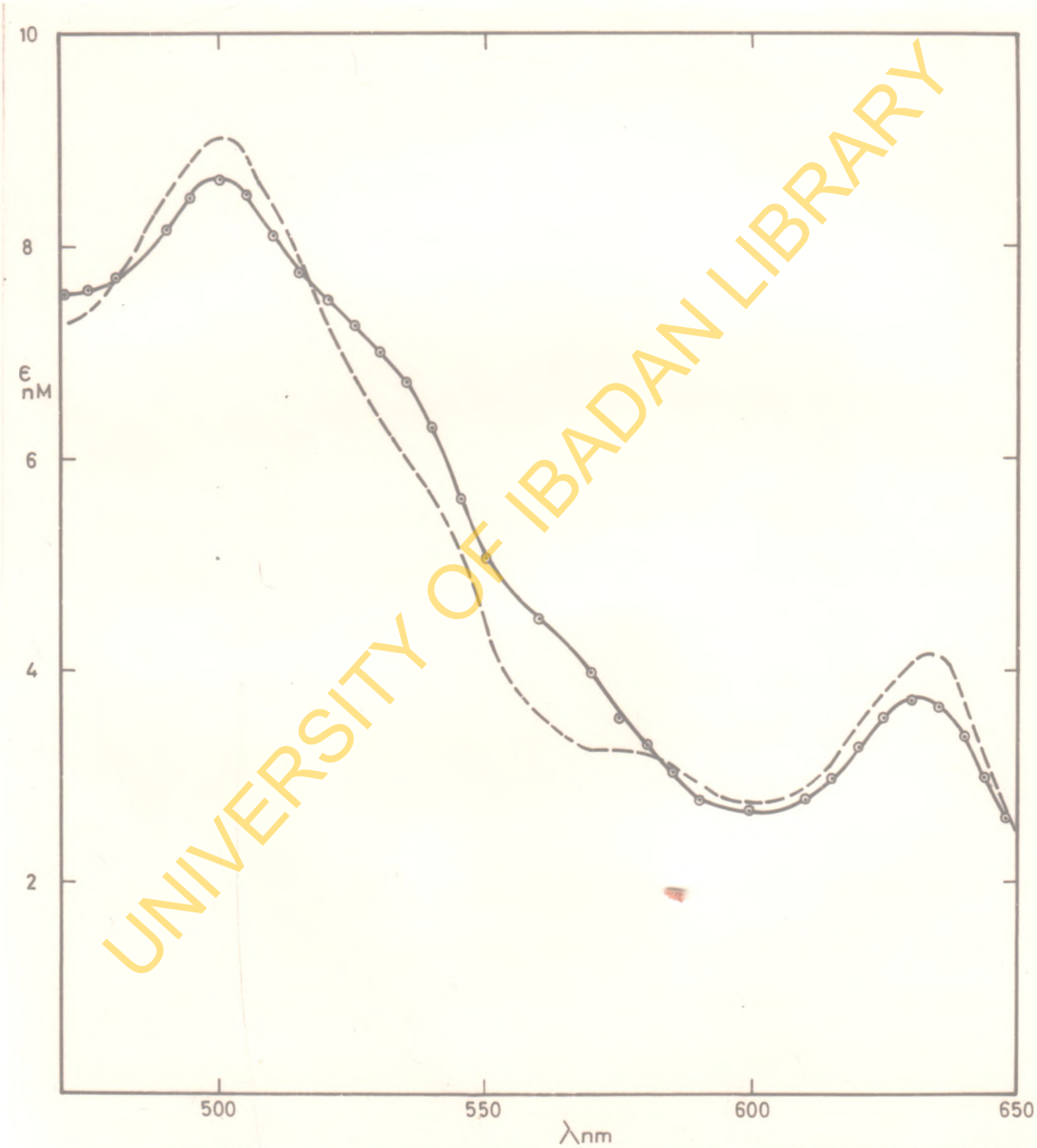


Fig. 6. SPECTRA OF MET-∝ CHAIN OF HUMAN HAEMOGLOBIN A ⊙-⊙-⊙; AND HUMAN METHAEMOGLOBIN A ——— IN 1M GLYCINE PH=6·0; I = 0·05; TEMP = 5 °C.

well as met canine haemoglobin except for differences in the magnitude of the extinction coefficients. The spectrum is reproducible for several independent preparations with minor changes in the extinction coefficient around the regions 520 - 540 nm and 620 - 640 nm.

TABLE 1

Values of the Hill constant 'n' for human and canine haemoglobins (obtained from the same chromatographic preparation as the hybrids) and the human-canine hybrids at 20°C.

Haemoglobin sample	PH	'n'
Hb A	6.01 7.02	2.80 2.76
Hb Canine	6.12 7.06	2.79 2.80
Hb $\alpha_2^A \beta_2^{Ca}$	5.85 6.58 7.01 8.20	2.65 2.70 2.63 2.60
Hb $\alpha_2^{Ca} \beta_2^A$	6.02 7.05 7.55 8.10	2.10 2.20 2.40 2.10

Table 1 above shows values of n; a measure of haem-haem interaction, for the human-canine hybrid haemoglobins and human and canine haemoglobins obtained from the same chromatographic

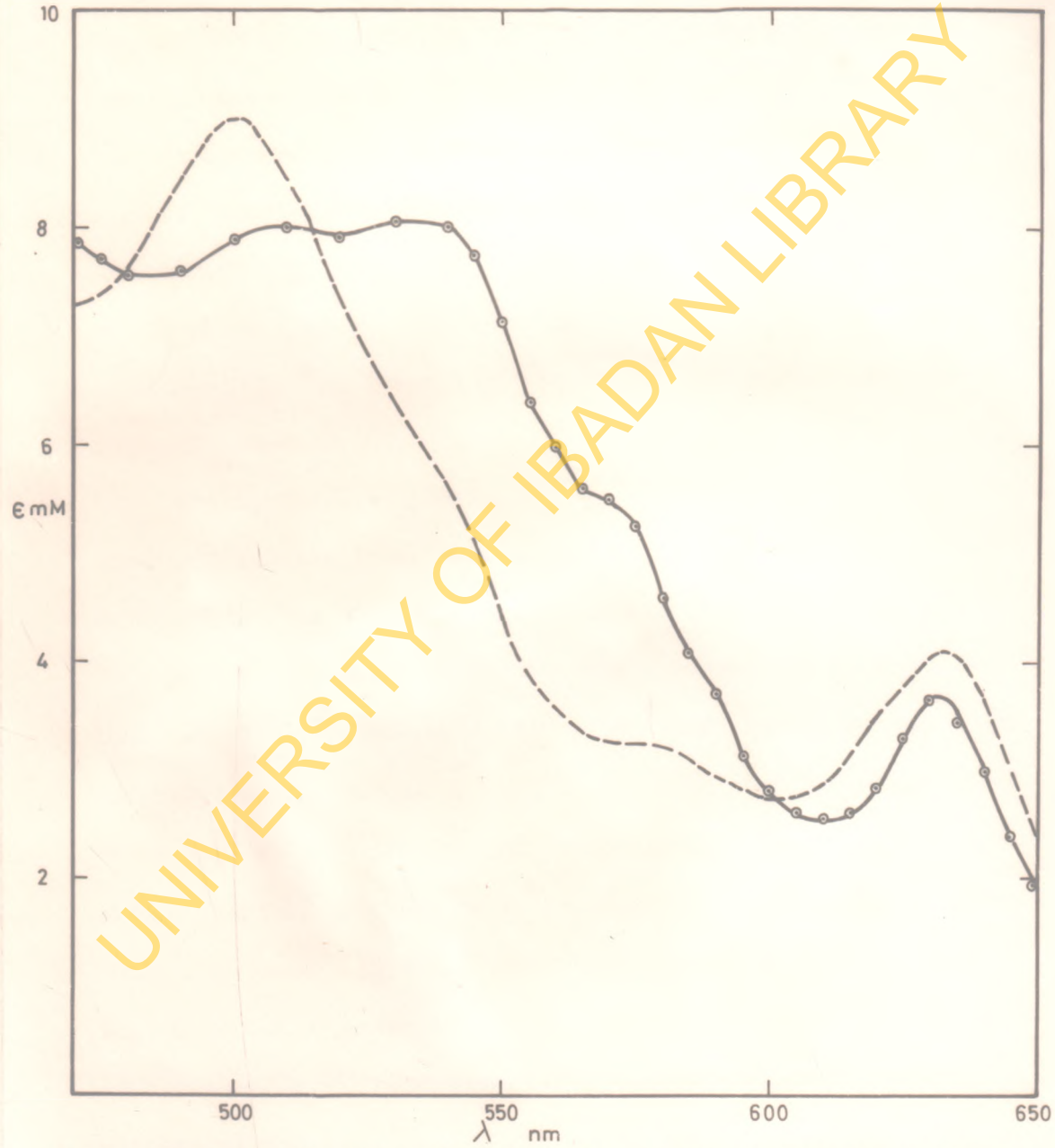


Fig. 7. SPECTRA OF MET — β CHAIN OF HUMAN HAEMOGLOBIN A ⊙—⊙—⊙; AND HUMAN METHAEMOGLOBIN A ———— IN 1M GLYCINE I = 0·05; TEMP = 5°C; PH = 6·0.

preparation as the hybrids. The value of n for human and canine haemoglobins obtained from the chromatographic separation of the hybrids is about 2.8. This is very close to the value of n usually reported for untreated normal human haemoglobin A (about 2.9) (Ige R.O., 1971; Antonini et. al. 1964). This result shows that the treatment involved in the hybridisation experiments has no drastic effect on the oxygenation properties of these haemoglobins.

The hybrid haemoglobin $\alpha_2^{Ca}\beta_2^{A}$ shows values of n which are significantly lower than normal at all the four pH's of the experiment. The values of n show an irregular change dependent upon pH. This result agrees with the observations of Enoki and Tomi ta (1968). The hybrid $\alpha_2^{A}\beta_2^{Ca}$ shows almost constant and slightly lowered values of n at the four pH's of the experiment, which implies that the form of the equilibrium curve does not vary with changes of pH. This is also in agreement with the result of Enoki and Tomita (1968). This result, however, differs from that obtained by Antonini et. al. (1965), who found that the value of n for $\alpha_2^{A}\beta_2^{Ca}$ haemoglobin decreases significantly with decreasing pH.

TABLE 2

Relative mobilities of human, canine and human-canine hybrid haemoglobins on starch gel electrophoresis.

pH = 8.3 Voltage = 300 Volts

Current = 16mA Duration = 12 hours.

Fractions in the Elution Pattern	Haemoglibin species	Distance moved on starch gel
II	A	4.0 cm
III	canine	3.7 cm
I	$\alpha_2^{Ca}\beta_2^{A}$	6.5 cm
IV	$\alpha_2^{A}\beta_2^{Ca}$	1.2 cm

TABLE 3

Relative mobilities of the isolated chains of haemoglobin A on starch gel electrophoresis pH = 8.3

Voltage = 300 Volts; Current = 16mA Duration = 12 hours.

Haemoglobin species	Distance moved on starch gel
A	4.1 cm
α_A	1.0 cm
β_A	6.7 cm

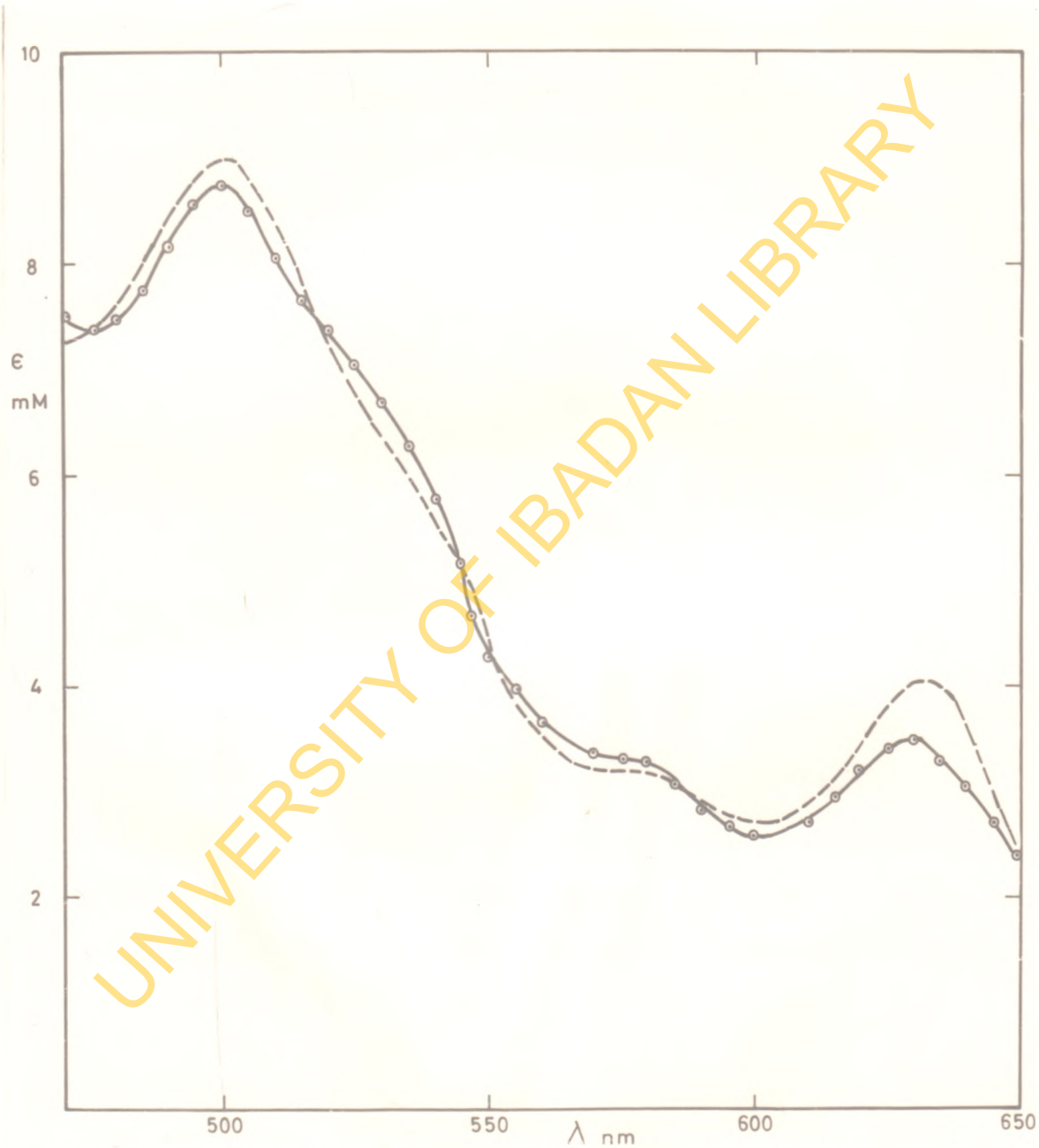


Fig. 8. SPECTRA OF MET - ∝ CHAIN OF CANINE HAEMOGLOBIN ⊙—⊙-⊙; AND CANINE HAEMOGLOBIN — — — IN 1M GLYCINE. PH 6·0; I = 0·05; TEMP. = 5 °C.

TABLE 4

Relative mobilities of the isolated chains of canine haemoglobin on starch gel electrophoresis pH = 8.3; Voltage = 300 volts; Current = 16mA Duration = 12 hours

Fractions in the Elution Pattern	Haemoglobin species	Distance moved on starch gel
II	Canine	3.7 cm
I	α_{Ca}	4.4 cm
III	β_{Ca}	2.5 cm

TABLE 5

Typical readings for the determination of pK_3' for $\alpha_3^{Ca}\beta_3^{A}$ methaemoglobin.

Temp. 13.0°C I = 0.05

pH	D at 405 nm	$D-D_\infty$	D_o-D	$\frac{D-D_\infty}{D_o-D}$	pK_3'
8.164	0.497	0.165	0.103	1.600	8.40
8.403	0.466	0.134	0.134	1.000	8.40
8.621	0.430	0.098	0.170	0.576	8.38
8.835	0.405	0.073	0.195	0.374	8.40
9.034	0.384	0.052	0.216	0.240	8.41
9.222	0.368	0.036	0.232	0.155	8.41

D_o = 0.600

D_∞ = 0.332

Mean pK_3' = 8.40 ± 0.01

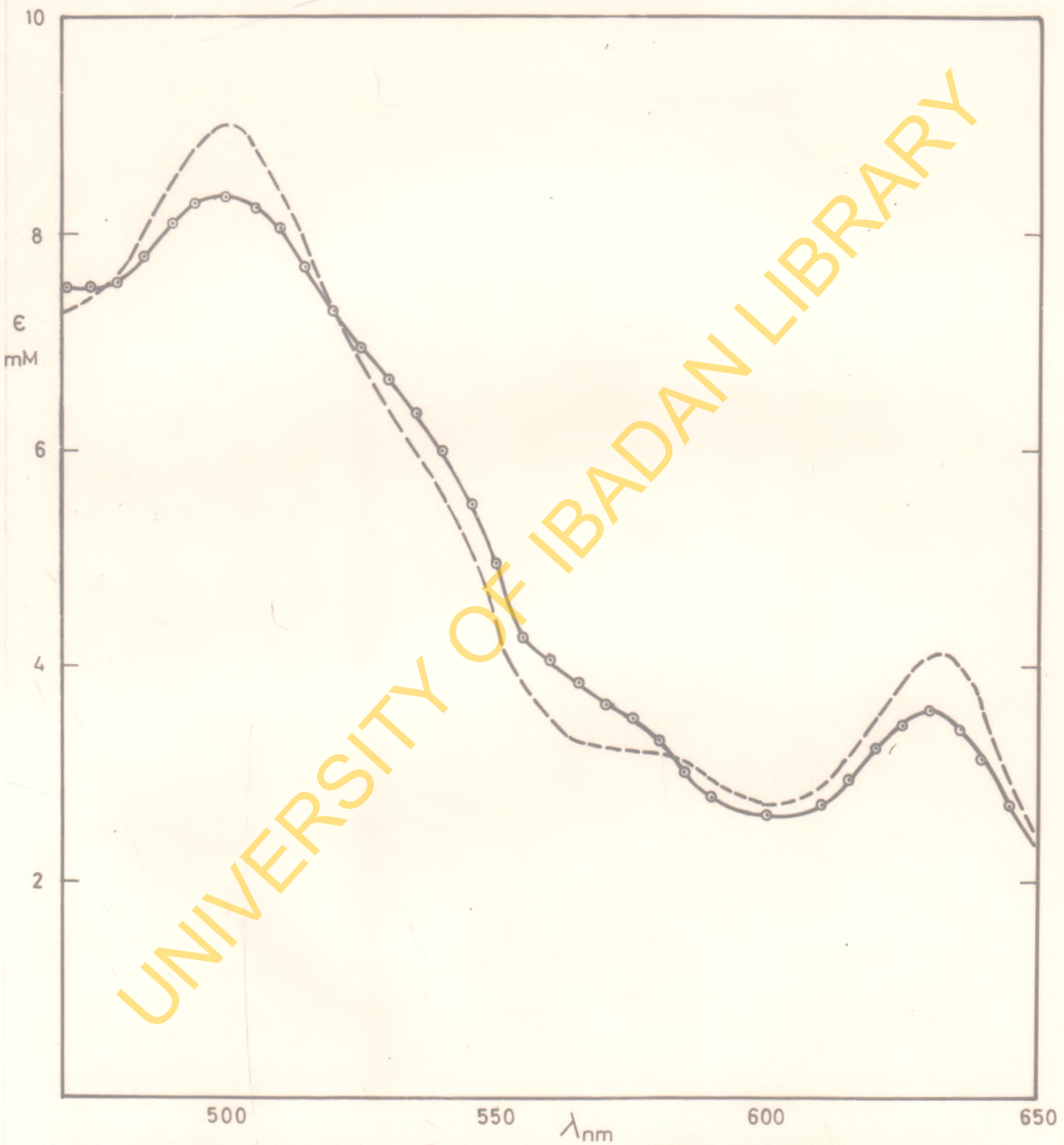


Fig. 9. SPECTRA OF MET – β CHAIN OF CANINE HAEMOGLOBIN ⊙–⊙–⊙, AND CANNE HAEMOGLOBIN – – – – – IN 1M GLYCINE PH 6·0; I = 0·05; TEMP = 5°C.

REACTIONS OF $\alpha_2^{Ca}\beta_2^{A}$ METHAEMOGLOBIN

TABLE 6

Values of pK_3' for $\alpha_2^{Ca}\beta_2^{A}$ methaemoglobin at various temperatures

Temp. ^{o}C	pK_3'
13.0	8.40 ± 0.01
20.0	8.34 ± 0.01
27.0	8.28 ± 0.01

TABLE 7

Typical readings for the determination of log K_{obs} for the formation of $\alpha_2^{Ca}\beta_2^{A}$ methaemoglobin azide complex.

Temp. = 13.0°C pH = 6.43 I = 0.05

D at 405 nm	$Hb_{Total} \times 10^{-6}$	$[N_3^-]_{Total} \times 10^{-6}$	$[N_3^-]_{unbound} \times 10^{-6}$	Log K_{obs}
0.400	3.098	1.998	0.995	5.68
0.347	3.091	3.988	2.356	5.68
0.315	3.085	5.970	3.964	5.67
0.298	3.079	7.944	5.744	5.64
0.282	3.073	9.911	7.529	5.66
0.271	3.067	11.870	9.366	5.67
0.265	3.061	13.821	11.256	5.66
0.260	3.055	15.764	13.150	5.65

D_o = 0.484

D_∞ = 0.227 Average log K_{obs} = 5.66 ± 0.01

D_{420}^{CN} = 0.357

TABLE 8

Reaction of $\alpha_2^{Ca}\beta_2^{A}$ methaemoglobin with azide

Temp. = 27.0°C I = 0.05

pH	log K_{obs}	log K_L
5.61	5.13 ± 0.01	5.13 ± 0.01
5.76	5.18 ± 0.01	5.18 ± 0.01
5.98	5.18 ± 0.01	5.18 ± 0.01
6.37	5.17 ± 0.02	5.18 ± 0.02
6.60	5.15 ± 0.01	5.16 ± 0.01
7.02	5.09 ± 0.01	5.11 ± 0.01
7.53	4.90 ± 0.01	4.98 ± 0.01
8.16	4.59 ± 0.02	4.83 ± 0.02
8.76	4.27 ± 0.02	4.87 ± 0.02

Temp. = 20.0°C I = 0.05

pH	Log K_{obs}	Log K_L
5.62	5.34 ± 0.01	5.34 ± 0.01
5.78	5.42 ± 0.01	5.52 ± 0.01
6.07	5.40 ± 0.01	5.40 ± 0.01
6.40	5.40 ± 0.01	5.40 ± 0.01
6.63	5.39 ± 0.01	5.40 ± 0.01
7.05	5.32 ± 0.01	5.34 ± 0.01
7.56	5.11 ± 0.01	5.18 ± 0.01
8.20	4.81 ± 0.01	5.05 ± 0.01
8.79	4.51 ± 0.01	5.10 ± 0.01

TABLE 8 (CONT'D)

Temp. = 13.0°C I = 0.05

pH	Log K_{obs}	Log K_L
5.62	5.54 ± 0.01	5.54 ± 0.01
5.88	5.62 ± 0.01	5.62 ± 0.01
6.06	5.66 ± 0.01	5.66 ± 0.01
6.43	5.66 ± 0.01	5.66 ± 0.01
6.64	5.62 ± 0.01	5.63 ± 0.01
7.09	5.54 ± 0.01	5.56 ± 0.01
7.57	5.36 ± 0.02	5.42 ± 0.02
8.23	5.05 ± 0.02	5.27 ± 0.02
8.83	4.73 ± 0.01	5.30 ± 0.01

TABLE 9

Values of ΔH^{o} for the formation of $\alpha_a{}^{Ca}\alpha_a{}^{A}$ methaemoglobin azide complex.

pH	$-\Delta H^{o}$ k cal./mole
5.6	11.5 ± 0.1
5.8	11.8 ± 0.1
6.0	13.3 ± 0.1
6.2	13.8 ± 0.2
6.4	13.7 ± 0.2
6.6	13.4 ± 0.1
7.0	12.8 ± 0.1
7.6	12.2 ± 0.1
8.2	11.9 ± 0.1
8.8	11.6 ± 0.2

TABLE 10

The isoionic point of $\alpha_2^{Ca}\beta_2^{A}$ methaemoglobin at 20°C (I = 0.05) is 7.240.

REACTIONS OF $\alpha_2^{A}\beta_2^{Ca}$ METHAEMOGLOBIN

TABLE 11

Values of pK_3' for $\alpha_2^{A}\beta_2^{Ca}$ methaemoglobin at various temperatures

Temp. °C	pK_3'
13.0	8.35 ± 0.01
20.0	8.30 ± 0.01
27.0	8.23 ± 0.01

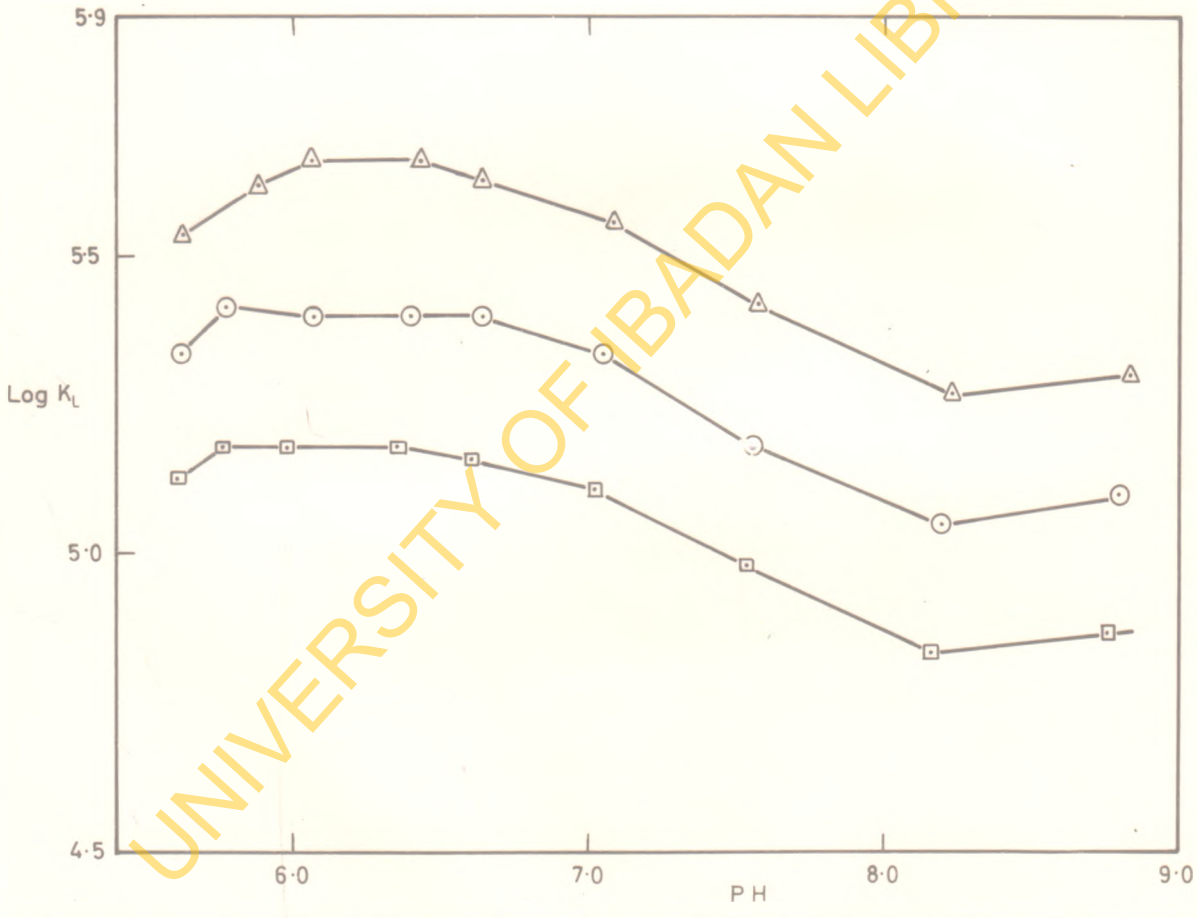


Fig. 10. PLOTS OF Log K_L AGAINST P H FOR THE REACTION OF $\alpha_2^{Ca}\beta_2^{A}$ METHAEMOGLOBIN WITH AZIDE: ⊡—⊡ 27°; ⊙—⊙ 20°; △—△ 13°

TABLE 12

Reaction of $\alpha_2^A\beta_2^{Ca}$ methaemoglobin with azide

Temp = 27.0°C I = 0.05

pH	log K_{obs}	log K_L
5.77	5.27 ± 0.01	5.27 ± 0.01
6.49	5.24 ± 0.01	5.25 ± 0.01
7.02	5.15 ± 0.02	5.18 ± 0.02
7.53	4.98 ± 0.01	5.06 ± 0.01
7.74	4.83 ± 0.01	4.95 ± 0.01
8.00	4.71 ± 0.01	4.91 ± 0.01
8.15	4.65 ± 0.01	4.91 ± 0.01
8.80	4.27 ± 0.02	4.94 ± 0.02

Temp. = 20.0°C I = 0.05

pH	log K_{obs}	log K_L
5.78	5.50 ± 0.01	5.50 ± 0.01
6.44	5.51 ± 0.01	5.52 ± 0.01
7.08	5.43 ± 0.01	5.46 ± 0.01
7.55	5.25 ± 0.01	5.32 ± 0.01
7.78	5.15 ± 0.01	5.26 ± 0.01
8.00	5.03 ± 0.01	5.21 ± 0.01
8.20	4.92 ± 0.01	5.18 ± 0.01
8.84	4.53 ± 0.01	5.18 ± 0.01

TABLE 12 (CONT'D)

Temp. = 13.0°C I = 0.05

pH	Log K_{obs}	Log K_L
5.84	5.71 ± 0.01	5.71 ± 0.01
6.46	5.74 ± 0.01	5.74 ± 0.01
7.11	5.72 ± 0.01	5.74 ± 0.01
7.57	5.57 ± 0.01	5.64 ± 0.01
7.82	5.45 ± 0.01	5.56 ± 0.01
8.06	5.34 ± 0.02	5.52 ± 0.02
8.24	5.24 ± 0.02	5.49 ± 0.02
8.86	4.85 ± 0.01	5.47 ± 0.01

TABLE 13

Values of ΔH^{o} for the formation of $\alpha_a{}^{A}\alpha_b{}^{Ca}$ methaemoglobin azide complex.

pH	$-\Delta H^{o}$ K cal./mole
6.0	13.1 ± 0.1
6.4	15.5 ± 0.1
6.8	15.2 ± 0.1
7.2	16.5 ± 0.1
7.6	17.4 ± 0.1
7.8	17.9 ± 0.2
8.0	17.7 ± 0.2
8.2	16.7 ± 0.1
8.4	15.5 ± 0.1
8.8	15.1 ± 0.1

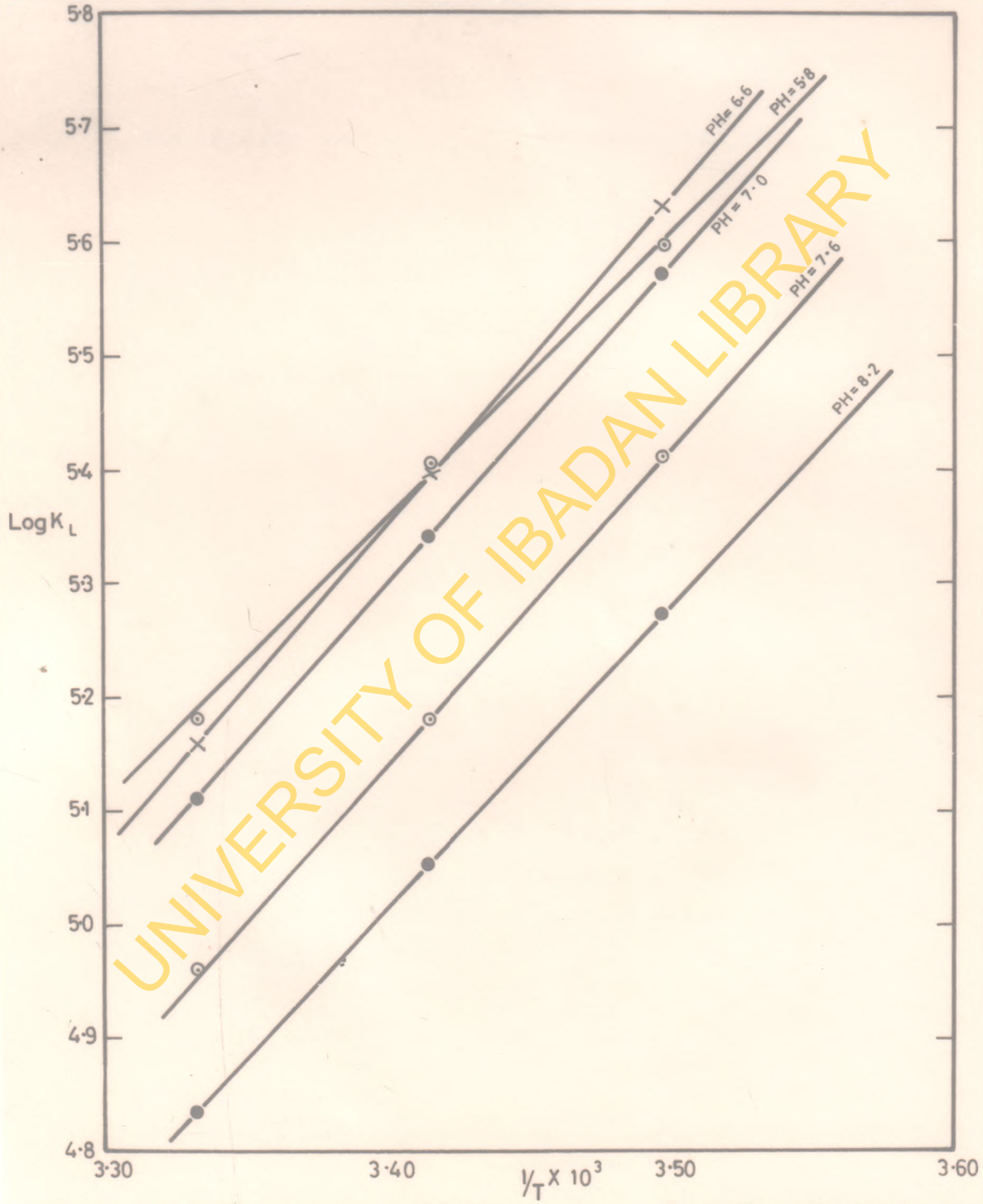


Fig. 11. PLOTS OF Log k_L AGAINST $1/T$ FOR THE REACTION OF $\alpha_2^{Ca}\beta_2^{A}$ METHAEMOGLOBIN WITH AZIDE.

TABLE 14

The isoionic point of $\alpha_2^A\beta_2^{Ca}$ methaemoglobin at 20°C (I = 0.05) is 7.775

REACTIONS OF METHAEMOGLOBIN A

TABLE 15

Reaction of methaemoglobin A with azide

Temp. = 27.0°C I = 0.05

pH	log K_{obs}	log K_L
5.97	5.39 ± 0.01	5.39 ± 0.01
6.40	5.34 ± 0.01	5.35 ± 0.01
7.06	5.18 ± 0.01	5.22 ± 0.01
7.40	5.09 ± 0.01	5.17 ± 0.01
8.02	4.84 ± 0.01	5.11 ± 0.01
8.83	4.31 ± 0.01	5.13 ± 0.01

Temp. = 20.0°C I = 0.05

pH	log K_{obs}	log K_L
6.01	5.63 ± 0.01	5.63 ± 0.01
6.43	5.57 ± 0.02	5.58 ± 0.02
7.08	5.48 ± 0.01	5.52 ± 0.02
7.40	5.37 ± 0.01	5.44 ± 0.01
8.04	5.15 ± 0.01	5.37 ± 0.01
8.86	4.60 ± 0.02	5.40 ± 0.02

TABLE 15 (CONT'D)

Temp. = 13.0°C I = 0.05

pH	log K_{obs}	Log K_L
6.03	5.84 ± 0.02	5.84 ± 0.02
6.45	5.83 ± 0.01	5.83 ± 0.01
7.10	5.76 ± 0.02	5.79 ± 0.02
7.42	5.65 ± 0.02	5.72 ± 0.02
8.07	5.42 ± 0.01	5.66 ± 0.01
8.89	4.85 ± 0.01	5.62 ± 0.01

TABLE 16

Values of ΔH° for the formation of human methaemoglobin A azide complex

pH	ΔH° K cal./mole
6.0	12.5 ± 0.1
6.4	13.8 ± 0.1
6.8	15.4 ± 0.2
7.0	16.1 ± 0.1
7.2	15.9 ± 0.1
7.4	15.5 ± 0.1
7.8	15.2 ± 0.2
8.4	14.9 ± 0.2
8.8	14.4 ± 0.2

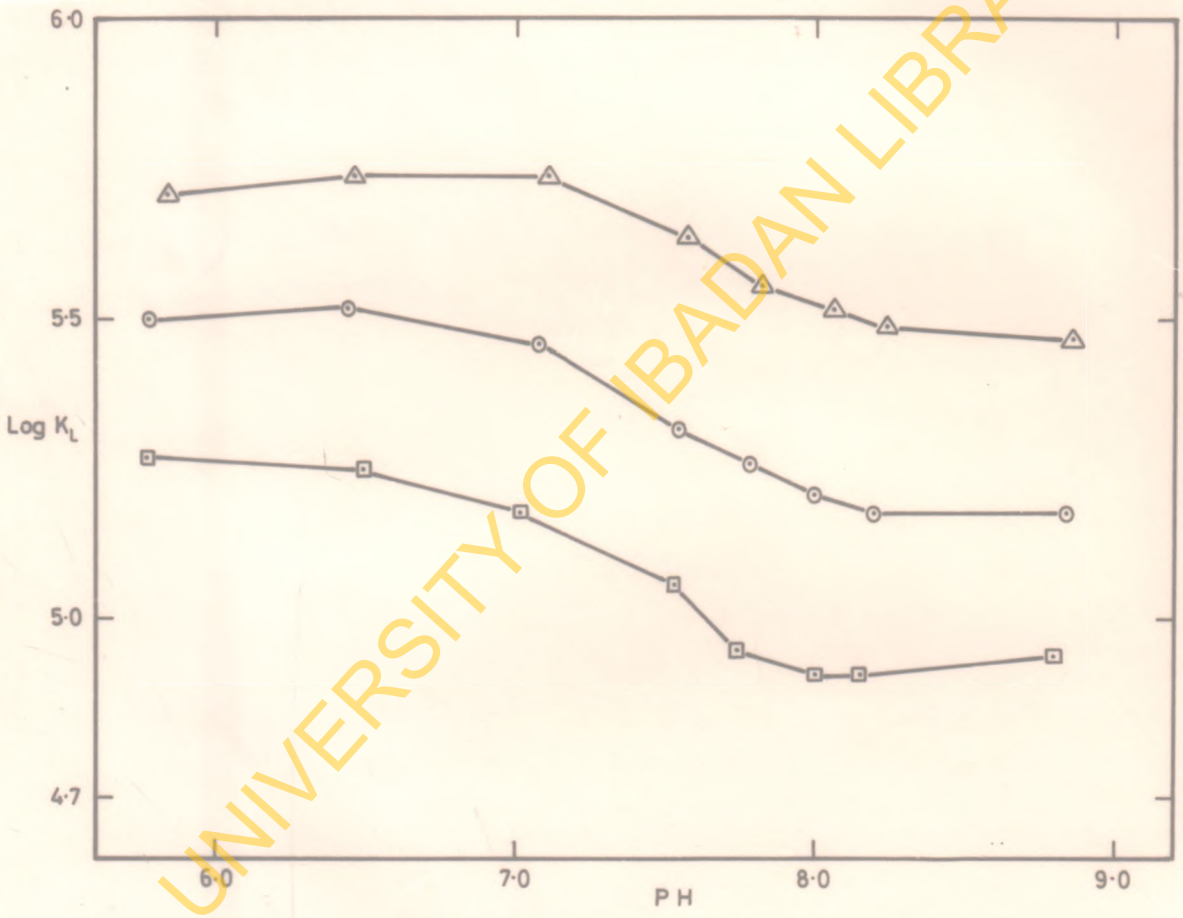


Fig. 12. PLOTS OF Log K_L AGAINST P H FOR THE REACTION OF $\alpha_2^A\beta_2^{Ca}$ METHAEMOGLOBIN WITH AZIDE: -⊡-⊡- 27°; -⊙-⊙- 20°; △-△ 13°

REACTIONS OF METHAEMOGLOBIN A OBTAINED FROM THE SAME CHROMATOGRAPHIC PREPARATION AS THE HYBRIDS

TABLE 17

Reaction of methaemoglobin A (obtained from the same chromatographic preparation as the hybrids) with azide.

Temp. = 27.0°C I = 0.05

pH	log K_{obs}	log K_L
6.00	5.38 ± 0.01	5.38 ± 0.01
7.06	5.18 ± 0.02	5.22 ± 0.02
8.04	4.84 ± 0.02	5.11 ± 0.02

Temp. = 20.0°C I = 0.05

pH	log K_{obs}	log K_L
6.01	5.62 ± 0.01	5.62 ± 0.01
7.08	5.46 ± 0.01	5.50 ± 0.01
8.06	5.10 ± 0.01	5.35 ± 0.01

Temp. = 13.0°C I = 0.05

pH	log K_{obs}	log K_L
6.04	5.84 ± 0.01	5.84 ± 0.01
7.10	5.77 ± 0.01	5.80 ± 0.01
8.08	5.43 ± 0.01	5.67 ± 0.01

TABLE 18

Values of ΔH° for the reaction of methaemoglobin A (obtained from the same chromatographic preparation as the hybrids) with azide.

pH	pH	$-\Delta H^{\circ}$ K cal./mole
	6.0	12.9 ± 0.1
	7.1	16.3 ± 0.1
	8.1	14.9 ± 0.2

REACTIONS OF METHAEMOGLOBIN A IN 1 MOLAR GLYCINE + 0.05M PHOSPHATE

TABLE 19

Values of pK_a' for methaemoglobin A in 1 molar glycine + 0.05M phosphate.

Tepm. °C	pK_a'
27.0	8.27 ± 0.01
20.0	8.38 ± 0.01
13.0	8.52 ± 0.01

TABLE 20

Reaction of methaemoglobin A with azide in 1 molar glycine + 0.05M phosphate

Temp. = 27°C I = 0.05

pH	log K_{obs}	Log K_I
5.88	5.35 + 0.01	5.35 + 0.01
6.30	5.33 + 0.01	5.33 + 0.01
6.74	5.30 + 0.01	5.31 + 0.01
6.97	5.23 + 0.01	5.25 + 0.01
7.33	5.10 + 0.01	5.15 + 0.01
7.64	4.97 + 0.01	5.06 + 0.01
8.02	4.75 + 0.02	4.94 + 0.02

Temp. 20°C I = 0.05

pH	log K_{obs}	log K_I
5.92	5.56 ± 0.01	5.56 ± 0.01
6.34	5.57 ± 0.02	5.57 ± 0.02
6.80	5.53 ± 0.02	5.54 ± 0.02
6.99	5.48 ± 0.01	5.50 ± 0.01
7.42	5.30 ± 0.01	5.36 ± 0.01
7.76	5.15 ± 0.02	5.25 ± 0.02
8.04	5.03 ± 0.01	5.19 ± 0.01

TABLE 20 (CONT'D)

pH	log K_{obs}	log K_L
5.95	5.80 ± 0.01	5.80 ± 0.01
6.39	5.80 ± 0.01	5.80 ± 0.01
6.86	5.77 ± 0.01	5.78 ± 0.01
7.13	5.74 ± 0.01	5.76 ± 0.01
7.43	5.62 ± 0.01	5.65 ± 0.01
7.78	5.42 ± 0.01	5.52 ± 0.01
8.07	5.23 ± 0.02	5.41 ± 0.02

TABLE 21

Values of ΔH° for the reaction of methaemoglobin A with azide in 1 molar glycine + 0.05 M phosphate.

pH	ΔH° K cal./mole
6.0	12.5 ± 0.1
6.4	13.2 ± 0.1
6.8	14.4 ± 0.1
7.0	15.2 ± 0.1
7.2	14.7 ± 0.2
7.6	13.8 ± 0.1
8.0	13.6 ± 0.2

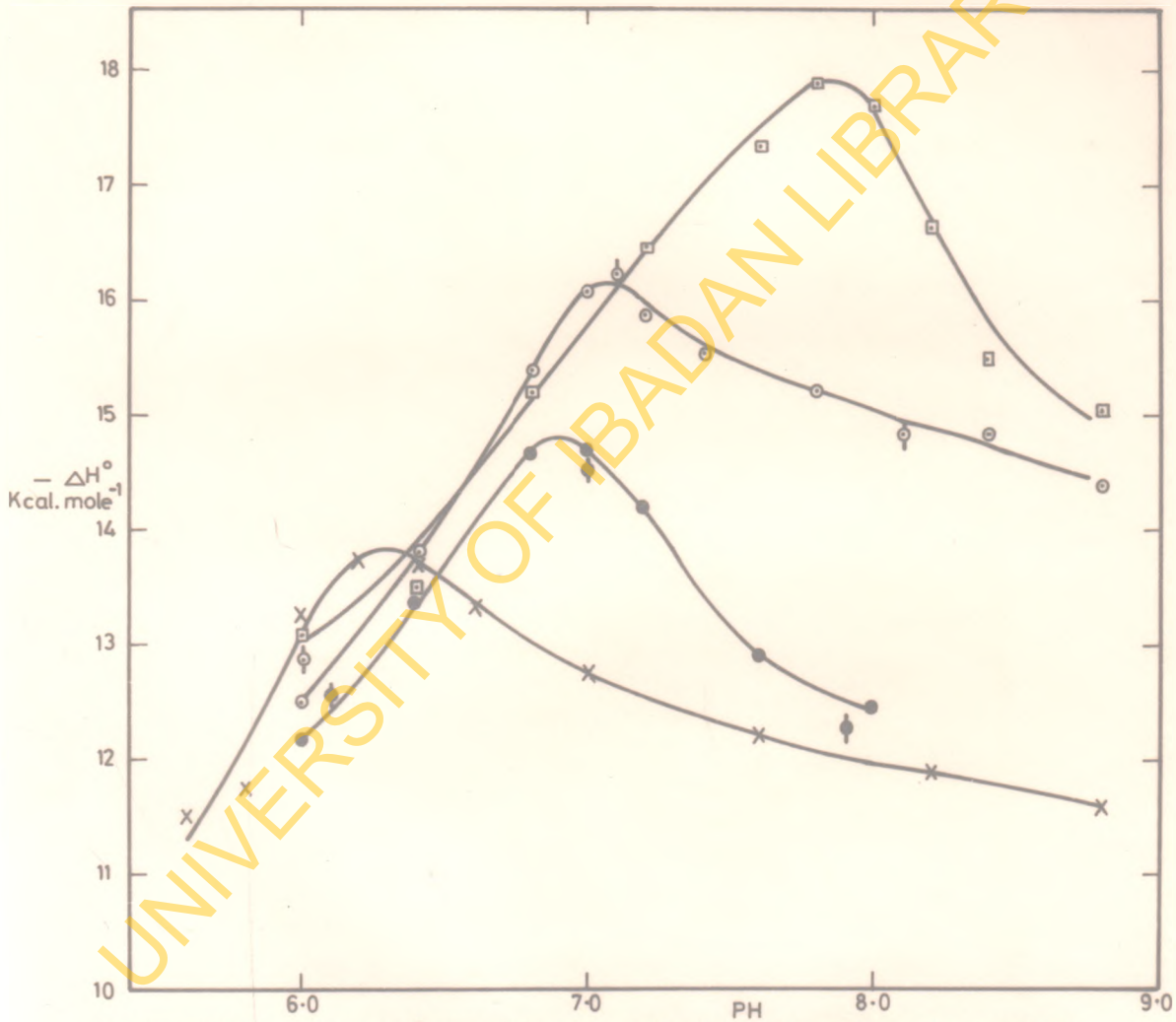


Fig. 13. PLOTS OF $-\Delta H^{\circ}$ AGAINST PH FOR THE REACTION OF VARIOUS METHAEMOGLOBINS WITH AZIDE: $\alpha_2^{Ca}\beta_2^{A}$ X ; $\alpha_2^{A}\beta_2^{Ca}$ ⊡ ; HUMAN A ⊙; DOG ● ; HUMAN A OBTAINED FROM CHROMATOGRAPHIC SEPERATION ⊕; DOG OBTAINED FROM CHROMATOGRAPHIC SEPERATION ⧫.

REACTIONS OF DOG METHAEMOGLOBIN

TABLE 22

Reaction of dog methaemoglobin with azide.

Temp. 27.0°C I = 0.05

pH	log K_{obs}	log K_L
5.78	5.15 ± 0.01	5.15 ± 0.01
6.19	5.15 ± 0.01	5.15 ± 0.01
6.61	5.12 ± 0.02	5.13 ± 0.02
7.06	5.00 ± 0.02	5.03 ± 0.02
7.50	4.93 ± 0.02	5.02 ± 0.02
8.16	4.68 ± 0.01	4.98 ± 0.01

Temp. = 20.0°C I = 0.05

pH	log K_{obs}	log K_L
5.79	5.36 ± 0.02	5.36 ± 0.02
6.21	5.37 ± 0.02	5.37 ± 0.02
6.62	5.36 ± 0.01	5.37 ± 0.01
7.08	5.27 ± 0.01	5.29 ± 0.01
7.50	5.18 ± 0.01	5.25 ± 0.01
8.16	4.94 ± 0.02	5.19 ± 0.02

Temp. = 13.0°C I = 0.05

pH	log K_{obs}	log K_L
5.81	5.56 ± 0.01	5.56 ± 0.01
6.25	5.61 ± 0.01	5.61 ± 0.01
6.64	5.62 ± 0.01	5.63 ± 0.01
7.08	5.52 ± 0.02	5.54 ± 0.02
7.57	5.41 ± 0.01	5.47 ± 0.01
8.24	5.16 ± 0.01	5.40 ± 0.01

TABLE 23

Values of ΔH^{o} for the formation of dog methaemoglobin azide complex.

pH	$-\Delta H^{o}$ K cal/mole
6.0	12.2 ± 0.1
6.4	13.4 ± 0.1
6.8	14.7 ± 0.2
7.0	14.7 ± 0.1
7.2	14.2 ± 0.2
7.6	12.9 ± 0.1
8.0	12.5 ± 0.1

REACTION OF DOG METHAEMOGLOBIN (OBTAINED FROM THE SAME CHROMATOGRAPHIC PREPARATION AS HYBRIDS)

TABLE 24

Reaction of dog methaemoglobin, (obtained from the same chromatographic preparation as hybrids) with azide.

Temp. 27.0°C I = 0.05

pH	log K_{obs}	log K_L
6.08	5.15 ± 0.01	5.15 ± 0.01
7.02	4.97 ± 0.01	5.00 ± 0.01
7.92	4.71 ± 0.01	4.99 ± 0.01

TABLE 24 (CONT'D)

Temp. 20.0°C I = 0.05

pH	log K_{obs}	log K_L
6.10	5.37 ± 0.01	5.37 ± 0.01
7.03	5.25 ± 0.01	5.27 ± 0.01
7.95	5.00 ± 0.01	5.21 ± 0.01

Temp. 13.0°C I = 0.05

pH	log K_{obs}	log K_L
6.14	5.58 ± 0.01	5.58 ± 0.01
7.06	5.50 ± 0.01	5.52 ± 0.01
7.98	5.26 ± 0.02	5.42 ± 0.02

TABLE 25

Values of ΔH° for the reaction of dog methaemoglobin, (obtained from the same chromatographic preparation as the hybrids) with azide

pH	$-\Delta H^\circ$ K cal./mole
6.10	12.6 ± 0.1
7.00	14.5 ± 0.1
7.90	12.3 ± 0.1

REACTIONS OF DOG METHAEMOGLOBIN IN 1 MOLAR GLYCINE + 0.05 M PHOSPHATE

TABLE 26

Values of pK_3' for dog methaemoglobin in 1 molar glycine at various temperatures (I = 0.05)

Temp. °C	pK_3'
13.0	8.62 ± 0.01
20.0	8.45 ± 0.01
27.0	8.30 ± 0.01

TABLE 27

Reaction of dog methaemoglobin with azide in 1 molar glycine + 0.05 M phosphate buffer.

Temp. 27.0°C I = 0.05

pH	log K_{obs}	log K_L
5.84	5.15 ± 0.01	5.15 ± 0.01
6.18	5.14 ± 0.01	5.14 ± 0.01
6.60	5.13 ± 0.02	5.14 ± 0.02
7.00	5.03 ± 0.02	5.05 ± 0.02
7.50	4.90 ± 0.02	4.96 ± 0.02
8.00	4.67 ± 0.01	4.85 ± 0.01

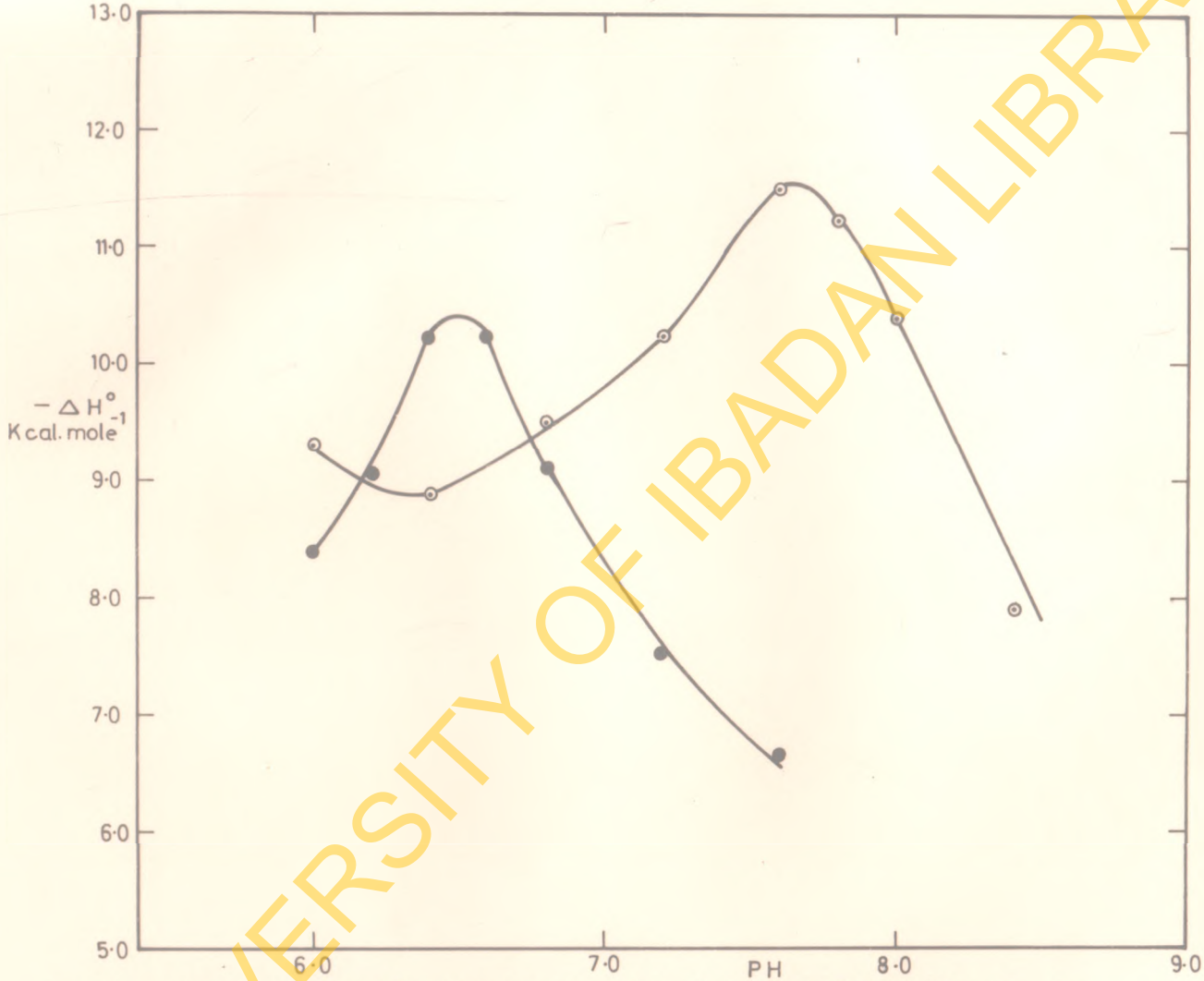


Fig. 14. PLOTS OF $-\Delta H^{\circ}$ AGAINST PH FOR THE REACTION OF α_A ⊙; AND β_A ● ; METHAEMOGLOBINS WITH AZIDE

TABLE 27 (CONT'D)

Temp. 20.0°C I = 0.05

pH	log K_{obs}	log K_L
5.88	5.35 ± 0.01	5.35 ± 0.01
6.20	5.36 ± 0.01	5.36 ± 0.01
6.60	5.35 ± 0.01	5.36 ± 0.01
7.01	5.29 ± 0.01	5.31 ± 0.01
7.51	5.12 ± 0.02	5.17 ± 0.02
8.01	4.89 ± 0.02	5.02 ± 0.02

Temp. 13.0°C I = 0.05

pH	log K_{obs}	log K_L
5.90	5.55 ± 0.01	5.55 ± 0.01
6.20	5.61 ± 0.01	5.61 ± 0.01
6.62	5.62 ± 0.01	5.62 ± 0.01
7.01	5.53 ± 0.01	5.54 ± 0.01
7.52	5.36 ± 0.01	5.39 ± 0.01
8.02	5.14 ± 0.01	5.24 ± 0.01

TABLE 28

Values of ΔH° for the reaction of dog methaemoglobin with azide in 1 molar glycine + 0.05 M phosphate buffer.

pH	$-\Delta H^{\circ}$ kcal./mole
6.0	11.7 ± 0.1
6.4	13.2 ± 0.1
6.8	14.3 ± 0.1
7.0	14.3 ± 0.1
7.2	13.3 ± 0.1
7.6	12.3 ± 0.1
8.0	11.7 ± 0.2

REACTIONS OF MET α CHAINS OF DOG HAEMOGLOBIN (α^{+}_{Ca})

TABLE 29

Values of pK_3' for α^{+}_{Ca} in 1 M glycine + 0.05 M phosphate buffer at various temperatures.

Temp. °C	pK$_3'$
20.0	8.47 ± 0.01
13.0	8.58 ± 0.01
6.0	8.67 ± 0.01

TABLE 30

Reaction of α^{++}_{Ca} with azide in 1 M glycine + 0.05 M phosphate buffer

Temp. 20.0°C I = 0.05

pH	log K_{obs}	log K_L
5.96	5.14 ± 0.01	5.14 ± 0.01
6.20	5.09 ± 0.02	5.09 ± 0.02
6.54	5.04 ± 0.02	5.04 ± 0.02
6.71	5.04 ± 0.02	5.05 ± 0.02
7.30	5.00 ± 0.02	5.03 ± 0.02
7.70	4.97 ± 0.01	5.04 ± 0.01
8.00	4.91 ± 0.02	5.04 ± 0.02
8.40	4.76 ± 0.01	5.03 ± 0.01

TABLE 30 (CONT'D)

Temp. 13.0°C I = 0.05

pH	log K_{obs}	log K_L
5.98	5.36 ± 0.02	5.36 ± 0.02
6.21	5.35 ± 0.01	5.35 ± 0.01
6.54	5.34 ± 0.01	5.34 ± 0.01
6.88	5.26 ± 0.02	5.27 ± 0.02
7.38	5.25 ± 0.01	5.28 ± 0.01
7.71	5.20 ± 0.01	5.25 ± 0.01
8.03	5.19 ± 0.01	5.28 ± 0.01
8.43	5.02 ± 0.02	5.25 ± 0.02

Temp. 6.0°C I = 0.05

pH	log K_{obs}	log K_L
6.00	5.63 ± 0.02	5.63 ± 0.02
6.22	5.65 ± 0.02	5.65 ± 0.02
6.52	5.60 ± 0.01	5.60 ± 0.01
6.94	5.50 ± 0.01	5.51 ± 0.01
7.38	5.46 ± 0.01	5.47 ± 0.01
7.72	5.42 ± 0.01	5.47 ± 0.01
8.03	5.40 ± 0.02	5.49 ± 0.02
8.43	5.30 ± 0.02	5.53 ± 0.02

TABLE 31

Values of ΔH° for the reaction of α^{+}_{Ca} with azide in 1 M glycine + 0.05 M phosphate buffer.

pH	$-\Delta H^{\circ}$ kcal./mole
6.0	13.4 ± 0.1
6.1	13.9 ± 0.1
6.2	15.2 ± 0.1
6.4	14.7 ± 0.1
6.6	14.1 ± 0.1
6.8	13.4 ± 0.1
7.2	12.0 ± 0.1
7.6	11.6 ± 0.1
8.0	12.0 ± 0.2
8.3	12.9 ± 0.2

REACTIONS OF MET β CHAINS OF DOG HAEMOGLOBIN (β^{+}_{Ca})

TABLE 32

Values of pK'_{s} for β^{+}_{Ca} in 1 M glycine + 0.05 M phosphate buffer at various temperatures.

Temp. °C	pK'_{s}
20.0	8.42 ± 0.01
13.0	8.53 ± 0.01
6.0	8.62 ± 0.01

TABLE 33

Reaction of β^{+}_{Ca} with azide in 1.M glycine + 0.05 M phosphate buffer.

Temp. 20.0°C I = 0.05

pH	log K_{obs}	log K_L
5.95	5.23 ± 0.01	5.23 ± 0.01
6.52	5.19 ± 0.01	5.19 ± 0.01
7.00	5.16 ± 0.02	5.17 ± 0.02
7.60	5.11 ± 0.02	5.17 ± 0.02
8.01	5.10 ± 0.02	5.24 ± 0.02
8.40	4.99 ± 0.01	5.28 ± 0.01

Temp. 13.0°C I = 0.05

pH	log K_{obs}	log K_L
5.96	5.50 ± 0.01	5.50 ± 0.01
6.55	5.40 ± 0.01	5.40 ± 0.01
7.04	5.43 ± 0.02	5.44 ± 0.02
7.62	5.43 ± 0.02	5.48 ± 0.02
8.03	5.37 ± 0.02	5.49 ± 0.02
8.41	5.29 ± 0.02	5.53 ± 0.02

Temp. 6.0°C I = 0.05

pH	log K_{obs}	log K_L
5.99	5.79 ± 0.01	5.79 ± 0.01
6.54	5.71 ± 0.01	5.71 ± 0.01
7.06	5.70 ± 0.02	5.71 ± 0.02
7.65	5.73 ± 0.02	5.78 ± 0.02
8.04	5.67 ± 0.01	5.77 ± 0.01
8.42	5.53 ± 0.01	5.74 ± 0.01

TABLE 34

Values of ΔH° for the reaction of β^{+}_{Ca} with azide in 1 M glycine + 0.05 M phosphate buffer.

pH	$-\Delta H^{\circ}$ k cal./mole
6.0	15.3 ± 0.1
6.4	14.3 ± 0.1
7.0	14.3 ± 0.2
7.4	15.5 ± 0.2
7.6	16.4 ± 0.1
7.8	15.3 ± 0.1
8.0	14.0 ± 0.1
8.4	12.0 ± 0.3

REACTIONS OF MET α CHAINS OF HUMAN HAEMOGLOBIN A (α^{+}_{A})

TABLE 35

Values of pK_3' for α^{+}_{A} in 1 M glycine + 0.05 M phosphate buffer at various temperatures.

Temp. $^{\circ}C$	pK_3'
20.0	8.33 ± 0.01
13.0	8.55 ± 0.01
6.0	8.75 ± 0.01

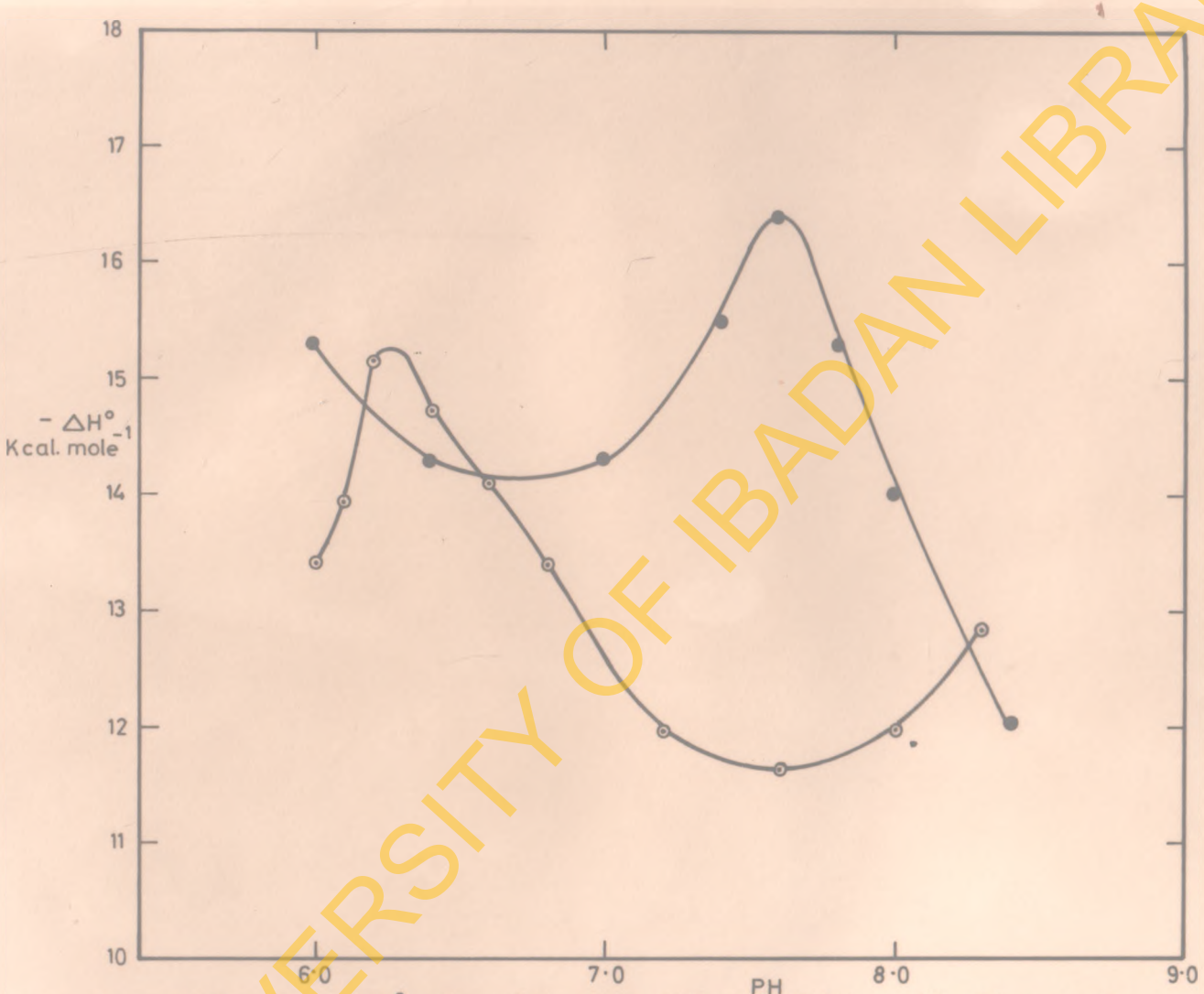


Fig. 15. PLOTS OF $-\Delta H^{\circ}$ Vs PH FOR THE REACTION OF α_{CANINE} ⊙ AND β_{CANINE} ● METHAEMOGLOBINS WITH AZIDE

TABLE 36

Reaction of α^{+}_{A} with azide in 1 M glycine + 0.05 M phosphate buffer.

Temp. 20°C I = 0.05

pH	log K_{obs}	log K_L
5.81	5.26 ± 0.01	5.26 ± 0.01
6.40	5.13 ± 0.01	5.13 ± 0.01
7.00	5.20 ± 0.02	5.22 ± 0.02
7.30	5.14 ± 0.02	5.18 ± 0.02
7.60	5.03 ± 0.02	5.10 ± 0.02
8.00	4.86 ± 0.02	5.03 ± 0.02
8.39	4.70 ± 0.02	5.03 ± 0.02

Temp. 13°C I = 0.05

pH	log K_{obs}	log K_L
5.83	5.43 ± 0.01	5.43 ± 0.01
6.43	5.28 ± 0.01	5.28 ± 0.01
7.01	5.35 ± 0.02	5.36 ± 0.02
7.31	5.33 ± 0.02	5.35 ± 0.02
7.62	5.28 ± 0.01	5.33 ± 0.01
8.01	5.11 ± 0.01	5.22 ± 0.01
8.40	4.94 ± 0.02	5.18 ± 0.02

Temp. 6.0°C I = 0.05

pH	log K_{obs}	log K_L
5.96	5.59 ± 0.02	5.59 ± 0.02
6.46	5.45 ± 0.02	5.45 ± 0.02
7.01	5.55 ± 0.02	5.56 ± 0.02
7.32	5.54 ± 0.01	5.56 ± 0.01
7.66	5.49 ± 0.01	5.52 ± 0.01
8.05	5.33 ± 0.01	5.41 ± 0.01
8.42	5.14 ± 0.02	5.31 ± 0.02

TABLE 37

Values of ΔH^{o} for the reaction of α_A^+ with azide in 1 molar glycine + 0.05 M phosphate buffer.

pH	$-\Delta H^{o}$ k cal./mole
6.0	9.3 ± 0.1
6.4	8.9 ± 0.3
6.8	9.5 ± 0.1
7.2	10.2 ± 0.2
7.6	11.5 ± 0.2
7.8	11.2 ± 0.1
8.0	10.4 ± 0.3
8.4	7.9 ± 0.2

REACTIONS OF MET β CHAINS OF HUMAN HAEMOGLOBIN A (β_A^+)

TABLE 38

Values of pK_s' for β_A^+ at various temperatures in 1 M glycine + 0.05 M phosphate buffer.

Temp. °C	pK_s'
20.0	8.38 ± 0.01
13.0	8.48 ± 0.01
6.0	8.59 ± 0.01

TABLE 39

Reaction of β_A^+ with azide in 1 M glycine + 0.05 M phosphate buffer.

Temp. = 20.0°C I = 0.05

pH	log K_{obs}	log K_L
5.80	5.44 ± 0.01	5.44 ± 0.01
6.21	5.43 ± 0.01	5.43 ± 0.01
6.50	5.27 ± 0.01	5.28 ± 0.01
6.80	5.25 ± 0.02	5.26 ± 0.02
7.20	5.25 ± 0.02	5.28 ± 0.02
7.60	5.24 ± 0.01	5.31 ± 0.01

Temp. 13.0°C I = 0.05

pH	log K_{obs}	log K_L
5.83	5.57 ± 0.01	5.57 ± 0.01
6.22	5.58 ± 0.01	5.58 ± 0.01
6.51	5.50 ± 0.02	5.50 ± 0.02
6.81	5.41 ± 0.02	5.42 ± 0.02
7.21	5.40 ± 0.01	5.42 ± 0.01
7.62	5.37 ± 0.02	5.43 ± 0.02

Temp. 6.0°C I = 0.05

pH	log K_{obs}	log K_L
5.85	5.73 ± 0.01	5.73 ± 0.01
6.25	5.77 ± 0.02	5.77 ± 0.02
6.52	5.68 ± 0.02	5.68 ± 0.02
6.81	5.59 ± 0.01	5.60 ± 0.01
7.23	5.54 ± 0.02	5.56 ± 0.02
7.63	5.51 ± 0.01	5.56 ± 0.01

TABLE 40

Values of ΔH^{o} for the reaction of β_A^+ with azide in 1 M glycine + 0.05 M phosphate buffer.

pH	$-\Delta H^{o}$ K cal./mole
6.0	8.4 + 0.1
6.2	9.1 + 0.1
6.4	10.2 + 0.1
6.6	10.3 + 0.2
6.8	9.1 + 0.2
7.2	7.6 + 0.1
7.6	6.7 + 0.1

TABLE 41

Values of $\frac{1}{2}\left\{ \Delta H^{o} (\alpha^{Ca}) + \Delta H^{o} (\beta^{Ca}) \right\}$

pH	$-\Delta H^{o}$ K cal./mole
6.0	14.4
6.4	14.5
6.6	14.1
7.0	13.4
7.2	13.4
7.6	14.0
8.0	13.0
8.4	12.5

TABLE 42

Values of $\frac{1}{2}\left\{\Delta H^{o}\left(\alpha^{A}\right) + \Delta H^{o}\left(\beta^{A}\right)\right\}$

pH	$-\Delta H^{o}$ K cal./mole
6.0	8.9
6.4	9.6
6.8	9.3
7.2	8.9
7.6	9.1

TABLE 43

ΔH^{o} values calculated by averaging values of log K_L obtained for the reaction of the isolated α and β chains of methaemoglobin A with azide.

pH	$-\Delta H^{o}$ K cal./mole
5.8	8.5
6.2	9.5
6.4	9.9
7.0	9.1
7.6	9.2

TABLE 44

Observed and calculated values of pH_{ch}: The calculated pH_{ch}'s were obtained from the equation $pH_{ch}(\alpha_2\beta_2) = \frac{1}{2}\{pH_{ch}(\alpha) + pH_{ch}(\beta)\}$

Haemoglobin species	pH_{ch} (observed)	pH_{ch} (calculated)
α (canine)	6.3	
β (canine)	7.6	
α (human A)	7.7	
β (human A)	6.5	
Human A	7.1	7.1
Canine	6.9	6.95
$\alpha_2^{A}\beta_2^{Ca}$	7.8	7.65
$\alpha_2^{Ca}\beta_2^{A}$	6.3	6.4

RESULTS OF BOHR EFFECT MEASUREMENTS

TABLE 45

The Bohr effect of $\alpha_2^{Ca}\beta_2^{A}$ haemoglobin.

Temp. = 25.0°C I = 0.05

pH	Δh^+
8.23	+ 0.172
7.93	+ 0.246
7.53	+ 0.375
7.01	+ 0.517
6.72	+ 0.443
6.37	+ 0.241
6.27	+ 0.119
6.08	- 0.063
5.72	- 0.261
5.51	- 0.446
5.17	- 0.621

TABLE 46

The Bohr effect of $\alpha_2^A \beta_2^{Ca}$ haemoglobin.

Temp. 25.0°C I = 0.05

pH	Δh^+
8.39	+ 0.129
7.83	+ 0.307
7.41	+ 0.451
7.11	+ 0.521
6.59	+ 0.473
6.07	+ 0.315
5.72	+ 0.133
5.39	- 0.011
5.01	- 0.110

TABLE 47

The Bohr effect of haemoglobin A obtained from the same chromatographic preparation as the hybrids

Temp. 25.0°C I = 0.05

pH	Δh^+
7.82	+ 0.349
7.08	+ 0.531
5.82	- 0.078
5.35	- 0.315

TABLE 48

The Bohr effect of dog haemoglobin, obtained from the same chromatographic preparation as the hybrids.

Temp. 25°C I = 0.05

pH	Δh^+
7.98	+ 0.362
7.39	+ 0.508
6.28	+ 0.205
5.67	- 0.219

CHAPTER IV

DISCUSSION

The thermodynamics of the ligand binding reactions of several methaemoglobin species have been studied by several workers in this laboratory (Anusiem, Beetlestone and Irvine 1966, 1968a, 1968b; Bailey, Beetlestone and Irvine, 1969a; Bailey, Beetlestone, Irvine and Ogunmola, 1970a). The aim of these investigations is to demonstrate the effect of changes in the amino acid composition of the protein on the reactivity of the iron atom in methaemoglobin and to attempt to set up a model by which changes in reactivity could be quantitatively correlated with changes in structure. Furthermore, these authors explored the possibility of finding any correlation between the ligand binding reactions of methaemoglobin and the physiologically significant binding of oxygen to haemoglobin. The significant findings of these authors have been discussed in Chapter I of this thesis.

Fig. 16 shows a plot of log K_L at 20°C against pH for the hybrid haemoglobins and their parent haemoglobins i.e. canine and human haemoglobins. Inspection of Fig. 16 shows that there is no large variation of ΔG° with pH for all the four haemoglobin species

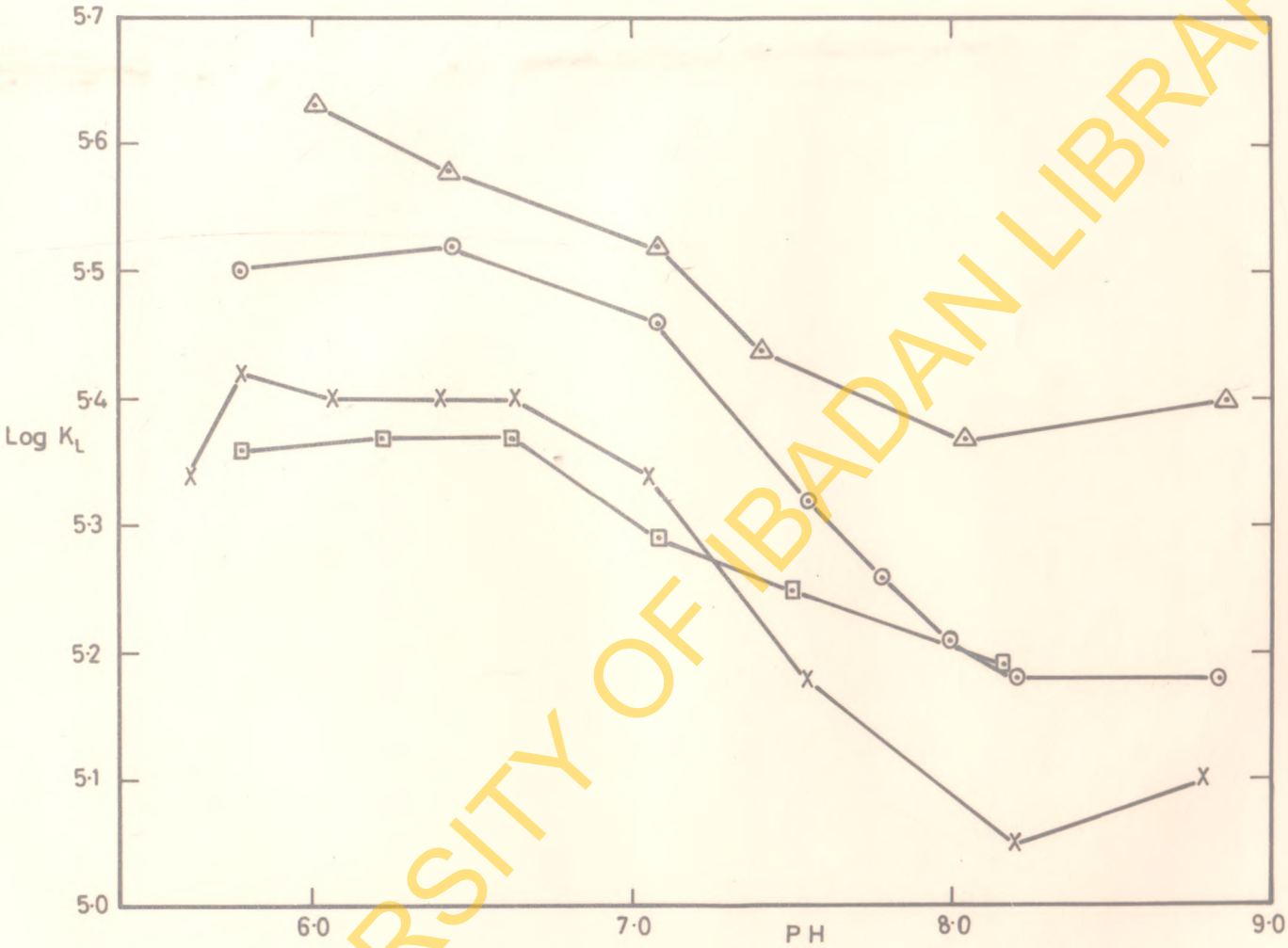


Fig. 16. PLOTS OF Log K_L AGAINST PH AT 20° FOR THE REACTION OF VARIOUS METHAEMOGLOBINS WITH AZIDE: $\alpha_2^A\beta_2^{Ca}$ ⊙; $\alpha_2^{Ca}\beta_2^A$ X; HUMAN A △; DOG ⊡

which is in agreement with earlier results on several other methaemoglobin species. The small differences between log K_L for canine and human methaemoglobins precludes any quantitative comparison but it is apparent that at all pH's, the values for the hybrids lie between those for canine and human methaemoglobins, and that for both hybrids log K_L lies closer to that parent methaemoglobin to which the α chain in the hybrid belongs. Log K_L values were determined on samples of canine and human methaemoglobins obtained from the same chromatographic separation as the hybrids at three different pH's. These values do not differ significantly from values obtained using untreated material (Tables 17 and 24). This confirms that the treatments for preparing the hybrids do not induce any drastic alteration in the functional properties of these haemoglobins.

A comparison of $-\Delta H^{\circ}$ variation with pH for the hybrid methaemoglobins and their parents are shown in Fig. 13. The following features of these curves are noteworthy.

(i) Large variations of $-\Delta H^{\circ}$ with pH are observed for all four haemoglobin species. In haemoglobin $\alpha_2^{Ca}\beta_2^{A}$, $-\Delta H^{\circ}$ varies by about 3 kcal/mole over the pH range 5.6 - 8.8 while for haemoglobin $\alpha_2^{A}\beta_2^{Ca}$, $-\Delta H^{\circ}$ varies by about 5 kcal/mole over the pH range 6 - 8.8.

Similarly, for canine haemoglobin $-\Delta H^{\circ}$ varies by about 3 kcal/mole while in haemoglobin A $-\Delta H^{\circ}$ varies by about 4 kcal/mole over the pH range 6 - 8.4. $-\Delta H^{\circ}$ values determined at three pH's on samples of canine and human methaemoglobins obtained from the same chromatographic preparation as the hybrids show no significant differences from the values obtained on unchromatographed samples.

(ii) The hybrids show the typical behaviour of a native methaemoglobin with a well-defined characteristic pH.

(iii) Below the pH_{ch} the two hybrid haemoglobins and the parent haemoglobins show a similar behaviour; the increasing sides of the Oval-shaped curves being very much superimposable. The large differences observed between the different haemoglobins arise as a result of the different pH_{ch}'s. In other words, below the pH_{ch}, the hydration structure is the same for all four haemoglobins whatever the configuration may be; but above the pH_{ch} the hydration structure is different and hence gives rise to the different ΔH° values.

(iv) The pH_{ch} for canine haemoglobin is 6.9 while for haemoglobin A it is 7.1. In spite of the similar pH_{ch}'s of the parent methaemoglobins pH_{ch} for $\alpha_2^{Ca}\beta_2^{A}$ methaemoglobin is 6.3 while for haemoglobin $\alpha_2^{A}\beta_2^{Ca}$ pH_{ch} is 7.8. This is in agreement with the

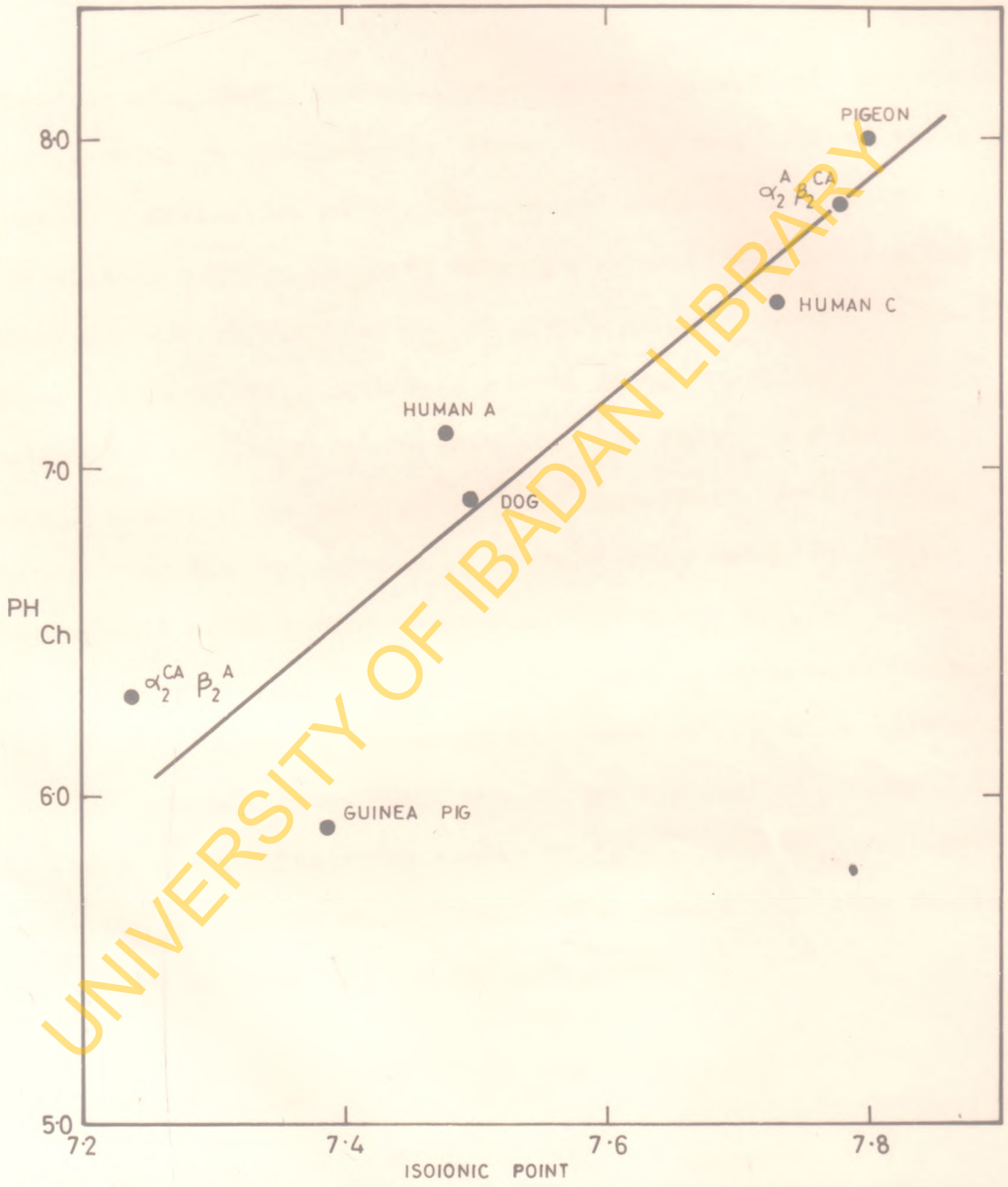


Fig. 17. A PLOT OF PHch (THE CHARACTERISTIC PH) AGAINST ISOIONIC POINT (AT 20 °c) FOR VARIOUS METHAEMOGLOBINS.
(EXCEPT FOR THE HYBRID HAEMOGLOBINS, DATA ARE FROM ANUSIEM, BEETLESTONE AND IRVINE, 1968c)

observed electrophoretic mobilities of the hybrids and the parent haemoglobins at alkaline pH. Since the sequence of mobilities is largely a reflection of the sequence of isoionic points and a correlation between pH_{ch} and isoionic point of the protein has been observed, the sequence of the pH_{ch}'s is to be expected. Fig. 17 shows a plot of pH_{ch} against isoionic point for several methaemoglobins. It can be seen that the points for the two hybrid haemoglobins agree with the correlation. Unfortunately we do not know the composition of charged amino acids in canine haemoglobin so we cannot ascertain whether the pH_{ch}'s of the hybrids is in agreement with the correlation between amino acid composition and pH_{ch}'s (Bailey, Beetlestone and Ogunmola, 1970a). However, if this correlation is an exact one it implies that the contributions of the α and β polypeptide chains to determining pH_{ch} are additive, since the α chain of haemoglobin A also agrees with this correlation. That is to say that for any methaemoglobin

$$pH_{ch}(\alpha_2\beta_2) = \tfrac{1}{2}\left\{pH_{ch}(\alpha) + pH_{ch}(\beta)\right\}$$

where $pH_{ch}(\alpha_2\beta_2)$, $pH_{ch}(\alpha)$ and $pH_{ch}(\beta)$ are respectively the characteristic pH_{ch}'s of the tetramer, the α chain and the β chain. If this equation is correct then we should be able to calculate pH_{ch} for

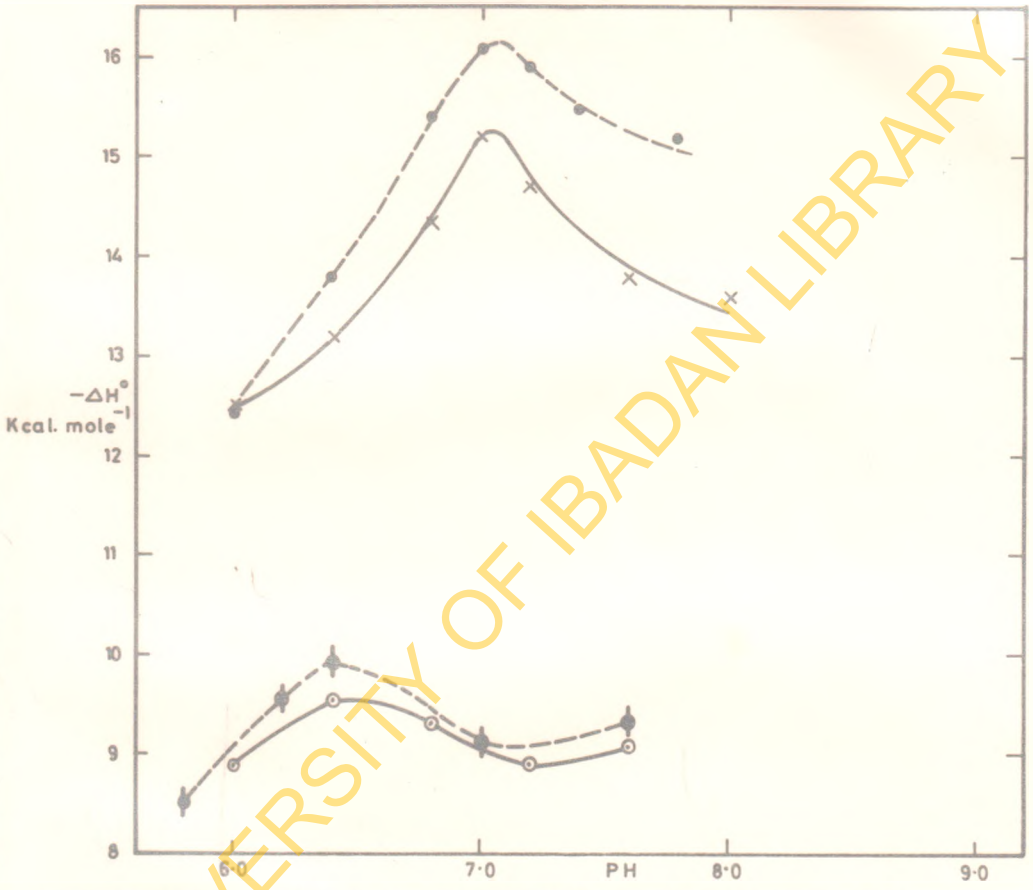


Fig 18. PLOTS OF $-\Delta H^{\circ}$ AGAINST PH FOR THE REACTION OF AZIDE WITH METHAMOGLOBIN A. IN 1 MOLAR GLYCINE + 0·05m PHOSPHATE BUFFER X; METHAEMOGLOBIN A IN 0·05m PHOSPHATE BUFFER ●; $\frac{1}{2}\left\{\Delta H^{\circ}(\alpha^{A}) + \Delta H^{\circ}(\beta^{A})\right\}$ ⊙; ΔH° VALUES CALCULATED BY AVERAGING VALUES OF Log K_L OBTAINED FOR THE ISOLATED α AND β CHAINS OF METHAEMOGLOBIN A ⊕.

canine, human and the hybrid haemoglobins from a knowledge of pH_{ch} for the isolated chains of canine and human haemoglobins. Bailey et. al.(1968) have reported the determination of pH_{ch} for the isolated α chain of human haemoglobin but subsequent work has shown that the native form of the met-α chain is unstable and that their experiments were carried out on modified α chain. However, it has now been shown that the isolated met-α and β chains are sufficiently stable in 1 molar glycine solution to permit the determination of pH_{ch} (Banerjee and Cassoly, 1969).

Tables 30, 33, 36 and 39 show values of log K_L against pH for the separate α and β chains of canine and human haemoglobins. Plots of values of $-\Delta H^{\circ}$ calculated in the usual way from these data, against pH, are shown in figs. 14 and 15. Each chain shows a typical variation of $-\Delta H^{\circ}$ with pH, with a distinct characteristic pH. It is relevant to mention that while the absolute values of $-\Delta H^{\circ}$ for the isolated met-α chains of human haemoglobin are slightly different from those reported by Bailey et. al. (1968) (between 0.5 and 1.0 kcal/mole over the pH range 6 - 9), the characteristic pH's reported in both cases are the same. In order to be able to compare observed $-\Delta H^{\circ}$ versus pH profiles with those of tetrameric human and canine haemoglobins, the reactions of canine and human methaemoglobins in

1 molar glycine are also studied. Figs 18 and 19 show respectively plots of $-\Delta H^{o}$ vs. pH for the reaction of azide with human and canine methaemoglobins in 1 molar glycine as compared with the profiles in ordinary phosphate buffers. The characteristic pH's of these haemoglobins are unchanged and the absolute values of $-\Delta H^{o}$ are changed only slightly.

Table 44 shows the observed values of pH_{ch} for the four chains, the two parent haemoglobins and the two hybrid haemoglobins, together with the values of pH_{ch} for the tetrameric species calculated from the equation

$$pH_{ch}(\alpha_2\beta_2) = \tfrac{1}{2}\{pH_{ch}(\alpha) + pH_{ch}(\beta)\}$$

The observed and calculated values agree within experimental error. Hence the contribution of the α and β polypeptide chains to determining pH_{ch} are additive.

Both of the possibilities suggested for the nature of the postulated configurational change (page 37 of this thesis) could lead to an equation as the one above, but if possibility (2) is correct then under certain conditions, this equation could break down as the following argument shows. Consider human methaemoglobin at pH 6.0. If we increase the pH to 6.5, we reach the pH_{ch} of the

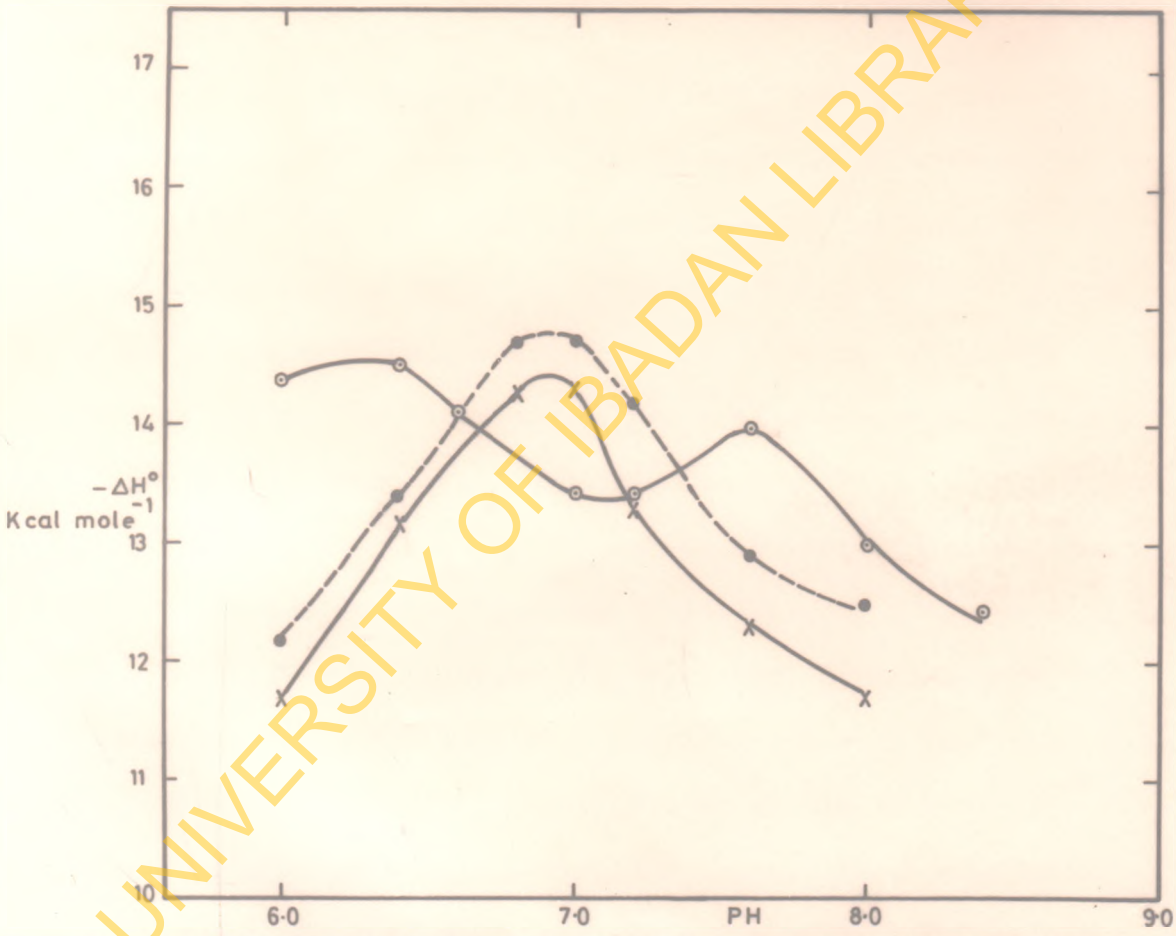


Fig. 19. PLOTS OF $-\Delta H^{\circ}$ AGAINST PH FOR THE REACTION OF AZIDE WITH CANINE METHAEMOGLOBIN IN ½M GLYCINE + 0·05m PHOSPHATE BUFFER X; CANINE METHAEMOGLOBIN IN 0·05m PHOSPHATE BUFFER ●;

$\frac{1}{2}\left\{\Delta H^{\circ}(\alpha^{Ca}) + \Delta H^{\circ}(\beta^{Ca})\right\}$ ⊙

β chain. However in the tetramer a configurational change in the tertiary structure of the β chains would be constrained by the presence of the α chains. Further increase of the pH to 7.1 would give rise to a situation where the configurational change of both the α and the β polypeptide chains could occur together if the decrease in the free energy arising from the release of the β chains from their constrained configuration was equal to the free energy required to force the α chains into their high pH form from 0.6 units below their characteristic pH. However, if in some way we disrupt the native structure of one of the chains we should no longer expect these two energies to be equal and the characteristic pH of the tetramer would be closer to that of the unmodified chain. Recent studies on the ligand binding behaviour of human methaemoglobin modified by reaction with Cysteine, Cystamine and Iodoacetamide at the β 93 sulphydryl group show that the characteristic pH of the modified haemoglobin is very near the characteristic pH of the isolated α chain. (Okonjo, 1971).

In Chapter I of this thesis, it was mentioned that the typical bell shaped curve for the variation of $-\Delta H^{\circ}$ with pH is a function of the tetramer and not an average for the behaviour of the constituent chains. If this is the case, then the form of the

curve obtained by averaging the ΔH° profiles for the α and β chains of either canine or human methaemoglobin should be different from the observed behaviour of the parent haemoglobins. Figs 18 and 19 confirm that this is the case. It could be argued that ΔH° for the parent tetramer is calculated from values of log K_L which are themselves the average of different log K_L's for the constituent chains. In order to examine this possibility, values of ΔH° as a function of pH have been calculated from values of log K_L obtained by averaging the values obtained for the isolated α and β chains of human haemoglobin. The calculated curve obtained is compared with the observed curve in fig. 18 and confirms that the observed $-\Delta H^{\circ}$ variation with pH for human or canine haemoglobin is indeed a function of the tetramer.

Bohr effect of Hybrid haemoglobins.

Fig. 20 shows the variation with pH of Δh^{+} for the reaction of oxygen with stripped human and canine haemoglobins and the two hybrid haemoglobin species (i.e. Hb $\alpha_2^{Ca}\beta_2^{A}$ and Hb $\alpha_2^{A}\beta_2^{Ca}$). The

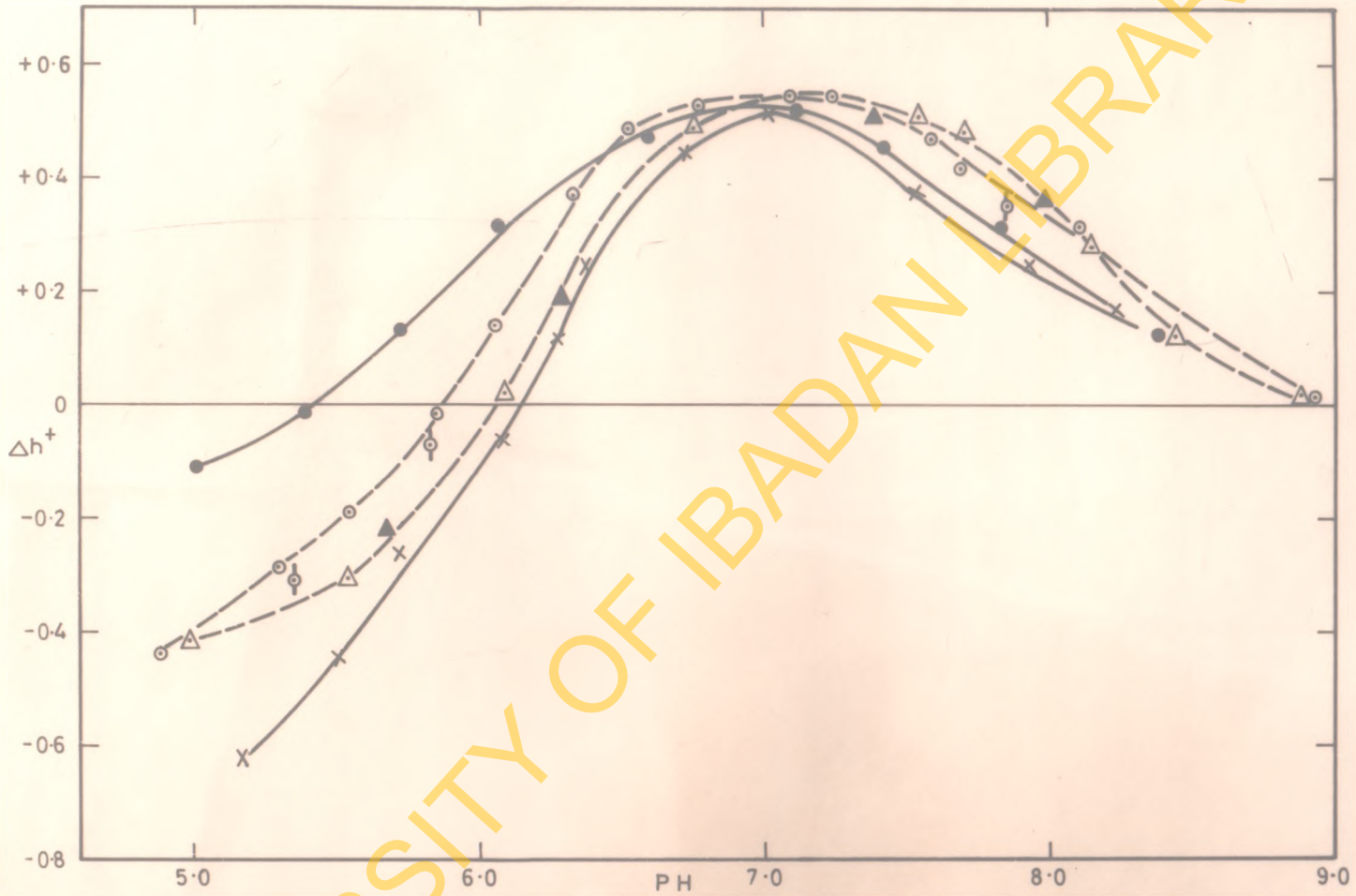


Fig. 20. PLOTS OF Δh^+ AGAINST PH FOR THE REACTION OF SEVERAL HAEMOGLOBINS WITH OXYGEN AT 25°C, I = 0·05 M: $\alpha_2^{Ca}\beta^A$ X ; $\alpha_2^A\beta_2^{Ca}$ ● ; HUMAN A ⊙ ; HUMAN A OBTAINED FROM CHROMATOGRAPHIC SEPARATION ϕ ; DOG △ ; DOG OBTAINED FROM CHROMATOGRAPHIC SEPARATION ▲.

(DATA FOR HUMAN A AND DOG HAEMOGLOBINS ARE OBTAINED FROM BAILEY, BEETLESTONE AND IRVINE, 1970b)

values for haemoglobin A and canine haemoglobin are taken from the data of Bailey et. al. (1970b). The values of Δh^+ were determined at a few pH's on human and canine haemoglobins obtained from the same chromatographic preparation as the hybrid haemoglobins. It can be seen from fig. 20 that there is no significant difference in the Bohr effect of these samples and the untreated samples. These results agree with the results of earlier studies by Antonini et. al. (1965), and Enoki and Tomita (1968), and confirm that the treatments for preparing the hybrids do not induce any drastic alteration in the structure and function of the haemoglobins.

It can be seen from fig. 20 that the general form of the variation of Δh^+ with pH is similar for the hybrid haemoglobins and their parents. The magnitude of the Bohr effect of both hybrids as well as their parent haemoglobins are essentially the same in the alkaline region, which is in agreement with the work of Antonini et. al. (1965). Bailey et. al. (1970b) have found that the magnitude of the alkaline Bohr effect is similar for several mammalian haemoglobins studied. However, the magnitude of the acid (reverse) Bohr effect varies quite significantly. The acid Bohr effect in $\alpha_2^{Ca}\beta_2^{A}$ haemoglobin is slightly bigger than for the parent haemoglobins while $\alpha_2^{A}\beta_2^{Ca}$ haemoglobin has a very small acid Bohr effect.

Previous workers (Antonini et. al. 1965; Enoki and Tomita, 1958) have claimed that while the acid Bohr effect in $\alpha_2^{Ca}\beta_2^{A}$ haemoglobin is slightly increased, it is completely absent in $\alpha_2^{A}\beta_2^{Ca}$ haemoglobin. The present study, however, shows that the acid Bohr effect in $\alpha_2^{A}\beta_2^{Ca}$, although very small, is not completely absent. This discrepancy may be due to the difference in the two methods of measuring Bohr effect. Previous measurements of Bohr effect were made from oxygenation curves while in the present study, the number of moles of hydrogen ion (Δh^+) produced when one mole of oxygen reacts with haemoglobin is directly measured. The method used in measuring Bohr effect in the present study is more sensitive and more accurate than the older method.

Since the canine-human hybrid haemoglobins have widely separated characteristic pH's they could provide a further test of the previously reported correlation between pH_{ch} and the magnitude of the acid Bohr effect (Bailey et. al. 1970b). Fig. 21 shows a plot of the magnitude of the acid Bohr effect at pH 5.3 against pH_{ch} for some haemoglobin species. The values for $\alpha_2^{A}\beta_2^{Ca}$ and $\alpha_2^{Ca}\beta_2^{A}$ haemoglobins are obtained from the present study while the values for the other haemoglobins are from Bailey et. al. (1970b). A value of 5.3 for the pH was chosen as the reference

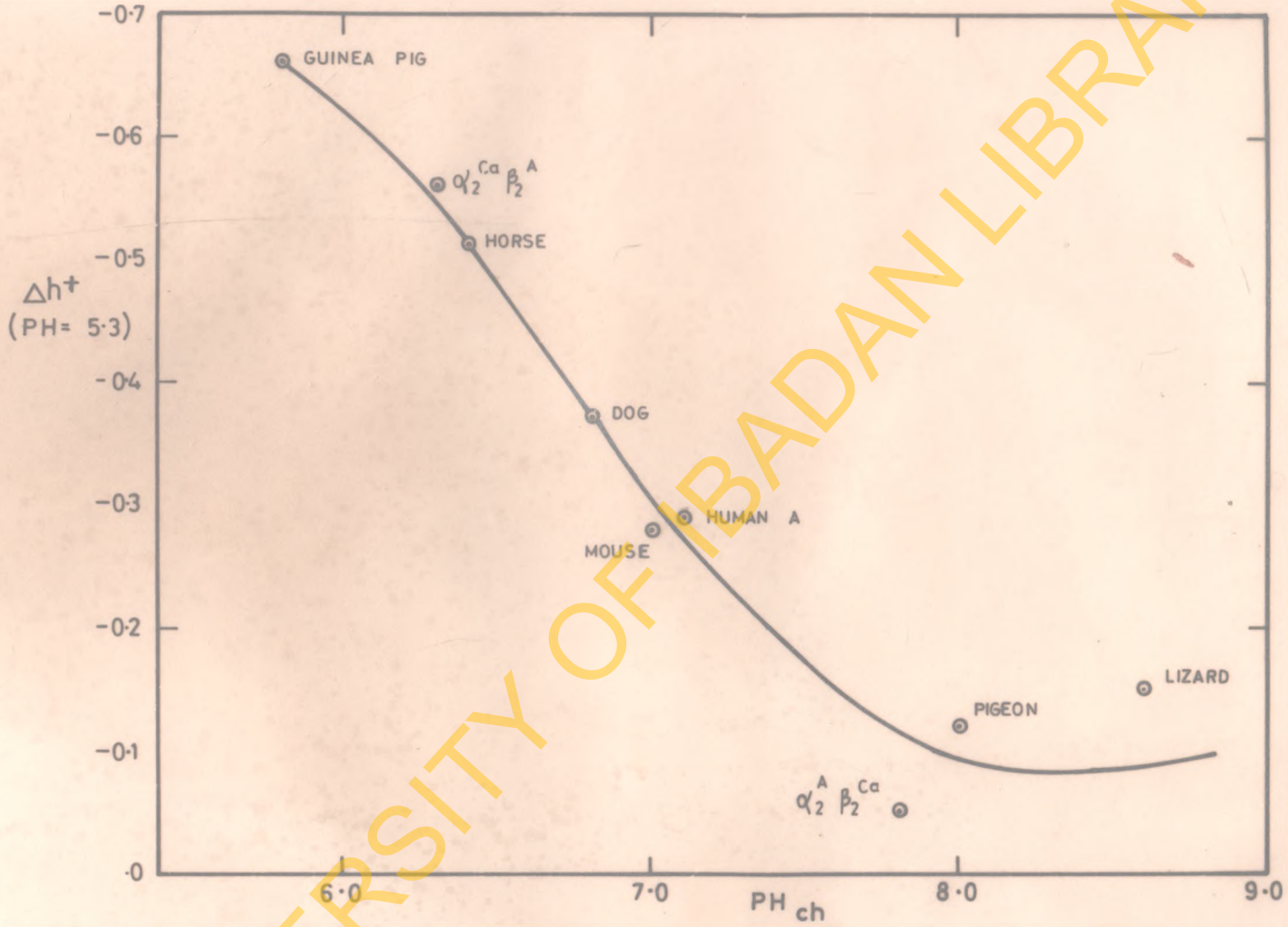


Fig. 21. A PLOT OF Δh^+ (PH = 5·3) AGAINST THE CHARACTERISTIC PH (PH ch) FOR VARIOUS HAEMOGLOBINS.

(EXCEPT FOR THE HYBRID HAEMOGLOBINS, DATA ARE FROM BAILEY, BEETLESTONE AND IRVINE, 1970b)

point because, (i) it is the lowest pH at which stable pH values can be obtained and (ii) it is sufficiently removed from the region in which the alkaline Bohr effect operates for this effect to have a negligible effect on Δh^+. It can be seen from fig. 21 that the points for the two hybrid haemoglobins agree quite well with this correlation. Thus no specific mechanism need be postulated to account for the low value of the acid Bohr effect of $\alpha_2^A\beta_2^{Ca}$; it arises as a consequence of its high characteristic pH.

CHAPTER V

REACTION OF CARBON MONOXIDE WITH OXYHAEMOGLOBIN

Introduction

The work described in the first part of this thesis as well as other reactivity studies on methaemoglobins (A.C. Anusiem et. al. 1966, 1968a, 1968b; J.G. Beetlestone et. al. 1964, 1965 1969) have been mainly concerned with establishing a model by which reactivity differences between different haemoglobins could be interpreted. The reason for choosing the reactions of met-haemoglobins is the apparent simplicity of these reactions as compared with the reactions of haemoglobin which are rendered complex by the phenomenon of haem-haem interaction. The ligand binding reactions of haemoglobin are structurally different from those of methaemoglobin in that ligation consists of filling the empty sixth coordination position and that a gross change in the quarternary structure accompanies ligation (Perutz. 1970) and phenomenologically in that the formation curves of the complex are sigmoid rather than hyperbolic. However, it is anticipated that the model thus created to explain methaemoglobin reactions could lead to a useful model for interpreting haemoglobin reactions.

As mentioned earlier (page 29 of this thesis), the large variation of ΔH° with pH observed for the ligand binding reactions of methaemoglobin has been interpreted in terms of hydration changes. Such hydration changes could arise if complex formation with certain ligands is accompanied by a shift of a proton on the distal imidazole from the nitrogen atom on the surface of the molecule to the nitrogen atom which points towards the iron atom. The question which then arises is whether one gets this 'hydrogen shift' in the reactions of haemoglobin itself. If the reaction of oxygen with deoxy-haemoglobin is accompanied by this 'hydrogen shift' then one should observe a similar ΔH° variation with pH as in the ligand binding reactions of methaemoglobin. Unfortunately however, the ligand binding reactions of haemoglobin are complicated by the phenomenon of haem-haem interaction. Hence we have to look at a much simpler reaction of oxyhaemoglobin which is easy to study. Thus in this section of the thesis, we have investigated the reaction of carbon monoxide with oxyhaemoglobin. This is indeed a simple reaction since, structurally there is no change in the quarternary structure of the protein (Perutz, 1970), and one ligand replaces another in the same coordination position rather than there being a change of coordination number as in the reaction

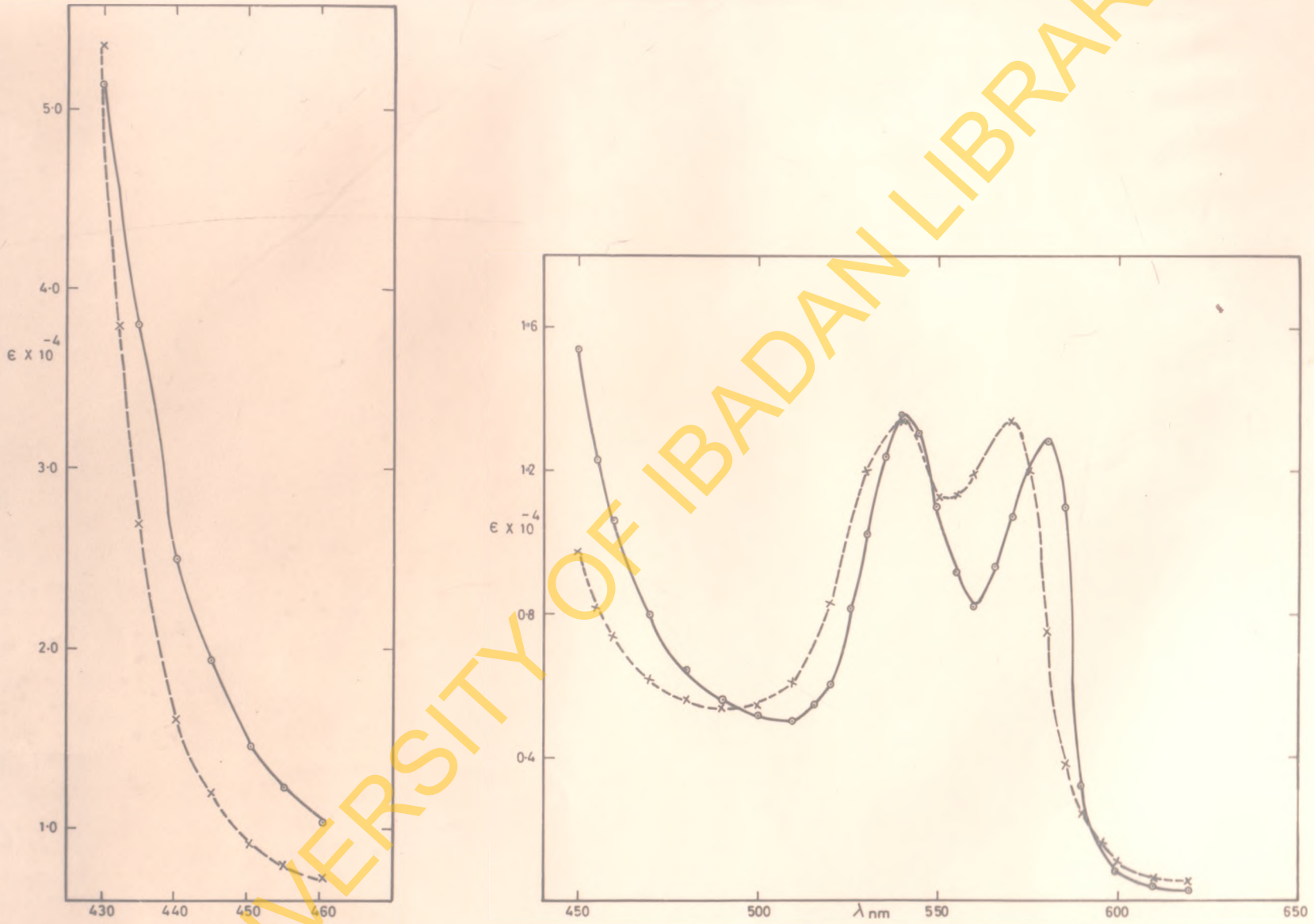


Fig. 22. SPECTRA OF CARBONMONOXY –HAEMOGLOBIN, X–X–X, AND OXY–HAEMOGLOBIN ⊙–⊙–⊙, PH= 6·0, TEMP = 20°C, I = 0·05.

of either carbon monoxide or oxygen with haemoglobin. Also, the formation curve of this reaction is hyperbolic unlike the reactions of oxygen or carbon monoxide with haemoglobin which are sigmoidal. Hence the reaction can be described, within experimental error, by a single equilibrium constant (Haldane and Smith, 1897; Douglas and Haldane, 1912).

The replacement reaction of oxygen by carbon monoxide from oxyhaemoglobin can be represented as

$$HbO_2 + CO \rightleftharpoons HbCO + O_2$$

(where HbO_2 and HbCO represent respectively, oxyhaemoglobin and carbon monoxy haemoglobin), since although there are four haem groups in the molecule, they apparently react independently. We define an equilibrium constant (Partition coefficient) K_m as

$$K_m = \frac{[HbCO]}{[HbO_2]} \frac{[O_2]}{[CO]}$$

where $[HbCO]$, $[HbO_2]$, $[O_2]$ and $[CO]$ represent respectively the concentrations of carbon monoxyhaemoglobin, oxyhaemoglobin, oxygen and carbon monoxide. The value of K_m determined experimentally will be an average of similarly defined equilibrium

constants for the haems associated with the α and β polypeptide chains in the tetramer.

This reaction was first studied quantitatively by Barcroft and his coworkers (Anson, M.L. et. al. 1925). They determined the value of K_m for a number of haemoglobin species. They deomonstrate that K_m differs markedly between species and they established an empirical relationship between log K_m and the 'span' of the haemoglobin. They defined the 'span' as the shift of the α band in the spectrum of oxyhaemoglobin when the oxygen is replaced by carbon monoxide. However most of this work was carried out on crude haemolysates and on occasions large differences between individuals of the same species were observed. The variation of K_m with species have been confirmed by other workers (Allen and Root, 1957, Roughton, 1954).

Values of K_m had usually been obtained by gasometric measurements (Haldane et. al. 1924; Roughton, 1954) but recently Brunori et. al. (1966) have determined K_m spectrophotometrically for human A and the isolated individual chains of human haemoglobin; while Antonini (1958) has determined K_m spectrophotometrically for myoglobin.

In the present study, the values of K_m have been determined spectrophotometrically and a simple tonometer has been devised for

this purpose. With this method, accurate and reproducible values of K_m could be obtained. It is therefore possible to investigate enthalpy, entropy and free energy changes in this reaction as a function of pH for a number of haemoglobin species by determining K_m as a function of pH and temperature.

EXPERIMENTAL

(a) Materials:

Blood samples

Blood samples containing haemoglobin A were obtained from the Blood Bank of the University Hospital, Ibadan and had been characterised as such by paper electrophoresis in the routine screening of blood taken from donors. Blood of rabbit was obtained from two different animals while in the case of pigeon and guinea pig the blood was obtained by pooling the blood from several animals. The anticoagulant used was either oxalate or citrate.

Preparation of haemoglobin

The method used was the same as the one described on page 43 of this thesis.

Carbon Monoxide

Cylinders of pure carbon monoxide were obtained from Industrial gases Ltd. Apapa, Nigeria.

Buffer solution

Phosphate buffers (I = 0.05) were used in the pH range 5.8 - 8.0 while above pH 8.0, borate buffers (I = 0.05) were used.

(b) EQUIPMENTS

Optical density measurements were made on a Cary Model 16 spectrophotometer with the constant temperature cell compartment. Measurement of pH was done using a Radiometer pH meter 4d. Hypodermic needles were obtained from Hamilton Co. California U.S.A.

The Tonometer

The tonometer devised for the measurement of K_m is shown in fig. 23. It consists essentially of a glass bulb B about 60 ml in volume with a neck stoppered with a rubber diagphragm D. The end E of the glass bulb is grounded and it fits tightly into a silica cell C. Thus the tonometer is detachable from the silica

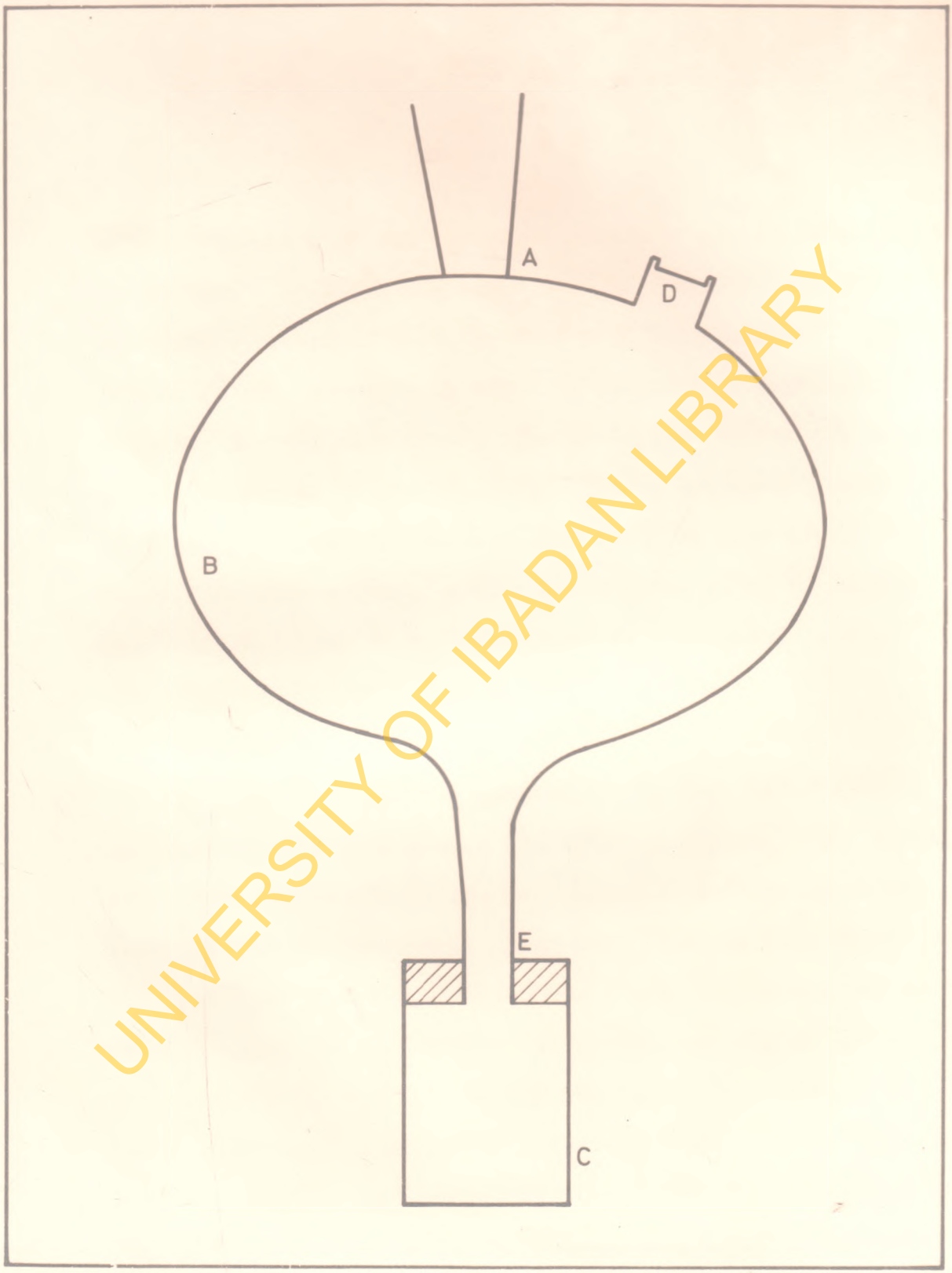


FIG. 23.

TONOMETER

cell. The end A of the tonometer is closed and it is connected to the shaft of a driving motor. It is therefore possible for the entire equipment to be rotated by a driving motor. The volume of the tonometer is found by weighing it empty and weighing it full with water. The difference in the two weights gives the volume of the tonometer. Three different tonometers with volumes 53.85 ml, 68.14 ml and 61.12 ml were used. The tonometers were darkened with a black paint so as to prevent photo dissociation of the carbon monoxy haemoglobin formed.

(c) PROCEDURE

Spectra. The visible spectra of the oxy- and carbon monoxy derivatives of haemoglobin A are shown in fig. 22. The maximum absorption difference in the two spectra occurs at about 450 nm hence spectrophotometric readings were taken at this wavelength.

Fresh haemoglobin solution was slowly passed through a Dintzis column to remove ionic contamination. An haemoglobin solution with an optical density reading between 0.8 and 0.9 (at λ = 450 nm) was prepared in the appropriate buffer solution. The concentration of such haemoglobin solutions was usually about 6×10^{-5} molar. The pH of the haemoglobin solution was then

determined.

2.5 ml of the haemoglobin solution was introduced into the silica cell C with a pipette and the tonometer was then tightly fitted to the cuvette. The apparatus was then tilted so that the haemoglobin solution now lay inside the glass bulb B. The end A of the tonometer was then connected to the shaft of a driving motor which rotated the whole apparatus in a water bath. After temperature equilibration, (about 20 minutes) the motor was stopped, and the haemoglobin solution was tilted into the cell. The silica cell together with the tonometer was carefully wiped dry and the optical density of the haemoglobin solution read (D_o). Optical density readings were taken with the tonometer tightly fixed to the silica cell. A special lid was constructed for the spectrophotometer to cover the entire apparatus.

An agla syringe fixed to an hypodermic needle was filled with carbon monoxide by means of the device shown in fig. 24. Carbon monoxide from the cylinder passed through the glass bulb T and the flow meter M into the atmosphere. When the glass bulb T was completely filled with carbon monoxide (about two minutes), the agla syringe was filled with carbon monoxide through the rubber diaphragm. The agla syringe was then fitted to a micrometer screw

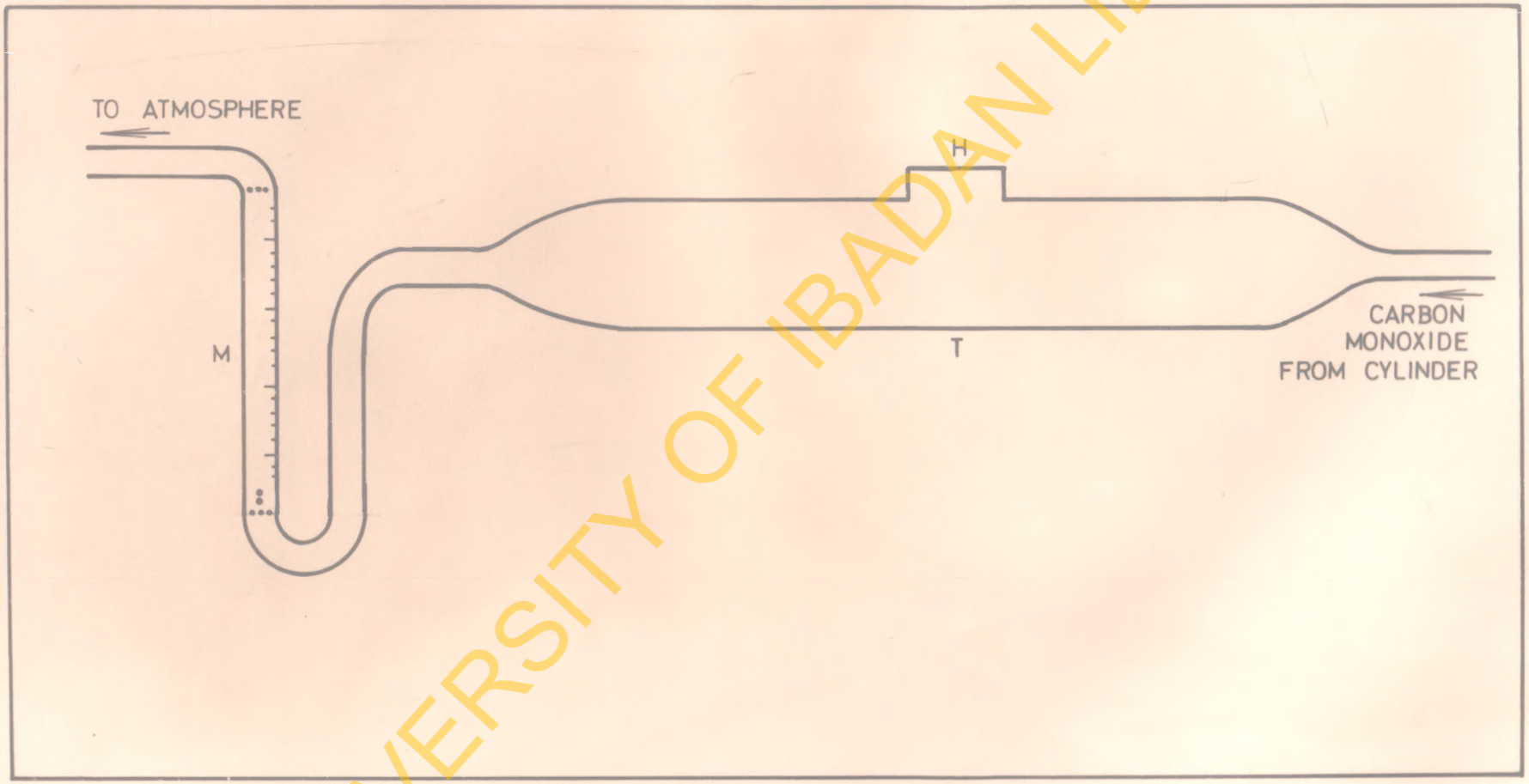


FIG. 24.

guage and the needle of the syringe covered with a rubber cap to prevent diffusion of the carbon monoxide from the syringe. The pressure of the carbon monoxide in the syringe was assumed to be the pressure of the atmosphere.

An accurately known volume of carbon monoxide (about 0.02 ml) was injected into the haemoglobin solution in the tonometer through the rubber diaphragm D (fig. 23) at atmospheric pressure. The haemoglobin solution, now in the cell. was tilted into the bulb B and the whole apparatus rotated for about 20 minutes in the water bath; care being taken to ensure that the haemoglobin solution did not fall back into the cell during equilibration. After temperature equilibration, the optical density (D) of the haemoglobin solution was measured. This was repeated for several aliquots of carbon monoxide to give between 30 and 80 percent formation of carbon monoxy haemoglobin.

The tonometer was then removed from the silica cell and the haemoglobin solution saturated with carbon monoxide. The optical density (D_∞) of the haemoglobin solution was then measured after equilibration at the particular temperature. Readings were taken at three different temperatures viz: 20°C, 27°C and 34°C. Since three tonometers of different volumes were used, the effect of

changing the volume of the tonometer was investigated. As table 50 shows, the value of K_m is independent of the volume of the tonometer used. Also the effect of dilution of the oxyhaemoglobin solution on the value of K_m was investigated (table 51).

Calculation of K_m

If we express K_m in terms of the partial pressures of the gas then we have that

$$K'_m = \frac{[HbCO]}{[HbO_2]} \cdot \frac{P_{O_2}}{P_{CO}}$$

where PO_2 and P_{CO} are the partial pressures of oxygen and carbon monoxide respectively and K'_m is the partition coefficient described in terms of the partial pressures of the gases. From the meanings of D_o, D and D_∞ as stated above, it is readily shown that, at equilibrium,

$$K'_m = \frac{D_o - D}{D - D_\infty} \times \frac{P_{O_2}}{P_{CO}}$$

This expression assumes that the pressure of displaced oxygen is negligible compared with P_{O_2} and similarly that the pressure of dissolved carbon monoxide is negligible compared with P_{CO}.

In a room of normal ventilation, the content of oxygen in the air can be taken to be 20.93%. Therefore $P_{O_2} = \frac{20.93}{100} \times (B-a)$ where B is the atmospheric pressure and 'a' is the partial pressure of the water vapour at the particular temperature. If the total volume of the tonometer is Vml and vml of carbon monoxide is introduced into it, the partial pressure of carbon monoxide is given by $\frac{v}{V} \times (B-a)$

Hence $K'_m = \frac{D_o - D}{D - D_\infty} \frac{V}{v} \times \frac{20.93}{100}$

K'_m can thus be calculated for different values of v. A typical set of calculations is shown in table 49. The values of K'_m can be converted to the equilibrium constant expressed in terms of the relative concentrations of the gases in solution (K_m) by converting the ratio P_{O_2}/P_{CO} to concentration units i.e. $[O_2]/[C_O]$. The relative concentration of $[O_2]/[C_O]$ dissolved in solution at a particular temperature is obtained by multiplying the relative partial pressures P_{O_2}/P_{CO} at that temperature by the ratio of the absorption coefficient (α) of the two gases at that temperature. (The absorption coefficient (α) is defined as the volume of gas when reduced to 0^oC and 760 mm is dissolved in one volume of water when exposed to one atmosphere pressure of the gas). The

absorption coefficients are given in books of physical constants and the values of α for carbon monoxide and oxygen at the three temperatures of the experiment are given in table 32.

Calculation of ΔH°_m, ΔG°_m and ΔS°_m

Values of log K_m are plotted against pH at the three temperatures of the experiment fig. 25. Extrapolated values of log K_m at a particular pH are plotted against 1/T and ΔH°_m is calculated from the slope of such a plot. In this calculation it is assumed that ΔC_p is zero in the range of temperatures used in these experiments. The good linearity obtained by plotting log K_m against 1/T (fig. 26) justifies this assumption. ΔG°_m is calculated from the relation, $\Delta G^{\circ}_m = -2.303RT \log K_m$ and ΔS°_m is obtained from the relation

$$\Delta G^{\circ}_m = \Delta H^{\circ}_m - T\Delta S^{\circ}_m.$$

EXPERIMENTAL RESULTS

Table 49

A typical calculation of log K_m for Haemoglobin A

pH = 5.76 I = 0.05 t = 27.0°C

Volume of tonometer = 68.14 ml.

Volume of carbon monoxide (ml.)	D at 450nm	$\frac{D_o - D}{D - D_\infty}$	$\frac{D_o - D}{D - D_\infty} \times \frac{P_{O_2}}{P_{CO}}$	K_m	log K_m
0.02	0.8840	0.278	198.14	261.5	2.42
0.04	0.8322	0.558	198.14	261.5	2.42
0.06	0.7948	0.850	207.12	273.7	2.44
0.08	0.7642	1.185	212.02	279.9	2.45
0.10	0.7540	1.326	188.85	249.4	2.40
0.12	0.7350	1.643	195.40	257.9	2.41

D_o = 0.9642

D_∞ = 0.5955

Average log K_M = 2.42 ± 0.01.

Table 50

Values of log K_m obtained with different tonometers for HbA.

pH = 7.00 I = 0.05 t = 20.0°C.

Volume of Tonometer	K_m	log K_m
53.85 ml	330.1	2.52
68.14 ml	322.7	2.51
61.12 ml	326.8	2.51

Table 51

Effect of dilution on the value of log K_m (haemoglobin A).

Concentration	pH	log K_m at 20°C
1.2×10^{-4}M	5.80	2.52 ± 0.01
	7.00	2.52 ± 0.02
6×10^{-5}M	5.78	2.53 ± 0.01
		2.52 ± 0.01
3×10^{-5}M	5.80	2.52 ± 0.02
	7.01	2.51 ± 0.01

Table 52

Absorption Coefficients (α) of oxygen and carbon monoxide at 20°C, 27°C and 34°C.

	α at 20.0°C	α at 27.0°C	α at 34.0°C
CO	0.02319	0.02080	0.01877
O_2	0.03102	0.02736	0.02440
$\frac{\alpha_{O_2}}{\alpha_{CO}}$	1.34	1.32	1.30

Pef. Hand Book of Chemistry and Physics 36th Edition 1954-55 p.1608.

Table 53

Reaction of oxyhaemoglobin A with carbon monoxide.

Temp. = 20.0°C I = 0.05

pH	log K_m
5.78	2.54 ± 0.01
6.40	2.51 ± 0.01
7.01	2.52 ± 0.01
7.60	2.52 ± 0.02
8.20	2.54 ± 0.02
8.80	2.54 ± 0.01

Table 53(Contd.)

Temp. = 27.0°C I = 0.05

pH	Log K_m
5.76	2.42 ± 0.01
6.38	2.41 ± 0.01
7.00	2.40 ± 0.01
7.59	2.42 ± 0.)1
8.17	2.43 ± 0.01
8.78	2.42 ± 0.01

Temp. = 34.0°C I = 0.05

pH	Log K_m
5.74	2.32 ± 0.01
6.35	2.29 ± 0.01
6.98	2.31 ± 0.01
7.56	2.31 ± 0.01
8.13	2.32 ± 0.01
8.72	2.32 ± 0.02

Table 54

Values of ΔH°_{m} for the reaction of oxyhaemoglobin A with carbon monoxide.

pH	$-\Delta H^{\circ}_{m}$
5.8	6.1 ± 0.2
6.4	6.1 ± 0.3
7.0	5.8 ± 0.2
7.6	5.8 ± 0.3
8.0	6.0 ± 0.2
8.4	6.1 ± 0.1
8.6	6.1 ± 0.2

Table 55

Reaction of pigeon oxyhaemoglobin with carbon monoxide

Temp. = 20.0°C I = 0.05

pH	log K_m
5.80	2.52 ± 0.01
6.41	2.52 ± 0.01
7.00	2.54 ± 0.02
7.61	2.54 ± 0.01
8.20	2.51 ± 0.02
8.82	2.52 ± 0.01

Table 55 (Contd.)

Temp. = 27.0°C	I = 0.05
pH	Log K_m
5.79	2.42 ± 0.01
6.39	2.40 ± 0.01
7.01	2.42 ± 0.01
7.60	2.42 ± 0.02
8.19	2.40 ± 0.01
8.81	2.41 ± 0.01

Temp. = 34.0°C	I = 0.05
pH	Log K_m
5.79	2.32 ± 0.01
6.38	2.29 ± 0.01
6.99	2.32 ± 0.01
7.60	2.32 ± 0.01
8.15	2.29 ± 0.01
8.76	2.31 ± 0.01

Table 56

Values of ΔH^{o}_{m} for the reaction of pigeon oxyhaemoglobin with carbon monoxide.

pH	$-\Delta H^{o}_{m}$
5.8	5.6 ± 0.2
6.4	6.4 ± 0.1
7.0	6.1 ± 0.2
7.6	6.1 ± 0.1
8.0	6.3 ± 0.1
8.4	6.1 ± 0.1
8.6	5.8 ± 0.2

Table 57

Reaction of Guinea pig oxyhaemoglobin with carbon monoxide.

Temp. = 20.0°C I = 0.05

pH	Log K_m
5.80	2.49 ± 0.01
6.39	2.50 ± 0.02
7.00	2.50 ± 0.02
7.60	2.49 ± 0.01
8.20	2.52 ± 0.01
8.79	2.51 ± 0.02

Table 57 (Contd.)

Temp. = 27.0°C	I = 0.05
pH	Log K_m
5.77	2.37 ± 0.01
6.40	2.39 ± 0.02
6.99	2.39 ± 0.02
7.60	2.38 ± 0.01
8.19	2.40 ± 0.02
8.77	2.39 ± 0.01

Temp. = 34.0°C	I = 0.05
pH	Log K_m
5.73	2.27 ± 0.02
6.35	2.28 ± 0.01
6.94	2.28 ± 0.01
7.59	2.28 ± 0.01
8.16	2.30 ± 0.02
8.72	2.29 ± 0.01

Table 58

Values of ΔH°_{m} for the reaction of guinea pig oxyhaemoglobin with carbon monoxide.

pH	$-\Delta H^{\circ}_{m}$
5.8	6.1 ± 0.2
6.4	6.1 ± 0.2
7.0	6.1 ± 0.3
7.6	6.0 ± 0.1
8.0	6.1 ± 0.1
8.4	6.1 ± 0.1
8.6	6.1 ± 0.2

Table 59

Reaction of Rabbit oxyhaemoglobin with carbon monoxide

Temp. = 20.0°C I = 0.05

pH	Log K_m
6.08	2.33 ± 0.01
6.56	2.31 ± 0.01
7.03	2.31 ± 0.02
7.60	2.33 ± 0.01
8.08	2.32 ± 0.01
8.60	2.30 ± 0.01

Table 59 (Contd.)

Temp. = 27.0°C	I = 0.05
pH	Log K_m
6.06	2.21 ± 0.02
6.56	2.21 ± 0.01
7.01	2.20 ± 0.01
7.59	2.22 ± 0.01
8.06	2.21 ± 0.01
8.58	2.19 ± 0.02

Temp. = 34.0°C	I = 0.05
pH	Log K_m
6.04	2.10 ± 0.01
6.53	2.10 ± 0.01
7.01	2.08 ± 0.01
7.58	2.12 ± 0.01
8.05	2.11 ± 0.02
8.53	2.09 ± 0.02

Table 60

Values of ΔH°_{m} for the reaction of rabbit oxyhaemoglobin with carbon monoxide

pH	$-\Delta H^{\circ}_{m}$
6.2	6.1 ± 0.1
6.6	5.8 ± 0.2
7.0	6.4 ± 0.2
7.4	6.0 ± 0.1
7.8	5.8 ± 0.1
8.2	5.9 ± 0.1
8.5	5.8 ± 0.3

DISCUSSION

More than fifty years ago, Haldane and his coworkers (Douglas et. al. 1912) showed that carbon monoxide combines with haemoglobin at the same site as does oxygen and in a similar way. They observed not only that the shape of the oxygen and carbon monoxide equilibrium curves of whole blood were identical within experimental error, but also that corresponding oxygen and carbon monoxide equilibrium curves obtained under different conditions could be superimposed by altering the scales of gas pressure by a constant factor. Also, the remarkable magnetic changes accompanying combination of haemoglobin with oxygen are duplicated in the case of carbon monoxide.

These findings suggested that the combination of carbon monoxide with haemoglobins involved exactly the same interactions as the combination with oxygen despite the differences in the absolute values of the affinity constants. We may therefore regard the two functions; combination with oxygen and combination with carbon monoxide as identically linked and look for a partition equilibrium involving the two gases. The existence of such an equilibrium in the case of mammalian haemoglobins was discovered

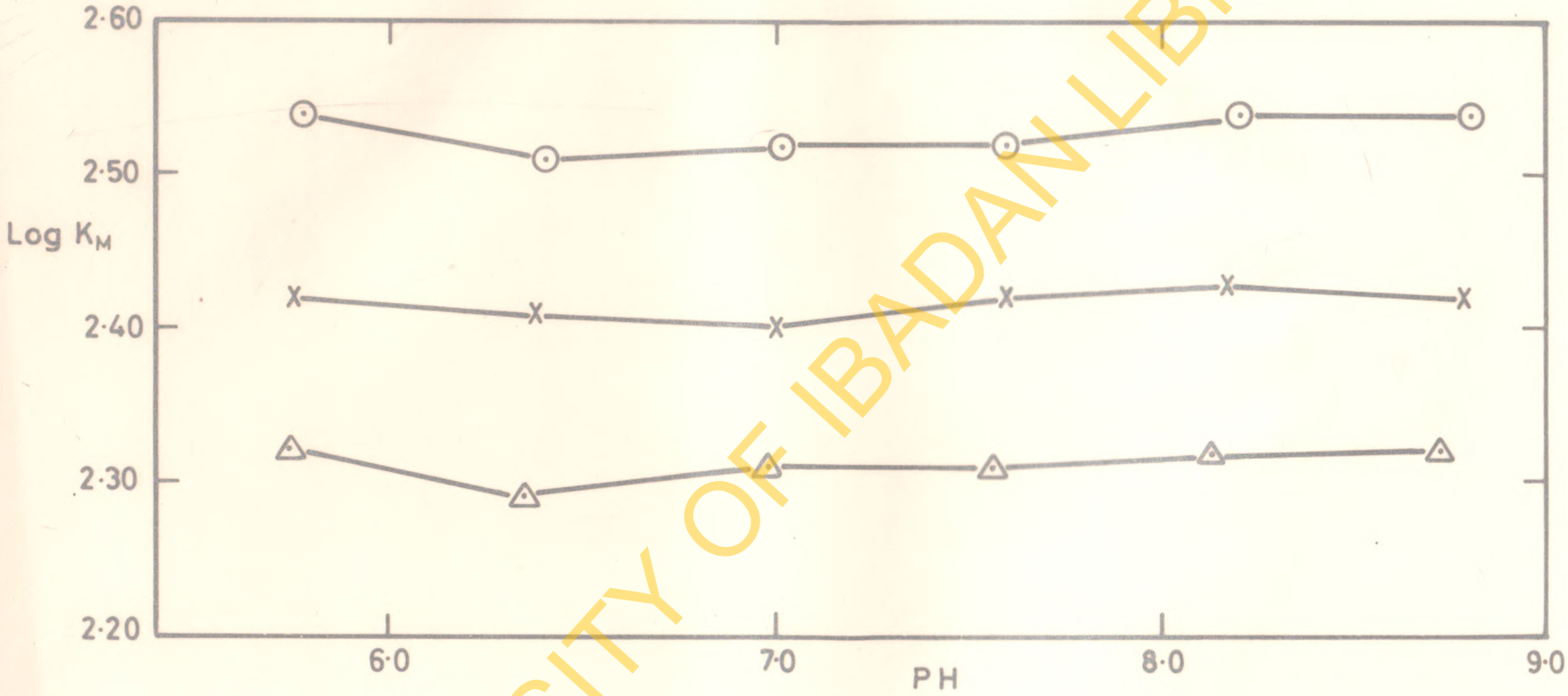


Fig. 25. PLOTS OF Log K_M AGAINST PH FOR THE REACTION OF CARBON MONOXIDE WITH OXYHAEMOGLOBIN A: ⊙ 20°c, X 27°c, △ 34°c.

and investigated in a series of fundamental experiments by Haldane and his associates (Douglas et. al, 1912; Haldane, J.S. 1895a, 1895b, 1896; Haldane and Smith, 1897), whose work has been substantiated by subsequent workers over a wider range of conditions. The partition coefficient (K_m) has been shown to be the factor by which the carbon monoxide pressure scale had to be multiplied to make the carbon monoxide dissociation curve coincide over the whole range of Y (the fractional saturation with ligand) with the oxygen dissociation curve in the absence of reduced haemoglobin. (Douglas C.G.et al. 1912). Recently, Roughton (1954) has also shown that using the Adair model, the value of K_m is really determined only by the ratio of the affinity constants for the combination of the fourth haem with carbon monoxide and oxygen.

Fig. 25 shows a plot of log K_m versus pH for human haemoglobin A at three temperatures. It can be seen that, within experimental error, log K_m is independent of pH. This is in agreement with the work of Barcroft (1925) on whole blood. The same behaviour is observed for the other haemoglobin species studied (fig. 27). A similar pH independence of log K_m has been observed by Antonini et. al. (1963) on 'Stripped' haemoglobin A. The fact that the partition coefficient is independent of pH implies that the Bohr

effect is the same for both oxygen and carbon monoxide. This in turn indicates that combination with carbon monoxide leads to identical effects on the same set of acid groups as combination with oxygen. This is confirmed by experiments of Hastings et. al. (1924) which show that combination with either oxygen or carbon monoxide increases the acidity of the solution by exactly the same amount at any given pH. This result has been confirmed by Antonini et. al. (1963) who showed that the replacement of oxygen by carbon monoxide in fully liganded haemoglobins leads to no change in the number of protons bound by the haemoglobin at any pH over the range studied (pH 5 - 9).

Fig. 27 shows a plot of log K_m against pH at 20°C for the four haemoglobin species studied. Within the experimental error of $\pm$ (0.01 - 0.02), the values of log K_m for human, pigeon and guinea pig haemoglobins are identical and pH invariant with an average value of 2.52 corresponding to a standard free energy change of -3.38 kcal $mole^{-1}$ at 20°C. On the other hand the average value of log K_m is about 2.31 for rabbit haemoglobin which is significantly different from the value obtained for the other haemoglobin species. Barcroft (1925) obtained a similar species variation and pH independence of the values of log K_m.

Fig. 26. PLOTS OF Log K_M AGAINST $\frac{1}{T}$ FOR THE REACTION OF CARBON-MONOXIDE WITH OXYHAEMOGLOBIN A. ⊙ at PH 8·4; ● at PH 6·4

However, there are significant differences in the magnitude of log K_m obtained by Barcroft and the one obtained in the present study. For example Barcroft obtained a value of 2.70 for haemoglobin A at 20°C as compared with a value of 2.52 obtained in the present study. He also observed much larger species variation in log K_m. These discrepancies could be due to the fact that Barcroft worked on whole blood while in the present study, 'Stripped' haemoglobin has been used. Antonini et. al. (1963), working on 'stripped' haemoglobin A obtained an average value of 2.48 for log K_m which is quite close to the average value obtained in the present study. It could therefore be suggested that such discrepancies might be due to co-factors present in whole blood. The identity of such co-factors is subject to further experiments.

Fig. 28 shows a plot of the standard enthalpy change (ΔH°_m) accompanying the reaction of oxyhaemoglobin with carbon monoxide against pH for the four haemoglobin species studied. Within the experimental error of $\pm$ 500 cal. mol^{-1} all the haemoglobins show the same pH independent ΔH° for the reaction. As mentioned above (page 147 of this thesis) large variations of ΔH° with pH would be expected if this reaction is accompanied by a shift of a proton

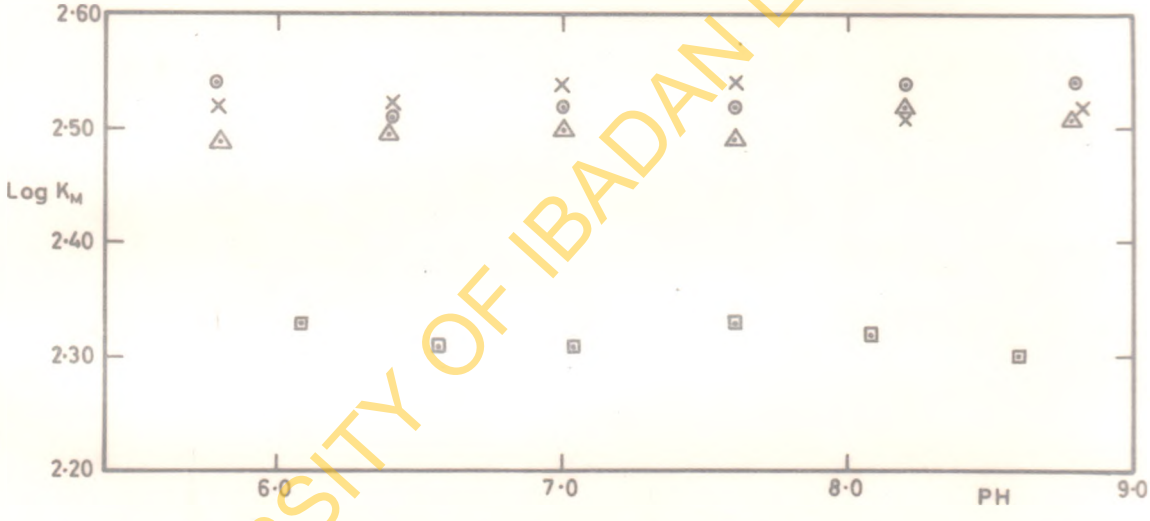


Fig. 27. PLOTS OF Log k_M AGAINST PH FOR THE REACTION OF CARBON MONOXIDE WITH OXYHAEMOGLOBIN A ⊙—⊙; PIGEON X—X; GUINEA PIG △—△ AND RABBIT ⊡—⊡ AT 20°C.

on the distal imidazole from the nitrogen atom on the surface of the molecule to the nitrogen atom which points towards the iron atom. Since there is no significant variation of ΔH° with pH, it follows that this reaction is not accompanied by this 'proton shift'. Hence we can presume that the hydrogen atom is either 'in' in both cases or "out" in both cases. The average value of ΔH°_{m} is -6.0 kcal mole^{-1}. Barcroft has shown that for human whole blood, lowering the temperature from 38° to 15°C increases K_m by ⅓ of its value. On the basis of this and correcting for the difference between the heat of solution of oxygen and that of carbon monoxide Barcroft's data give ΔH°_{m} of -4.3 kcal mol^{-1}, (Rossi Fanelli et. al. 1964) which is significantly different from -6.0 kcal mole^{-1} obtained in the present study. However, the value obtained in this work can be compared to the value as directly measured by Brown and Hill (1923) on sheep blood namely -6.6 kcal/mole and for ox blood, -5.9 kcal/mole.

Taking the average value of ΔH°_{m} as -6.0 kcal/mole and the standard free energy change (ΔG°_{m}) as -3.38 kcal/mole, it follows that the standard entropy change (ΔS°_{m}) for the reaction is -8.9 e.u. Since the partial molar entropies of oxygen and carbon monoxide are almost equal, this implies that the partial molar

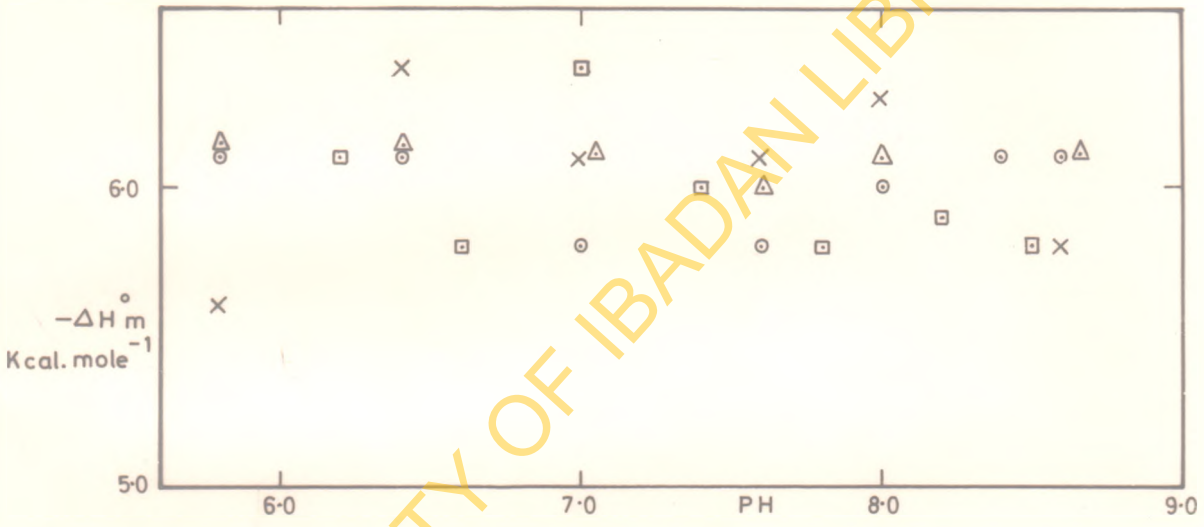


Fig. 28. PLOTS OF $-\Delta H^{\circ}_{m}$ AGAINST PH FOR THE REACTION OF CARBON MONOXIDE WITH OXYHAEMOGLOBIN ; ⊙ HUMAN A ; X PIGEON; △ GUINEA PIG; ⊡ RABBIT.

entropies of carbon monoxy-haemoglobin and oxyhaemoglobin differ by this amount. The negative entropy change suggests that the carbon monoxy-haemoglobin is more restricted than oxyhaemoglobin. This might probably mean that the oxyhaemoglobin has more degrees of freedom than the carbon monoxy haemoglobin.

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