

HAEMOGLOBIN

A Study of Its Stability in Stored Blood

by

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THESIS

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ABSTRACT

INTRODUCTION A Bursary of the National Research Council of Canada made this work possible.

PORPHYRINS

The Blood Bank of the Department of Biochemistry was the source of all blood samples.

IRON I am deeply indebted to Dr. O. F. Denstedt, who directed my research. He gave generously of both his time and advice, and our many discussions were invaluable.

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THE NATURE OF THE IRON AND HEME LINKAGES

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The Role of Carbon Dioxide

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Some Other Respiratory Pigments

COMBINATION OF HEMOGLOBIN WITH

METHAEMOGLOBIN

Cyanosis due to Sulfhemoglobin

Spontaneous Methaemoglobinemia

"Natural" Occurrence

ABSORPTION SPECTRA

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INTRODUCTION

The study presented in this thesis arose as a side-issue of a main investigation on the preservation of whole blood assigned to the Department of Biochemistry, by the National Research Council, shortly after the outbreak of war in 1939.

During the recent Spanish Civil War, the extensive use of stored blood in the treatment of wounded soldiers and civilians had demonstrated the practicability and the usefulness of stored blood in modern warfare. As a result, a widespread interest developed in many countries in the improvement of methods of preservation. The idea of the Blood Bank was introduced in the United States by Dr. B. Fantus in 1937, and within a short time the bank had become an established facility in many of the larger hospitals of North America.

When the second World War finally broke out there arose a sudden need, particularly in bombed areas, for large stores of blood. Much blood was collected in anticipation of the "blitzkrieg", but it could be kept only for a week or so and then had to be discarded. Since little research had been done up to the time on the improvement of storage methods, many groups including the one at McGill University, undertook an investigation of this subject. Rapid progress was made, and in the course of two or three years the average time of preservation has been extended to six weeks; nevertheless, most hospitals use as fresh blood as possible.

In the treatment of war casualties, the most important element of blood is the erythrocyte. Accordingly, in the preservation of blood attention is focussed mainly on the preservation

of the red cells. The fate of the immunological factors, the white cells, and other factors is of secondary importance. The red cell is the unit of transportation whereby oxygen is carried to the tissues. It is necessary to keep this unit intact, since free haemoglobin, liberated by hemolysis of the corpuscle, is rapidly removed from circulation. This rapid elimination greatly decreases the value of liberated haemoglobin, although it can carry oxygen.

The stability of haemoglobin in the red cell is of prime importance in considering the usefulness of stored blood, however; obviously there is little point in storing blood beyond the time at which haemoglobin deteriorates even if the erythrocyte does remain intact. Accordingly information was required concerning (1) haemoglobin deterioration in stored blood, (2) the length of time during which haemoglobin retains its normal oxygen-carrying properties, (3) time of survival of the erythrocyte.

That haemoglobin may be altered early in storage was suspected from the progressive darkening of the sedimented red cells. Within two or three weeks they assume a venous colour indicating that any oxyhaemoglobin originally present has been reduced. Whether the oxyhaemoglobin merely lost the oxygen or whether it was altered in the process of darkening was not clear. Since the blood could be oxygenated again quite easily in the presence of air, it was obvious that the oxygen-carrying function was retained. Any change that had occurred, had affected chiefly the rate at which oxygen could be taken up and given off.

The question resolved itself, therefore, into a study of the influence of storage on the rate of equilibration of haemoglobin with oxygen. Possible factors involved are (1) Alteration of haemoglobin itself, (2) Change in the electrolyte environment, due to an increased permeability of the red cell membrane, or a change in pH. (3) Change in the oxygen tension due to escape of the gas. (4) Inactivation of carbonic anhydrase.

Barkan and co-workers observed that a small part of the iron in red blood cells was split off more easily by dilute acids than that of the remainder (haemoglobin iron). Barkan and Walker had shown later that when erythrocytes are incubated at 37°C in plasma, the plasma bilirubin and iron increase slightly. They suggested that the source of the E.S.I. (Easily-Split-off Iron) and the bilirubin was a bile-pigment complex accompanying haemoglobin in the erythrocyte. Legge and Lemberg confirmed the finding regarding labile iron. They named the complex "choleglobin". The only difference between choleglobin and haemoglobin is that in the former the heme ring is split; the iron is still present but is now more easily detached by acid, leaving bile-pigment and globin. They also noted that not all the E.S.I. comes from choleglobin, and this finding confused the matter somewhat. However, the greater part of the E.S.I. does come from this haemoglobin-like compound, and by measuring the labile iron one can estimate the amount of choleglobin present. Choleglobin, presumably a product of haemoglobin breakdown, is present in a concentration of about 1% in the red cell, and E.S.I. is less than 2%.

Barkan et al had suggested that the greater part of the E.S.I. came from older cells. Red cells are always being produced

and destroyed with an average life of about 80 days. One might expect, therefore, that there may be a steady increase in E.S.I. in preserved blood with increased time of storage. The first part of the present investigation was confined to studying the E.S.I. (and therefore the choleglobin) content in stored blood. Choleglobin cannot be separated from haemoglobin and therefore its concentration was estimated by measuring the E.S.I.

Haemoglobin may be inactivated without the formation of choleglobin. To see whether the pigment was altered in some other way, haemoglobin determinations were carried out by two methods: (1) The Van Slyke oxygen-capacity method and (2) The photo-colorimetric method (Evelyn). Since one measures active haemoglobin and the other measures total pigment, any inactivation should be revealed by differences in the values of the two methods for the same blood sample.

The question of whether oxygen uptake and liberation by haemoglobin is altered during storage can be studied by determining the oxygen-dissociation curve by the van Slyke method at different intervals during storage. In this way it should be possible to detect any change in oxygen-carrying properties.

At the same time rates of hemolysis in various preserving solutions were followed, since free haemoglobin is more prone to inactivation than is haemoglobin in the corpuscle.

Since blood contains a mixture of young and old erythrocytes (some of the latter about to be discarded) one might expect that all the cells have not equal stability, and that perhaps the haemoglobin of older cells may not function as well as haemoglobin of new cells. Therefore it is important to know what

variations may be expected in the behaviour of bloods of different individuals.

Blood of older people may be less stable than that of young persons. Another question is whether the blood of diseased individuals is abnormal, even if they are not suffering from some blood dyscrasia. In the present study emphasis was placed on determining the stability of haemoglobin under these conditions.

THE CHEMISTRY OF HAEMOGLOBIN AND RELATED COMPOUNDS.

PORPHYRINS

Haemoglobin is a conjugated protein consisting of a prosthetic group (ferroheme) and the protein globin. Ferroheme consists of a porphyrin combined with iron, while the globin is usually described as a histone. For convenience the porphyrins will be described first, and a discussion of iron, globin and haemoglobin will follow.

The Porphyrins.- The porphyrins are coloured compounds which are found widespread in both plants and animals. The pigments are iron-free and contain the porphin nucleus which is made up of four pyrrole groups united by four methene bridges (Fig. 1).

All porphyrin derivatives, with or without iron, show a strong absorption band (the Soret band) in the region of the limit between the visible and ultra-violet spectrum (1). This band and the colour of the compounds are due to the planar conjugated system of double bonds in the porphyrin ring.

By substituting the hydrogens attached to C's 1-8 in the porphin nucleus by various groups and radicals, porphyrins are obtained. There are four artificial isomeric porphyrins in which these hydrogens are substituted by methyl and ethyl groups in equal number (2). These are the etioporphyrins I-IV and they are the reference compounds for all porphyrins (Fig. 2). They have all been synthesized, and their structure is accurately known. Of these four, types I and III are of importance since all the naturally-occurring porphyrins are derived from them. The four

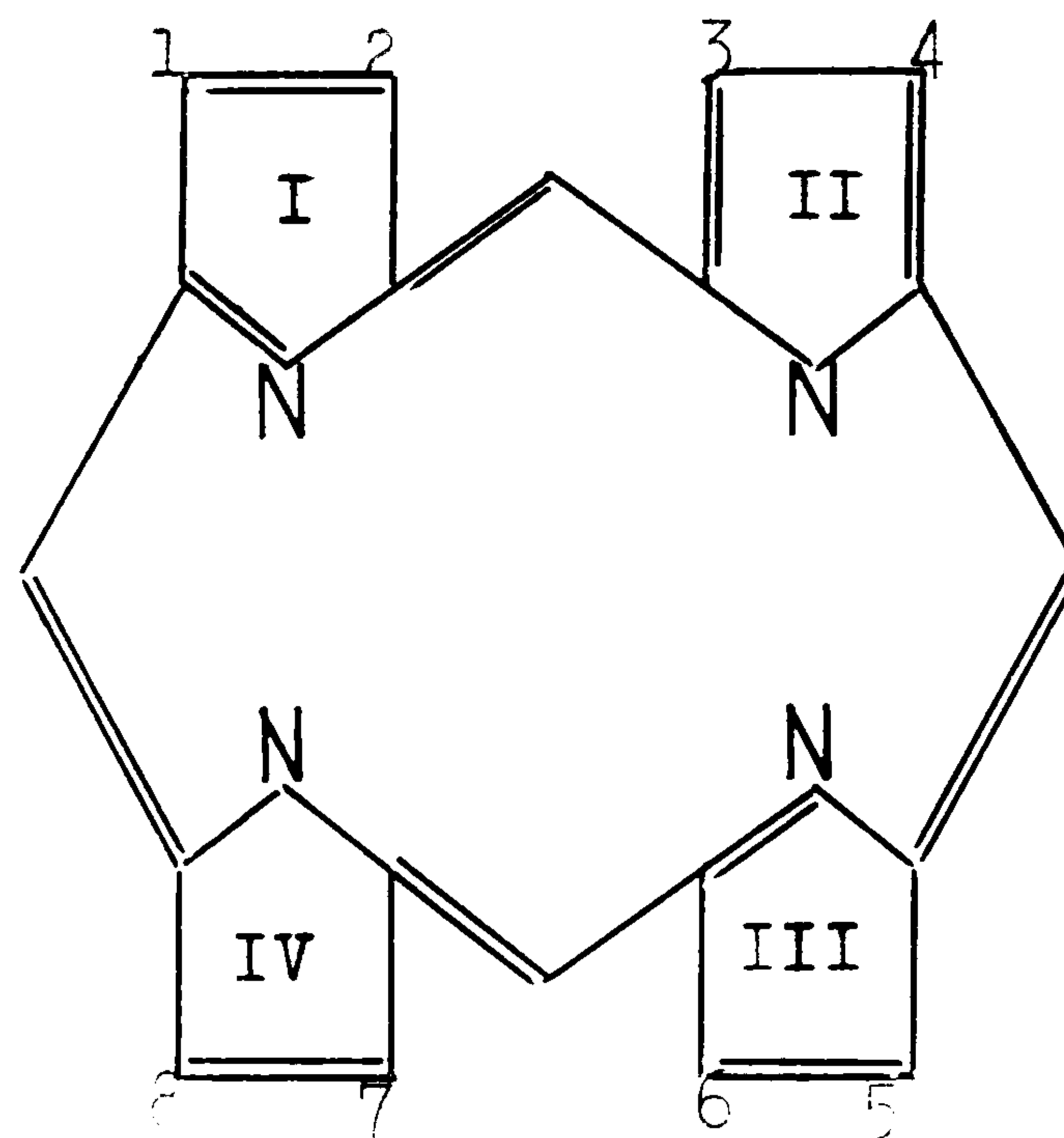
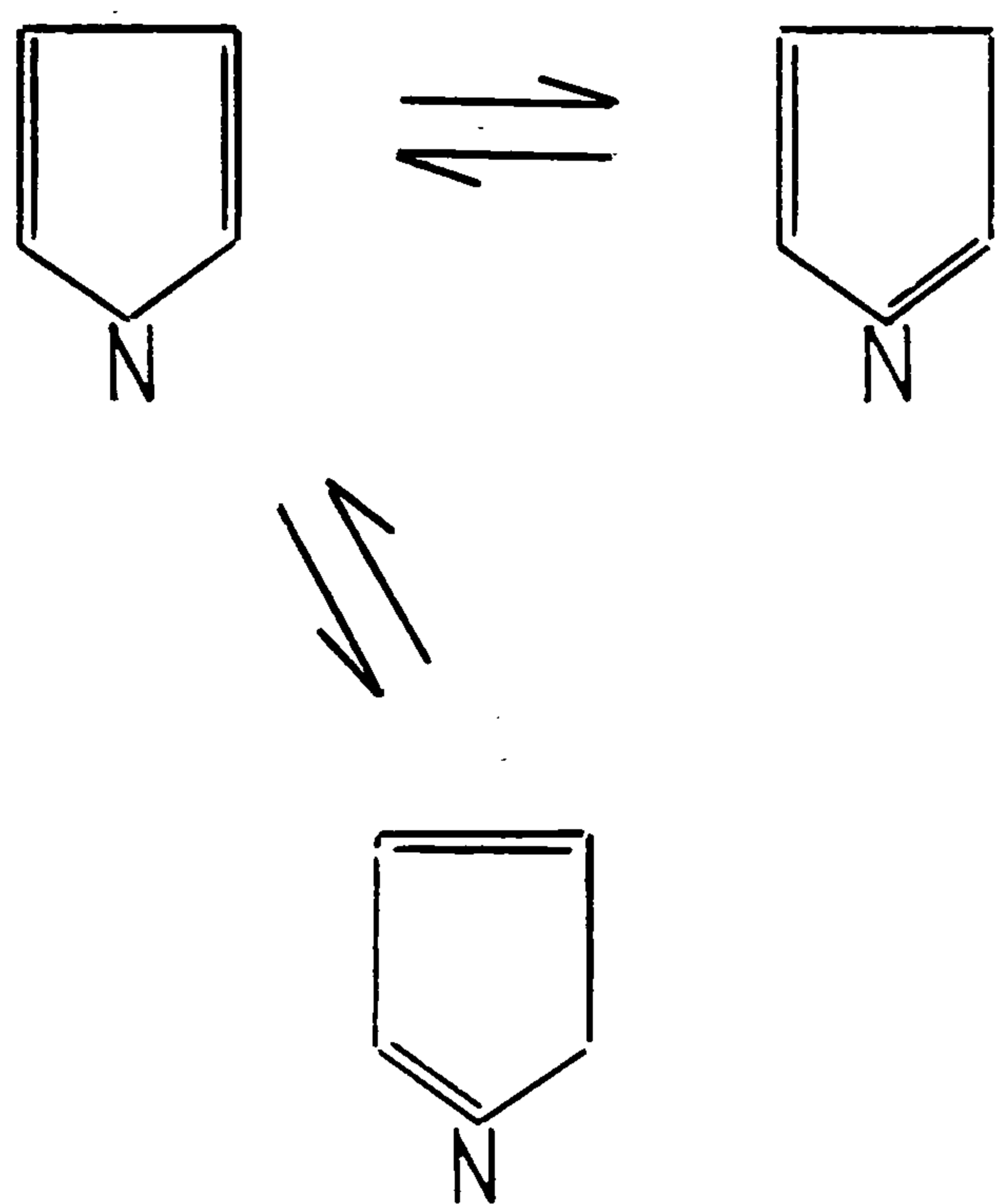
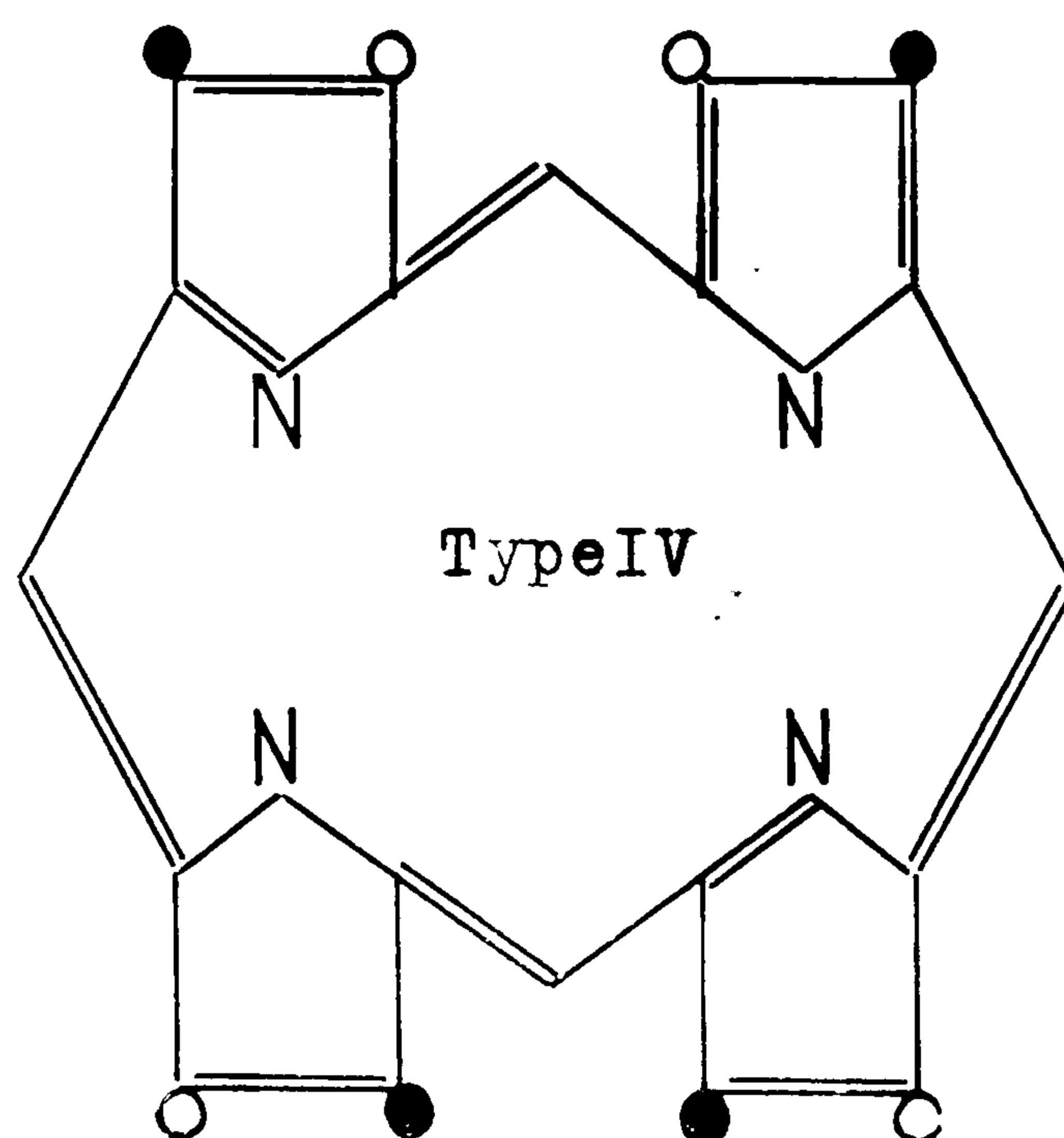
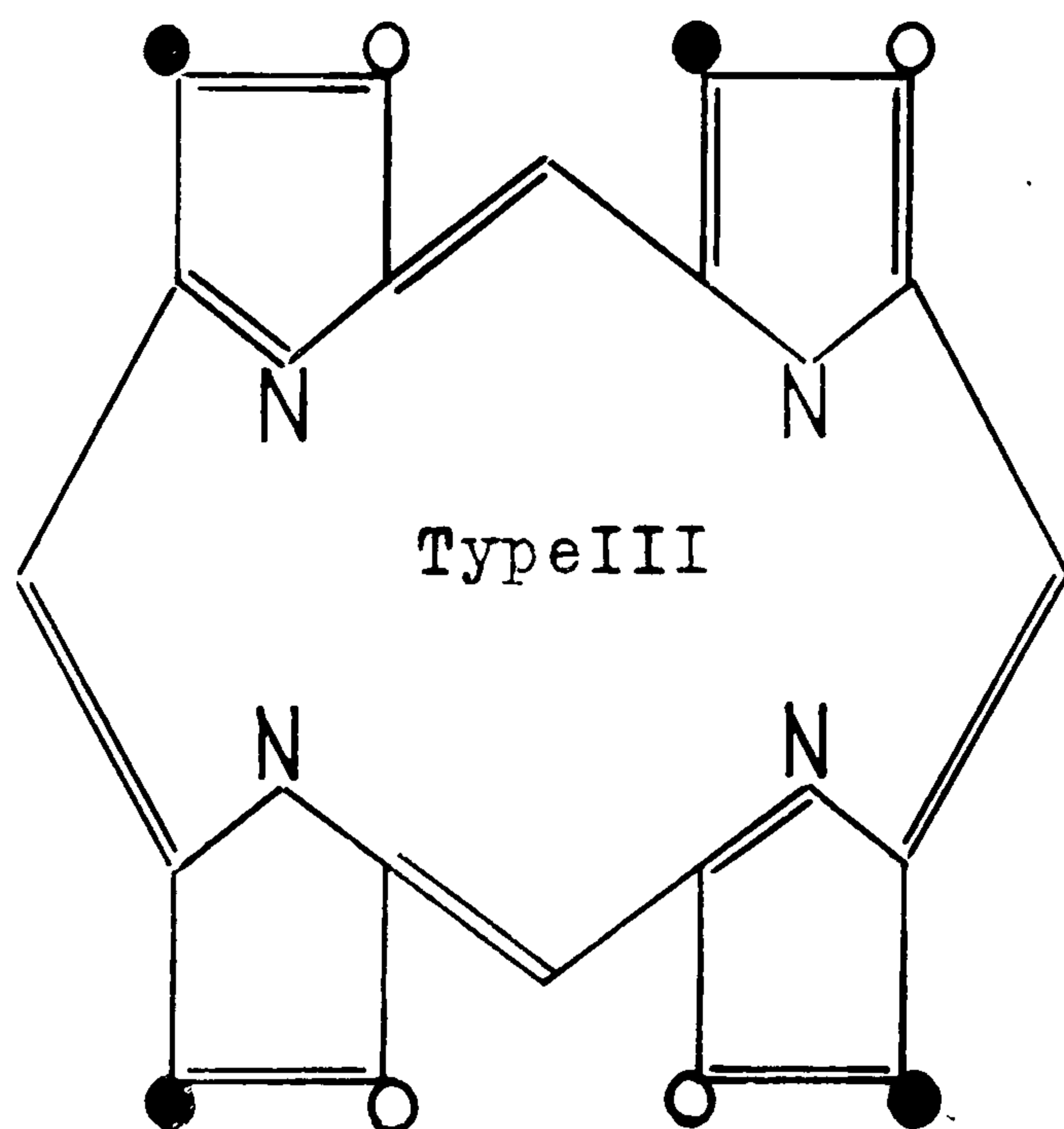
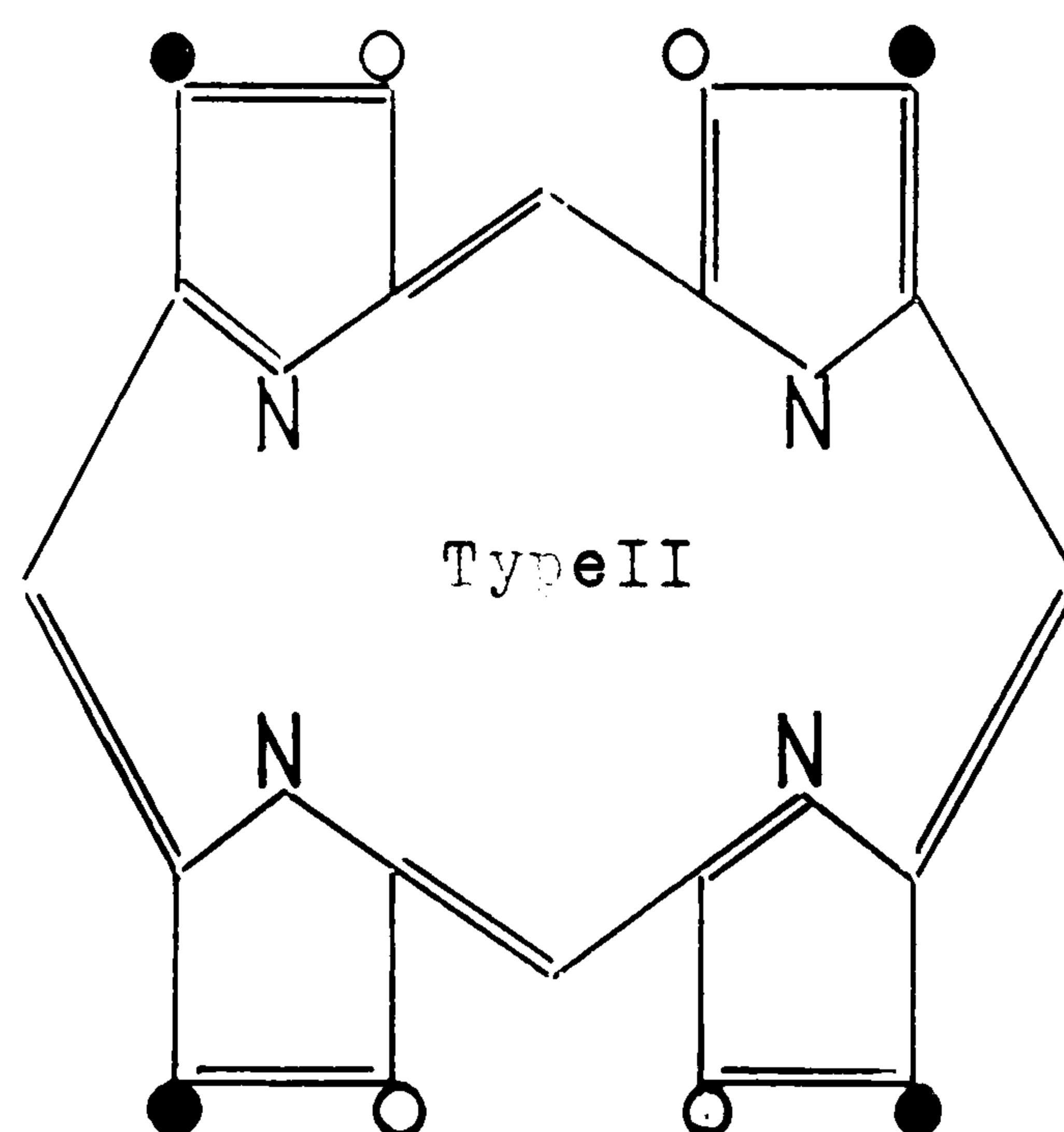
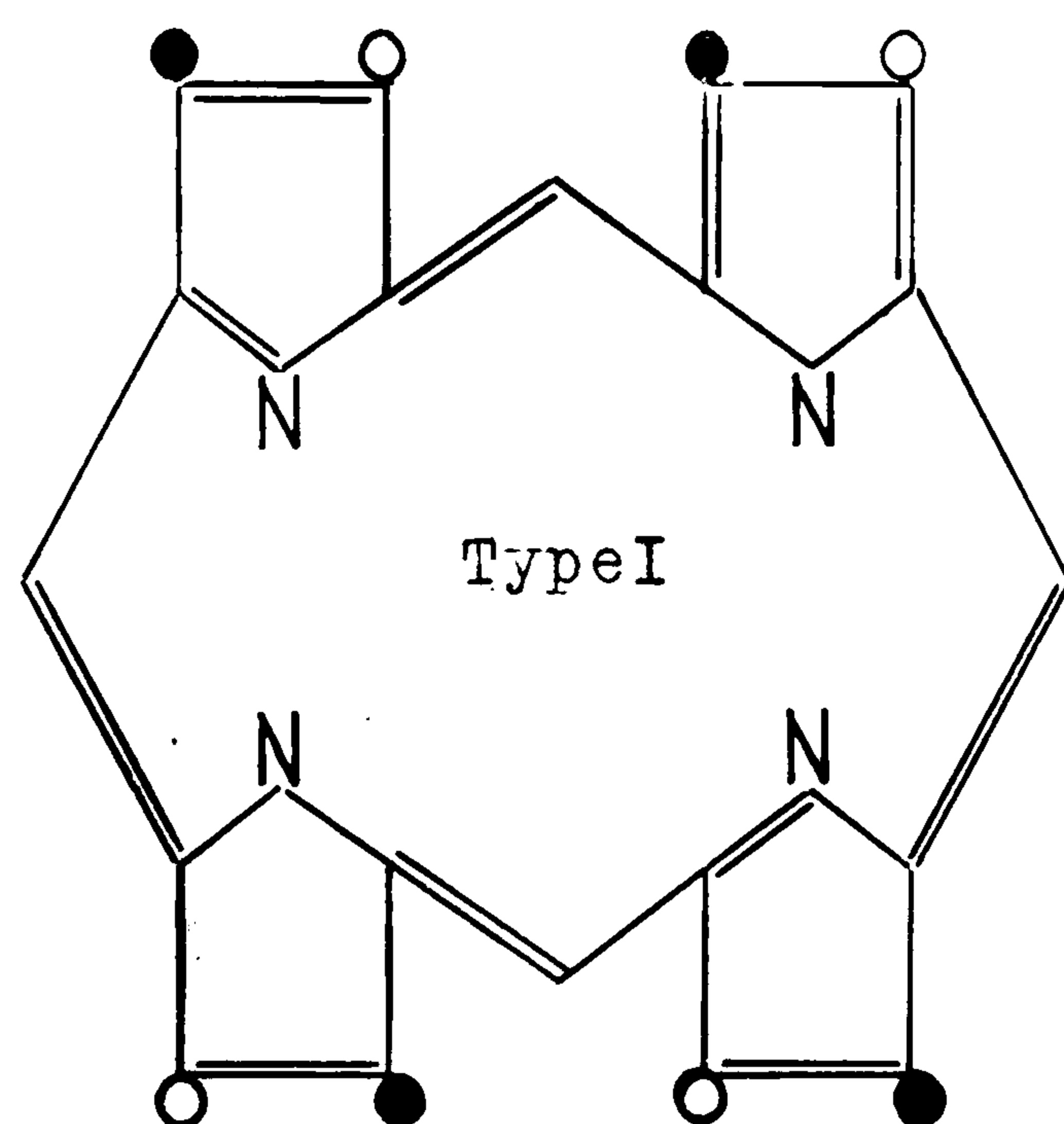


FIGURE 1 - The Pyrrole Rings

The Porphin Nucleus.



○ = C₂H₅

● = C H₃

FIGURE 2 - The Four Isomeric Etioporphyrins.

nuclei are numbered clockwise beginning in the upper left.

Haemoglobin, myoglobin, cytochrome, catalase, bilirubin, urobilin and chlorophyll are all derived from type III (3). The bile pigments do not have a ring nucleus like the porphyrins, but instead are made up of a chain of four pyrrole nuclei (Fig. 3). Chlorophyll contains magnesium and phytol while haemoglobin contains iron and globin.

The number of possible porphyrins and derivatives is very large, e.g., protoporphyrin and hematoporphyrin each have fifteen isomers. Protoporphyrin type III (Fig. 4) can be obtained from haemoglobin, and has been combined with iron and protein to form haemoglobin and myoglobin. Various writers disagree as to the exact location of the double bonds in the various types. In this discussion, the formulas of Hans Fischer will be followed.

Porphyrin Metabolism.-

The porphyrins are assumed to be synthesized from pyrromethenes (Fig. 4) each of which consists of two pyrrole groups connected by a methene bridge. These pyrromethenes condense to form types I and III porphyrins. Pyrromethenes have been synthesized and derived from the bile pigments (4). Simpler building units have not been found, nor is it known why only these two types are made. It has been suggested (5) that enzymes synthesize these particular types only, and are responsible also for the relative amounts of each. Fischer's theory of the "dualism of the porphyrins" is based on the observation that the types are not interconvertible in the laboratory. Probably there is an independent biological synthesis for each. This theory of

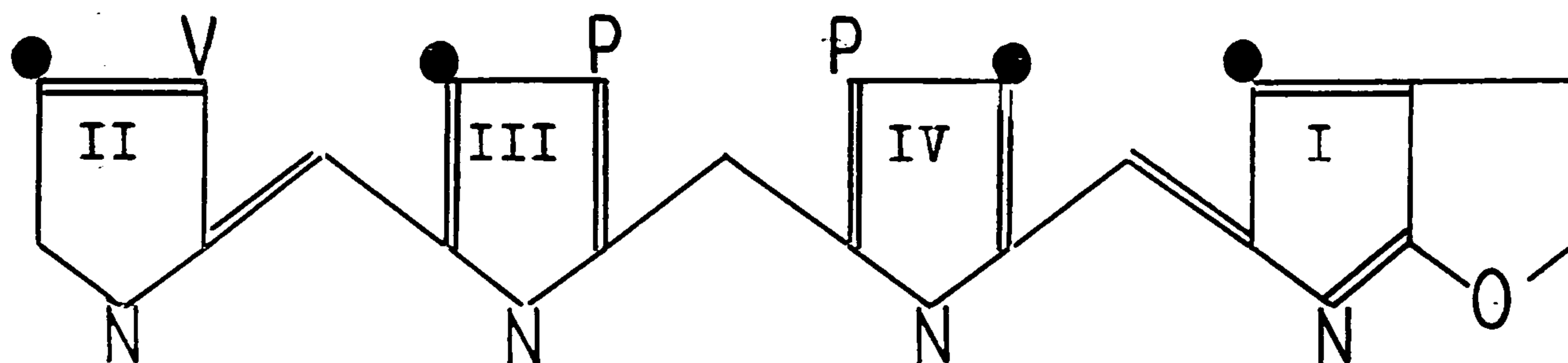


FIGURE 3 - BILIRUBIN.

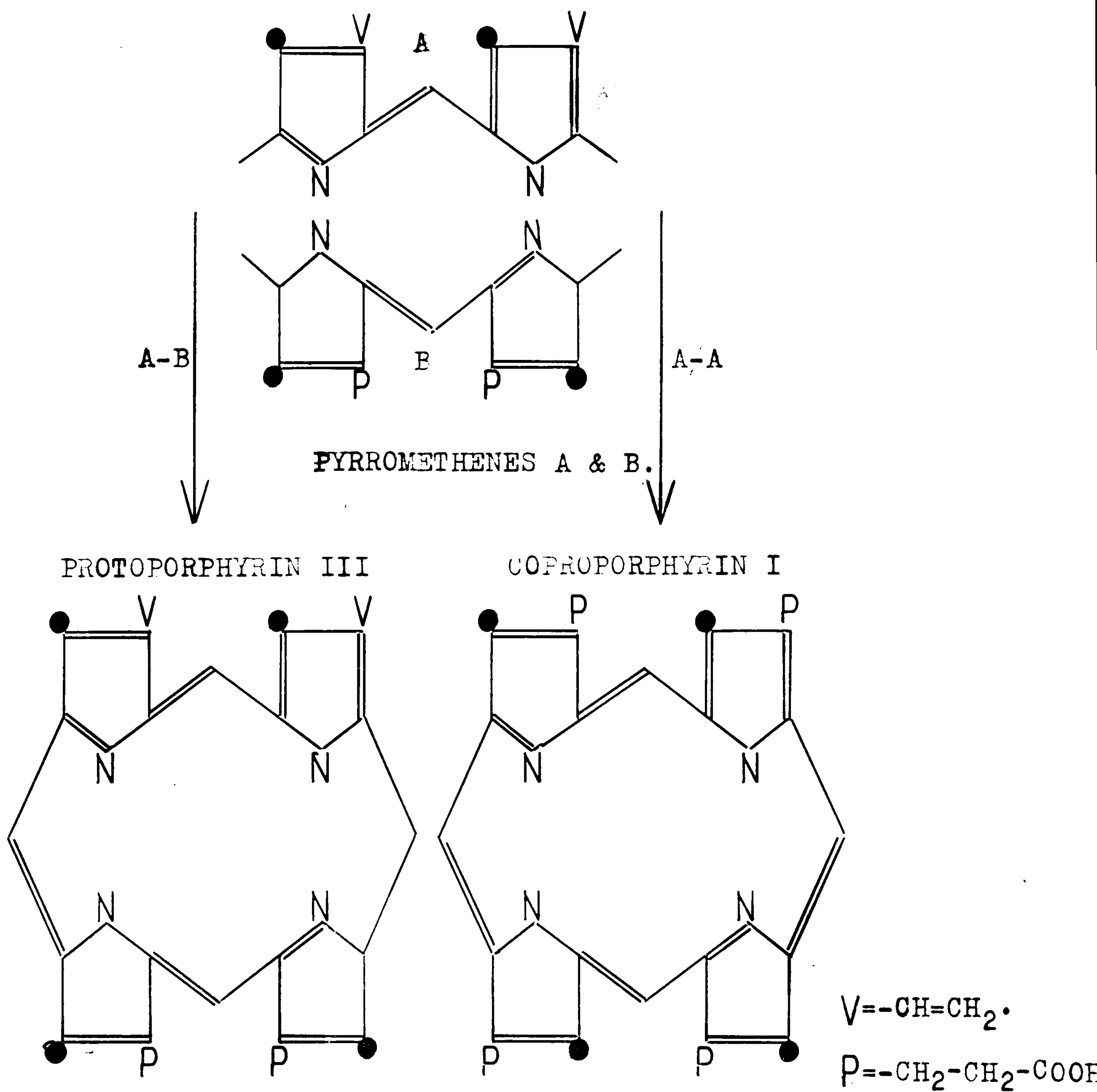


FIGURE 4 - Simultaneous Synthesis of Types I & III Porphyrins.

separate synthesis has been supported by the work of Rimington (5), Dobriner (6), Vigliani (7), and others who have used it to explain normal and abnormal excretion of both types of compounds.

Normally, coproporphyrin type I is excreted in the urine and bile, though as mentioned previously, haemoglobin etc. belong to type III. Watson (2) believes that type III coproporphyrin is also normally present but in much smaller amounts. The explanation is that the greater portion of synthesized porphyrin is type III, which is built into various compounds, including haemoglobin. The slight amount of type I is useless, and is excreted in the oxidized form of coproporphyrin.

In congenital porphyria, hemorrhage, and pernicious anemia, there is an abnormally great secretion of type I compounds (coproporphyrin and uroporphyrin). Congenital porphyria is a rare inborn error of metabolism possibly inherited as a Mendelian recessive character. Symptoms are (1) Excretion of much porphyrin - especially of uroporphyrin type I, the formation of which is limited to this disease. (2) Discoloration of the teeth and bones by impregnation with uroporphyrin. (3) Sensitivity of the skin to light in spring and summer. (4) Urine coloured Burgundy red.

The above conditions are associated with increased haemoglobin formation, and the normal ratio of I/III synthesis is believed to be altered so that excessive amounts of type I are formed.

Uroporphyrin I is found normally in the calcified parts of the bones of the fox-squirrel and stains them red. Combined with copper it forms turacin, a pigment in the feathers of the turaco,

a South African bird.

In acute porphyria and in lead (8), arsenic, mercury, and sulfanilamide (9) poisoning coproporphyrin III is found in large amounts. Acute porphyria is another metabolic disturbance, inherited as a Mendelian dominant. No light sensitivity occurs. Although this disease is actually chronic, it is termed "acute" because during the abdominal or paralytic attacks much porphyrin is excreted and the urine is red.

In these disturbances liver function is often disturbed (10) and since the porphyrins are of type III, deranged haemoglobin formation is involved. Possibly iron cannot enter the ring.

Porphyrin Compounds.-

Porphyrins unite with metals such as Fe, Co, Ni, Ag, Mn, Sn, and Zn to form metallo-porphyrins (11). According to Holden (12, 13) porphyrins can combine with proteins in the absence of metal, but for globin to show its specific effects, a metal is necessary. He found that globin changes the ultra-violet absorption spectra of protoporphyrin and of hematoporphyrin, while casein has no such effect. The differences observed indicate the formation of porphyrin-protein compounds analogous to haematin-protein compounds. Haurowitz & Waelisch (14) believe that only iron-porphyrins can combine with globin.

Porphyrins not only combine with proteins but also form adsorption compounds with them and with lipids (15). The existence of these complexes explains the phenomena of photosensitization, action on paramoecia, and transport in blood serum. Alexander (16) found that both protoporphyrin and hemin penetrate cholesterol and amine monolayers quite strongly.

Heme is iron combined with protoporphyrin III; this complex has been synthesized by Fischer & Zeile (17). Heme is the prosthetic group in haemoglobin, cytochrome, myoglobin, heliocorubin and actineohematin; all these are respiratory pigments. The exact nature of the iron linkage is not known.

Nomenclature:- Heme ($C_{34} H_{32} N_4 O_4 Fe$) can be oxidized to hematin ($C_{34} H_{32} N_4 O_4 Fe OH$). In this reaction the ferrous iron becomes ferric. Hemin ($C_{34} H_{32} N_4 O_4 Fe Cl$) is the hydrochloride of heme (18).

The properties of reduced or oxidized heme are greatly altered by combination with nitrogenous substances in the form of hemochromogens. These nitrogenous compounds include albumin, pyridine, nicotine, piperidine, hydrazine, cyanide, ammonia, glycine, and denatured globin.

Haemoglobin itself is oxidized quite slowly to methaemoglobin by molecular oxygen, as Brooks found (19). He measured the decrease in haemoglobin in a solution containing dissolved oxygen due to the formation of methaemoglobin. The rate of oxidation is directly proportional to a power of the hydrogen ion concentration. Heme, on the other hand, is very easily oxidized by minute traces of the gas (20).

Cyanide can combine with free reduced heme to form a hemochromogen containing one cyanide group per heme molecule, and then combine with this further just as cyanide or carbon monoxide combines with globin hemochromogen (21). Hogness et al (22) studied the reaction of ferriheme hydroxide (hematin) with cyanide. They concluded that the hematin was polymerized in solution, but could not find a structural explanation. Haurowitz (23) found that

hemin is easily destroyed by hydrogen peroxide with the formation of a di-hydroxy pyrromethene, "pentdyopent". Later (24) he claimed that hemin can also be destroyed by linoleic or linolenic acid in air at 38°C. The products of the reaction were not porphyrins, bile pigments, or pentdyopent. Though heme alone can destroy hydrogen peroxide, when it is combined as catalase, its activity is increased 10^7 times (25).

Herzog (26) believes that hematoprosthetin, not heme, is the natural blood pigment. The former contains one less carbon atom.

Heme in the free state has been found in wheat flour, oatmeal, and other unexpected sources. It is widely distributed throughout the animal and vegetable kingdoms. In the course of evolution, heme was developed before chlorophyll since it occurs in the most elemental forms of plant life in which chlorophyll does not exist. Respiratory pigments containing heme have been found in the lowest forms of plant life as well as in some invertebrate and all vertebrate animals.

The distribution of haemoglobin is much more restricted. It occurs in certain larvae, but not in the body fluids of any adult insect, and it is believed to be confined to the animal kingdom. The heme of all haemoglobins is the same; only the globins vary and these variations are responsible for differences in haemoglobins of different species (Page 22).

IRON

Iron is an essential constituent of plant and animal protoplasm. There is only about one part in 25,000 or one tenth of an ounce in the normal adult but it is of fundamental importance. The greater part of the iron is contained in haemoglobin but the remainder is of equal importance. This smaller portion is present in the cytochrome system, myoglobin, various enzymes containing heme such as catalase, and the chromatin substances which are concerned with the most essential cell activities (27); it is not decreased in anemia unlike haemoglobin and stored iron (28). Fischer and Hultzsich (29) mention the existence of masked iron in phosphoproteins and nucleic acid. This differed from inorganic dissociable iron and also from organically bound iron.

Blood makes up about 7% of the body weight and contains at least 70% of all the iron (27). It contains its iron in three chief forms, (I) haemoglobin iron, (II) plasma iron, (III) easily-split-off iron (E.S.I.).

The iron content of haemoglobin is about 0.335% for most animals. This gives a minimum molecular weight of 17,000 for haemoglobin, assuming one iron atom per haemoglobin molecule. Actually four iron atoms are present, as shown by Adair (30), who studied the osmotic pressure of haemoglobin, and by Svedberg (31) using the ultracentrifuge.

The level of iron in plasma is very low, 0.1 - 0.3 mgs.% and its source is probably the reticulo-endothelial system. According to Barkan (33) plasma iron is not ultrafiltrable but becomes so after incubation of the plasma with dilute hydrochloric

acid at 37°C. It is in the ferric form in vivo, and the hydrochloric acid treatment reduces it to the ferrous form which is ultrafiltrable. Tompsett (34) found that the dialysis of ferric iron, not of ferrous iron, was inhibited by phosphatides and phosphoproteins.

The plasma iron level can fluctuate widely unlike E.S.I. The latter is obtained from a haemoglobin-like compound in the red cell which may represent the beginning of haemoglobin breakdown. It yields its iron upon treatment with dilute acid, unlike haemoglobin (Page 60). The E.S.I. level is constant. Plasma iron is abnormally high when haemoglobin is being destroyed rapidly, as in congenital hemolytic jaundice, or when iron utilization is low, as in aplastic anemia or pernicious anemia (28). On the other hand, when iron utilization increases the plasma level falls, as it does in deficiency.

Some authors claim that E.S.I. is involved in iron transport and they consider plasma iron the medium of exchange, but this is doubtful (35). E.S.I. amounts to about 2% of the blood iron.

Radioactive iron has been employed by Whipple et al (36) to show that plasma is the means of transport of iron. Starkenstein (37) writes that the absorbed ferrous iron forms a ferric-globulin complex by oxidation with oxyhaemoglobin. The iron thus passes in an inert form to the site of hemopoiesis. In the cells concerned with manufacture of haemoglobin, the ferric iron is reduced to the ferrous form and by action of the cell nucleus is introduced into

the hemochromogen complex.

Daily iron requirements are 10-15 mgs. Females, as they mature, store more iron than males and also lose more. Requirements increase in pregnancy and lactation (28). Not all the iron in foods can be absorbed. More than half that in meat, liver, wheat and oats is utilizable, while less than a quarter of the iron in spinach is available. Sherman (27) states that fruit and vegetables, despite low nutritive value, are useful as sources of iron because they need not replace staple foods of high caloric and protein value. Fruits and vegetables may well be the most important sources of iron. The unavailable iron is usually in the form of heme. Such organically bound metal is not liberated by tryptic or peptic digestion and, since presumably only ferrous iron can be absorbed (34), heme iron is unavailable. Hydrochloric acid helps liberate iron and also converts it to the ferrous form. Accordingly, in achlorhydria iron absorption is impaired and ferrous salts have a specific effect. Milk contains very little of the element, only 1-2 mgs. per litre. To compensate for this, most animals are born with an extra supply of iron in the liver. It is used while milk is the chief or only food. An increased iron intake does not raise the milk iron level, and milk diets are used experimentally to produce anemia in rats.

Iron is absorbed best in the upper part of the small intestine. The amount absorbed depends upon the acidity of the medium, as was explained above, and upon the calcium content of the diet (34); the calcium facilitates absorption. Ferric iron is firmly bound to organic components of plasma and similar compounds may prevent absorption from the intestine.

Regardless of the degree of dosage it is never completely absorbed (38) and none is taken in when none is needed, e.g., when the body stores are full. The control of the amount of iron in the body is by regulation of absorption, not of excretion. For this reason iron is known as a one-way substance since only traces are excreted. McCance and Widdowson (39) found that large doses of iron could overrule this control. Taniguchi (40) gave inorganic iron continuously to his animals and found abnormal bone growth in the region of the epiphyses, and deformity of the joints. These symptoms were prevented by vitamins A and D. Iron absorption in bile-fistula dogs has been investigated by Smith and Crandall (41). The animals remained anemic without oral administration of bile, yet if an iron compound was given alone or added to the diet they absorbed it. Fat prevents the absorption of iron in the absence of bile due to the formation of insoluble iron soaps. The anemia was not considered to be secondary to iron deficiency however, but to lack of the erythrocyte maturation factor.

There is now available a radioactive isotope of iron which can be used to follow the element in the course of its absorption, transport, exchange, mobilization and excretion. This isotope is prepared by bombarding iron with deuterons and has a half-life of 47 days. Iron was known to be stored chiefly in the liver, also in the spleen and kidney. Austoni and Greenberg (42) employed the isotope and found that the specific accumulation/gm. rat tissue was greatest in bone-marrow, blood, spleen, liver and heart. These results agree with accepted views. According to Fontes and Thivolle (43) $\frac{4}{9}$ of the iron in dogs is combined in haemoglobin, $\frac{1}{9}$ in liver and spleen, and $\frac{4}{9}$ in other tissues.

When haemoglobin is broken down, its iron is not excreted. The traces appearing in urine and feces cannot account for anything like the amount liberated in the body each day. It is reutilized in the production of haemoglobin. Hahn, Bale, and Balfour (44) tagged red blood cells by feeding the iron isotope. When disintegration occurred, by ageing or trauma, the labelled iron from the haemoglobin was almost immediately reutilized by new cells, since the circulating radioactivity remained constant. Fontes and Thivolle (43) suggest that iron from haemoglobin enters the stores, the rest (from food) being used directly for formation of haemoglobin.

Cruz, Hahn, and Bale (45) using the isotope, destroyed labelled haemoglobin in vivo with pyrodin (acetyl-phenyl-hydrazine). The iron liberated was utilized "readily and nearly quantitatively" for the regeneration of haemoglobin, since the radioactivity remained constant during the period of blood destruction and recovery.

X Hahn et al believe that reserve iron stores are not used normally, instead the iron from broken down cells is utilized. The turnover of iron is quite rapid (38); traces appear in red blood cells 4 hours after feeding to the fasting anemic dog. The dog can turn out as haemoglobin nearly all the absorbed iron in two to three days, when red cell production has been accelerated by iron feeding and diet factors. The authors found later (46) that in vitro neither red blood cells nor free haemoglobin of anemic animals could absorb significant amounts of radioactive iron.

Kuhn et al (47) have found a ferric nucleoprotein of very high iron content in the spleen.

Hemosiderin.-

In hemochromatosis, a condition of abnormal iron metabolism, there is found a yellow iron-containing pigment known as hemosiderin. It is seen in the liver, spleen and other tissues; the Kupffer cells contain large amounts of it. Hemofuscin is a similar pigment found occasionally in connective and muscle tissue. In this disturbance, hemosiderin is not believed to be derived from haemoglobin, since there is no abnormal red cell breakdown. This substance is also found in chronic hemolytic states such as pernicious anemia where it probably does come from haemoglobin. The pigment is not a well-defined substance and may have more than one origin.

Markoff (48) mentions a case of hemochromatosis and pernicious anemia where there was considerable storage of hemosiderin, "particularly in the reticular cells of the sternal bone marrow". The iron content of the bone marrow was low, and erythropoiesis impaired; liver extract was without effect.

Lorenz and Menkin (49) injected Fe Cl_3 into monkeys and other animals and found "cytosiderin", the same pigment that occurs in hemochromatosis. It was not dissolved by H_2O_2 or KOH . They found the compound in pernicious anemia and chronic passive congestion of the lung as well, but the pigment in the spleen of a malaria patient was different. Since they produced it in a tissue-culture of lymph nodes, these authors rule out its origin as a product of haemoglobin breakdown.

Muir and Niven (50) injected homologous red blood cells subcutaneously into mice and rats. These cells were rapidly phagocytosed, and hemosiderin formed. Hematoidin crystals (bilirubin

probably) appeared on the seventh day. Similar results were obtained with haemoglobin solutions:- the formation of hemosiderin was diffuse first and then granular. Niven (51) used tissue cultures of mouse liver and spleen, and subcutaneous tissue. These contained macrophages which ingested red blood cells and often produced hematoidin. Isaacs (52) noted that hemosiderin is found in cells of the reticulo-endothelial system, especially after transfusions. Hemolysis is presumably the first step, but there is no definite evidence to prove it.

GLOBIN

Globin - the protein portion of haemoglobin makes up 94% of the weight of the molecule. It is usually described as a histone, but while histones usually contain arginine as the principal diamino acid, globin contains more histidine. Bauman (53) notes several differences between globin and histones:-

(1) The average content of arginine and histidine in eight histones was 24.8% and 4.1% respectively, and in globin 10.3% and 17.1%. (2) Histones treated with gastric juice break down to histopeptone and globin does not. (3) Globin is non-toxic unlike histones. (4) It is not a basic but a neutral protein more similar to the albumins than to the histones and (5) correspondingly native globin does not precipitate at its isoelectric point (pH 6.8) unlike histones.

The importance of histidine in the structure of globin is discussed by Bauer and Strauss (54). They suggest that the protein is made up of six units, each weighing about 11,600 and that the imidazole groups of the histidine link these units together. Wyman et al (55-57), employing various physical techniques (including (a) differential acid-base titration of ferrohaemoglobin and oxyhaemoglobin (58), and of oxyhaemoglobin and ferrihaemoglobin (55), (b) titration of oxyhaemoglobin over a pH range 4-10 by a thermal method (56) and (c) a study of the connection between the heat of oxygenation and base-binding groups (57)), concludes that the imidazole group of histidine is involved in the oxygenation equilibrium and in the union of globin and heme. This group was identified by various properties, e.g., (1) Its activity

in the pH range 5.5-8.5 (56). (2) The heat of oxygenation (related to the heat of dissociation of base-binding groups interacting with oxygen) which was similar to the heat of dissociation of the imidazole group (57). (3) The unchanging potential for ferrohaemoglobin - ferrihaemoglobin at pH 5-6 as measured by Taylor and Hastings (59).

Specificity.- Haemoglobin has the biological characteristics of adaptation and variation, and it differs from species to species (60). Since the prosthetic group of all haemoglobins is the same, variations in the protein are responsible for species-specificity. These variations are in amino acid composition and possibly in the manner of their intramolecular combination.

In analyses of three haemoglobins (horse, sheep and dog), Block (61) found the following ratio:-

Iron : arginine: histidine: lysine = 1:3:8:9. The same ratio was formed by Bergmann and Niemann (62) whose table appears below:-

Constituent	Horse	Sheep	Cattle	Dog
Iron	4	4	4	4
Cysteine	2	3	3	6
Non-Cysteine-Sulphur	6	12	-	6
Arginine	12	12	12	12
Histidine	32	32	32	32
Lysine	36	36	36	36

The numbers represent periodicity.

The amino acid content, especially in regard to cystine, shows slight differences. Beach and co-workers (63) state that the different blood globins show specific differences in total sulphur, cystine and methionine content. Differences in the molecular ratios of methionine to cystine are characteristic of species, according to Birkofer and Taurins (64).

Techniques used to demonstrate specificity among animal haemoglobins include crystallography(65), solubility tests(66), spectroscopic analysis(67), affinity for gases(68) and alkali denaturation(69). Haemoglobin was the first protein to be crystallized. Incidentally, crystallization of proteins is no criterion of purity unlike the case in inorganic compounds. Different globins were responsible, no doubt, for Reichert and Brown's discovery that oxyhaemoglobin crystals differ with the species of animal from which they are obtained. The ease of crystallization varies greatly as well, being pronounced in the case of rat haemoglobin, and much more difficult in human haemoglobin. Denaturation curves are quite different where spectral absorption curves are almost identical for the same haemoglobins, indicating again that the heme portions are all the same. The rate of denaturation varies with temperature, concentration of alkali and type of haemoglobin (69,70,71). Boor and Hektoen (72) confirmed the species-specificity of haemoglobin and its derivatives by studying the antigenic properties of carboxy haemoglobin. Closely related species - e.g., duck, chicken, and turkey, also beef and sheep - gave cross reactions.

Different haemoglobins in the same animal.- Bohr (1892) suggested that more than one haemoglobin may be present in the same animal. Geiger (73) claimed that he had illustrated the presence of two different oxyhaemoglobins in the blood of ox, sheep, ass, dog, and sometimes in man. The separation of the two forms occurred at the isoelectric point of haemoglobin with one form approaching the anode, and the other approaching the cathode at the same time. However, Dummet and Bowden (74) found that the observed effects

vanished if the solution of haemoglobin was passed through a filter-candle.

Brinkman et al (71) studied the alkaline denaturation of normal human haemoglobin, employing a sensitive photo-electric arrangement. The denaturation proceeded as two monomolecular reactions with entirely different velocity constants, suggesting the presence of two kinds of haemoglobin in human blood. In 1935 Brinkman and Jonxis (70) using the same method found three kinds of haemoglobin present at different times in man. The first occurred in foetal blood, the second seven months after birth, and the third three years after birth and was then the only one. They later introduced an improved method in which denatured haemoglobin is precipitated while unchanged haemoglobin remains in solution (75). Foetal haemoglobin of the cow and goat were less resistant than the adult form to the action of alkali; foetal haemoglobin of rabbit and man was more resistant, while no difference was found in the cat.

Hall (76), Elliot, Hall and Hugget (77), and Barcroft (78) suggest that differences between foetal and adult haemoglobin (in respect to oxygen-affinity) may be explained by the differences in sources of the pigment. In most animals, several organs form blood at various times. As the animal develops, hemopoiesis becomes more concentrated. The liver and spleen only make all the red cells by the middle of foetal life in man, and eventually the bone-marrow takes over production. Ramsey (69) suggests that the two types of haemoglobin which she found in man, rabbit, rat, pigeon and alligator, may be the products of two or more different hemopoietic sites.

THE NATURE OF THE IRON AND HEME LINKAGES IN HAEMOGLOBIN

Measurements of the magnetic properties of certain metal compounds, particularly those of iron, cobalt and nickel, determine whether the metal is bound by ionic or covalent linkages (79). Pauling (80) has measured the molal paramagnetic susceptibility of haemoglobin, hemin, cytochrome-c and their derivatives. From these measurements the number of unpaired or unbalanced electrons in the various compounds studied have been calculated, and the nature of the bond present has been deduced.

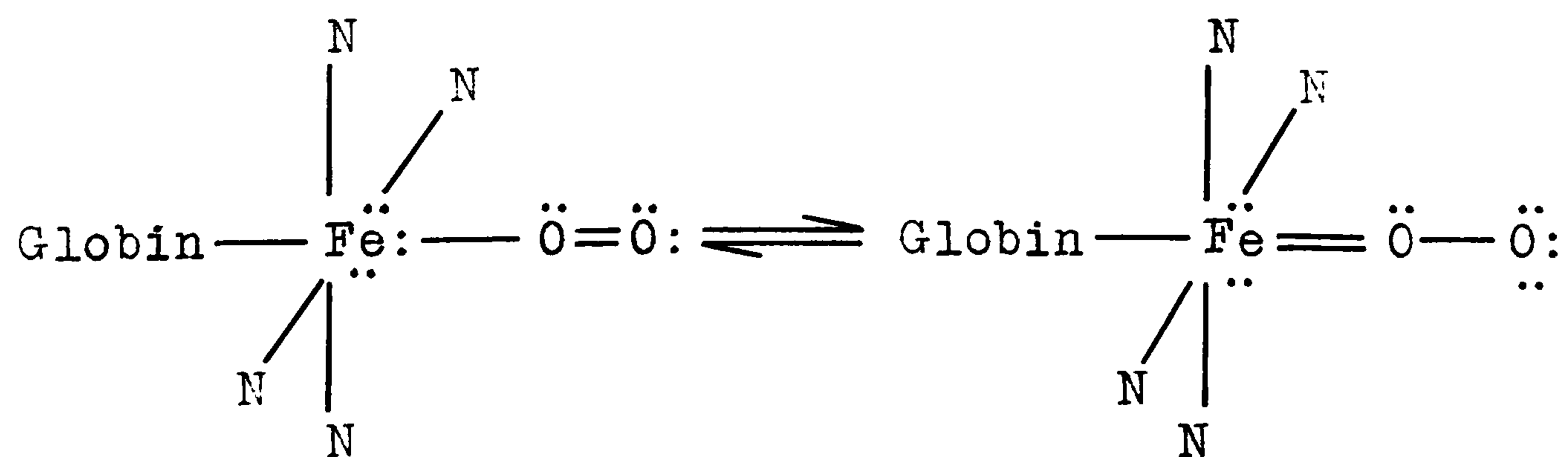
Theory.- When a magnetic field is applied to substances they may become (I) diamagnetic (repelled by the magnet) or (II) become diamagnetic but be inherently paramagnetic (attracted by the magnet). The latter group undergo a paramagnetic polarization which is associated with the presence of atoms, ions, or even molecules containing unpaired electrons. The magnetic field has an accelerating effect upon electrons in all substances, but in the absence of unpaired electrons a diamagnetic polarization results which has been useless in the study of chemical bonds.

The unbalanced permanent magnetic dipole moment responsible for paramagnetic polarization is made up of the spin magnetic moments of the unpaired electrons and the moments due to their orbital motion. These dipoles orient themselves with the magnetic field and the stability of the orientation depends upon the temperature.

The paramagnetic polarization is inversely proportional to the absolute temperature in ideal systems, i.e., where the interaction between dipoles is small. The paramagnetic contribution to the total magnetic susceptibility can be measured and from it

deductions drawn as to molecular structure. This contribution is due to unpaired electrons which orient themselves with the magnetic field. Ferric iron has five unpaired electrons in the 3d orbitals which give it the maximum possible magnetic moment (5.92 Bohr magnetons). Therefore complexes of iron which have similarly high moments are considered to be "essentially ionic" in nature. Ionic bonds between atoms or groups are electrostatic and result from Coulomb attraction of the excess electric charges of oppositely charged ions. The term "essentially ionic" implies that the bonds have not extreme ionic nature.

Pauling (81) found that reduced haemoglobin has four unpaired electrons per heme unit. The iron atom is present as the ferrous ion, and the bonds to the neighbouring atoms are essentially ionic. Oxyhaemoglobin and carboxy-haemoglobin have no unpaired electrons - that is, they are diamagnetic - and each iron atom is attached to the four porphyrin nitrogen atoms, the globin molecule and the oxygen (or carbon monoxide) molecule by covalent bonds. When haemoglobin takes up oxygen, the two unpaired electrons of the oxygen as well as the unpaired electrons of the iron atom disappear and thus, despite the lability of its attachment to haemoglobin, the oxygen molecule undergoes a profound change in electronic structure:-



Oxyhaemoglobin

It is surprising that the haemoglobin molecule undergoes such an extreme structural change on the addition of oxygen or carbon monoxide. The remarkable facility with which the bond types can change has been seen only in heme compounds and is no doubt partially responsible for their biological importance. The four heme groups in haemoglobin have magnetic moments independent of each other. Unlike the case in haemoglobin, the ferrous hemochromogens are diamagnetic - that is, the iron in them is linked by covalent bonds to the four porphyrin nitrogens, as well as to two nitrogens of the nitrogenous compound with which heme is combined.

Pauling suggested that the four heme groups lie at the corners of a square. This is supported by Bernal, Fankuchen and Perutz (82) whose X-ray data showed that the four heme groups in methaemoglobin are arranged in pairs related by a twofold axis. Such parallelism suggests that they lie at the corners of a square. In horse oxyhaemoglobin their arrangement appears to be the same as in methaemoglobin. According to Haurowitz (83), reduced horse haemoglobin crystallizes in the form of hexagonal plates which on contact with oxygen change spontaneously into the monoclinic needles of oxyhaemoglobin. This change of symmetry indicates that a rearrangement of the heme groups may take place on reduction. Perutz (84) investigated the absorption spectrum of single haemoglobin crystals in polarized light and concluded that methaemoglobin and oxyhaemoglobin have four parallel heme groups.

Ross (85) found that carboxy-haemoglobin is more resistant to digestion by pancreatic enzymes than oxyhaemoglobin. The rôle of the four hemes in holding the molecule together was shown by a greatly retarded initial attack on haemoglobin as compared to that

on globin. Carbon monoxide therefore strengthens the union of heme and globin, and reduces the availability of linkages to enzymes. In carboxy-haemoglobin most of the protein was digested away, leaving the heme associated with the amino acid residues to which it was originally combined. In a later paper (86), Ross states that the stabilizing influence which carbon monoxide may have is insufficient to prevent breakdown of that linkage in pancreatic hydrolysis, and found the following order for the rates of digestion:- globin, methaemoglobin, oxyhaemoglobin, haemoglobin, carboxy-haemoglobin (the first being attacked to the greatest extent). Since globin weighs half as much as haemoglobin, therefore for the same weight more area is available for digestion.

If globin were a hemispherical body, two heme groups, placed diametrically opposite on the semi-planar surface, would enable union between two such halves to give rise to a haemoglobin molecule in which four hemes are located in the central plane of a square (Pauling). The two globin halves might then be held together both by the two co-ordinate valencies of the heme iron not saturated by porphyrin nitrogen and by a second kind of linkage, possibly hydrogen bonds, still active when the first type is occupied with a foreign molecule such as oxygen. Reduced haemoglobin, with both kinds in force, would be more resistant to breakdown than oxyhaemoglobin (86). The linkage of heme to globin is not through the unsaturated side chains of the heme (18).

The heme molecule is flat and this flatness is involved in the heme-protein linkage. The large plate of heme, when

united to a protein by one valence, partly covers the surface of the protein molecule - e.g., haemoglobin, catalase and peroxidase none of which can be reduced to a hemochromogen unless the protein part is denatured. When this type of linkage occurs, one place of co-ordination is available to oxygen (in haemoglobin) or to hydrogen peroxide (in catalase and peroxidase).

In the hemochromogens, on the other hand, heme is bound by two valencies and is inserted in crevices which can be produced by denaturing the protein.

THE REACTION OF HAEMOGLOBIN WITH OXYGEN

Haemoglobin can combine with oxygen - two atoms of oxygen for each iron atom - to form the labile compound oxyhaemoglobin. This reaction is not a true oxidation since the iron remains ferrous, but rather an "oxygenation". Neither heme alone nor denatured haemoglobin can be oxygenated, though both can be oxidized. This illustrates the importance of globin though the reaction occurs in the prosthetic group. The oxygenation is reversible and the relative amounts of oxyhaemoglobin and reduced haemoglobin depend on the oxygen tension as well as on the pH, temperature, and salt concentration (84,85). Hartridge and Roughton (86) showed that only a fraction of a second is needed for the actual combination. Roughton (87) demonstrated that the oxygen uptake by haemoglobin in the corpuscle is much slower than by haemoglobin in solution, due chiefly to time for diffusion within the corpuscle.

One gram of haemoglobin can combine with 1.34 cc. oxygen at S.T.P. and the total amount of blood in the body will hold about 1200 cc. The iron content of the pigment is 0.336%, which gives a minimum molecular weight of 16-17,000. Molecular weight determinations show, however, that the intact molecule is made up of four equal fragments, the total weight approximating 68,000.

The Oxygen-Dissociation Curve.- By determining the volume of oxygen combined with haemoglobin at various partial pressures of oxygen, an "S"-shaped oxygen-dissociation curve is obtained. Oxyhaemoglobin is more acidic than haemoglobin, and therefore

increasing the pH increases the affinity for oxygen. Adair (88) tried to explain the "S"-shape by assuming that haemoglobin combined with oxygen in steps to form Hb_4O_2 , Hb_4O_4 , Hb_4O_6 and Hb_4O_8 . Ferry and Green (89) obtained accurate and extensive data on oxygen equilibrium for horse haemoglobin in phosphate and borate buffers. They used a four-constant equation corresponding to the conception that four oxygen mols are added successively with different equilibrium constants. Conant and McGrew (90) found that as oxyhaemoglobin was deoxygenated, the fully oxygenated form did not disappear, as it should if haemoglobin were forming intermediates. They suggested that if intermediate oxidation products were formed, they were present only in very small quantities.

Pauling (81) derived a two-constant equation for this equilibrium. Coryell, Pauling and Dodson in 1939 (91) wrote that the theory of intermediate compounds gave the only explanation of the experimental data that was compatible with the degree of aggregation (Hb_4) of haemoglobin. The oxygenation of haemoglobin (as well as the reactions with CO and NO) involved a considerable change in structure which was accompanied by noticeable changes in magnetic properties. The concentration of various intermediates in the oxygenation reaction was calculated as a function of the degree of reaction, and their results could explain the sigmoid oxygen-saturation curve.

Holden and Hicks (92) investigated the absorption of ultra-violet radiation by haemoglobin and derivatives. An intense absorption band in the region of 4,000 Å is shown by haemoglobin, methaemoglobin, oxy- and carboxy-haemoglobin, and most strongly

by hemin in an organic solvent. The addition of a gaseous compound leads to the appearance of two well-defined bands at 3400 Å and 2500 Å, the exact positions differing for the two gases (oxygen and carbon monoxide). The intense main band is present only when the pigment molecules are widely separated or when all the valencies of the iron are utilized.

According to Forbes and Roughton (93) dilute haemoglobin gives an oxygen-dissociation curve like that of blood. Therefore the "S"-shape is due to haemoglobin itself, not to its high concentration in blood. They suggest that the four iron atoms have not the same affinity for oxygen.

Barcroft (94) observed that in foetal haemoglobin, the oxygen-dissociation curve is shifted (to the left) so that it takes up oxygen more readily from maternal blood (in which the oxygen-dissociation curve shifts to the right). The former effect is due to a different haemoglobin, and this has been confirmed by Trought (95) and others. Such an arrangement is of obvious benefit to the foetus.

Hill and Wolvekamp (96) prepared dissociation curves for diluted haemoglobins from different animals. Different variations were shown from the respective corpuscles, but dilution always results in a greater affinity for oxygen. Christensen and Dill (97) reported that the dissociation curve of bird blood is similar to that of human blood, but that it has a lower affinity for oxygen. The product of hemolysis of bird erythrocytes with water is not homogeneous and the supernatant solution (after centrifuging) has much less affinity for oxygen than whole blood at the same pH. Amphibian blood was studied by McCutcheon

and Hall (98). They concluded that each of the several hemopoietic organs produces a different haemoglobin which results in a progressive change in the haemoglobin composition of the blood, as shown by oxygen-dissociation curves. The oxygen-affinity depends on other factors as well - e.g., respiratory mechanism, limitations of environment and metabolic activity.

The Rôle of Carbon Dioxide.- As an acid (HCO_3^-) carbon dioxide helps liberate oxygen from oxyhaemoglobin. It also combines with haemoglobin to form a carbamino compound, carbhaemoglobin, which further reduces the capacity of haemoglobin to form oxyhaemoglobin (99). Ferguson and Roughton (100) observed that carbon dioxide combines directly with haemoglobin at pH 7 and above. They believed that unchanged amino groups were involved. In 1937 Stadie and O'Brien (101) found that little of the carbamino form existed at the isoelectric point of haemoglobin, but it increased as the available base increased. Carbonic anhydrase (102,103) present within the red cell, accelerates the breakdown of bicarbonate and enables carbon dioxide to leave the blood quickly when the latter is oxygenated. While haemoglobin may carry both oxygen and carbon dioxide the amount carried by carbamino compounds is less than 1% of the total carbon dioxide transported in the blood.

Myoglobin - is a red respiratory pigment found in striated muscle (and heart muscle). It has been isolated in crystalline form by Theorell. Its isoelectric point is at pH 6.99 and its absorption spectrum differs from that of haemoglobin.

Roche and Vieil (104) found the molecular weight of horse myoglobin to be 16,850, and therefore it is a "monomer haemoglobin" containing only one iron atom per molecule. The prosthetic group is heme but the protein portion differs slightly from globin. A comparison of the content of various amino acids in horse haemoglobin and myoglobin shows little difference (105). Similarity is further suggested by the same spreading properties of their monolayer films (106).

Hill (107) found that sheep, dog, and goose myoglobin gave hyperbolic oxygen-dissociation curves which indicated a greater affinity for oxygen than that shown by haemoglobin. Though it combines faster with oxygen, it releases the gas just as easily as does haemoglobin. It can therefore remove oxygen from blood and create an oxygen reserve. The pigment shows less affinity for carbon monoxide than does haemoglobin but is more unstable, forming the met- derivative easily. Its oxygen-affinity is unaffected by pH.

Myoglobin has been found recently in the urine of victims of crush injury (108). After the weight is removed from the individual, shock develops and may prove fatal. The shock is accompanied by a mild haemoglobinuria, and in such cases death results from renal failure.

Some other Respiratory Pigments.- In the molluscs and crustaceans, the blue copper-containing hemocyanins carry oxygen. These molecules are very large - e.g. the weight of undissociated hemocyanin (*Helix pomatia*) is in the neighbourhood of 6.6-8.9 millions (109). They are aggregates of smaller units bound together by relatively weak forces.

Millikan (110) investigated the reaction of oxygen with hemocyanin; it occurs with the same order of speed as that with haemoglobin.. The oxygen-dissociation curve is monomolecular independently of the shape of the equilibrium dissociation curve.

Chlorocruorin, a green oxygen-carrier in polychaete worms, also has a molecular weight of the order of millions (111). The active group here resembles haemoglobin rather than hemocyanin since the characteristic element is iron, not copper.

The hemoerythrins (from *Supunculus nudus*, *Phascolosoma vulgare* and *P. elongatum*) all contain the same prosthetic group, according to Florkin (112). These differ from the heme pigments in (i) spectrographic properties, (ii) no combination with carbon monoxide, (iii) oxygen/iron ratio and (iv) no porphyrin structure. There is little evidence to show any relationship to hemocyanins.

COMBINATION OF HAEMOGLOBIN WITH OTHER GASES

Carbon monoxide easily displaces oxygen from oxyhaemoglobin to form carboxy-haemoglobin, one molecule of carbon monoxide combining with one iron atom. The reaction is reversible but the affinity of haemoglobin for carbon monoxide is 200-250 times that for O_2 -ie, 0.1% carbon monoxide in air, resulting in a carbon monoxide/oxygen ratio of 1/210, will cause half the haemoglobin to combine with each. This explains the danger of exposure to small amounts of carbon monoxide. Though the carboxy-haemoglobin compound can dissociate, the rate is quite slow, even in an atmosphere of pure oxygen. Artificial respiration and the use of 95% oxygen and 5% carbon dioxide are valuable, nevertheless, in treating carbon monoxide poisoning. Both the venous and arterial blood become cherry-red when carbon monoxide poisoning occurs. Obviously no cyanosis is present and the cheeks and lips of the victim become bright red.

Death results because no oxygen is available to the tissues. The carbon monoxide not only decreases the amount of functional haemoglobin but, as carboxy-haemoglobin, depresses considerably the dissociation of any oxyhaemoglobin present. Ordinarily a person with 40% oxyhaemoglobin and 60% haemoglobin would not suffer at rest. But if he had 40% oxyhaemoglobin and 60% carboxy-haemoglobin, no oxygen could be liberated from the remaining oxyhaemoglobin nor could haemoglobin take up oxygen.

Haemoglobin combines with hydrogen sulphide to form sulphaemoglobin, which is chocolate-coloured. Drugs such as acetanilid and phenacetin supposedly sensitize haemoglobin so that it combines

more readily with the gas which is absorbed from the intestine. The complex is not crystallizable, and requires oxygen for its formation. There is some controversy regarding the prosthetic group of this compound. Barkan and Schales (113) suggest that an open-ring structure is present. Their evidence is (i) the green colour of haemoglobin solutions treated with hydrogen sulphide, (ii) the absorption band present in the red and (iii) the increase in E.S.I. The mechanism of sulfhaemoglobin formation is similar to bile-pigment-haemoglobin formation and since oxygen is involved, peroxide probably forms as an intermediate (113,114).

On the other hand, Lemberg and co-workers (115) do not think that sulfhaemoglobin has an open ring. Sulfhaemoglobin-haemoglobin mixtures treated with alkali or pyridine give spectra typical of reduced globin-hemochromogen or pyridine ferroprotoporphyrin (116,117), and therefore not of chole- or verde-hemochromogens with bands in the red. Haurowitz (118) demonstrated that sulfhaemoglobin does not lose iron on treatment with dilute acid and concludes that the porphyrin rings remain intact.

METHAEMOGLOBIN

Mild oxidizing agents convert haemoglobin into methaemoglobin quite easily. The ferrous iron of heme is oxidized to the ferric form and the colour of the compound changes from red to chocolate-brown. This electromotively active (reversible) reaction is quite unlike the oxygenation of haemoglobin, where the iron remains ferrous. Methaemoglobin is sometimes written $HbOH$, also HbO . Crystalline methaemoglobin is prepared by adding ferricyanide to oxyhaemoglobin, dialysing, and then adding amm. sulphate or phosphate buffer (21).

Methaemoglobin, unlike haemoglobin, does not combine with oxygen or carbon monoxide. It does combine with cyanide, however, to form cyanmethaemoglobin. Accordingly, methaemoglobin-producing drugs are given in cyanide poisoning. Such treatment is effective if used soon enough. Methaemoglobin is produced in vivo by methylene blue, which is therefore frequently used in the treatment of cyanide-poisoning.

The list of oxidizing agents includes ozone, pot. permanganate, pot. ferricyanide, and such compounds as nitrites, chlorates, sulphates, acetanilid, bismuth subnitrate and nitrobenzene. These chemicals, by inactivating haemoglobin, cause "toxic cyanosis". The general reaction is: reduced haemoglobin oxidized to methaemoglobin - e.g., ferricyanide acts by displacing the oxygen in oxyhaemoglobin and oxidizing the reduced haemoglobin. Nitrites behave differently. Nitric oxide-haemoglobin is a ferrous compound (120) and nitrite-methaemoglobin is not simple methaemoglobin (121). The nitrite can combine with the

ferric iron and diminish its oxidizing power, besides reacting with and denaturing the globin of haemoglobin derivatives (122).

Sulfanilamide administration often causes a cyanosis which may be due to (1) a change in the haemoglobin, or (2) according to some authorities, staining of the red cells with some metabolic product of the drug.

Since the sulfonamide drugs are aniline derivatives, they could cause cyanosis by the formation of sulfhaemoglobin and methaemoglobin. Rimington and Hemmings (123) find no relation between therapeutic efficiency of the drugs and porphyrinuric action. All compounds which caused porphyrinuria also produced methaemoglobin in vivo. These workers consider the presence of a free or potentially free amino group attached to an aromatic nucleus to be important. This group may possibly be oxidized to a hydroxylamine derivative, or converted to a reversibly oxidizing system (p. or o-iminoquinone).

Various workers have shown that methaemoglobin is often present (124-132), as is sulfhaemoglobin in the blood of patients receiving sulfanilamide (124-126, 129-133). Fox and Cline (134) used a Hardy recording spectrophotometer to demonstrate methaemoglobin and sulfhaemoglobin in these cases (206). Wendel (131, 132) reported the presence of methaemoglobin in every blood specimen containing more than 4 mgs. sulfanilamide/100 cc. and he also found that the functionally active haemoglobin was often reduced 15-30%. Hartmann, Perley and Barnett (127) demonstrated the presence of methaemoglobin in every case of toxic cyanosis which they examined. They confirmed Wendel's observation that methylene blue given intravenously reduced methaemoglobin to

haemoglobin and caused the cyanosis to disappear. However, these abnormal blood pigments do not invariably form, and when they do there is often little correlation between the concentration of these pigments and the degree of cyanosis (128, 130, 134, 135). For this reason the formation of coloured derivatives has been postulated (130, 132, 136, 137). Sulfanilamide cannot act directly on haemoglobin, but, after being exposed to various tissues, it forms some compound which could produce methaemoglobin. It is rather difficult to oxidize corpuscular haemoglobin to methaemoglobin in vivo. Generally hemolysis precedes the oxidation.

Spontaneous methaemoglobin formation.-

Conditions favouring the spontaneous formation of methaemoglobin in haemoglobin solutions are those in which the haemoglobin is partly reduced and partly oxygenated (137). Neill (138) found that oxygen was necessary for its spontaneous formation in blood or haemoglobin solutions at room temperature and at 55°. Methaemoglobin was so rapidly produced in sterile blood in vitro that some mechanism must inhibit it in vivo - e.g. the reducing power of the tissues. Brooks (139) noted that glucose prevents methaemoglobin formation.

Methaemoglobin can be reduced by ascorbic acid in vitro (140). This reaction occurs at 0° and at 25-30° according to thermodynamic prediction (83-95% reduction). Methylene blue in catalytic amounts greatly accelerates the reaction. Keilin and Hartree (141) described a red addition compound formed between methaemoglobin and hydrogen peroxide (or ethyl hydrogen

peroxide). This complex, on standing, changed to acid methaemoglobin.

Bernheim and Michel (142) studied methaemoglobin production in vitro in the presence of various tissues. Haemoglobin alone shaken in air at 37° formed some methaemoglobin and the amount produced was a function of the oxygen-uptake. For the same amount of oxygen, kidney tissue was more effective in producing methaemoglobin than was heart, liver, brain, or muscle.

Tyramine and l-alanine (which form hydrogen peroxide when oxidized) increased methaemoglobin formation. Methaemoglobin could be reduced to haemoglobin anaerobically only.

"Natural" Occurrence.-

Ammundsen (143) claims that in 82 examinations of people who had taken no methaemoglobin-producing remedies, there was found an average of 3.5% "inactive haemoglobin" believed to be methaemoglobin, though not identified. Cox and Wendel (143) state that methaemoglobin is not present in spectroscopically detectable amount (4%) in mammals. They could not demonstrate methaemoglobin in incubated sterile blood for many hours. This lag in formation was shown by Warburg, Kubowitz and Christian (145) to be due to intrinsic enzymes of the erythrocytes which reduce methaemoglobin to haemoglobin. The reducing agents formed in vitro from glucose and lactic acid accounted only for 25-50% of the methaemoglobin reduced to haemoglobin.

ABSORPTION SPECTRA

Solutions of haemoglobin and its derivatives when exposed to white light absorb certain wavelengths and thus produce characteristic absorption spectra. It should be pointed out that free porphyrins have definite absorption bands, as have metallo-porphyrins, i.e. neither the presence of metal nor protein is a prerequisite for the possession of these bands.

The absorption spectra of compounds containing ferrous covalent iron (oxy- & carboxy-haemoglobin) show two definite bands, but when the iron is linked by ionic forces (reduced haemoglobin) only one absorption band is present (79).

Spectroscopic analysis is widely used for the identification of organic compounds, and it may be employed quantitatively. Methods used are (i) photographic (ii) visual and (iii) dependent on the photocell, e.g. the Evelyn colorimeter and the high-speed recording spectrophotometer developed by Stanley (146).

Sidwell and co-workers (147) have used photometric observation to study the effect of concentration of salt on the oxygenation of haemoglobin. They consider the band at 735 $m\mu$ to be the best for oxyhaemoglobin determination.

The absorption of ultra-violet light by haemoglobin has been mentioned (page 31).

Adams et al (148) made the interesting discovery that the Soret band (420-415 $m\mu$) is absent in the spectrum of corpuscular oxyhaemoglobin, and he concluded that haemoglobin outside the corpuscle is unsaturated while in the cell it is combined with the stroma. Drabkin (149) points out that the absence of this

band is suggestive of open-ring compounds, and it is unlikely for intra- and extra-cellular haemoglobin to differ so greatly. The absence of the Soret band was confirmed by Keilin and Hartree (150) but, since they can duplicate the effect by preparing emulsions of oxyhaemoglobin in paraffin oil, they do not hold intracellular combination with stroma responsible. Fox and Cline (134) used the Hardy recording spectrophotometer to demonstrate the presence of methaemoglobin and sulfhaemoglobin in the blood of patients receiving sulfanilamide. Michel and Harris (151) have described a method for measuring methaemoglobin in the presence of oxyhaemoglobin.

Solutions need not be crystal-clear for spectroscopic analysis; Drabkin and Singer (152) have recently developed a quantitative spectroscopic method for the study of whole blood.

The following table gives the centres of the absorption bands of haemoglobin and a few related compounds:-

<u>Compound</u>	<u>Centre at</u>	<u>Compound</u>	<u>Centre at</u>
Reduced Hb	559 $m\mu$	Acid Met Hb	634 $m\mu$
Oxy-Hb	579, 542	Methaemalbumin	623, 540, 500
Carboxy-Hb	570, 542	Reduced choleglobin	628-630

Though the information from absorption spectra is of considerable value in the determination of structure and identification of organic compounds, as yet it is impossible to deduce accurately the organic structure from absorption spectra alone (153).

FORMATION OF HAEMOGLOBIN

"An unsatisfied curiosity exists concerning the source and history of this pigment in the body" (11). Whipple wrote:- "Haemoglobin is a peculiar basic protein and its fabrication may be quite different from that of other proteins."

The Prosthetic Group.-

In the body heme is made from pyrrole nuclei, and Whipple (154) believes that the pyrrole nucleus itself can be synthesized. Heme is present in many foods, especially in meats, and might appear to be an inexhaustible source of blood pigment, but the iron in heme is quite useless.

Theoretically, porphyrins in food differing from the porphyrin in haemoglobin might be broken down in the body and the pyrrole groups put back as protoporphyrin. The value of green vegetables in blood formation is based on this idea (155). In the frog, periodic injections of haemoglobin, hematin, hematoporphyrin, ferropyrrole and pyrrole have resulted in a notable increase in the amount of haemoglobin and in the red cell counts, according to Ferrari (156). Hughes and Latner (157) found that rabbits can convert most porphyrins in the diet to haemoglobin. Large doses of chlorophyll were toxic, probably because of the effect of magnesium on the bone-marrow.

Anemic rats deficient in copper but receiving iron were given chlorophyll, protoporphyrin, and bilirubin - orally and parenterally (158). None had a good effect on hemopoiesis and 50-60% of the chlorophyll was excreted unchanged. The rest appeared as proboporphides A (or C) and B. The dog (and man)

cannot use chlorophyll.

If whole blood or haemoglobin is fed (to man), only the digested protein portion is useful for the synthesis of haemoglobin. If haemoglobin is injected the heme is destroyed, producing bilirubin and iron. The bilirubin is excreted, but the iron and globin are retained (159,160).

Bencsik et al (161) suggest that bilirubin regulates the red cell count. Small amounts of bilirubin (orally or subcutaneously) to mice, rats, and rabbits led to an increase in the number of erythrocytes; large amounts led to a decrease. Continual feeding maintained the high level, therefore the cells were not merely released from the spleen or bone-marrow. Popper (162) considers the haemoglobin content of the blood, not the cell count, to be involved. Zih (163) noted that intravenous bilirubin caused an accelerated erythropoiesis in hypochromic anemia. Bomford (164) injected bilirubin and iron into dogs made chronically anemic by repeated hemorrhage. He claims that each substance stimulated the formation of haemoglobin and that the two together were especially effective. Vannotti and Siegrist (165) studies haemoglobin formation and destruction in vitro in bone-marrow preparations. Haemoglobin was made, and bilirubin and iron disappeared. As the preparation aged, bilirubin and iron were set free. Cultures from animals with lead poisoning showed slight ability to form haemoglobin, but they did form porphyrins in large amounts.

The Protein Portion.- Globin is much more important than porphyrin as a limiting factor in the synthesis of haemoglobin. Most meats contain globin - of these liver is the

most effective, not only because of its globin but because it contains iron and various factors important in hemopoiesis. Casein, egg-albumin, soya-bean oil meal, and rat blood are good sources of amino acids useful in building the respiratory pigment; corn gluten and wheat gluten are poor, while gliadin and gelatin are almost worthless as sources of these amino acids (166).

Histidine and phenylalanine are very good building units. Hamada (167) produced anemia in rats with a tryptophane-free diet; the recovery was hastened by subcutaneous injection of the amino-acid. Feeding l-cystine or dl-methionine in 1 gm. daily doses to experimentally anemic dogs stimulated haemoglobin production, and of these two acids, cystine was more effective. D, l, and iso-valeric acid also led to an increase in haemoglobin in the standard anemic dog (168). If dogs are starved they will use their own protein for synthesis of haemoglobin (169).

Milk is a poor source of building material, and carbohydrates have no regenerative power.

The amount of haemoglobin produced from liver and iron varies directly with the degree of anemia (170).

Liver:-

The liver plays an important part in hemopoiesis, and infection or other damage depresses hemopoiesis. This organ contains an anti-anemic principle which is absent in pernicious anemia. The principle is the product of the interaction between the "extrinsic factor" of the diet and the "intrinsic factor" of normal gastric juice. This complex can be given preformed by feeding liver or liver extract. Pernicious anemia is usually due to

absence of the intrinsic factor and is accompanied by a characteristic achlorhydria and atrophy of the stomach.

The liver probably contains more than one hemopoietic principle.

The red marrow is the only site of red cell formation in the normal individual. It is found in the vertebrae, the ribs, the sternum, the diploe of the bones of the skull, and in the ends of the shafts of the long bones. The total red marrow volume approximates 1400 cc.

The circulation in the marrow is closed and the blood flows through sinusoids, many of which may collapse, producing areas of low oxygen tension which stimulates hemopoiesis.

The Cells involved.-

The hemocytoblast $\longrightarrow$ 2 basophilic erythroblasts which have no haemoglobin. These give rise to polychromatophil erythroblasts which are the first cells to contain haemoglobin. X As they age, the haemoglobin increases. The older ones $\longrightarrow$ normoblasts. These are slightly larger than red cells but retain their nuclei. The nuclei are extruded after an unknown number of divisions and the resulting erythrocyte passes into the general circulation.

The cell is actually greenish-yellow but when millions are seen together the cumulative effect is red. The erythrocyte is an opaque colloidal mixture in which haemoglobin forms approximately 95% of the dried weight (171).

Copper.-

Copper is found in all plant and animal tissues. The

average man contains 100-150 mgs. or about 34 mgs./kg. dried tissue (172). Chou and Adolph (173) believe that the daily requirement of man is about 2 mgs. Hart et al (174) stressed its importance in curing a rat anemia produced by an exclusive milk diet; .01-.02 mgs./rat/day was found to be the curative dose. Iron was present in the livers of the anemic rats but was not utilized until copper salts were added to the diets. Purified iron alone was ineffective. Schultze, Elvehjem, and Hart (175) showed that iron deficiency may produce anemia in young pigs. Iron alone cured the anemia only when the animals had retained their copper.

In dogs made anemic by bleeding there is no copper deficiency, according to Whipple and Rabscheit-Robbins (176). Copper added to the standard diet often caused a moderate increase in haemoglobin production in the anemia. There is no real deficiency since much of the element is present in the liver and spleen. They admit that copper may be related to enzyme complexes involved in production of haemoglobin. It may influence the mobilization of iron deposits and their utilization in hemopoiesis (177).

Sherman (155) considers copper an essential element in nutrition, and considers its action in hemopoiesis to be physiological, not like that of a drug.

Yoshikawa, Hahn, and Bale (178) using radioactive copper found that it appeared soon in the red cells, but this may be only greater adsorption to the red cell.

Mann and Keilin (179) have isolated in crystalline form a blood protein containing copper which they named haemocuprein.

It is blue, contains 0.34% Cu. 14.35% N. and 1.12% S. All of the erythrocyte copper is in this form. Since its M. Wt. is 35,000 it contains two copper atoms per molecule. Hepatocuprein is another copper-protein found in liver. It is almost colourless and also contains 0.34% Cu. But only a fraction of the copper in the liver is in this form. Neither compound has any catalytic properties and their function is unknown.

Agner (180) at one time believed he had found a copper-protein which was part of the catalase system. Later (181) he discovered that the complete removal of the copper with dithiocarbamate did not seriously impair the activity of the enzyme.

Cobalt.-

Many workers have claimed that cobalt is important in hemopoiesis, but "the specific rôle of cobalt in various area troubles which respond to its administration has not yet been established." - Maynard (182).

Orten (183,184) found that cobalt given to rats which were anemic due to a low protein diet produced an increase in cell count and in concentration of haemoglobin. However, attempts to produce cobalt deficiency in rats were unsuccessful (185). Kleinberg et al (186) injected 50 mgs. of hydrated cobaltous nitrate daily into anemic rabbits; the cell count rose and presumably the element acted by stimulating the formation of "erythrogenic precursors" in the bone-marrow. In dogs, cobalt has no stimulating effect on hemopoiesis but is actually inhibitory if more than the minimal dose is given (187).

Kato (188) gave iron with cobalt to anemic children, and

found the mixture to give better results than iron alone.

Unfortunately, there is often no correlation between the state of cobalt nutrition and the level of haemoglobin (189). In some areas copper is absent too, and may be responsible for the anemic symptoms in animals, since the physical symptoms of cobalt deficiency can be produced without the anemia (190).

INACTIVE HAEMOGLOBIN

Early workers found that attempts to purify haemoglobin usually resulted in the production of an inactive form (191). Wu and Ling (192) noted in 1927 that purified haemoglobin was more prone to inactivation on shaking than diluted blood. Blood stored for some days at 0° often changed gradually into a form optically similar to oxyhaemoglobin - therefore not methaemoglobin, - soluble at neutral pH - therefore not cat-haemoglobin, - and not acted upon by sod. hydrosulphite and carbon monoxide. Denaturation was believed responsible. The compound did not take up oxygen.

Though haemoglobin was crystallized long ago, the crystals, when dried, retained little or no activity capacity to equilibrate with oxygen. Bohr called the inactive compound "beta-haemoglobin", and Hufner found methaemoglobin with small amounts of oxyhaemoglobin present.

Hill (193) once prepared haemoglobin crystals. They were dried and when dissolved in water gave the spectrum of oxyhaemoglobin. However, no oxygen could be liberated by ferricyanide. Van Slyke et al (194) dried haemoglobin at high vacuum and in the cold. it was almost completely inactivated, though in solubility and colour still resembled oxyhaemoglobin. They found a slow loss of activity occurring even while the dissolved crystals were in solution. Later, Morrison and Hisey (195) presented a method for the preparation of reduced haemoglobin in a dried and stable condition. This dried haemoglobin was bright red in thin films by transmitted light and could be preserved "indefinitely" in vacuo. According to Haurowitz

(196) reduced horse haemoglobin crystallizes in the form of hexagonal plates which on contact with oxygen change spontaneously into the monoclinic needles of oxyhaemoglobin. The author claims that a rearrangement of the heme groups may take place on reduction.

Wyckoff and Corey (197) studied X-ray diffractions from haemoglobin and other crystalline proteins. The protein crystallizes with water of crystallization which is very readily lost. The band pattern common to haemoglobin and other proteins is produced by apparently amorphous materials resulting from the efflorescence of the crystalline compounds. If crystal decomposition is prevented, these proteins give typical powder patterns rich in sharp lines. Some of the observed spacings were much longer than those found from insoluble proteins of silk, hair, and tendon.

The problem of inactive haemoglobin in vivo is connected with labile iron and bile-pigment precursors (198). Van Slyke and Hiller (199) demonstrated that the oxygen and carbon monoxide uptake are identical by the common methods. However, Ammundsen (143,200) states that if the blood is reduced with hydrosulphite the carbon monoxide capacity may increase by about 5% in human and bovine (201) bloods. Though ferric iron is suggested, the inactive form does not seem to be methaemoglobin alone (Oxygen-capacity after reduction cannot be measured due to the formation of strong oxidizing agents).

Legge and Lemberg (202) confirmed Barkan And Schales' finding (198) that easily-split-off iron (E.S.I) represents

about 5% of the total blood iron; only one third as much was obtained in the presence of carbon monoxide, the rest in the presence of oxygen. The larger fraction is an artefact produced by oxidation of the prosthetic group by the oxygen liberated from oxyhaemoglobin in the presence of acids. Actually no more than 2% of the blood iron is E.S.I. and therefore it does not account for all the inactive haemoglobin.

DENATURATION OF HAEMOGLOBIN

Soluble proteins are globular or corpuscular in solution. However, many of the "globular" ones are ellipsoidal or rod-shaped. Haemoglobin and serum albumin are not spherical, but have similar molecular weights, volume and dissymmetry, according to Svedberg (203). On the other hand, keratin and collagen are extremely elongated fibres (109).

Mirsky and Pauling (204) consider one large polypeptide chain to make up the protein molecule. This chain is folded into a definite configuration which is kept in that shape by hydrogen bonds between peptide nitrogen and oxygen atoms, and also between free amino and carboxyl groups of diamino and dicarboxylic amino acids. The hydrogen bond N-H-O is made up of a hydrogen atom linking two electronegative atoms together and is electrostatic. The nitrogen and oxygen are thus held closer together than if the bond were absent.

According to these authors, denaturation of proteins involves a drastic change in the configuration characteristic of native protein. The breaking of hydrogen bonds (and probably other bond types between the chains) which results in the disorganization, enables the molecule to rearrange itself in several possible ways. This type of change could explain the loss of the specific properties of the protein; haemoglobins from different animals possess varying crystal structures etc., and these differences vanish on denaturation. In the same way, antigenic properties are greatly altered. No denatured protein has been crystallized.

Though it is assumed that denaturation destroys the orderly protein structure, sometimes there seems to be an increase in the regularity of some of the structure, or in certain aspects of the framework (205,206,207).

Haurowitz (208) thinks that, on denaturation, internal positive and negative groups are brought to the surface and facilitate aggregation at the isoelectric point. Fischer (209) believes that denaturation is a chain reaction.

Many reagents (water, acid, alkali, alcohol, acetone, urea, thiocyanate) can denature proteins; other agents such as heat, ultra-violet light, high pressure, and shaking are also effective. However, different denaturing agents act in different ways and do not lead to the same product.

Acid and alkali bring protein out of the region of its isoelectric point, and produce their effect quite easily. No visible effect occurs but when the altered protein is brought back to the same isoelectric point, it precipitates. This change in solubility is one of the most important effects of denaturation.

Haemoglobin, because of its colour and solubility has been employed frequently in the study of denaturation. Anson and Mirsky (210) made the important discovery that the denaturation of haemoglobin can be reversible. Globin denatured by acid-acetone treatment, trichloroacetic acid or heat, can be redissolved to give the native form which, combined with heme, gives haemoglobin (210,211). They also made methaemoglobin from hemin and native globin which was prepared by dissolving coagulated globin (212). Later they observed the disappearance of disulphide

groups on the reversal reaction. Roche and Donnat (213) could not reverse the process of coagulation of haemoglobin by trichloroacetic acid. Nagaoka (214) prepared a soluble form of denatured haemoglobin by heating it at 52° at its isoelectric point; the product had greater buffering power than the native protein, whereas with heat-coagulated protein buffering power is diminished. Heymann (215) found that denaturation without coagulation increases protein volume, which is correlated with changes in hydration after coagulation.

Holden (216) observed that haemoglobin denatured in the presence of hydrogen by acid, ethyl alcohol, and sod. salicylate, can be renatured; the second and irreversible step in the destruction of globin is an oxidation. Oxyhaemoglobin and haemoglobin are more difficult to denature than is methaemoglobin.

Steinhardt (217) found that haemoglobin is completely dissociated into half-molecules in the presence of high concentrations of urea and other amides. The effect is partly reversible and despite the alteration, no loss of function occurs. Therefore the "disaggregation" appears to be independent of denaturation. Presumably the forces binding the protein together are weakened by amides. Drabkin (218) does not believe that the liberation of sulfhydryl (-SH) groups accounts for the splitting in urea, since this splitting occurs in acetamide where no exposure of -SH groups is found. Dog haemoglobin, unlike horse haemoglobin, does not disaggregate with urea (219); both, however, are denatured. It should be mentioned that native globin has half the weight of haemoglobin, i.e. the four prosthetic groups (hemes) bind two globins together for a resulting weight

of 68,000. Gralén (220) split haemoglobin with acids to globin and heme, which could be recoupled to form native haemoglobin.

The combination of haemoglobin with ions of copper, zinc, and mercury was investigated by Rawlinson (221). Oxyhaemoglobin and native globin were precipitated within pH limits. Cupric copper changed oxyhaemoglobin to parahematin; the iron was oxidized and the protein denatured. The denaturation may be an oxidation coupled with the oxidation of the iron atom, because native globin did not react in this way.

The rates of alkali denaturation have been employed by Brinkman and co-workers, and by Ramsey (69-71) to study different haemoglobins. Alkali changes globin in haemoglobin to globin-hemochromogen. (The rate of denaturation is determined by the type of haemoglobin, among other factors). Herzog (222) found that the absorption spectrum of methaemoglobin in alkaline solution changes with time depending on the strength of alkali. The change is confined to the pigment portion, however, with no dissociation from the globin involved.

The reaction between haemoglobin and NaOH, according to Drabkin (218) is:

1. Ferro Hb (HbO_2) $\not\rightarrow$ NaOH $\longrightarrow$ globinoferriporphyrin (oxidized denatured globin hemochromogen)
2. Globinoferriporphyrin $\not\rightarrow$ NaOH $\longrightarrow$ ferriporphyrin hydroxide (alk. hematin) $\not\rightarrow$ denat. globin.

Dow and Matthews (223) subjected haemoglobin solutions (and red cell suspensions) to high pressures -5,000 atmospheres for 2-4 hours. The haemoglobin coagulated into a red-brown rubbery mass and no crystallization was observed. The authors do not

consider that the effects of pressure is simply mechanical. One of the results is comparable to the addition of an -OH group to the haemoglobin. The reaction seems to be a reduction and the product is similar to that obtained by using diethyl ether and ethyl alcohol.

"A substance will form a monomolecular film on water if its molecules contain a soluble and an insoluble group. Spreading is impossible when the molecule as a whole is soluble because it contains too many polar groups (e.g. glucose) and spreading does not occur when the molecule is insoluble, because it is built up of non-polar groups only (e.g. paraffin)." Gorter (224).

Compounds which cannot spread (heme) can be kept at a surface by being bound to surface-active molecules such as proteins. The protein functions as a "boat" or "carrier" fixing the reactive prosthetic group at the water surface.

A Study of the area of monolayers of different substances has shown that, in general, the molecules have the form and dimensions indicated by their structural formulas and as derived from X-ray or electron diffraction measurements. Proteins behave unexpectedly. If they spread completely, they all occupy a surface corresponding to a film thickness of 10Å. Apparently, the chain structure of the spherical molecule unfolds to form a flattened disc-like mass. Proteins, by unfolding and spreading in this manner, become denatured and insoluble.

Jonxis (225) prepared films from various haemoglobins. The maximum area in the compressed state was found to be the

same in all cases, namely, 9400 sq. cm./mg. Reduced haemoglobin spreads more rapidly than oxyhaemoglobin.

The author concluded that each haemoglobin is built up from the same number of amino-acids per Svedberg unit.

INTERMEDIATE COMPOUNDS IN THE FORMATION OF BILE PIGMENTS

If blood is incubated at 37°, an increase occurs in the iron content of the plasma (226). Under similar conditions a comparable increase in the bilirubin content of the plasma occurs - noted by von Czike (227). Barkan (228) thought that the source of bilirubin might not be the haemoglobin itself, as von Czike believed, but that it was related to the E.S.I. ("leicht abspaltbares Bluteisen") discovered by Barkan in 1925. At this time the nature of E.S.I. was not known, but it was realized that it might be an intermediate stage in the conversion of haemoglobin to bilirubin. Barkan and Berger (229) had identified two forms of E.S.I., one ferrous and the other ferric, and in 1937 Barkan and Schales (230) demonstrated that the two fractions were haemoglobin-like compounds in which the protein was unaltered globin, while the prosthetic group was a heme derivative with an opened porphyrin ring. The name "pseudo-haemoglobin" was suggested for these chromoproteins. In 1939 Barkan and Walker (231) demonstrated that a simultaneous increase of plasma iron and bilirubin occurs in incubated blood; they believed that the actual cleavage occurred within the red cell and held hydrogen peroxide responsible. The rates observed could not account for more than a small fraction of the daily production of bile-pigment.

Lemberg, Lockwood, and Legge (232) also have found biliverdin and biliviolin in red blood cells. Their analyses suggest that less than 1% of the haemoglobin is of the bile-pigment nature and therefore it cannot account for all the labile iron of

blood.

Verdohemochromogens and Related Substances.-

Lemberg. (233) began the work in this field by showing that the porphyrin ring of protohemin can be split oxidatively to form verdohemin, the ferrichloride of biliverdin. This compound had been made previously by Warburg and Negelein (234) who called it "green hemin". Nitrogenous derivatives of verdohemin after reduction were named verdohemochromogens. This term has been employed also for any compound containing the type of open-ring iron porphyrin which can combine with globin to form verdoglobin (235,236). Recently Lemberg (237, 238) et al have introduced two new precursors of the pigments called choleglobin and cholehemochromogen. The authors believe that the chole-class of compounds are not quite identical with the verdo-group, on the basis of spectroscopic evidence.

Choleglobin is a combination of native globin with an open-ring iron prophyrin. It is easily denatured by alkali and a reducing agent to form cholehemochromogen (237) which is analogous to denatured globin-hemochromogen. The "pseudohaemoglobin" of Barkan and Schales is not identical with choleglobin; the former was prepared by treating oxyhaemoglobin with H_2O_2 and KCN, followed by reduction with sod. hydrosulphite, and it is a ferrous denatured globin cyancholehemochromogen. Havemann's verdoglobin (236) was also produced by means of peroxide in the presence of cyanide and is probably identical with pseudohaemoglobin. According to Havemann the following reaction occurs:-

$$\text{Haemoglobin} + 3 \text{ peroxide} \longrightarrow \text{verdoglobin} + CO_2 + 2H_2O.$$

Lemberg et al (238) consider the presence of cyanide to

be an unnecessary complicating factor, while Havemann and Jung (239) believe that cyanide, and any other reagent which inhibits oxidation enzymes, promotes the formation of choleglobin-like compounds.

All these compounds - verdohemochromogens, choleglobin and pseudohaemoglobin - are green pigments containing broken porphyrin rings with iron still attached. This iron is labile and is easily split off by dilute acids. The tetrapyrrolic residue is bile-pigment (biliverdin and biliviolin (232)). Bile-pigments and bile-pigment precursors lack the Soret band and this can be used as proof of structure. The band is due to the closed ring system of conjugated double bonds.

Choleglobin Production.-

Lemberg (240,237) uses a coupled oxidation of ascorbic acid with haemoglobin (or pyridine-heme) in the presence of air. The initial reaction velocity of choleglobin formation is about twice as large at pH 8.5 as it is at pH 7.2. The temperature coefficient is high ($Q_{10} = 3.5-4$ between 25° and 37°). In the initial stages choleglobin is the only product of the oxidation and its absorption band in the red (maximum at $630\text{ m}\mu$) is soon visible. Choleglobin cannot be separated from haemoglobin, but its production can be followed quantitatively using a spectrophotometric method (241). In air, about ten molecules of ascorbic acid are oxidized per molecule of choleglobin formed. The increase in E.S.I. during this coupled oxidation is parallel to the choleglobin increase, as might be expected (202). As the oxidation proceeds, secondary changes occur in the choleglobin

resulting in a mixture of compounds. The method of analysis used by Lemberg et al is based upon a difference in absorption spectra of carbonyl hemochromogens and of carbonyl cholehemo-chromogens (238); the former have two absorption bands in the green region, the latter have an absorption band in the red.

Choleglobin unites with carbon monoxide but the absorption band remains at 630 $m\mu$ (48). There is insufficient evidence to show a reversible equilibrium with oxygen.

Spectroscopic observations:-

Reduced choleglobin	628-630 $m\mu$	CO-choleglobin	628 $m\mu$
Cholehemochromogen	618-620	Cholehemochromogen	628
Fe choleglobin or	670		
Oxycholeglobin			

The bands of the carbon monoxide compound in the chole-series do not differ widely from that of reduced choleglobin, unlike the proto-series.

It was found that reduction forms choleglobin as rapidly as does ascorbic acid, while thiolacetic acid acts more slowly, and glutathione and cysteine act very slowly, if at all (241).

Choleglobin can be made by using hydrogen peroxide in low concentrations with or without Vitamin C.

Postulated mechanism of reaction:- Ascorbic acid gives up hydrogen to oxyhaemoglobin $\longrightarrow$ Hb-H₂O₂ complex which is ferrous. The compound undergoes an intramolecular peroxidative reaction, the porphyrin ring being oxidized by the oxygen. Ring opening is believed to be a secondary reaction. It is not known whether ring cleavage is necessary here for the colour change from red to green.

Green pigments can be prepared without breaking the

porphyrin ring. Libowitzky and Fischer (242) made an ester of the ferrichloride of α -hydroxyisocoproporphyrin I. This compound is green, has an absorption band at 655 *m μ* , and is a precursor of bile pigments. Libowitzky (243) also found a green derivative of the ferric chloride of α -oxycoproporphyrin in which the ring was intact. He noted that ferrous complexes of coproporphyrin esters yield green pigments with peroxide; ferric complexes react only with very high concentrations of peroxide to form propentdyopent (244); pentdyopent occurs in nature (79). Iron porphyrin derivatives in alkaline solution are decolourized and the iron quantitatively liberated when peroxide is added (245).

BILIRUBIN

Bilirubin ($C_{33}H_{36}N_4O_6$) is the chief excretory product of haemoglobin breakdown (and possibly of myoglobin (246)). One gm. of haemoglobin yields about 35 mgs. bilirubin (247), which is derived from the prosthetic group heme. Iron is absent, and the four pyrrole rings of the pigment are not in a porphyrin ring, since the compound shows no absorption bands in the visible spectrum. Fischer (248) showed it to consist of two pyrrole-methenes joined by a methylene bridge, and it is believed to form by the splitting-open of the porphyrin ring of heme between pyrrole nuclei I and II. Biliverdin is possibly an intermediate in the reaction. The formula suggested for bilirubin is shown in fig. 3. (249).

This compound is produced in the cells of the reticulo-endothelial system. The greatest portion forms in the liver, but in dogs the liver can be cut out of the circulation without production ceasing (250). Mann, Bollman, and Magath (251) also removed the liver in dogs and found that bilirubin increased in the plasma. This result cannot be obtained in birds where the reticulo-endothelial system is confined to the liver.

Doljanski and Koch (252) observed that tissue extracts can split haemoglobin with the formation of bilirubin. Xanthorubin was an intermediate if living cells were present. Niven (51) drew attention to the macrophages in tissue cultures of liver, spleen and subcutaneous tissue (mouse); these could ingest red blood cells and produce "hematoidin" (crystalline bilirubin).

Bilirubin is found in normal serum as a result of the activity of the reticulo-endothelial system mentioned above; the normal level is about 0.2-0.8 mgs.%. The van den Bergh test for this bile pigment (see Malloy and Evelyn in appendix for method) is made up of two reactions:- (1) the direct and (2) the indirect - requiring alcohol. The first may be immediate - 20-30 secs., or delayed - i.e. showing no change until a minute or more has passed.

The pigment differs (i) physically or (ii) chemically in these forms:- (1) The indirect form may be adsorbed to or combined with the serum proteins or lipids, while the other form is free. Possibly bile salts can break this union, explaining why the direct reaction predominates in obstructive jaundice and liver injury, while in hemolytic jaundice the indirect form is in excess. Bennhold (253) in 1932 showed that blood bilirubin is adsorbed by the albumin fraction; this specific effect did not take place with egg albumin. The different amino-acid distributions in these proteins were held responsible by Pedersen and Waldenstrom (254) who demonstrated by means of the ultracentrifuge that both forms of plasma bilirubin were bound to serum albumin. The objection to free bilirubin is its insolubility in blood.

Stenhagen and Rideal (255) studied the penetration of protein monolayers by bilirubin. Small pH changes greatly affect penetration and they consider this phenomenon to have some biological significance. Pedersen and Waldenstrom (254) found that in bile, bilirubin is adsorbed onto a colloidal

carrier which increases the solubility. The solubility is decreased as the pH falls, and gallstone formation is often associated with a low pH of the bile (256).

(ii) Collinson and Fowweather (257) consider the two carboxyl groups of bilirubin to be important. They believe that the formation of a water-soluble alkali salt is responsible for the direct reaction, since bilirubin is insoluble at blood pH. It is, however, alcohol-soluble and could give the indirect test.

The liver passes bilirubin and a slight amount of biliverdin into the bile. The bilirubin is excreted into the intestine where bacteria reduce it to colourless urobilinogen (11). Part of this is oxidized to yellow urobilin. Both urobilinogen and urobilin are present in feces, and both are reabsorbed to a certain extent, re-entering the circulation. The reabsorbed fraction is passed into the bile and its cycle begins again. Any that may reach the general circulation is eliminated by the kidney.

Urobilinogen is colourless, but becomes yellow on oxidation (urobilin) after contact with air and hence the darkening of the urine. Normally the colour of urine is not due to bilirubin or urobilin, but to urochrome.

THE DESTRUCTION OF HAEMOGLOBIN

Three recognized routes of elimination of haemoglobin are:- (1) Absorption by the reticulo-endothelial system, (2) Intravascular catabolism and (3) Excretion via the kidney. (258).

(1) The reticulo-endothelial system normally attacks damaged red blood cells and can absorb free haemoglobin as well. In both cases bilirubin and hemosiderin are produced. The haemoglobin is presumably oxidized to a bile-pigment complex, choleglobin, which then loses its iron and protein (Page 61). The latter two may combine to form hemosiderin. Biliverdin is the first product of the breakdown and it is reduced to bilirubin in the reticulo-endothelial system and in the liver. Hawkins and Johnson (259) injected bile-fistula dogs with haemoglobin solutions. They observed that the pigment portion is split off to form bile-pigment while the iron and globin are used again in the manufacture of haemoglobin. This is the only "physiological" mechanism for the destruction of haemoglobin and it is responsible for the hyperbilirubin-aemia observed after a transfusion of old cells.

(2) When hemolysis is marked intravascular catabolism occurs together with (1). Extracorporeal haemoglobin is treated as a foreign substance which is attacked and eliminated (258). Its fate is determined by its concentration in the plasma. Some of the haemoglobin in the circulation is broken down to globin and heme; the latter is oxidized to hematin

which combines with serum albumin to form methaemalbumin. Another possible intermediate step is methaemoglobin formation, but Fairley believes that the previous sequence occurs.

In investigating blackwater fever, in which extensive intravascular hemolysis occurs, Fairley (260) found that methaemoglobin was not responsible for the brown colour of the plasma. The brown pigment was methaemalbumin, the formation of which has already been described.

Rimington (3) injected hematin and observed an increase in fecal porphyrin. The hematin of methaemalbumin is probably excreted by the liver as type III porphyrin.

(3) Renal excretion. Plasma haemoglobin must exceed a concentration of 0.12% or 16.1 mgs.% for hemoglobinaemia to exist. Lower values are not significant. Baker and Dodds (261) injected oxyhaemoglobin into rabbits. It was easily excreted in the normally alkaline urine of these animals. If the urine were made acidic (as it is in man), methaemoglobin and acid hematin were found in the kidney. Hematin was deposited in the tubules as such or combined with some nitrogenous compound producing anuria and perhaps death.

Anuria with uraemia is the cause of death usually, where there is a severe intravascular hemolysis. For this reason, preliminary alkalization of urine before transfusions is advised (261,262).

Fairley injected haemoglobin solutions into patients whose urine was kept alkaline. In one case 14 gms. haemoglobin did not produce any reaction. Within about 10 mins. the

Schumm test (appendix) for hematin was positive. Since free hematin does not occur in plasma, this was taken to indicate the presence of methaemalbumin. Injected heme was oxidized to hematin and combined with albumin also.

Miscellany.-

The urochrome pigments are supposedly products of globin breakdown, possibly from the tryptophane (18). Urochromogen is colourless and is present in urine; it can be oxidized to the yellow urochrome and, on further oxidation, to uromelanin. Whipple (263) claims that urochrome contains pyrrole groups but that its derivation is doubtful.

Lanaurin (264) is a pyrrole pigment which imparts a yellow colour to wool. It is produced from haemoglobin due to hereditary hyperactivity of the reticulo-endothelial system of certain sheep, and it is excreted via the kidney and sweat glands.

In vitro experiments.-

Chargaff et al (265) showed that cephalin, but not lecithin, can disrupt the heme-globin linkage in oxyhaemoglobin and carboxy-haemoglobin. The concentration of cephalin is quite low (0.005 M) unlike the high concentrations required for urea and salicylate. Mechanism proposed:- oxyhaemoglobin / cephalin $\longrightarrow$ cephalin-globin / hematin. The cephalin-globin compound is a soluble complex at pH 7.

Bingold (266) treated oxyhaemoglobin with hydrogen-peroxide and obtained an iron-free substance. This is not a

porphyrin and is precipitated by saturated amm. sulphate. It is also made by peroxide-producing bacteria. Bingold found it in a urine when bilirubin is present, b liver cirrhosis without bilirubin and c in old pleural exudates. It may be produced in the liver and kidney.

Zeynek and Kittel (267) mention that blood pigments, exposed to sulphur dioxide and light, lose iron and become porphyrin proteoses. Bingold (268) believes that blood catalase and hydrogen peroxide can convert haemoglobin to pentdyopent (a dihydroxy-pyrromethene), bilirubin, and urochrome.

Unsaturated fatty-acids may be involved in haemoglobin destruction. Haurowitz (24) treated haemoglobin with linoleic and linolenic acids in the presence of air, and the haemoglobin was destroyed.

E X P E R I M E N T A L

THE EXPERIMENTAL WORK

The greater part of the work consisted of an investigation of the stability of haemoglobin and the red cell in preserved blood. Abnormal bloods and derivatives of haemoglobin were also studied.

Section 1:

E.S.I. determinations were carried out on several preserved bloods using Barkan and Walker's concentration of HCl (1.2% = 0.328 N). The results were considered unsatisfactory and it was decided to find the effect of varying the strength of acid. Concentrations from 0-.1N were first employed as described in the appendix. No other change was made and the incubation time of 24 hours was kept constant. The effect of 1 hr. incubation is shown in figure 6. Several bloods were incubated for more than 24 hours but no increase in iron was observed, while shorter periods did give lower iron values.

The results of varying the acid strength are seen in fig. 5. In all cases (9 blood samples) the yield of iron increased up to about .07 N HCl and always decreased with increasing acidity. The peaks of the curves are all in the same region because the haemoglobin concentration was always 9-10 gms.%. The maximum values for iron are all greater than the amount obtainable with the prescribed 1.2% acid. The pH's corresponding to the maximum E.S.I. in two blood samples were 5.19 and 5.63.

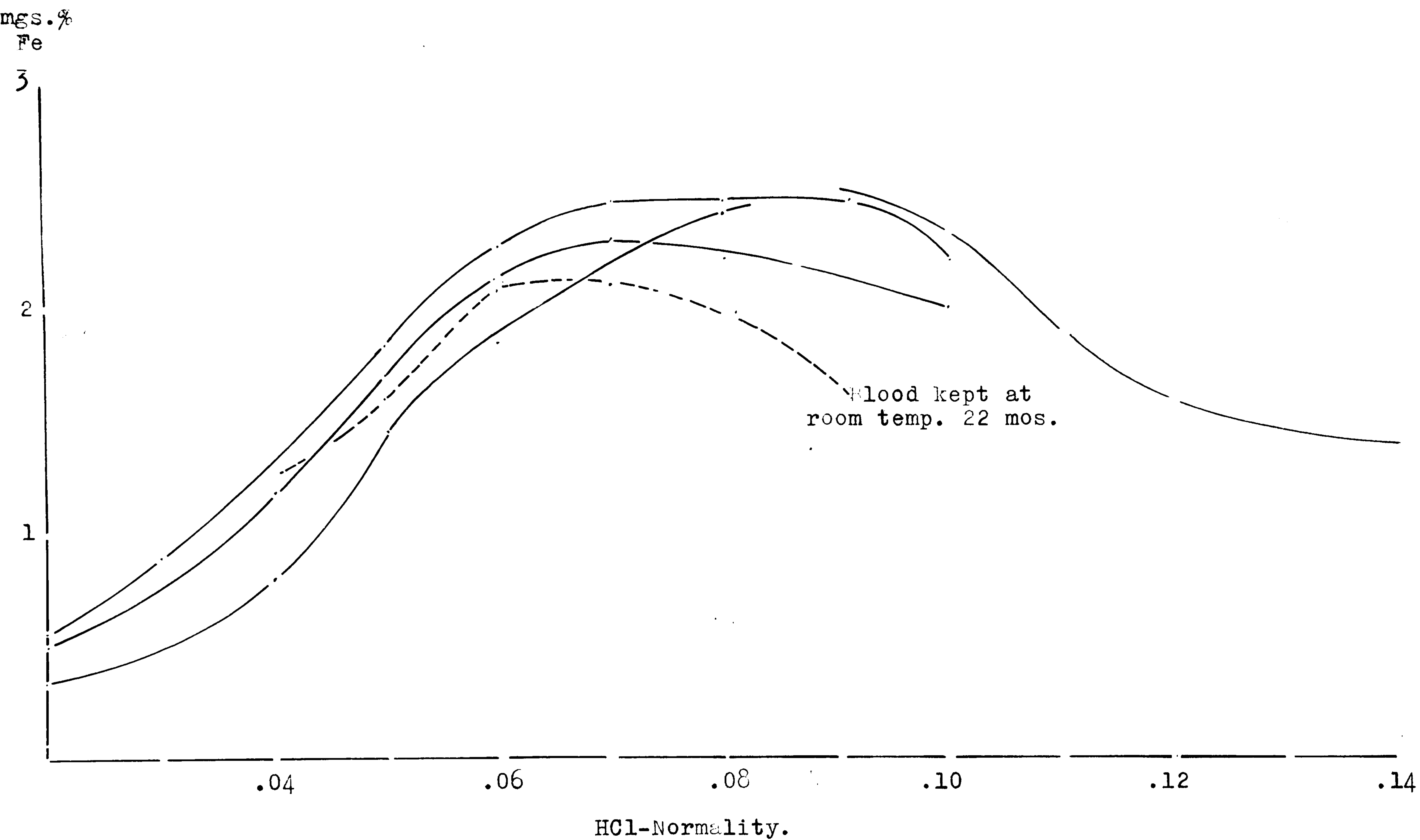


FIG.5 - Effect of Increasing Acidity on Yield of Iron.

FIGURE 6 - Effect of One Hour Incubation.

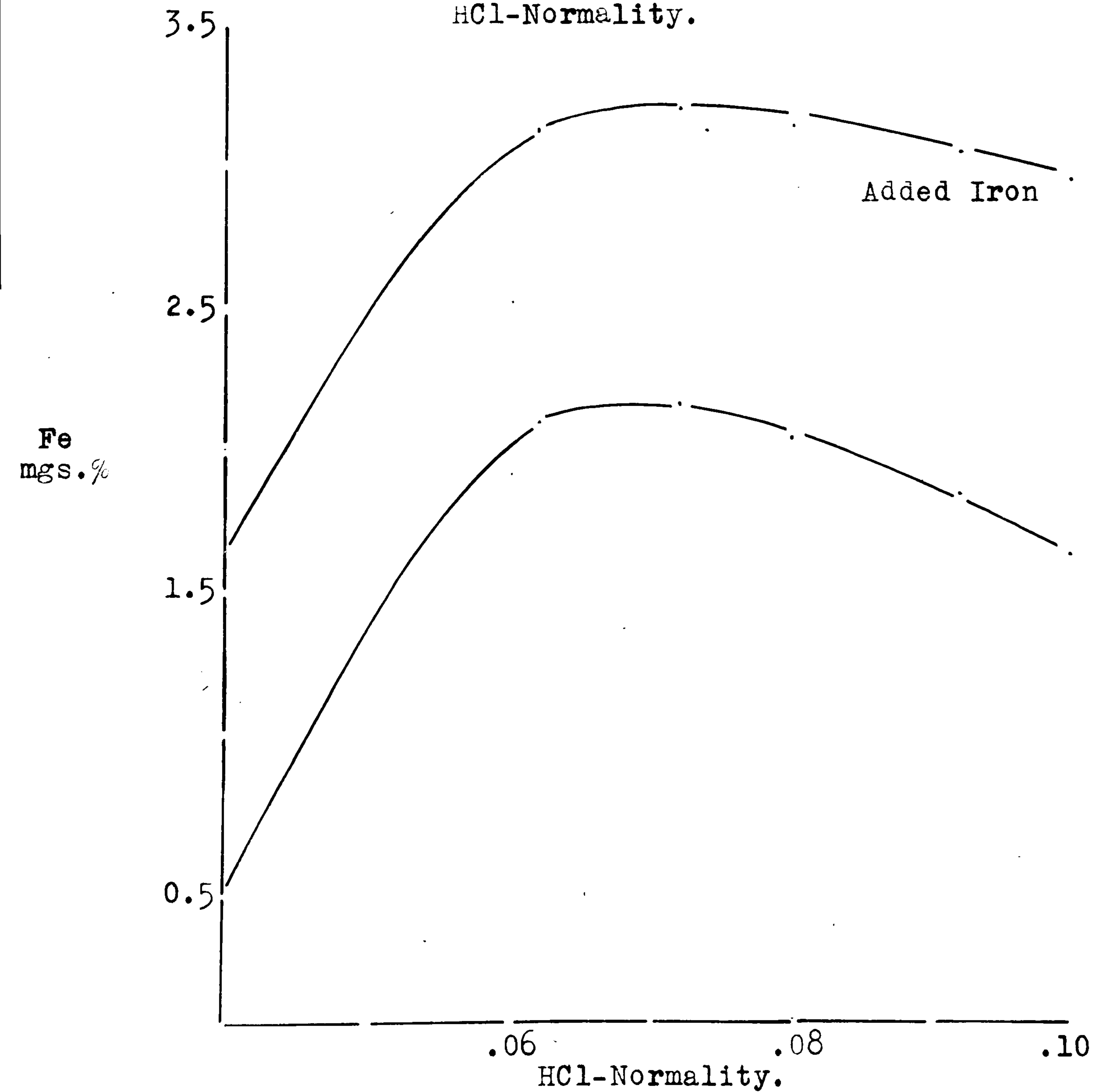
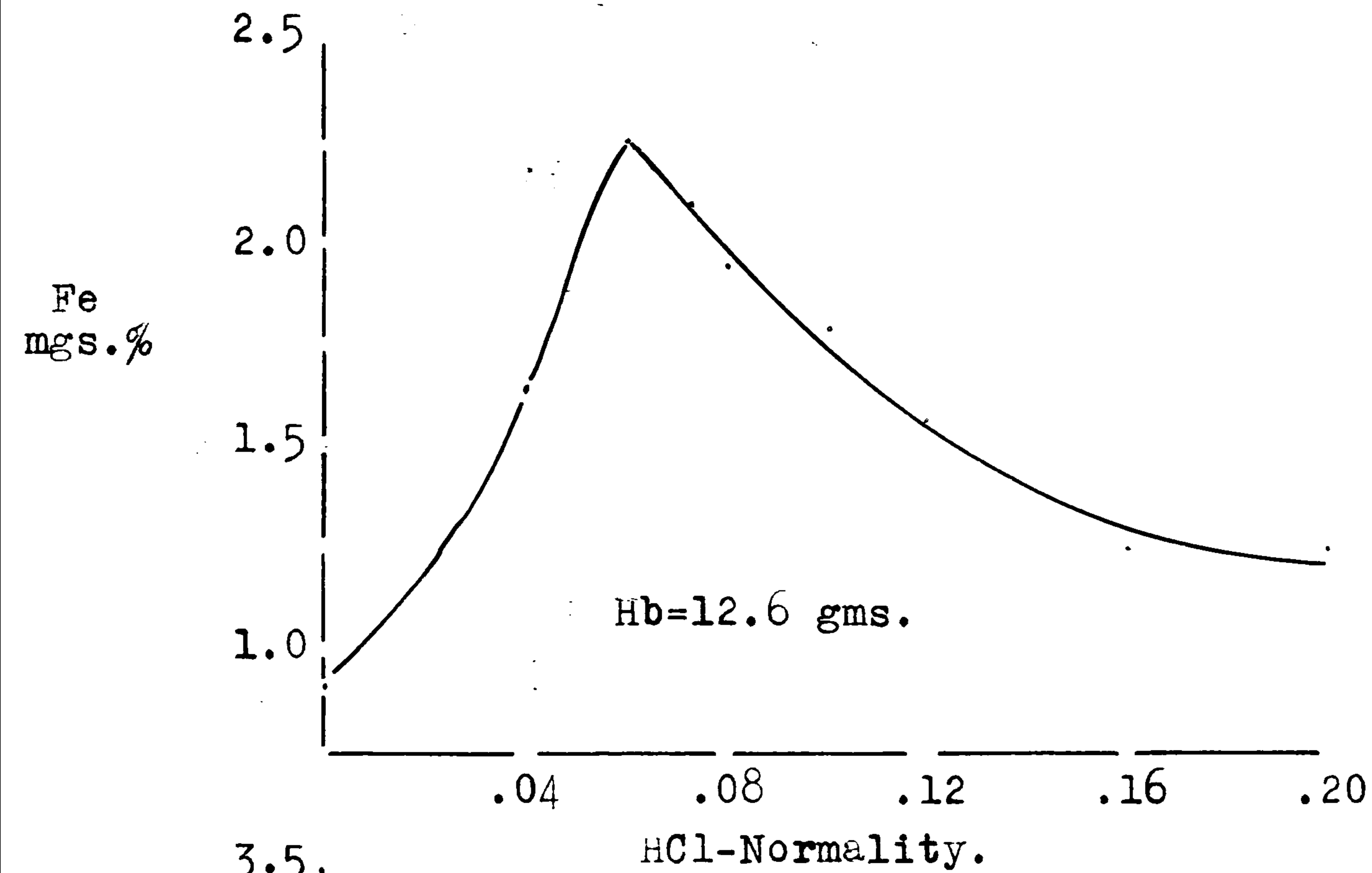


FIGURE 7 - Recovery of Added Iron.

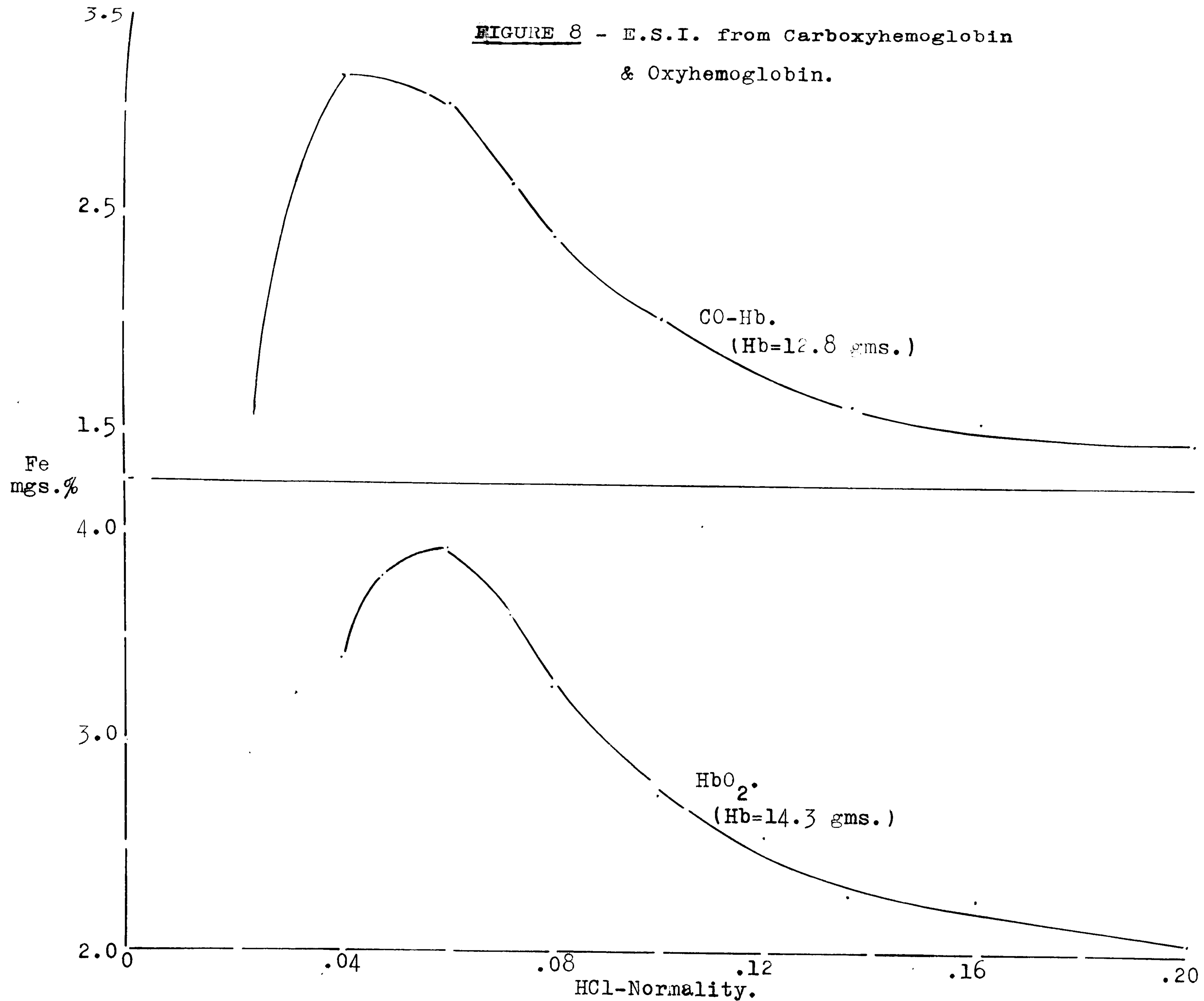
Once it was established that the results were characteristic of blood, higher concentrations of acid were used. From 0.1-0.2N HCl, the iron level continued to fall slightly but noticeably (fig. 5). Concentrations of .2-.5N and 1-3N acid were employed, but over these ranges of acidity no significant difference in iron was observed.

Added ferric iron was recovered to the extent of 90%, the curve running parallel to the control on blood alone (fig. 7). This suggests that the lower concentrations of acid do not leave any inorganic iron behind, i.e. the higher acidities not only measure E.S.I. but also produce some from intact haemoglobin, as shown by Lemberg. Presumably the protein precipitate bound the remaining 10%, though a trichloroacetic precipitate is not supposed to retain any iron.

Washed red blood cells were analysed for E.S.I. Determinations on oxyhaemoglobin and carboxy-haemoglobin showed similarity to the results obtained from whole blood. Therefore the only effect of the plasma is to partly neutralize the hydrochloric acid, since the peaks are to the right of those on washed cells (fig. 8). Serum iron - 0.1 mgs.% - is so little as to be negligible.

Both oxyhaemoglobin and carboxy-haemoglobin were treated with 0.5% ascorbic acid and much more iron was obtained than with hydrochloric acid (fig. 10). This treatment was used originally by Lemberg to obtain choleglobin. Bile-pigment was also present. Though the curve is similar to that obtained with HCl, it is unlikely that methaemoglobin formed in the

FIGURE 8 - E.S.I. from Carboxyhemoglobin
& Oxyhemoglobin.



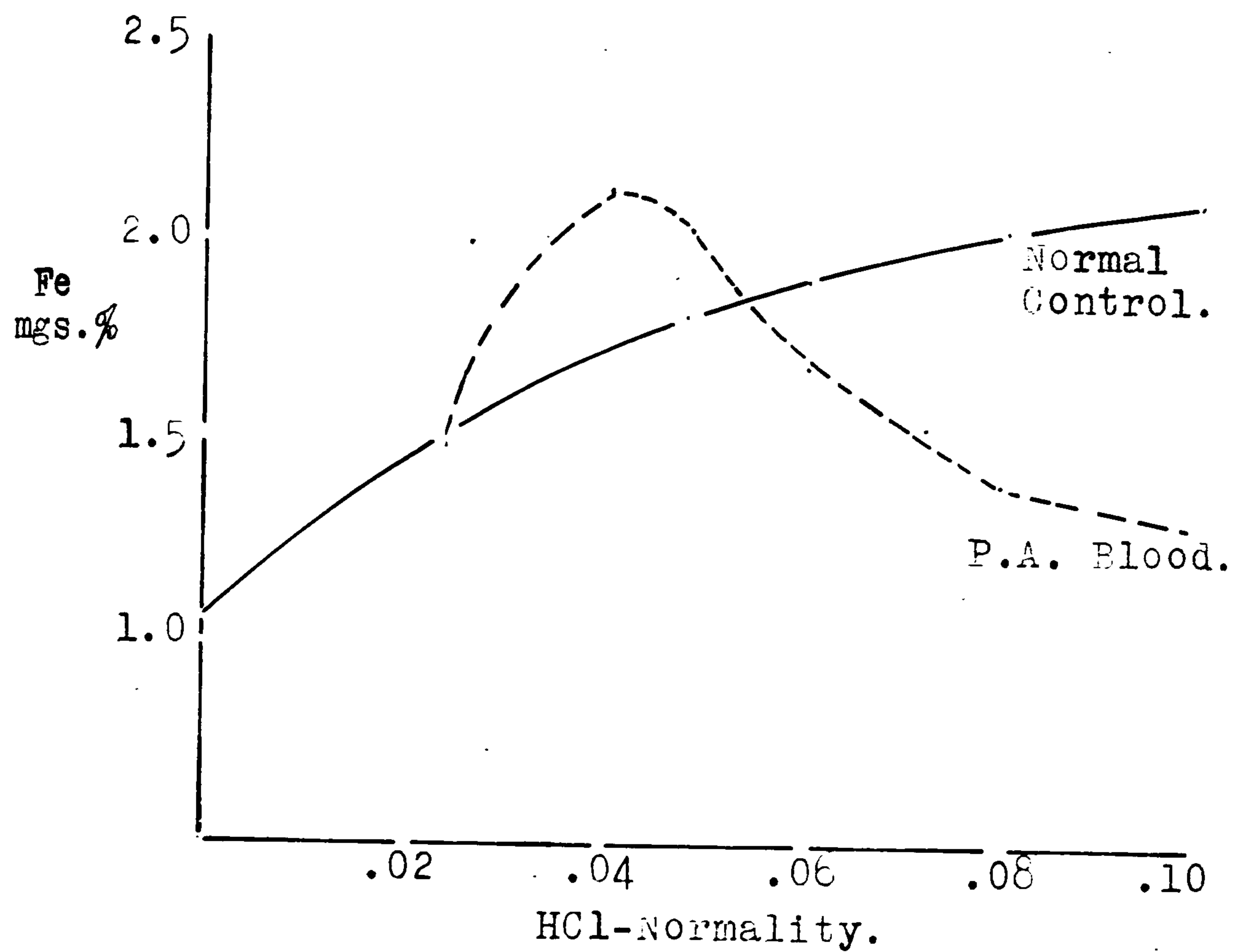


FIGURE 9 - Pernicious Anemia Blood.

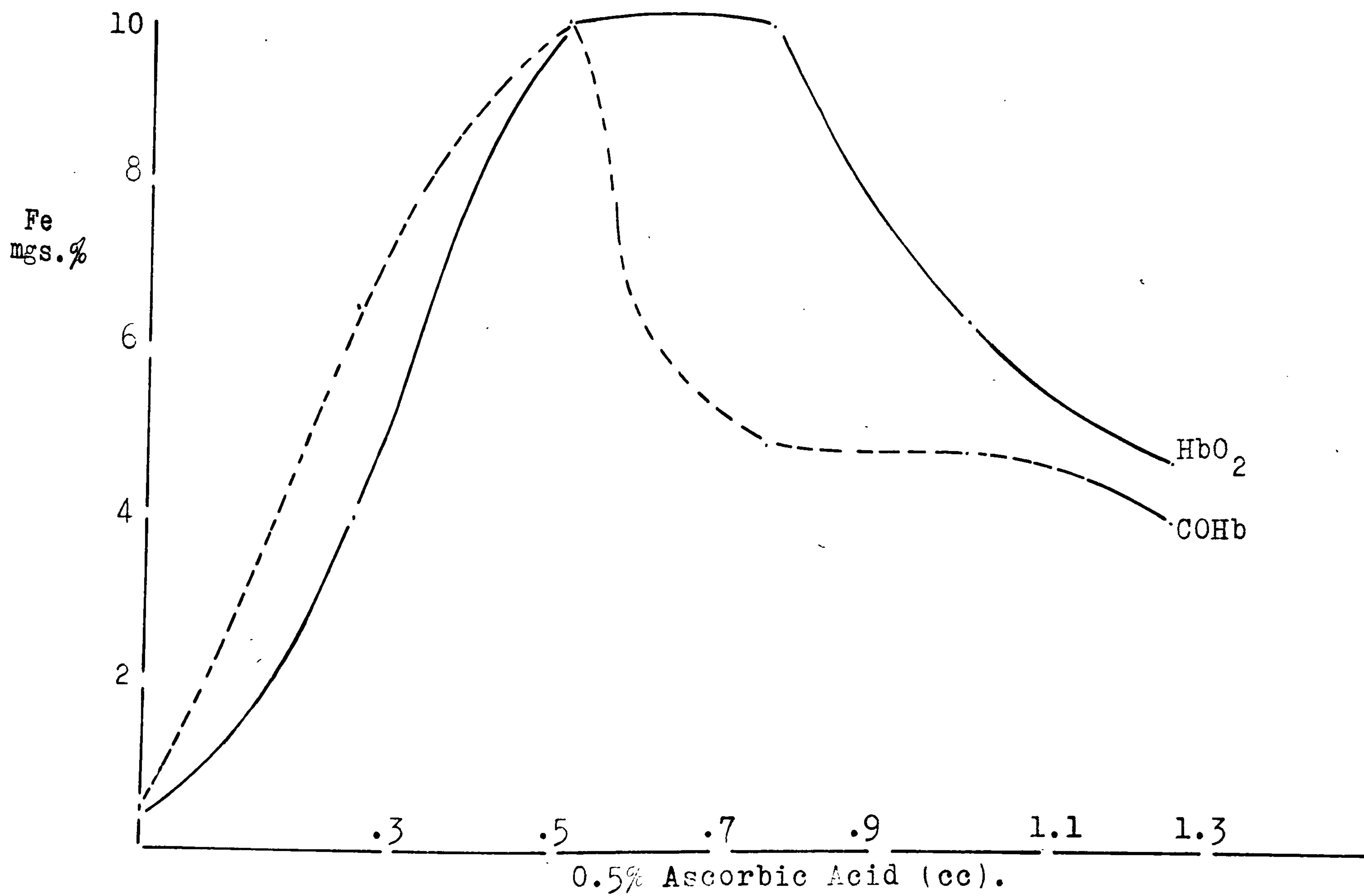


FIGURE 10 - Production of E.S.I. by Ascorbic Acid.

Spectroscope
Reading.

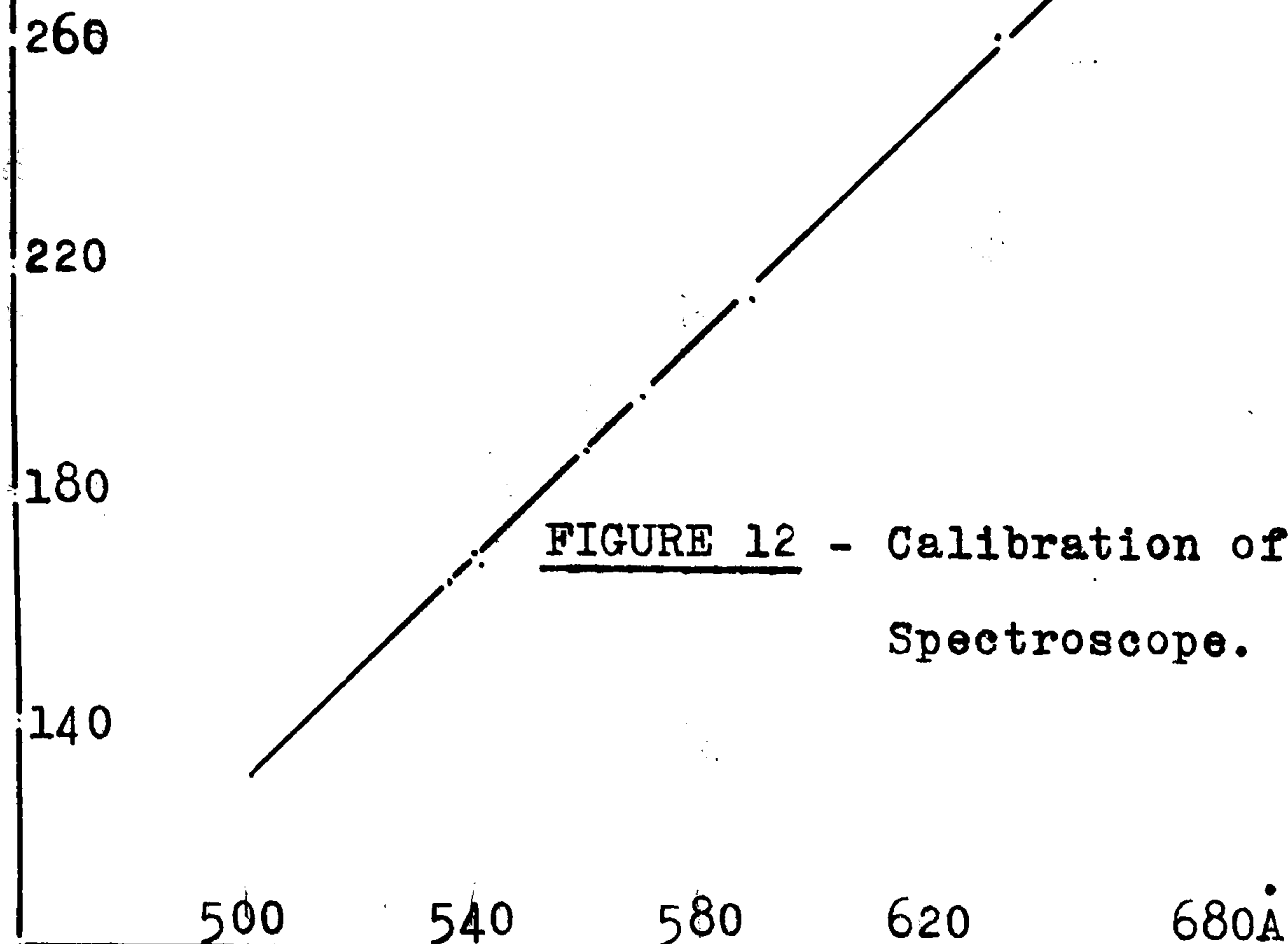


FIGURE 12 - Calibration of Spectroscope.

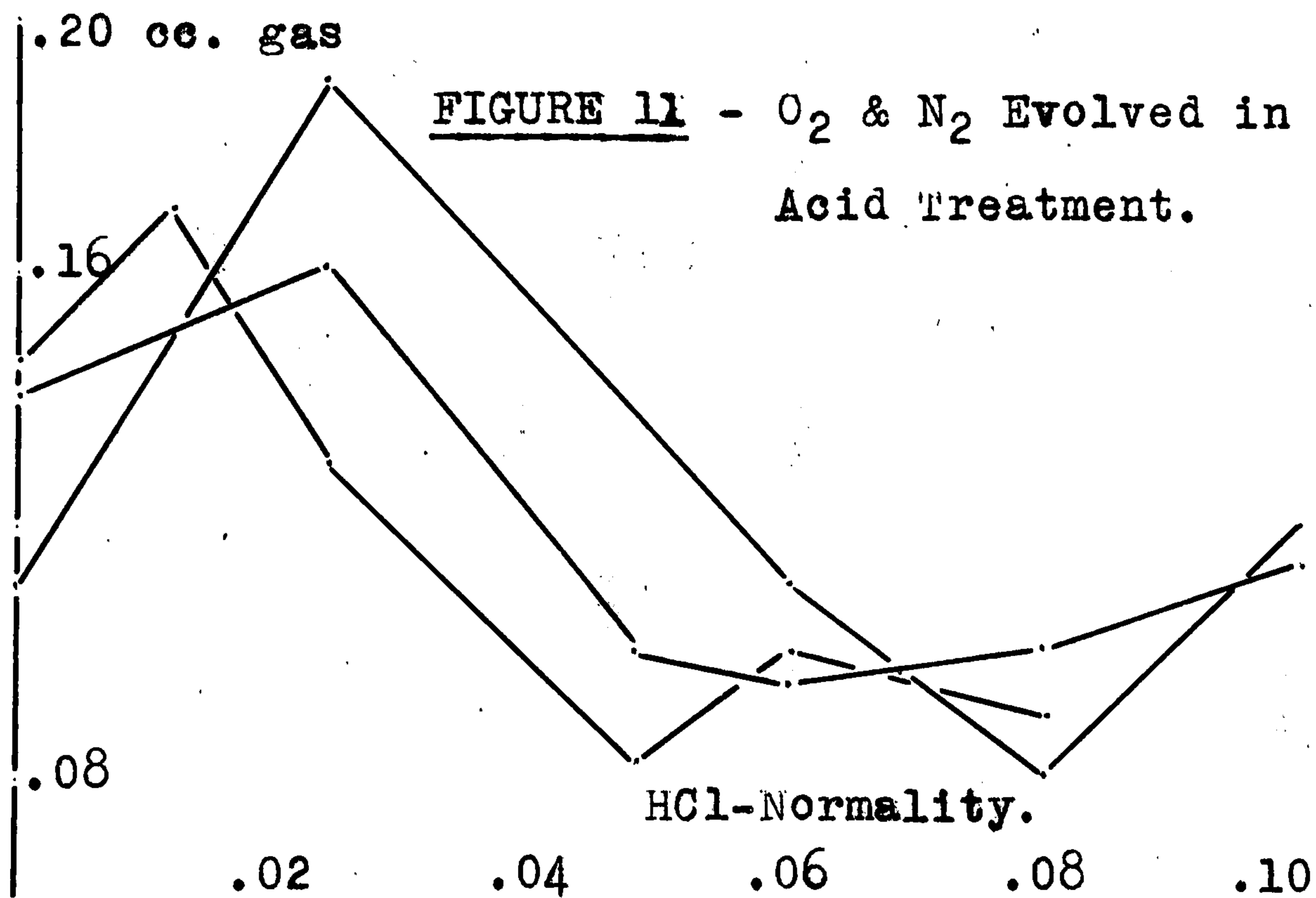
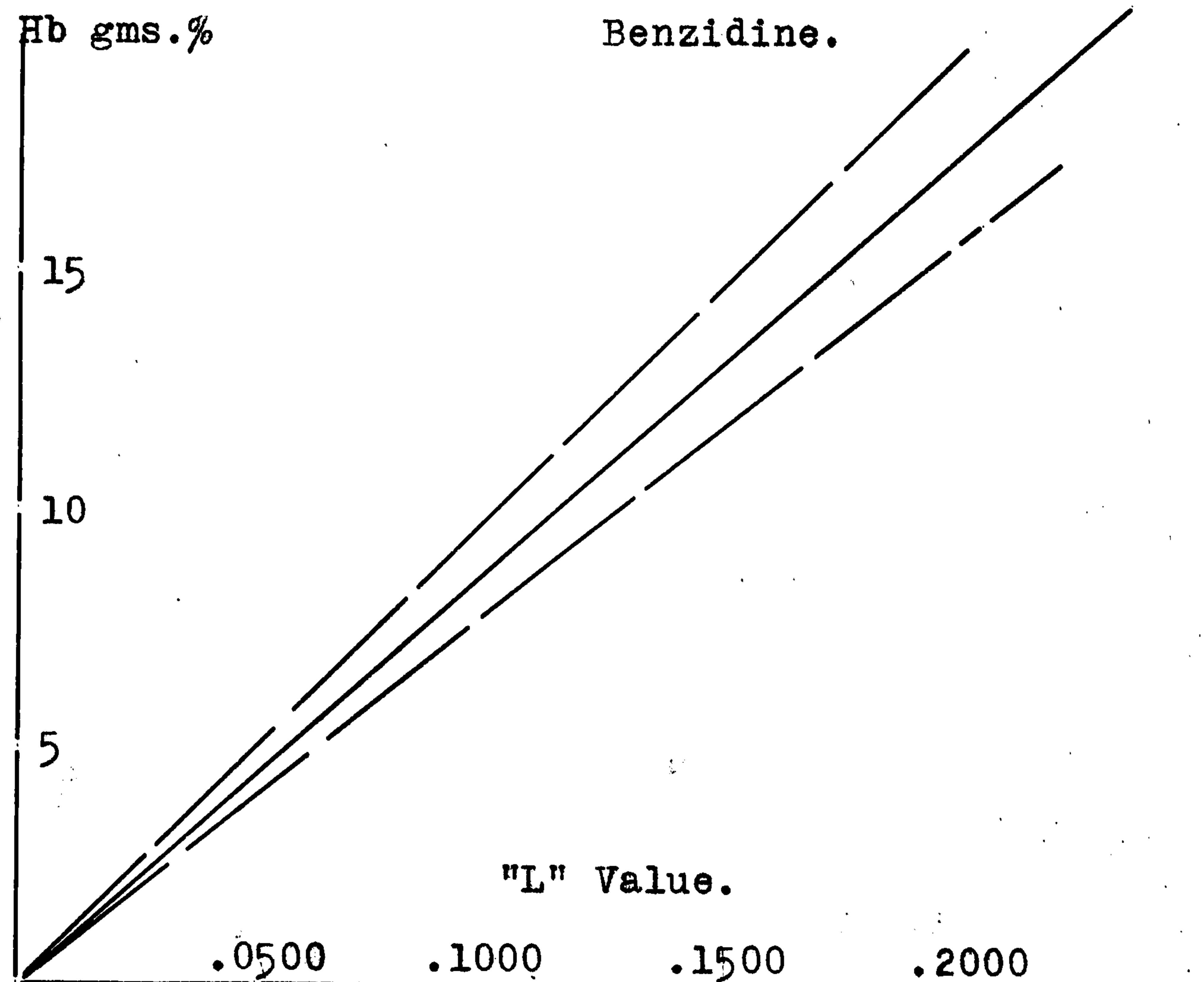


FIGURE 11 - O_2 & N_2 Evolved in Acid Treatment.

FIGURE 13 - Standard Curve for Benzidine.



presence of such a strong reducing agent.

Instead of ferricyanide, hydrochloric acid of varying strengths was used in the oxygen-capacity method of van Slyke. The amount of oxygen immediately liberated varied inversely with the iron split off, suggesting that oxygen is absorbed in the reaction (fig. 11).

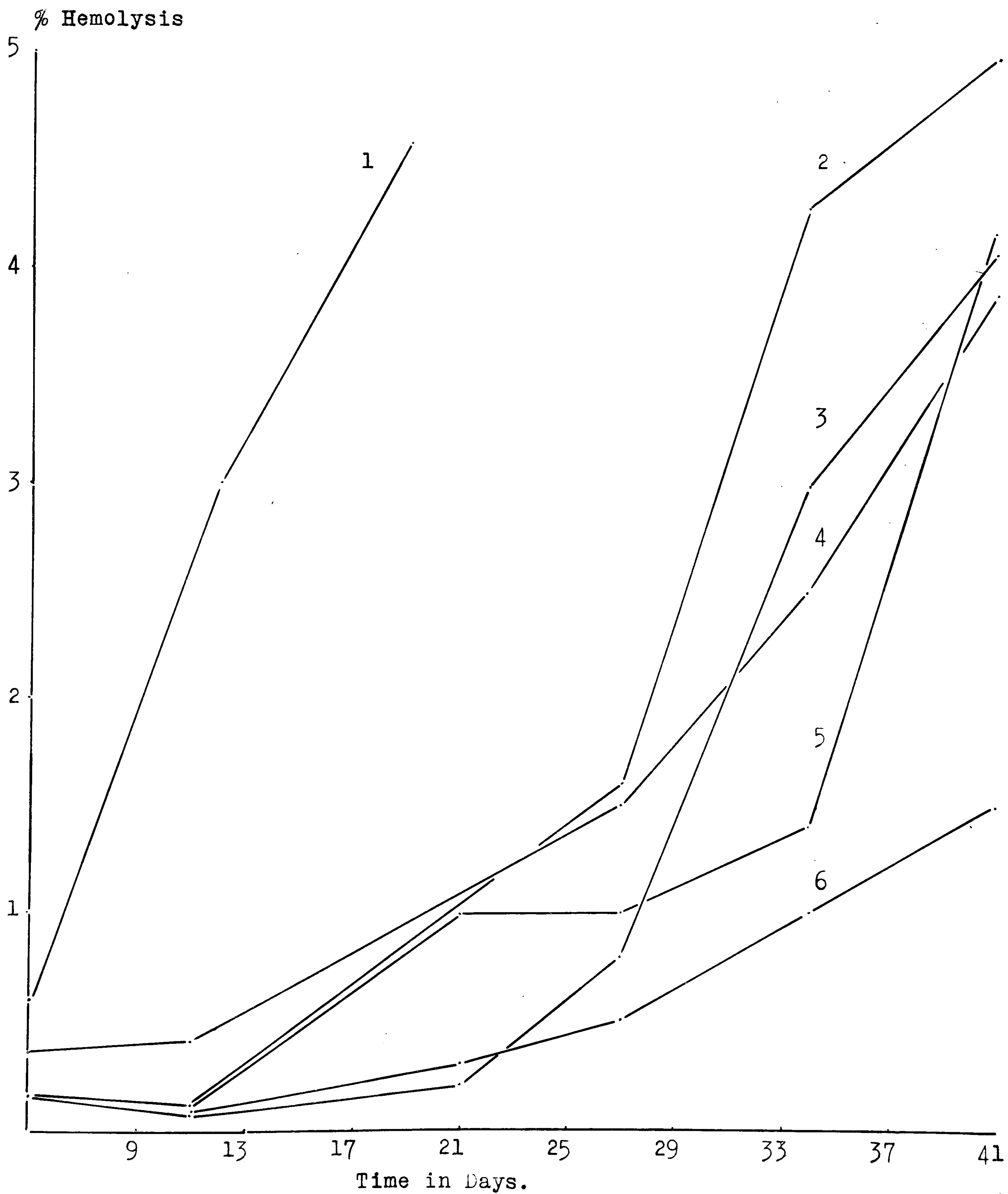
Section 2: Abnormal Bloods.-

Bloods taken from rabbits on alkaline and acid diets showed no difference. Dried blood was unaffected by the range of acidity, i.e. it gave a constant value of iron, not in curve form.

An old blood - kept in liquid form at room temperature for 22 months - gave the curve of normal blood. Oxygen-capacity determination showed that 36% of the original haemoglobin was still capable of being oxygenated. Haemoglobin is accordingly quite stable when preserved at low temperatures (fig. 5).

The first sample of pathological blood - from a pernicious anemic - gave an abnormal iron curve (fig. 9). Since pernicious anemia is often difficult to diagnose, the difference in the curve suggested that an E.S.I. determination, as carried out above, could be of value in differentiating pernicious anemia from other anemias. Five more samples of P.A. blood were obtained but unfortunately the haemoglobin in these behaved as did normal blood. The abnormal result was obtained in a patient who had received no liver therapy, unlike the others, and this may be important. A placental and leukemic sample were also treated with acid. Again no abnormalities were observed.

FIGURE 14 - Hemolysis During Storage.



Section 3 - Haemoglobin Derivatives.-

Washed red blood cells treated with H_2O_2 and KClO_3 at 40° yielded 33% of total iron as E.S.I. Though the solution was brown, there is no reason for believing that only methaemoglobin was present. Methaemoglobin made by adding ferricyanide to oxyhaemoglobin (11 gms.%) was unaffected by the various acidities, yielding the same slight amount of iron (0.84 mgs.%). Sulphhaemoglobin (9 gms.%) similarly gave no curve but a constant amount of 2.4 mgs.%. Carboxy-haemoglobin, however, behaved just like oxyhaemoglobin (fig. 8). Nitrite-haemoglobin was abnormal.

Oxygen seems to be necessary for the splitting off of iron with acids and the iron must be ferrous.

Section 4.- Spectroscopic Examination of the Oxy-haemoglobin showed that (i) with increasing acidity methaemoglobin formed more quickly. (ii) Only the bands of oxyhaemoglobin were detectable in the weaker acidities at first, but after 24-hr. incubation at 37° only bands of methaemoglobin were seen. This, together with the evidence in section 3, suggests that methaemoglobin formation may be responsible for the drop in the curve.

Section 5.- Choleglobin is not necessarily the only product of haemoglobin inactivation. It was decided to follow any progressive inactivation by measuring the oxygen-capacity and comparing it with the total pigment present as measured by the Evelyn colorimeter.

Blood No	van Slyke Hb	Colorimeter Hb	Age in Days
76-0	16.68	16.62	48
	16.77	16.85	56
	16.34	16.96	76
76-1	13.2	13.16	48
	12.63	12.62	51
	13.13	13.16	57
	13.76	13.8	72
	12.71	14.5	84
76-2	13.2	13.54	51
	13.6	14.12	57
	13.	14.12	76

As can be seen, the values check precisely over a period of ten weeks. During this period, therefore, no inactive haemoglobin had formed. After this time both the van Slyke and E. colorimeter values rose: (i) Evaporation in the ice-box was quite possible. (ii) Fibrin and stroma precipitation in the colorimeter tubes occurs to a greater extent in old bloods, and the cloudiness would give an incorrect high reading. This was noticed at the end of the experiment, otherwise the colorimeter tubes would have been centrifuged. (iii) The relatively higher colorimeter values might have been due to inactive haemoglobin, of course, but no other evidence supported this.

It was considered important to follow the rate of hemolysis of the preserved blood since free haemoglobin can be inactivated much more easily than the corpuscular form. Here the preservative used is of obvious value. The results (fig.14) show less than 1% hemolysis in 25 days and therefore free haemoglobin was not important. The amount of free haemoglobin was measured by the benzidine method (Appendix 91). Preserving

solutions consisted of:-

- | | |
|--------------------------------------|---|
| <u>1</u> .Citrate. | <u>4</u> .Citrate,glucose. |
| <u>2</u> .Citrate,glucose,phosphate. | <u>5</u> .Citrate,glucose,phosphate & CO ₂ . |
| <u>3</u> .Citrate,glucose. | <u>6</u> .Citrate,glucose & CO ₂ . |

For further details see appendix.

Work on the oxygen-dissociation curve of preserved blood is incomplete, and therefore it will not be discussed.

DISCUSSION

The behaviour of haemoglobin towards acid.-

Acid splitting of E.S.I. involves the oxygen in oxyhaemoglobin. The reaction is by no means clear-cut and the products obtained vary with the concentration of acid used.

Lemberg showed that acid not only frees iron from choleglobin but oxidizes the prosthetic group of normal haemoglobin with the aid of oxygen liberated from oxyhaemoglobin. The acid then splits the iron from this artificially produced choleglobin; hence a considerable portion of the E.S.I. measured is an artefact. Wu (269) observed that about 1/4 of the oxygen evolved from oxyhaemoglobin by acid may be used to oxidize haemoglobin to methaemoglobin. Some oxygen is taken up in the denaturation of globin by acid, while the remainder is liberated. The writer found that the amount of oxygen liberated varies with the strength of acid and is minimal with the same concentration which gives a maximum of E.S.I. This suggests that oxygen is involved in the reaction, as was previously postulated by Lemberg.

Apparently this rise and fall in E.S.I. with increasing acidity has not been observed by other workers before. The fall may be due possibly to: (i) methaemoglobin formation (ii) hydrolysis of haemoglobin (iii) denaturation of the globin.

The results obtained with haemoglobin derivatives indicate that the porphyrin iron must be ferrous, if cleavage of the heme ring is to occur. Carboxy-haemoglobin behaves like oxyhaemoglobin, suggesting that carbon monoxide can replace oxygen in the cleavage.

Though oxyhaemoglobin absorption bands remain with the weaker concentrations of acid, a mixture of oxyhaemoglobin, methaemoglobin and probably denatured haemoglobin derivatives form readily with the stronger concentrations. After incubation for 24 hours at 37° no oxyhaemoglobin remained in any of the digests, and only methaemoglobin could be detected spectroscopically. This lends support to the suggestion that methaemoglobin (and other ferric derivatives) is responsible for the drop in the curve, since the acid treatment does not remove any iron from methaemoglobin.

Ascorbic acid liberates much more iron than does mineral acid (HCl). Since the haemoglobin in the ascorbic acid solutions remains red during incubation, it is unlikely that methaemoglobin formed. Nevertheless, the same rise and fall in E.S.I. is observed. Possibly globin may be denatured by the strong ascorbic acid and may fail to participate in the coupled oxidation.

Various authors have used the term "E.S.I" with different meanings. In 1935 Rosen-Runge (270) spoke of E.S.I. in serum. In 1936 Barkan (271) wrote that "labile iron" is present to the extent of 0.1 mgs. % in serum and 1.7 mgs. % in whole blood. The latter was not believed to come from haemoglobin. Josephs and Winocur (272) measured total iron, haemoglobin iron, and E.S.I. in various blood samples. The E.S.I. which they obtained by acid treatment amounted to 1-2 mgs. % and was found not to change (i) in infants during the first two months of life (ii) in infants with iron therapy (iii) during infections. Nevertheless the total iron was found to be greater than haemoglobin

iron in normal samples by 6 mgs.%, and in cases of increased blood formation and destruction by more than this amount. The above authors concluded that besides E.S.I. there is present another form of iron which may vary in pathological conditions. This form is present in an organic iron compound which does not carry oxygen (choleglobin?) or perhaps in a more "primitive type" of haemoglobin containing more than 0.336% iron.

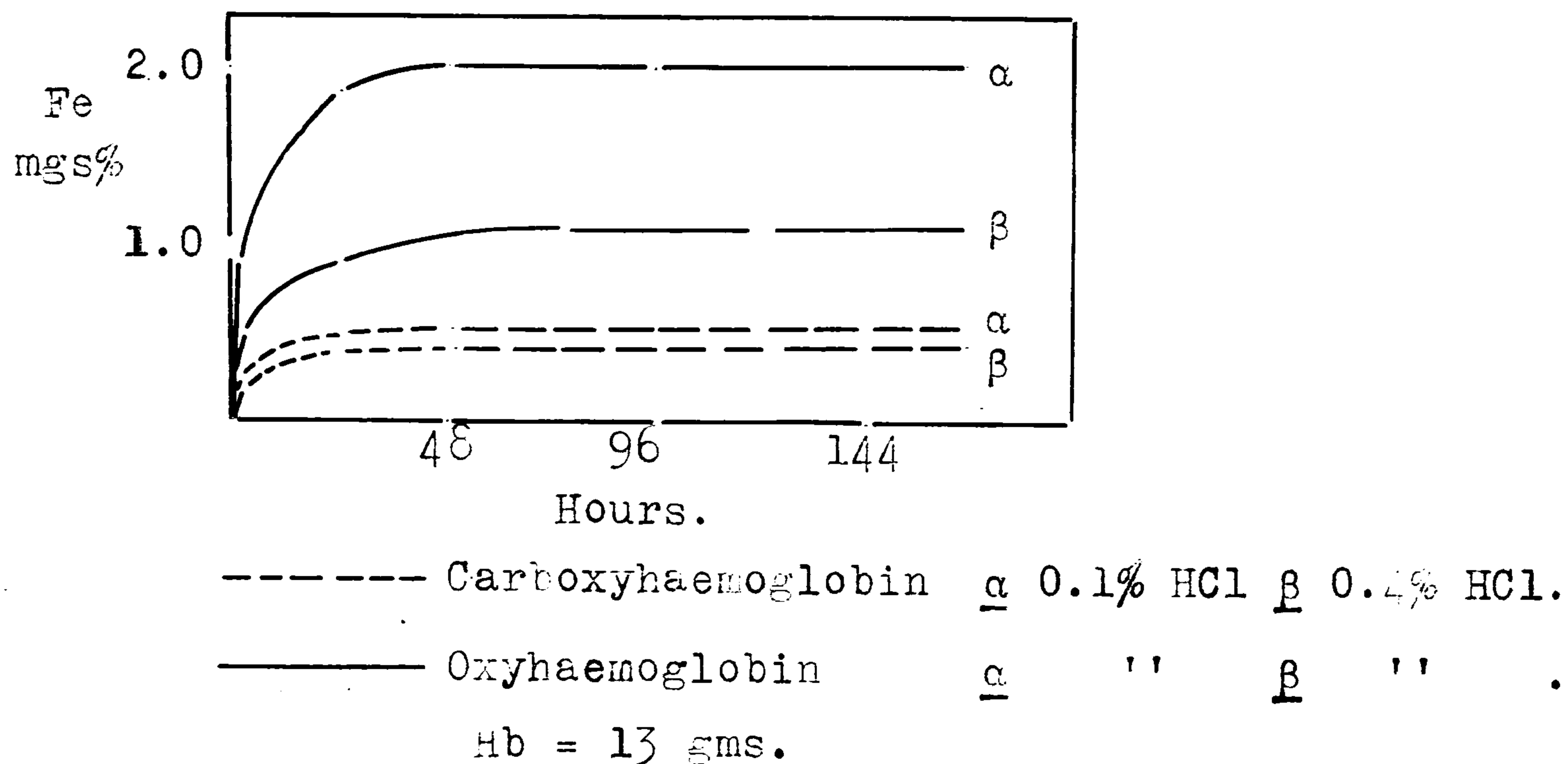
Moore et al defined E.S.I. as the amount of iron in whole blood which is changed to an ionized form by the action of dilute acids. He states that there is no conclusive evidence to show that it is non-haemoglobin. In 1941, however, Lemberg conclusively demonstrated that preformed choleglobin exists in red cells to the extent of about 1% while E.S.I. is less than 2%. He also showed that the rise in choleglobin (when haemoglobin was treated with ascorbic acid) is paralleled by an increase in E.S.I. It therefore seems safe to assume that measuring E.S.I. gives a fairly accurate method of finding the amount of choleglobin present. The term E.S.I. should be confined to labile iron of the erythrocyte alone.

Pathological bloods.- Too few samples were analysed to determine definitely whether choleglobin content varies abnormally in the conditions mentioned. Our results indicate, however, that it is not abnormal.

Stored bloods.- No increased formation of choleglobin took place during storage at 4°C. Bloods which had been preserved for months gave the same reaction to acid treatment as did fresh blood. It is unlikely therefore that choleglobin is confined to older erythrocytes in vivo. The agreement between the

van Slyke and Evelyn colorimetric methods shows that haemoglobin is not inactivated in any other way for at least two months. Though the rate of oxygenation may be slow, it is not due to changes in the haemoglobin; other factors should be considered - e.g., altered environment (electrolyte), pH changes, and inactivation of carbonic anhydrase.

Hemolysis was not an important factor in these experiments because its extent was negligible. The stability of the red cell was maintained by the low temperature (4°) and a suitable medium. Different degrees of hemolysis were observed with different preservatives (of which citrate alone was the worst) but despite these variations no difference in E.S.I. was observed.



While this thesis was being typed, a paper discussing the effect of dilute acid on haemoglobin was discovered. The journal in which this article appeared was unavailable in Montreal, but a copy was obtained from the National Research Council in Ottawa.

The author (Venndt) shows that low acidities split off more iron than higher ones, as can be seen in the above figure. However, he does not point out the rise in iron concentration before the fall, and the inference from his graph is that less iron is split off from carboxy-haemoglobin than from oxyhaemoglobin. If the acid strength were sufficiently low, more iron would be obtained (fig. 8).

Venndt H., Hoppe-Seyl. Z. 263:162 -1940.

APPENDIX

Iron Determination in serum or plasma - Barkan and Walker, J. Biol. Chem., 135: 37, -1940.

Principle.-

It involves incubation with dilute hydrochloric acid, reduction of ferric iron to the ferrous form with hydrazine, and the measurement in a photoelectric colorimeter of the ferrous complex of o-phenanthroline. The method may be used for the estimation of pseudohaemoglobin iron in whole blood. Part of the pseudohaemoglobin iron measured thus, however, is an artefact.

Reagents.-

1. HCl - 1.2%
2. HCl - 0.3%
3. CCl_3COOH -20%
4. CH_3COONa -sat.
5. 2M acetate buffer pH 4.5 by mixing 90 vols. of CH_3COONa and 110 vols. of 2M AcOH
6. Hydrazine sulphate - 1% solution in 2M acetate buffer (pH4.5). Prepare daily.
7. O-phenanthroline monohydrate, 0.1% - warm to accelerate solution. Reject if coloured.
8. Blank solution - 1 vol. of Reagent 1, 1 vol. of Reagent 3 and 2 vols. H_2O . Change blank every time new HCl or CCl_3COOH is prepared.

Method: (a) Serum Iron.-

Measure 2 cc. of serum or plasma into a short stout rimless test-tube (Pyrex 25 cc. is quite suitable). Add 1 cc. of 1.2% HCl. Keep stoppered at 37° for at least 1 hr. (or overnight). Cool to room temperature and add 1 cc. of 20% trichloroacetic acid; mix thoroughly and let stand 1 hr. at room temperature. Cover with tinfoil and centrifuge (3000 rpm.) for 15 mins.

Into a colorimeter tube (Evelyn) measure 2 cc. of the clear

supernatant fluid, 0.5 cc. sat. sod. acetate, 0.5 cc. of the buffered 1% hydrazine sulphate solution, and 0.5 cc. of 0.1% o-phenanthroline monohydrate solution.

Blank - same reagents to 2 cc. of "blank"; both to stand at room temperature at least 1 hr. Then add 2.5 cc. H₂O to each, mix and read in Evelyn colorimeter using 6 cc. aperture, filter 490, and setting blank at 100. Formula $100L/K_2 =$ mgs. iron per 100 cc. where K is 114.6. Double to find original value.

Remarks.-

Buffer maintains pH 4.5 in filtrate.

Citrate does not interfere with colour reaction.

CCl₃.COOH may be contaminated by iron.

Colour is stable for 8 days.

Normal plasma iron - 0.113 mgs. %.

(b) Pseudohaemoglobin iron.-

To 0.5 cc. blood in a test-tube is added 2 cc. water.

To the 2.5 cc. of diluted blood after oxygenation by shaking in air, are added 1.25 cc. of 1.2% HCl. The writer used varying concentrations of acid in order to obtain the iron curves previously under discussion. 0.1N HCl and H₂O were added to the diluted blood from two burettes as follows:-

cc. .1N HCl	cc. H ₂ O	Resulting normality
0	1.25	0
0.3	.95	.024
0.4	.85	.032
0.5	.75	.04
0.6	.65	.048
0.75	.50	.06
0.9	.35	.072
1.0	.25	.08
1.15	.10	.092
1.25	0	.10

The acidified solution was incubated for 24 hrs. at 37°. This time was best since further incubation did not increase the yield of iron, and less time liberated less iron.

After cooling to room temperature 1.25 cc. of 20% CCl₃.COOH are added and the mixture after thorough agitation is allowed to stand 1 hr. at room temperature. It is then filtered or centrifuged. In the latter case the supernatant fluid is drawn up through absorbent cotton placed over the end of the pipette to prevent any precipitate from entering.

The filtrate is then treated exactly as is the filtrate in the serum analysis; Evelyn tubes and the same blank solution are used. The result is multiplied by 10 instead of 2 due to original 1:5 dilution of the blood. Samples of filtrate of less than 2 cc. made up to 2 cc. with blank, may be used if due account is taken in calculations.

Micro-haemoglobin - Bing, F.C. J. Biol. Chem., 95: 387, -1932

Principle.-

The method is based on the pseudoperoxidase activity of haemoglobin.

Purification of Benzidine.-

Dissolve 20 gms. benzidine base in 200 cc. of warm EtOH. Filter off insoluble material. Add 1 gm. blood charcoal extracted with HCl and reactivated (or "Merck's Activated Charcoal -

Suitable for Decolourizing" which does not require extraction with HCl) to filtrate, stir and maintain at 50° - 10 for 15 mins. (electric hot plate or hot water). Filter while warm and wash charcoal with 10 cc. warm alcohol. Repeat charcoal treatment until filtrate is almost colourless.

To filtrate add distilled water, enough to make alcohol 50-60 vol.% and warm to obtain clear solution. Allow to stand in refrigerator 24-48 hrs. to obtain crystalline benzidine. Filter on a Buchner funnel with suction, wash with cold 50% alcohol and continue suction until crystals are dry. Preserve in dark bottle.

Reagent.-

(i) Dissolve 1 gm. in 20 cc. glacial acetic acid (0.5 gm. in 10 cc. was actually employed). Add 30 cc. water and 50 cc. 95% EtOH. to obtain the reagent. Do not decolourize if coloured.

Blank.- Place 2 cc. reagent in colorimeter tube, add 1 cc. distilled water, 1 cc. 0.6% H_2O_2 and mix. No colour should result in two hours. If it is diluted to 25 cc. with (ii) 20% acetic acid, it should be colourless.

Hydrogen Peroxide.- (iii) Commercial 3% peroxide may be diluted. It should be titrated with $KMnO_4$:- Dilute 10 cc. (of 3% H_2O_2) to 100 cc. mix and remove 10 cc. Dilute these 10 cc. to about 300 cc. add 10 cc. of 50% H_2SO_4 and titrate the entire amount with 0.1N $KMnO_4$. of which 1 cc. represents 0.0017 gms.

H_2O_2 . At least 17.6 cc. should be used.

3.161 gms. $KMnO_4$ are required for 1 liter 0.1N solution.

Method.-

Haemoglobin standard - Add .02 cc. blood to 10 cc. water in colorimeter tube and measure the haemoglobin concentration as usual. 1 cc. of the diluted haemoglobin is then diluted to 100 cc. giving a final dilution of 1:50,000. A few cc. of this dilute haemoglobin are placed in a pyrex tube, brought to the boiling-point, and cooled by immersion in water. To 2 cc. of the benzidine reagent in a colorimeter tube add 1 cc. of the cooled liquid, 1 cc. of 0.6% H_2O_2 and mix. Allow to stand for at least 2 hrs. Dilute to 25 cc. with 20% acetic acid and read in Evelyn colorimeter using 10 cc. aperture and filter 540.

The "L" value for the colorimeter tube is plotted against the haemoglobin value obtained originally by the routine colorimetric method. By using several haemoglobin concentrations a curve can be obtained (which is a straight line). This line shifts in slope occasionally (Fig. 15) and therefore a standard must be used with every group of unknowns.

Plasma Haemoglobin.-

The method described above has been used to measure free haemoglobin.

The degree of dilution of the plasma obviously depends on the degree of hemolysis of the blood. The following table may prove useful:-

Appearance	Dilution	If the plasma is diluted
Fresh plasma	1:25	1:50, then the haemoglobin value, as read from the standard curve, must be divided by 1000, and so on.
Older colourless plasma	1:50	
Very faint colour	1:100	
Pink	1:200	
Red-Pink	1:500	
Like red ink	1:5,000	

Bilirubin - Malloy and Evelyn, J. Biol. Chem., 119: 481, -1937.

Principle.-

The method is based on a diazo coupling reaction.

Reagents.-

1. Solution A. 1 gm. sulfanilic acid dissolved in a liter of water containing 15 cc. concentrated HCl.
2. Solution B. 0.5% sod. nitrite.
3. Diazo reagent - is prepared fresh each day by adding 0.3 cc. of solution B to 10 cc. of solution A.
4. Diazo blank solution - 15 cc. concentrated HCl diluted to 1 liter with water.
5. Absolute methyl alcohol.

Procedure.-

Serum or plasma may be used. Fairly accurate results can be obtained even in the presence of moderate degrees of hemolysis. The routine method using 2 cc. plasma is not described here.

Using 0.5 cc. plasma -

The 6 cc. aperture is used first, and the same tubes for direct and indirect reactions.

0.5 cc. plasma is added to 9.5 cc. water in a colorimeter tube and after mixing, 5 cc. of the diluted plasma is transferred to a second colorimeter tube. 1 cc. of diazo blank solution is added to tube 1, and 1 cc. of diazo reagent to tube 2. The contents of the tubes are shaken up and tube 2 is read with filter 540 and the 6 cc. aperture at the end of 30 mins., using tube 1 as blank. As soon as the reading

is made 6 cc. of absolute methyl alcohol are added to each tube, the 10 cc. aperture is replaced, and at the end of 10 mins. tube 2 is read again with tube 1 as blank.

Mgs. of direct bilirubin/100 cc. plasma = D = (100 x L value of tube 2 before alcohol) / 7.0

Mgs. of indirect bilirubin/100 cc. plasma = I = (100 x L value of tube 2 after alcohol) / 3.5

Spectroscopy.-

The Hartridge Reversion Spectroscope was used to detect the presence of methaemoglobin etc., in the blood digests, The instrument was calibrated by finding the readings for absorption and emission lines of known wavelength, and plotting these readings against the actual values (Fig. 12).

Schumm's Test.-

This test has been used to demonstrate hematin in plasma.

The technique used by Fairley follows:-

A given volume of plasma is covered with a layer of ether; one-tenth the volume of concentrated ammonium sulphide is then run in with a pipette and subsequently mixed by shaking.

A positive reaction is indicated by the appearance of a hemo-
X chromogen with a sharply defined band at 558 m μ in a depth up to 4 cm. of plasma.

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