" OBSERVATIONS ON HUMAN HAEMOGLOBIN."

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C O N T E N T S.

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P R E F A C E.

The work described herein has been carried out during the tenure of two clinical posts in the Department of Haematology, Manchester Royal Infirmary (1951 - 53).

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C H A P T E R I.

Introduction to Haemoglobin.

A. Historical Note.

The evolution of haematology as a science owes most to Paul Ehrlich,[62.] who in 1879 classified the blood cells and their specific granules after introducing the aniline dyes to histological work. Since then new facts have been added to this branch of science at an astonishing rate, necessitating constant revision of knowledge previously regarded as fairly settled.

Prior to this, in 1673, the Dutch microscopist, Antony van Leeuwenhock, "the father of microscopy," had carefully studied "the small round globules" which he found in the blood of man and many animals. After describing and drawing the cells, he estimated their size to be 7.5u, established their passage from artery to vein, and ascribed to them the colour of blood. William Hewson,[97.] exactly a hundred years later, again measured the cells and declared that they were "as flat as a guinea" and "must be of great use in the body economy," but it was 1846, before George Gulliver,[72.] who edited and annotated Hewson's work, really established the reliability of Hewson's observations.

The origin of these red corpuscles was the subject of much theorising at this time, and the most popular conception was that of Hewson[97.] who believed that

they were derived from leucocytes. However in 1868, Neumann[153] convinced many that colourless nucleated elements in the bone marrow were the precursors of the red corpuscles.

Karl Vierordt[215] in 1852 made the first blood count by diluting a measured quantity of blood, and counting the corpuscles contained in a certain volume of that dilution. His estimation of corpuscles in male human beings by a most laborious technique was five millions per cubic millimetre. At the same time he devised a spectroscopic method, and Welke developed a colorimetric method for measuring the colouring matter of blood. By 1871, Preyer[180] in the first monograph on haemoglobin was able to refer to several spectroscopic, chemical and colorimetric methods.

The presence of iron in the blood was demonstrated as early as 1747 by Menghini, but even in 1849, Reichert[182] after examining crystals extracted from red blood corpuscles, regarded the colouring matter as haemin or haematin. However two years later, Funke isolated haemoglobin in crystalline form, and in 1864 Hoppe-Seyler[102] clarified the functional significance of the pigment and the cells in which it was carried, by discovering haemoglobin's property of taking up and discharging oxygen, a property since studied extensively.

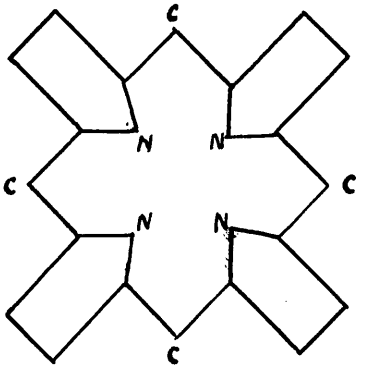
The term, haemoglobin, was only introduced at this time by Hoppe-Seyler, and it was adopted rapidly in favour of terms like haematoglobulin[30.] or haematocrystallin[137.] in common usage. A new term, cruorine,[206.] had been introduced in Britain the very same year, but this has only persisted with reference to invertebrate pigments.

B. Properties of Haemoglobin.

1. Structure: Haemoglobin, the first conjugated protein to be investigated, consists of haem, an iron containing pigment plus globin, a protein of the histone class, and its construction is deemed worthy of summary.

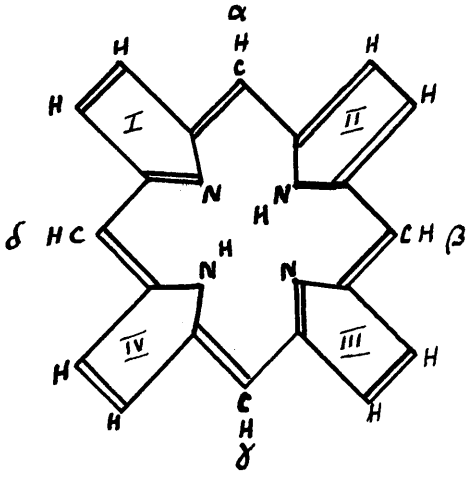
(a) Union of four pyrrole nuclei equals a porphyrin compound (Fig. 1)

Fig. 1.



In all porphyrins there is this linkage, in which the four pyrroles are kept together by four single carbon atoms in the form of a closed planar ring system. This basic conception may be amplified (Fig. 2), to show the stable type of ring system found in nature

FIG 2



The pyrrole nuclei are numbered I to IV as shown; the " substitute" hydrogen atoms are designated by figures 1 to 8, and the methene groups or "carbon atom bridges" by the Greek letters α, β, γ & δ

The porphyrins vary according to the side-chains replacing the substitute hydrogen atoms, thus while the aetioporphyrins contain four methyl and four ethyl groups in positions 1 to 8, the protoporphyrins contain four methyl, two vinyl and two propionic acid groups.

Protoporphyrin IX is found widely dispersed as the precursor of the most common ironporphyrins and haemoproteins in nature. In this protoporphyrin IX (Series III)

the methyl groups are in the positions, 1, 3, 5, 8, the vinyl groups in 2 and 4, and the propionic acid groups in the positions 6 and 7.

(b) Porphyrin plus various metals equal metalloporphyrins - thus protoporphyrin plus iron gives haem, the prosthetic group. Fischer and Zeile[66.] achieved the synthesis of this in 1929. In the iron-porphyrin complexes, one iron atom replaces the two central hydrogen atoms of Fig. 2, but is bound equally to all four nitrogen atoms.

(c) Haem, if containing the iron in the ferous state, plus globin equals haemoglobin.

The linkage between haem and globin has at various times been attributed to peptide, salt or ester linkages formed by the haem carboxyl groups with groups in the globin , or to coordinate linkage from iron to some group in the protein called for lack of any clearer term "haemaffine" groups.[49.] However the ease with which the prosthetic group may be removed or recombined with the protein makes strong chemical linkage most unlikely. The more recent conception is that the iron of the haem is held between two imidazole groups of the globin[222.] with the possibility that electrostatic bonds might also be present between

the ionised carboxyl side chains of the porphyrin and basic $-NH_3^+$ or -OH groups in the protein.

2. Molecular Weight: The minimum size of the haemoglobin molecule was first estimated by establishing the equivalent weight on the basis of the iron atom as 16,700. In 1924, Adair[1,2] by osmotic pressure measurements, correctly assessed the molecular weight as 66,800 (i.e. that the molecule consists of 4 sub units of weight 16,700), and this was soon corroborated by Svedberg[207,208] using an ultracentrifuge technique. Workers have since confirmed these results by other physical methods such as surface tension,[134] diffusion[131] and ultrafiltration[63] measurements.

3. Form of the Molecule: Sir Michael Foster, on the topic of haemoglobin, has been quoted by Prof. J. Barcroft,[20] his eminent successor at Cambride, as saying, "the growth of knowledge may be compared to the ascent of a spiral stair, from which the observer periodically surveys the same landscape, but each time from a higher level than the last."

Perutz has certainly followed this Cambridge tradition in his X-ray studies on the haemoglobin molecule.

His earlier conception of molecular size was 64Å long, 48Å wide and 36Å thick.[169,170]. After further extensive studies with co-workers[37]. on wet and dry crystals, he concluded that the molecule is a circular disc of 57Å diameter and 34Å thickness and that it consists of four equally spaced layers perpendicular to the axis. From earlier work[28]. he had postulated that these four layers represented four layers of polypeptide chains, and that the molecules consisted of two structurally and chemically identical halves with yet a twofold axis of symmetry perpendicular to the cylinder axis. The four haems, it was suggested, were arranged on the surface of the molecule grouped in two identical pairs with their planes parallel to the cylinder axis and the axis of symmetry; each haem united to an identical configuration of the globin, so that they are inherently alike.

Later when Porter and Sanger[179]. showed that haemoglobin consists of six separate polypeptide chains, apart from the possibility of cyclic chains, Perutz[173]. suggested that these chains are folded back in the molecule in a zig-zag fashion.

The next advance was when, after the X-ray work of Bamford et al,[17]. Pauling and Corey[162,163]. recognised

that certain X-ray diffraction patterns could be explained on their basis of the α.helix. Perutz after further work felt bound to accept this and also Crick's[51.] conception that the polypeptide chains may not continue in a straight line for longer than 16Å i.e. one quarter the length of a molecule, before turning a corner. In his most recent communication[174.] he describes Fourier projections already published,[38.] showing 3 inner heavy and 2 light outer layers of chains, each being arranged in approximately hexagonal close packing, with a distance of 10.5A between centres; yet he believes that a considerable fraction of the molecule is taken up by chains turning corners or running in directions other than that normal to the projection. These projections, it is claimed, remain unaltered at the various stages of swelling or shrinking of the crystal although Oncley[156.] has shown how so many conflicting results regarding molecular weight and size have been due to the great difficulty in the accurate estimation of hydration. In wet crystals, the protein molecules have been demonstrated to form rigid and coherent layers which alternate with layers of crystallisation with relative volumes of 47.6:52.4 respectively.

After the fresh approach with Bragg et al,[38.]

Perutz has abandoned his cylindrical conception of the haemoglobin molecule, and now postulates that the hydrated haemoglobin molecule can be represented as an ellipsoid with two short axes of 55Å and a longer axis of 56Å parallel to the direction of the polypeptide chains. Not only does this shape suit the molecules of many species, but the Patterson projections have shown striking similarities in the shape and distribution of peaks for horse, ox and man, which suggests at least a close similarity in the arrangement of the haems and the polypeptide chains. Foetal sheep haemoglobin has been shown not to fit this shape, suggesting that the protein has a different structure.

Workers in this field of study must wonder how much further they have to go to reach the elusive summit of this staircase.

4. Concentration of Haemoglobin within the Erythrocyte: The concentration of haemoglobin within an erythrocyte is approximately 34%, and the pH 7.4. This concentration in many species simply cannot be achieved in vitro. The investigations of Dervichian et al,[57.] and of Perutz[172.] show that the arrangement of the haemoglobin is of freely rotating molecules arranged in a "close packed lattice", the degree of regularity being comparable to metal ions in

a liquid metal. Close packing of the molecules is necessary to achieve the concentration of haemoglobin found; random packing would not suffice.

5. Buffering Property of Haemoglobin: In effect haemoglobin resembles other proteins in its amphoteric nature. Circulating blood has a higher pH than oxyhaemoglobin (isoelectric point 6.7) and haemoglobin (isoelectric point 6.8) and so Hb. behaves as an acid and combines with bases. In common with other weak acids it thus combines, under certain conditions, with strong bases such as sodium and potassium, which provides the blood with a useful ancillary buffering power. It is in accordance with the Donnan equilibrium that the red cells are always more acid than the plasma as haemoglobin acts as an excess of non-diffusible anion; and it is a consequence of this concentration of haemoglobin and its buffering effect that the blood is able to carry so much carbon dioxide. Furthermore it can explain the physiological occurrence of the Hamburger interchange viz. that as carbon dioxide is added to the blood, the plasma bicarbonate increases and the plasma chloride diminishes by passing into the red blood cells.

6. Absorption Spectra: Haemoglobin and its derivatives have characteristic and specific absorption spectra, which are of great value in the study of these pigments qualitatively and quantitatively. Both of these aspects have been employed in the work herein, and so a few points useful for the recognition of various pigments are presented.

Haemoglobin: a diffuse band at 555mu + absorption at 430mu

Oxyhaemoglobin: a strong band at 576-578mu, a weaker band at 540-542mu and absorption at 412-415mu.

Methaemoglobin: a four banded absorption spectrum with a band in the orange red at 630mu which may be detected in the presence of a twenty-fold excess of oxyhaemoglobin.

Ferrohaemochromogen: a strong band at 555-560mu, a weaker band at 528-530mu + absorption at 424mu.

It is the haem groups which are essentially responsible for this band system, and thus for the colour of the haemoglobins. The band which exists in the extreme violet is of special importance, the γ or Soret[201]. band, as this constitutes an essential property of the 16 membered porphyrin ring of conjugated double bonds. The various chemical variations of the haemoglobins give rise to the destructive positions of the bands; thus the oxyhaemoglobin

Soret band is maximal at 414.5mu and that of haemoglobin at 425mu. Changes in the prosthetic portion of haemoglobin are not the only influence on the Soret band as the nature of the protein moiety has some effect also, thus that of human oxyhaemoglobin is at 414.5mu which human oxymyoglobin is at 418mu.

7. Relationship with Oxygen: The outstanding property of haemoglobin is its extraordinary chemical relationship with oxygen, which enables the blood to carry more than 75 times the oxygen possible in simple solution. The combination with oxygen is a true linkage but not an oxidation in the electro-chemical sense, in that the iron in oxyhaemoglobin is in the ferrous state just as in reduced haemoglobin, and this remarkably labile bond with oxygen does not occur if the iron is in the ferric form as in methaemoglobin. Yet the iron in haemoglobin does yield fairly readily to oxidation by various reagents!

Among the more unique features of the binding of haemoglobin and oxygen are then (1) its rapid and reversible union, which is so nicely adjusted in the body that the blood leaves the lungs fully oxygenated yet practically all the oxygen can be released in the tissues (2) that a lowering of oxygen pressure does not result in a total

reduction of the haemoglobin but to a different state of equilibrium, in which there is a smaller amount of oxygen in combination (cf. $CaCO_3$, where a reduction in pressure from 2.7cm. Hg. to 1cm.Hg. would result in all the CO_2 coming off) and (3) how variations in temperature and the presence of electrolytes affect the reaction to the benefit of the body.

A rise in temperature results in haemoglobin retaining less oxygen,[19.] which is clearly of advantage to the warm blooded animal in the oxygenation of its tissues. That the different position of the equilibrium is due to heat accelerating the dissociation of oxyhaemoglobin was studied by Hartridge and Roughton (1923),[79,80.] who found that temperature has practically no effect on the velocity of the reaction $Hb + O_2 = HbO_2$ in dilute solution, whilst it increases the velocity of the reverse reaction $HbO_2 = Hb+O_2$ some 3.8 fold for each rise of $10^{o}C$.

The oxygen dissociation of pure haemoglobin can be expressed by the equation to a rectangular hyperbola;[104.] Bohr[34.] demonstrated however that the dissociation curve for haemoglobin as present in the blood is S-shaped, and that in the presence of salts, haemoglobin gives off its oxygen more readily. The effect of acid is even more

marked than that of salts, and as all cells produce CO_2 in activity, and muscle in particular produces lactic acid, this facilitation of supply of oxygen to the active cells is clearly of great value to the body.

Few of the many factors involved in the respiratory gas exchange have greater theoretical and practical importance than the dissociation curve of oxyhaemoglobin. Standard curves are still the basis for the estimation of blood pO_2, and conversely have been employed in the calculation of venous admixture in the lungs or of the degree to which blood exposed to high alveolar tensions is diluted with less well oxygenated blood from anatomical shunts and poorly ventilated alveoli.[141 & 186.]

Lambertson et al (1952)[132.] have reinvestigated the relationship of oxygen tension and oxygen saturation in the arterial blood of man, as, in spite of this widespread application of oxyhaemoglobin dissociation curves, two variances are in common use.[33,76.] The techniques used brought the results of spectrophotometric and gasometric studies into close alignment, proved the constancy of dissociation characteristics in the arterial blood of 15 men, and vindicated the early in vitro results of Bock, Field and Adair (1924).[33.]

Phase Rule: Early workers on the phenomenon of the oxygen combining capacity of haemoglobin were baffled somewhat by the non-observance of the "Rules" of the day; for example the Phase Rule as formulated by William Gibbs[69]. could not be applied, viz. the system should behave like the $CaCO_3$ system, $CaCO_3 \longrightarrow CaO + CO_2$ with one degree of freedom only. However experimental work showed the oxyhaemoglobin system to be bivariant, and that even on varying temperature and oxygen pressure, equilibrium could be achieved. It was assumed that the rule might not apply to the case of microheterogenous systems and that there might be a region in which transitional states between simple surface adsorption and true chemical combination were to be met.

Surface Adsorption: Wolfgang Ostwald (1908)[158]. suggested that oxygen might be taken up by haemoglobin, as gases are adsorbed by surfaces such as charcoal, but other observers at that time felt that it was impossible to reconcile the constant oxygen saturation level of haemoglobin with anything but a chemical compound as the final result. Barcroft (1914)[19]. closed this chapter by taking pure haemoglobin in solution, regarding the oxygen dissolved under various pressures as its concentration (Henry's Law), and finding that the relative amounts of haemoglobin and

oxyhaemoglobin under a given oxygen pressure were in accordance with the Law of Mass Action, with the curve a rectangular hyperbola. Under the hypothesis of adsorption a parabolic curve would have been expected.

Hufner Equation: Hufner[104.] had earlier attempted to describe the dissociation curve by an equation also expressing the equilibrium in terms of the Law of Mass Action, after satisfying himself that one molecule of oxygen combined with one atom of iron:

$$K = \frac{HbO_2}{(Hb)(O_2)} \quad \text{from which} \quad y = \frac{K_p}{1 + K_p} \quad \text{may be}$$

derived where p is the partial pressure of oxygen and K, the equilibrium constant. This equation, however, was quickly found wanting in blood, although it has been found to describe the gaseous equilibria of myoglobin.[211,99.]

Hill's Equation: Seven years later, 1910, A.V.Hill[98.] put forward the theory that the unit containing one atom of iron was capable of polymerisation to a rather indefinite size. This theory is not now held, as it is incompatible with kinetic data but the equation is still widely used, since it is convenient and formally related to the more modern theories.

Hill assumed that the reaction could be described by the equation $K = \frac{(HbnO_{2n})}{(Hb_n)(O_2)^n}$ from which the equation $y = \frac{K_p^n}{1 + K_p^n}$ may be obtained when n is the average number of molecules in the polymer.

Thus if n = 1, the equation is identical with that of Hufner. When "n" is more than one, there is interaction between at least two haems, such that the oxygenation of one is closely associated with the oxygenation of the other, and the y vs p. plot is S shaped. This equation is more commonly used logarithmically when it becomes $- \log K = n \log (O_2) + \log \frac{(Hb_n)}{(Hb_nO_{2n}}$ and values for n and K may be obtained from a plot of the values $\log \frac{(Hbn)}{(Hb_nO_{2n})}$ against $\log p\ O_2$. For the dissociation of oxyhaemoglobin in blood n = 2.5 and between 10% and 90% saturation, a very good fit can be obtained thus. These equations, so far, have been valuable by facilitating comparison of results by different workers, which has accelerated the discovery and fuller appreciation of other facts such as the effects of salt concentration.

Adair's Equation: The above two equations differ then in the mean size of the unit assumed to be present in solution; Hufner assumed a molecular weight of 16,800, while Hill suggested that the most frequently found particle contained between 2 and 3 of these units. Adair,[3.] having shown that the molecule contained indeed 4 molecules, formulated a new elaborate equation postulating the existence of intermediates between non-oxygenation, Hb_4, and full oxygenation, $Hb_4(O_2)_4$, viz. Hb_4O_2, $Hb_4(O_2)_2$, $Hb_4(O_2)_3$, the reaction proceeding stepwise with the affinity of the molecule for oxygen varying according to the stage of oxygenation.

Pauling's Equation: Pauling (1935)[159.] reinvestigated this work and by assuming that the haems lay at the corners of a square, he produced a more advanced and yet no more difficult equation which has since been compared with and related to Hill's equation by Coryell,[45.] who has thus facilitated the application of haem-haem interaction to a number of systems which have generally been described in terms of the latter equation.

Haem-Haem Interaction: It is thus purported that the S-shaped curve representing the oxygenation of haemoglobin is explained on the basis of facilitating interactions between

oxygen combining centres, haems, which are four in number, all identical and believed to be bound in the same manner to the protein. When a molecule of oxygen is taken up by one haem, this interaction leads to an increase in the likelihood of the oxygenation of a second haem and so on. The mechanism of such an interaction is far from clear, but various theories have been put forward.

One of these is that the interaction might have a steric origin viz. a steric hindrance possibly by imidazole groups. It is postulated[160,204] that the haem groups are buried within a haemoglobin molecule in such a way that a pushing apart of the molecule must occur to make space for the apposition of the diatomic oxygen molecule to the iron atom of a haem. The loosening of the structure caused by the conversion of the first haem to oxyhaem, makes the apposition of a second molecule less difficult, each addition of oxygen further decreasing the steric hindrance.

Another suggestion, closely related to the first, is that of Wyman,[224] who tentatively explains the reaction on the basis of entropy. He quotes X-ray diffraction studies to show that while horse oxyhaemoglobin shows an ordered pattern with an axis of symmetry, there is no

evidence of such an axis of symmetry in the reduced pigment. (In human haemoglobin no such axis of symmetry has been demonstrated in either form). It is suggested that as the first oxygen molecule enters a pair of haems, there is some kind of ordering of the haemoglobin molecule leading to a more regular arrangement in space such that, when a second molecule of oxygen enters, little further change of configuration is necessitated. His explanation for the difficulty of entry for the oxygen molecule is the entropy barrier involved in this ordering process. A similar but smaller effect serves to explain the interaction between haems of different pairs.

That the problem is not yet solved is evidenced by Rigg's recent observations,[184,185] which indicate that the sulphydryl (-SH) groups of haemoglobin are closely linked with the mechanism of haem-haem interaction in the oxygenation process. Free -SH groups are known to occur in human haemoglobin[105,107] and the blocking of these groups resulted in a decrease in the interaction between the haems; counteracted however if glutathione be added. Dialysis against a slightly alkaline buffer resulted in loss of interaction, again reversed by the addition of glutathione. No such loss of interaction was observed when

haemoglobin was stored without dialysis at the same pH and temperature. It thus appears that the sulphydryl groups of globin play a part in the mechanism of haem-haem interaction.

Much work in this field of study remains.

Relationship with Carbon Dioxide: Far back in 1837, it was shown by Magnus[145.] that the blood passing to the lungs contained more carbon dioxide and less oxygen than that passing away from the lungs and from the days of Bohr[35.] and Henriques,[19.] it was suspected that haemoglobin was capable of combining with carbon dioxide; one of the suggestions was that oxygen combined with the haem part of the molecule while carbon dioxide combined with free amino groups in the globin portion. Roughton[190.] felt that he had confirmed this anticipation and claimed that haemoglobin was responsible for 30% of the carbon dioxide transportation in the blood. Wyman[223.] in a recent review conceded that carbon dioxide was indeed carried by haemoglobin but suggested that 10% was a more accurate estimate of this combination, and that 90% of the carbon dioxide was carried as bicarbonate.

C. Distribution and Variation in Nature:

If haemoglobin is defined in this connection as a class of ferroporphyrin-protein compounds able to combine reversibly with oxygen without oxidation of the iron to the ferric state, it is found to be widely distributed in the animal kingdom. It has been demonstrated in as low a phylum as protozoa, in the cytoplasm of ciliate paramecia[192.] and becomes of greater importance with more highly evolved phyla, where it presents as myoglobin in the muscle in addition to haemoglobin in the red cell. These are the forms of presentation in the vertebrate species, but two other distinctive forms are found in invertebrates, chlorocruorin and crythrocruorin.

Milne-Edwards in 1839,[146.] first drew attention to the green blood of certain polychaetes, but it was Lankester,[133.] who named this pigment chlorocruorin, and determined that while it was green in dilute solution it was reddish when more concentrated, that the oxy-body has two absorption bands (much more to the red end of the spectrum than oxyhaemoglobin) and the reduced body but one. Fox[67.] showed that the main difference from haemoglobin was the porphyrin foundation, which is a type IX porphyrin with one vinyl group replaced by formyl.[65.] Chlorocruorin has

been the subject of study in many marine worms, and in all it is found in simple solution and not in corpuscles. The pigment of the Spirographis is the most completely studied, and it is known to have a molecular weight of about 3 million, to contain approximately 190 haems[209]. and to have 1.2% iron content in each molecule which amounts to four times that in haemoglobin.[68].

Erythrocruorin, resembles haemoglobin in that it has a prosthetic group of protoporphyrin[191]. but has a wide distribution in both plasma and cells. Great variances are encountered in its molecular weight and protein content with the species, and even within the species; it occurs amongst invertebrates without any seeming relation to evolution, worms, arthropods, insects, molluscs, etc.; and indeed two distinct erythrocruorins have been found to exist in the parasitic worm, Ascaris, which contains one in the body wall and another in the peri-enteric fluid.[209,123]. As a class the erythrocruorins have more acid iso-electric points than the haemoglobins, viz. 4.5-6,[167]. and the vast majority fall into the extracellular category with molecular weights perhaps even of a million or more.

This aggregation of the extracellular pigments

into large particles would appear to be another evidence of Nature's wisdom, allowing an increased oxygen capacity without incurring undesirably high osmotic pressures, which would obtain with a high concentration of smaller particles. The evolution of the erythrocyte with its high specific permeability allowing high oxygen capacity, and but slight interference with the osmotic pressure of the circulation, has led to the disappearance of respiratory proteins of large molecular weight.

In the case of animals containing haemoglobin in cells, evolution has on tne whole turned to the development of a great variety of respiratory organs for the more active animals, rather than modification of the respiratory pigment; while the haemoglobins of the various vertebrate species differ considerably in their protein composition,[214.] the individual variations are much less than among say the annelid erythrocruorins.[205.] Thus the haemoglobins of fish, birds and mammals all have almost identical molecular weights, contain four haems permolecule and have an iron content of approximately 0.35%. Nevertheless differences in the protein moiety do exist and serve to explain variations in solubility, crystalline structure and reactions to diverse denaturing agents.

There have been many claims in the past that the haemoglobins of different individuals of the same species may show variation. Oxygen affinity has oft been used as the basis for such assertions, but most recent results using more accurate techniques tend to deny their validity.[132.] Valer[213.] has claimed that the content of sulphur is not constant in either dogs or horses, while Belassa[27.] has carried this further in human beings to assert that each haemoglobin molecule in individuals of Blood Group A contains 14 sulphur atoms, while in those of Blood Group O only 13 sulphur atoms are detected.

Brinkman and Jonxis[40.] analysed graphically their experiments on alkali denaturation, and postulated the occurrence of 3 forms of human haemoglobin, a foetal resistant form which was replaced entirely by a less resistant adult form at 7 months and a third haemoglobin of an intermediate resistance, appearing at 3 years of age approximately, and remaining present in adult life. This latter pigment was said to vary considerably from day to day in the one individual and to have a distinctly lower iso-electric point (pH6.3), when estimated by the spreading technique.

Roche, over the years, has been interested in

this aspect, and recent solubility studies in phosphate media, mainly at pH 5.5, carried out in collaboration with Derrien[56.] on carboxyhaemoglobin, have led them to postulate the presence of five human haemoglobins which may be found in varying proportions in the newborn with three distinct forms specifically adult.

Schenck[193.] in 1930, declared that the globin of adults has no constant composition, which might appear very likely from the reports above, but it has been considered that his experimental data as presented was not entirely convincing.

It is obvious that fresh approaches with more accurate techniques are a vital necessity for some advance and agreement in this field of research.

This short discussion on the distribution and variation of haemoglobin in Nature has, so far, centred round the animal kingdom, but Kubo[129.] in 1939 reported a haemoglobin-like compound in root nodules, subsequently confirmed by Burris and Haas.[44.] Further research was carried out on this by Virtanen,[216,217.] who suggested a correlation between the content of haemoglobin in the root nodules and their ability to fix nitrogen, but Keilin and Smith[125.] have been unable to confirm his results.

D. Ontogenetic Variation.

The haemoglobin of the foetus has been found to differ distinctly from the haemoglobin of the adult, not only in human beings, but also in other mammalian species including the goat,[151,74.] the sheep,[22,122.] the cow[23.] and probably also the rabbit[40.] and the chicken.[74.] In human beings, a small percentage of adult haemoglobin is found in early premature babies, even at 20 weeks foetal age, and by 6 months - 24 months of age it has completely replaced the foetal form. Brinkman and Jonxis[41.] believed that this replacement occurred at 6 - 7 months, but newer techniques suggest that the foetal form may be present up to 24 months.[46.] In spite of the differences, now to be enumerated the molecular weight of the two forms is yet identical.[152.]

Alkali Resistance: Foetal haemoglobin exhibits a much greater resistance towards alkaline denaturation than does adult haemoglobin, and this very important distinction between the two forms was first described by Korber (1866)[126.] who worked with whole blood, adult and human placental. Using more or less pure solutions, this phenomenon was studied subsequently by von Kruger,[127,128.] by Wakulenko[218.] using umbilical cord blood, and in more detail by Bischoff,[32.]

Haurowitz[83,84.] and Brinkman and co-workers.[42.] More recently Singer and co-workers[199.] have evolved a technique, more straightforward and more accurate than the earlier methods, for the quantitative estimation of the two forms making use of this phenomenon and this procedure will be described below.

Considerable study has demonstrated that the two types of haemoglobin differ in many other properties but none of these are so pronounced as this original observation of the speed of denaturation by alkali, although the mechanism of this reaction is not yet understood.

Oxygen and Carbon dioxide Relationship: Foetal blood releases carbon dioxide more readily than the adult blood at all gas tensions,[61,138.] and this is obviously vital for placental interchange; equally helpful is the property of the foetal form at low gas tensions viz. 25 - 60mm. Hg., of absorbing oxygen more effectively than maternal blood. Early workers[61.] suggested that the differences could be explained by the laws governing the diffusion of these gases through a wet membrane, but Darling and co-workers[52.] concluded that "if the cell membrane of the foetal red cell has the same characteristics of permeability as the adult cell, then the foetal haemoglobin has different properties."

They had studied 2 premature and 8 full term infants and felt that the differences in salt concentration also were too insignificant to explain the shift in the foetal oxygen dissociation curve, which had been demonstrated to lie above that of the mother (i.e. to the left) in the human species by Hazelhurst and Stromberger,[81,82] and Liebson and co-workers.[138] The "n" of the Hill equation was noted to be lower for the foetal haemoglobin within the erythrocyte. McCarthy[151] followed by examining stroma free dialysed haemoglobins from maternal and foetal blood of the goat, and proved by gasometric analysis that the difference in the dissociation curves was a property of the haemoglobins. An interesting finding by Hill and Wolvekamp[100] has been that in weak solution foetal haemoglobin has actually a lower affinity for oxygen than the adult!

Solubility: Jope and O'Brien[119] have shown the adult carboxyhaemoglobin, oxyhaemoglobin and methaemoglobin in 2M. phosphate buffer pH6.7 have solubility-temperature curves with a form often shown by proteins in strong salt solutions, while those of the respective foetal pigments are in the same media more characteristic of proteins in dilute salt solutions. Both adult and foetal reduced

haemoglobin have very similar solubilities in this solvent, unaffected by temperature. Working with bovine blood, Wyman[225.] demonstrated that in strong phosphate buffers pH6.8, foetal carboxyhaemoglobin was more than six times as soluble as that of the adult.

Crystalline Form: It would appear from the diversity of accounts that the haemoglobins may crystallise out in varying forms according to the technique used, viz. Drabkin[59.] described tetragonal crystals of adult carboxy-, oxy- and met-haemoglobin, while Perutz[171.] and Jope and O'Brien[119.] have described them as orthorhombic. In a more recent publication Perutz describes the oxyhaemoglobin crystal as monoclinic.

In 1930, Perrier and Janelli[168.] first described a difference in the crystalline structure of neonatal and adult haemoglobin, and recently Jope and O'Brien[119.] drew up the following table to summarise the conclusions following their studies of the pigments by direct crystallisation and photomicrogaphy.

Table I.

Pigment.	Adult Crystal Form.	Foetal Crystal Form.
Carboxyhaemoglobin	Orthorhombic	Triclinic(or monoclinic) system still to be determined
Oxyhaemoglobin	"	"
Methaemoglobin	"	"
Reduced haemoglobin	Monoclinic and another form whose system is still to be determined.	Different from above three, possibly monoclinic.

Surface Studies: Jonxis[113,114.] has reported an interesting distinction between adult and foetal haemoglobin when spread in a mono-molecular layer on the surface of water in a trough. When the pH of the water is approximately at the iso-electric point of the haemoglobin (buffer solution = 3 millimolar concentration) the adult haemoglobin rapidly forms a stable mono-molecular layer with a thickness of about 8Å. Foetal haemoglobin however unfolds itself more slowly and takes about 10 minutes to spread completely into a stable layer. Brinkman and Jonxis[40.] using surface pressure curves in dilute buffer solutions at various hydrogen ion concentrations were able to show that the iso-

electric points were not identical; that of the foetal form was at pH6.7 and that of the adult at pH6.9.

Electrophoresis: Andersch, Wilson and Menton[4.] conducted an experiment into the electrophoretic mobility and the ultracentrifugal sedimentation constant of the two varieties of haemoglobin, testing specimens from infants and adults; significant differences were apparent although the series was small. From their data, they estimated that foetal haemoglobin co-existed with adult haemoglobin in 5, 9 and 90 day infants with a predominance of the former. Hoch[73.] repeated the electrophoretic studies under identical buffer conditions and his results were not so distinctive, although he still showed that between pH 7 and pH 8 human adult oxy-haemoglobin moves slightly faster than human foetal oxy-haemoglobin, and demonstrated that under conditions of large boundary anomalies, this could be used as a quantitative measure of the two pigments.

Amino-acid Composition: In 1930, Schenck purported to show that in the human there was a decrease in certain amino acids with age and produced an "age curve" of the arginine content of haemoglobin. Schenck also stated that these chemical differences between human globins corresponded to their different resistances to pepsin and hydrochloric acid.

Porter and Sanger (1948)[179.] investigated several sources of haemoglobin using the technique of partition-paper chromatography, and it was evident that there was considerable species differences both in the nature of the N-terminal residues and presumably in the number of open polypeptide chains. Assuming a constant molecular weight, human cord haemoglobin was estimated to have 2.6 terminal valyl residues as compared to a fixed number of 5 valyl residues in the adult form. If cord haemoglobin is presumed to contain 80% foetal and 20% adult haemoglobin, this would give a figure of 2 valyl residues for foetal haemoglobin. It was also noted that there were about 47 free-lysine E amino groups in foetal haemoglobin and only about 43 such groups in the adult.

Brand and Grantham (1946)[39.] noted related differences in a microbiological assay on bovine haemoglobin; the foetal form contained more isoleucine and less methionine and histidine than the adult form. Van der Linden (1949)[213.] introduced another microbiological method based on the need of lactic acid bacilli for certain essential amino-acids, which also appears to demonstrate differences in amino-acid composition.

Serological Differentiation: In the early years of this century, French workers[55,106,136.] were preparing precipitins from the serum of animals following injection of another type of haemoglobin. Heidelberger and Landsteiner (1923)[88.] demonstrated complement-fixing antibodies and anaphylactic antibodies to the haemoglobin injected. Hektoen and Schulhof[92.] and later Johns and Bradley confirmed that the specific antigenicity of the haemoglobin resides in the protein fraction, globin.

Darrow and his co-workers[53.] used this property of antibody formation to distinguish between human foetal and adult haemoglobin. Antisera was prepared from rabbits with human cord blood, and this showed positive precipitation reactions with both the adult and cord bloods. After full absorption with adult haemoglobin further precipitation occurred on adding cord haemoglobin, yet with another sample after full absorption with cord haemoglobin, no further precipitation was obtained with the adult. Antisera prepared with adult blood showed no further precipitation with either adult or cord haemoglobin after absorption with either haemoglobin. The authors concluded that in the human species, cord blood contains a mixture of 2 different and distinct haemoglobins, one of

which resembles the adult form, while in adult blood there was no evidence of the presence of foetal haemoglobin.

Spectral Absorption: Jungbloed[120.] believed that no distinction existed between adult and foetal haemoglobin, but Jope[118.] recently reinvestigated both the visible and ultraviolet absorption spectra of both forms and found that there was no variance in the α, β or γ (Soret), bands in common with all mammalian species i.e. the haems were indistinguishable. However the ultraviolet spectrum absorption for which the amino-acids are responsible produced an interesting distinguishing feature. By the use of a moving plate technique it was established that the tryptophane absorption band is found at 291mu in adult human haemoglobin but at 289.8mu in foetal haemoglobin. This distinction can be determined both in solution and in the intact red cells, which would appear to be of great value for cytological research. In the adult haemoglobins of several species the peak was in the same position as for the human adult, but the distinction between adult and foetal haemoglobin was not obtained with sheep or rat blood.

Further investigations by Beavan et al (1951)[26.] revealed that in human foetal haemoglobin the tryptophane longwave fine-structure feature is a resolved maximum,

while in the adult type it is an unresolved inflection. Comparison with parallel electrophoretic studies showed that this wavelength distinction could be utilised for at least approximate measurement of the proportions of the two pigments, - as with mixtures the peak was in an intervening position relative to the concentrations. The peaks of various standardised mixtures of the two pigments were estimated, and a graph between 291mu and 289.8mu was constructed. Over the composition range 0 - 50% adult type, the curve is relatively flat and of no practical value for quantitative measurement but between 50 - 100% adult haemoglobin, the experimental precision was within 10%.

Thus, in spite of a variety of differences between adult and foetal haemoglobin only 3 types of techniques are available for quantitative measurement. Two measure only large differences, the electrophoretic procedure and the ultraviolet absorption procedure. The third, however, has been improved over the years, and now constitutes a fairly accurate method viz. the alkaline denaturation technique of Singer et al.[199].

Preamble to Experimental Work.

Interest was aroused in this subject of haemoglobin after perusal of "Haemoglobin", a symposium of the contributions to the Conference at Cambridge to commemorate the old Master, Sir Joseph Barcroft; and this coincided happily with the reading of the work of Singer et al (1951) on foetal haemoglobin in sickle cell anaemia and other haematological conditions. The alkaline denaturation technique was something of a mystery. What is denaturation? What other agents result in denaturation of haemoglobin?

Gradually from these considerations a project took shape:

1). To compare the behaviour of adult and foetal haemoglobin in the presence of various denaturing agents;

2). To discover whether the work of Jonxis reported in "Haemoglobin" correlating foetal haemoglobin and erythroblastis foetalis was indeed valid ; and

3). To investigate the possible presence of foetal haemoglobin in the various adult haematological conditions common in this country.

These, therefore, constitute the objects of this investigation.

CHAPTER II.

A. Reaction of Adult and Foetal Haemoglobin to Denaturing Agents.

The term, denaturation, has been used loosely for many years, and Bancroft et Rutzler[18.] in 1931 presented an amusing review of the current conceptions on denaturation which concluded succinctly "the dogma of denaturation is deleted." Nevertheless the term is still in current use. No pithy or practical definition has covered all aspects without embracing other reactions, and recently the term has been reserved for a characteristic group of changes in observed properties, as distinct from a chemical reaction like the combination of native protein with a salt.

Neurath et al (1944) in their review[154.] provide a useful guide: "denaturation is any non-proteolytic modification of the unique structure of a native globin, giving rise to definite changes in chemical, physical or biological properties." Lemberg and Legge (1949)[139.] define it theoretically as "a change in the structure of a native protein without breaking the chain itself." This allows various degrees of denaturation from the disarrangement of a few amino acids to a complete unfolding of the chain. Attempts have been made to add more preciseness to the term

by separating the preliminary reactions or "activation", i.e. the breaking of hydrogen bonds or salt linkages between sidechains which precede and facilitate the disarrangement of the peptide chain, under terms like "perturbation" (Holden[101].). This idea however has not found general favour.

A precise description of the fundamental changes in denaturation is at present beyond the realms of our knowledge, as a prerequisite would be an exact analysis of the dislocation and relocation of the affected atoms and chemical groups. Nevertheless certain measurable changes in properties do exist, which are characteristic. Any of these changes may not be found in a particular denaturation, and furthermore certain of them may occur without denaturation in either chemical or physical changes e.g. in the dissociation of proteins into smaller units as with the erythrocruorins of Planorbis in acid solution. It is therefore perilous to place reliance on any single one of the criteria now to be discussed.

1. Decrease in Solubility:

An early recognised feature of denaturation was the decrease in solubility, either close to the iso-electric point, or perhaps on addition of a small amount of neutral

salts, which would normally have no effect on the native protein. Such was the constancy of this observation that Wu (1931)[221.] was led to express the view that "denaturation is a change in the natural protein whereby it becomes insoluble in solvents in which it was previously soluble." Further work has tended to confirm this view,[155.] but in certain instances this change is very slight if present at all e.g. in the tomato bushy stunt virus.[24.] That there should be always a decrease in solubility accompanying minor degrees of denaturation, and not involving gross changes in the internal structure of the molecule, is a finding not yet within understanding.

2. Loss of Crystallisability:

Although many native proteins are known in crystalline forms, crystallisation of a fully denatured protein has not been achieved from solution. On "reversal of denaturation" this crystallising ability may be regained, although the specific molecular configuration may not be regained and denaturation still exist. Mirsky[147.] has reported a very interesting finding, that various proteins crystals (e.g. horse serum albumen) on heat denaturation may retain their crystallin form, but from a solution of these denatured crystals, no crystallisation can be effected.

3. Changes of Molecular Size and Shape:

The specific size and shape of the native protein are of course liable to be grossly altered on denaturation, and thus in many instances these changes produce a measurable variation in the molar frictional ratio, viscosity and diffusion measurements etc. These results led, many years ago, to the concept that denaturation of haemoglobin led to the conversion of the closed polypeptide chain into an extended chain, and more recent work, including X-ray diffraction studies, has resulted in this view becoming well established. These studies are not so advanced as to enable a definition of denaturation using the molecular changes as criteria, but steady progress is being made.

4. Loss of Biological Activity:

This feature of denaturation is most marked perhaps with haemoglobin and enzymes. Denatured haemoglobin is unable to combine reversibly with oxygen and this may be measured by gasometric analysis. Bawden and Pirie suggest that this feature may reveal denaturation in the absence of other criteria.[24,25]. On the other hand, it has been observed that there was no loss of specific combination between certain antibodies and their specific

antigen following denaturation of the former by guanidene hydrochloride[64.] or surface forces.[189.]

5. Alterations in the Accessibility of Certain Groups:

Heffter (1907)[87.] and Arnold (1911)[12.] noted that in contrast to the negative reaction of the native egg albumen, the denatured form gives the nitroprusside colour test for SH groups. Since then it has been observed that after denaturation, disulphide or phenolic groups may be detected, where either they were not apparent or only to a small extent. This alteration in accessibility has been most fully studied with regard to SH groups, and many theories have been advanced in explanation from that of Linderstrom-Lang[142.] and his "thiazolidene" compound theory (a compound never proved to exist in protein), to the "accessibility theory" (Mirsky and Pauling (1936))[148.] which suggested that those groups which are inaccessible in the centre of the complex molecule become accessible on the opening up of the chains. Crammer and Neuberger (1943)[50.] pointed out that it is unlikely that the small hydroxyl ions find the interior of a native protein inaccessible, while it is known that larger molecules may enter within, viz. only 20% of the tyrosine groups of native egg albumen were found to react with the small hydroxyl

ions at pH 12, while Herriott[95,96] under similar conditions had found that 60% of the tyrosine groups reacted with the Phenol Reagent which has a molecular weight of about 41,000.[220] This has led up to the "bonding theory" which is propounded wittingly by Anson[9] as pure theory with no experimental basis. It suggests that the breaking of the non-peptide bonds between polypeptide chains may result in a general change in the reactivity of the protein groups quite apart from making them more accessible.

This phenomenon of alteration in accessibility has been of considerable value in determining and comparing the extent of denaturation under different conditions as many rapid and useful quantitative methods have been evolved, particularly for sulphydryl groups.[71,7,8,90]

6. Susceptibility to Proteolytic Enzymes:

Proteolytic enzymes attack the peptide bonds, and thus are more effective in proteolysis when the polypeptide rings are extended and more vulnerable. Thus trypsin attacks denatured haemoglobin at a speed approximately forty times as rapidly as it attacks native haemoglobin.[143] Similarly urea denatured serum albumen is much more susceptible to trypsin than the native protein.[29] The inference must therefore be that denaturation increases the suscepti-

bility to enzymatic proteolysis.

7. Electrochemical Changes:

In the case of denatured protein the electrophoretic mobility curve runs parallel to that of the native protein, but to the alkaline side.[167,150,88] Mirsky and Pauling[148] have attempted to explain this phenomenon by the liberation of paired amino and carboxyl groups, which should shift the iso-electric point to neutrality and might account for the very small move of pH (0.4 - 0.5) which does occur. However, they do not feel sure enough to refute the view that the gross change in molecular structure might in itself account for a change in amphoteric properties.

8. Absorption Spectra:

The change in spectrum resulting from denaturation of haemoglobin is the basis of a method used to estimate both rate and occurrence of denaturation, and this is utilised in work reported herein. The spectrum of haem is altered by the combination with native globin, and this latter spectrum is altered when the globin is denatured. Thus not only may observations be made on the disappearance of the specific absorption bands of the native protein, but attention may be directed to the appearance of the specific absorption bands of the denatured protein complex

(e.g. ferrihaemochromogen).

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A summary of known differences between adult and foetal haemoglobin has already been presented, and it is surprising that while the most striking distinction is in their reaction to denaturation by alkalis, apart from studies on reaction to acid[130]. where both pigments behaved similarly, no further comparative studies on denaturation were observed in the literature, although many agents are known to denature adult haemoglobin. Accordingly it was decided to submit adult and foetal haemoglobin to the action of a variety of agents known to denature adult haemoglobin; at the same time maintaining the thus denatured protein in solution. In accordance with the declaration above that one change viz. spectral absorption is not evidence of denaturation, each reaction was submitted to a second text, viz. evidence of haemochromogen production.

Urea:

Spiro[202]. in 1900 was the first to report the solvent action of urea solutions for protein, and some 30 years later, Anson and Mirsky[10]. and Burk and Greenberg[43]. reinvestigated this phenomenon, each using horse haemoglobin

and other proteins. These experiments led to the belief that the action of urea on haemoglobin resulted in an increase in solubility, the dissociation of the haemoglobin into smaller molecules,and finally denaturation of the globin component. Hands[75.] contended that these results were fallacious in that the viscosity measurements did not take account of the hydration of the haemoglobin molecule but subsequent workers on horse[13.] and ox[103.] haemoglobin confirmed the earlier work by osmotic pressure measurements. Taylor and Hastings[210.] reported an interesting confirmation of the splitting of the molecules of haemoglobin: that in the presence of urea a greater amount of oxygen is combined at a given oxygen tension than in its absence and the "n" of the Hill equation = 1.9.

The effect of varying concentrations of urea on horse haemoglobin was then studied extensively by Steinhardt (1938),[203.] who found, when working with horse methaemoglobin formed by the action of potassium ferricyanide, that within 20 hours at room temperature, the characteristic spectral absorption band in the red (630mu) fades, and is replaced by a diffuse band in the green (at approximately 534mu). It was concluded that the latter band was not due to ferrihaemochromogen since, on treatment

with sodium hyposulphite the typical absorption spectrum of ferrohaemochromogen did not appear.

Salicylate:

The denaturing effect of sodium salicylate on bovine methaemoglobin was examined in detail by Anson and Mirsky.[10,11] They measured the increase in the spectral absorption in the green, and found that when salicylate was added to native methaemoglobin, the absorption in the green at first increased rapidly, then remained constant, and finally increased again very slowly. The concentration of salicylate in their experiments varied from 0.05M to 0.5M, and they observed that with increasing concentration of salicylate, a greater proportion of denatured protein was produced and the speed of the reaction was also increased. Using 0.5M salicylate solutions, denaturation was complete in 30 minutes.

Formamide:

That formamide acted similarly but not so effectively on haemoglobin as does urea was referred to incidentally by Burk and Greenberg[43] in 1930 and studied in more detail by Steinhardt (1938).[203] The latter provided experimental evidence that formamide, when present in high concentration, also dissociates methaemoglobin into half

molecules, causes the characteristic band in the red (630mu) to fade and a diffuse band in the green to appear.

Cephalin:

While investigating differences between lecithin and cephalin Chargaff, Ziff and Hogg[47]. discovered the interesting phenomenon of the rapid disappearance of the specific absorption bands of human oxyhaemoglobin and canine carboxyhaemoglobin on treatment with neutral emulsions of purified cephalin. On the evidence that the subsequent addition of ammonium sulphide produced a typical spectrum of ferrohaemochromogen, although sodium hyposulphite did not, these workers did not assume that true denaturation had occurred, although rather taken to task by Lemberg and Legge[139]. on this point. They assumed rather that the effect was: oxyhaemoglobin + cephalin → cephalin-globin + haematin, and suggested that the reason for reduced haemoglobin not being thus affected might be that the iron is bound to the porphyrins by covalent bonds in oxyhaemoglobin and carboxyhaemoglobin, whereas ionic forces effect the linkage in haemoglobin, as postulated by Pauling and Coryell.

Detergents and Bile Salts:

Once again Anson appears to have been the first to exploit the denaturing effect of the synthetic detergents and bile salts[5,6]; although workers have since investigated solubility,[181.] dissociation[194.] and electrophoretic[144.] aspects of the former on a variety of proteins. Anson discusses detergents and bile salts together in view of their generally similar lyophobic-lyophilic structure, each consisting of a large lyophobic part with a small lyophilic part attached to it. An amazing selection of synthetic detergents are available commercially, but the only available preparation regarding purity solubility and suitability for spectrophotometric observations was for us a sodium dodecyl benzene sulphonate (NANSA marketed by Marchon Bros.) - a preparation comparable to the American "Nacconal". In his reports Anson states that the detergents act equally on oxyhaemoglobin and methaemoglobin, and presented as additional proof of denaturation a decrease in solubility with weak ammonium sulphate solutions and an increased susceptibility to tryptic digestion. For measurement of the denaturation of methaemoglobin, he followed the decrease of absorption in the red (630mu) and the increase of the absorption in the green which took place

during the reaction. Similar studies were made on the bile salts, sodium glycholate and sodium taurocholate, although the latter had a low degree of purity (40%).

From his experiments Anson had three distinct impressions; that it was most remarkable how little detergent or bile salt was necessary to procure denaturation as compared to previously observed denaturing agents; that in view of the great chemical multiplicity of detergents and bile salts, the denaturing process can only be presumed to be due to the solitary common property, the lyophobic-lyophilic structure; and that the striking similarity in the action of the synthetic detergents and the bile salts might explain the physiological importance of the latter; perhaps they are simply biological detergent molecules whose specific structures are of secondary importance.

Alcohol:

Alcohol has been known to denature protein for many years,[78.] but apart from the work of Booth (1930)[36.] little investigation has been carried out into this process. Booth observed the speed of the reaction with varying amounts of alcohol, noting that the pH always remained constant in any one reaction, and also estimated the effect

of temperature and pH changes by measurement of the unimolecular velocity constants throughout. These constants were found to increase in magnitude with increasing alcohol content under otherwise similar conditions.

Acetone:

This organic solvent is a known denaturing agent, but the earliest reference found was in the review by Neurath et al (1944)[154]. where it is bracketted with alcohol. No experimental work can be quoted in spite of considerable delving into books, journals and abstracts.

Experimental.

Material: Venous blood freshly withdrawn from healthy adults and fresh cord blood were used as sources of adult and foetal haemoglobin respectively and were received into screw-capped bottles containing appropriate quantities of crystallised Paul and Heller's anticoagulant solution, prepared according to the bulk formula

Ammonium oxalate	1.2gms.
Potassium oxalate	0.8gms.
Formaldehyde	1 ml.
Distilled water to	100 ml.

The cord blood was estimated by the technique of Singer et al (1951) to contain foetal haemoglobin in concentrations

varying from 75-95% of the total haemoglobin.

Pure haemoglobin solutions were prepared from the erythrocytes after repeated washings (4-6) with 0.9% w/v sodium chloride; thorough shaking with 1.2 - 1.5 volumes of distilled water and 0.4 volumes of toluol; and centrifugation at 3,000r.p.m. for 20 minutes. After partial removal of the top two layers, the clear pure haemoglobin solution was siphoned off and finally filtered.

Methaemoglobin was prepared from the above haemoglobin solutions by the use of potassium ferricyanide according to Anson and Mirsky (1931)[10A]. The reaction is stoichiometric and 1.1 molecule of the oxidising agent was added per atom of iron using a 1% w/v solution of $K_3Fe(CN)_6$.

1.0m sodium salicylate solution (Analar) was prepared, filtered and stored in the cold as suggested by Anson and Mirsky (1934).[11].

Cephalin was prepared according to the method of Hanger (1939)[77] using bovine brain. Fresh brain tissue was thoroughly minced and then dehydrated by six extractions with acetone. The dry material obtained was powdered and extracted three times with peroxide free ether. The ether extracts were concentrated in vacuo and crude cephalin was

precipitated by the addition of 4 volumes of absolute alcohol. The precipitate obtained was dissolved in the minimal amount of ether, left overnight in the cold to precipitate cerebroside impurities and centrifuged. The solution was siphoned off and treated with 4 volumes of absolute alcohol and chilled. The precipitate was then filtered off, washed with alcohol and acetone and finally dried. This material was believed to be almost pure cephalin and from this a 2½% w/v emulsion of cephalin was prepared. This emulsion was homogeneous and could not be separated by centrifugation or filtration.

Analar, preparations of urea, acetone, absolute ethyl alcohol, and formamide were used.

Sodium dodecylbenzene sulphonate was supplied in a dry pure state by Marchon Bros.

Sodium taurocholate: was supplied by DIFCO with a guaranteed purity of only 70%. The remaining 30% was of various other bile salts.

Methods:

Prior to dilution or to conversion to methaemoglobin the haemoglobin concentrations were determined in an E.E.L. photoelectric colorimeter.

The changes in the absorption during the experiment

following the addition of the denaturing agent were determined by the use of a Unicam diffraction grating spectrophotometer and a Hartridge reversion spectroscope was employed for the spectroscopic observations.

I. Experiment with Urea: Steinhardt's experimental conditions were applied. All solutions contained 0.14g. methaemoglobin per 100ml. and 0.1 M KCl. Two experiments were carried out with each blood sample. The concentration of urea was 4.4 M while the control solutions contained are equal volume of distilled water. The final volume of each solution was 10ml. The optical density was measured at 630mu and 540mh and readings taken every 2 hours for 24 hours.

Results: The results obtained, expressing the mean of six experiments are summarised in Table II. It shows that the rate of denaturation of foetal methaemoglobin was quicker than that of adult methaemoglobin. Each individual experiment followed very similar pattern and in no instance was the reading with the solution of foetal methaemoglobin at 540mu lower than with the adult pigment. At 8 hours, the denaturation of foetal methaemoglobin reached a stage which adult methaemoglobin attained only after 24 hours. The contrast in colour between the two solutions was apparent

TABLE II.

Rate of denaturation of adult and foetal methaemoglobin by 4.4 M. urea as determined by the increase in spectral absorption at 540 mu.

Time (hours)	Adult haemoglobin				Foetal haemoglobin			
	Solution 1. (with urea)		Solution 2. (control)		Solution 3. (with urea)		Solution 4. (control)	
	630 mu.	540 mu.	630 mu.	540 mu.	630 mu.	540 mu.	630 mu.	540 mu.
0	.54	.82	.49	.75	.54	.84	.49	.74
2	.53	.86	.49	.765	.50	.93	.50	.745
4	.51	.89	.49	.765	.45	.98	.50	.745
6	.50	.90	.49	.76	.43	.99	.50	.755
8	.50	.91	.51	.765	.42	1.0	.51	.76
12	.49	.92	.49	.76	.40	1.04	.50	.76
14	.49	.92	.49	.76	.39	1.05	.49	.75
16	.50	.94	.48	.76	.37	1.06	.48	.76
18	.48	.95	.48	.765	.37	1.07	.50	.77
20	.44	.98	.49	.765	.32	1.07	.49	.75
24	.42	1.0	.48	.765	.30	1.09	.48	.75

even to the naked eye.

Addition of sodium hyposulphite (dithionite) produced the typical ferrohaemochromogen spectrum in both the foetal and adult haemoglobin solutions contrary to Steinhardt's observations. There was, however, a marked time difference, typical ferrohaemochromogen appearing at eight hours in the foetal methaemoglobin solution, and at 24 hours in the adult methaemoglobin solution.

II. Experiment with Salicylate: Anson and Mirsky's (1934) experimental conditions were applied. All solutions contained 0.1g. methaemoglobin per 100ml. in 0.1 M phosphate buffer pH 6.8. Two experiments were carried out on each blood sample. Varying volumes of 1.0 M sodium salicylate solution were added to obtain solutions containing 0.0, 0.2, 0.4, 0.5 M salicylate. The final volume of each solution was 10ml. Readings were obtained at 540mu before and at 0.5, 3, 5, 10, 15, 30, 60 and 120 minutes after addition of salicylate.

Results: Table III summarises the results obtained and presents the mean of six experiments. This shows, in contrast, that the speed of denaturation of foetal methaemoglobin by salicylate was slower than that of the adult

TABLE III.

Rate of denaturation of adult and foetal methaemoglobin by salicylate as determined by the increase in spectral absorption at 540 mu.

Time (minutes)	Control		Salicylate concentration					
			0.2 M.		0.4 M.		0.5 M.	
	A.	F.	A.	F.	A.	F.	A.	F.
0.5	.41	.41	.50	.47	.61	.59	.66	.62
3			.535	.495	.66	.62	.665	.63
5	.41	.41	.55	.50	.66	.625	.665	.63
10			.56	.51	.66	.625	.665	.63
15	.415	.415	.56	.52				
30			.56	.53	.66	.63	.67	.63
60	.415	.415	.57	.54				
120	.415	.415	.575	.56	.67	.635	.68	.64

A = Adult. F = Foetal.

pigment in all concentrations. Each individual experiment led to the same conclusion, and in no instance were the results conflicting. For fuller appreciation of this fact from the table it must be noted that a difference of 0.03 in the optical density represents over 10% of the total denaturation.

When sodium hyposulphite was added at 60 minutes, the typical ferronaemochromogen spectrum was obtained in all solutions with methaemoglobin and salicylate.

III. Experiment with Formamide: Steinhardt's experimental conditions were applied. All solutions contained 0.1g. methaemoglobin per 100ml. and 0.1 M KCl. The concentration of formamide was 5M, while the control solutions contained an equal volume of distilled water. The final volume of each solution was 10ml. The optical density was measured at 630mu and 540mu and readings were taken at 0.5, 1, 3, 5, 10, 15, 30, 40, 60 and 90 minutes. Two experiments were carried out with each sample of blood.

Results: Table IV summarises the results obtained and presents the mean of six experiments. It shows that no appreciable difference exists in the rate of denaturation of adult and foetal methaemoglobin by 5M formamide as

TABLE IV.

Rate of denaturation of adult and foetal methaemoglobin by 5 M. formamide as determined by the increase in spectral absorption at 540 mu.

Time (minutes)	Adult haemoglobin				Foetal haemoglobin			
	Solution 1. (with formamide)		Solution 2. (control)		Solution 3. (with formamide)		Solution 4. (control).	
	630 mu.	540 mu.	630 mu.	540 mu.	630 mu.	540 mu.	630 mu.	540 mu.
0.5	.35	.655	.44	.69	.35	.65	.44	.69
1	.34	.67			.34	.67		
3	.335	.70			.33	.70		
5	.32	.74	.445	.69	.32	.74	.445	.695
10	.30	.805			.305	.80		
15	.28	.845	.44	.69	.285	.85	.44	.69
30	.265	.90			.265	.90		
40	.255	.93	.44	.695	.255	.93	.445	.695
60	.24	.97			.235	.975		
90	.23	.98	.445	.695	.23	.98	.445	.695

measured by spectral absorption. Slight variations were noted in individual experiments but these were always negligible. When sodium hyposulphite was added at 90 minutes and the specimen shaken, the typical ferrohaemochromogen spectrum was obtained in all solutions, again very contrary to Steinhardt's observations on horse methaemoglobin.

IV. Experiment with Cephalin: Chargaff, Ziff and Hogg's experimental conditions were applied. Fresh haemoglobin solutions prepared for each experiment were fully oxygenated by passing oxygen through the solution. All solutions contained 0.14g. oxyhaemoglobin per 100ml. and 1ml. of 2½% cephalin suspension was added to 6ml. haemoglobin solution in Soln.1. of each series. 1 ml. distilled water was added to the control solutions. A second control was also run with 1ml. cephalin suspension plus 6ml. distilled water. The optical density was measured at 575mu and 540mu taking readings at 0.5, 5, 10, 15, 20, 25, 30, 40, 60, 75 and 90 minutes.

Results: Table V, expressing the mean of six experiments, presents the results obtained with the oxyhaemoglobin solutions. It had been initially checked that no variance

TABLE V.

Rate of denaturation of adult and foetal oxyhaemoglobin by a 2.5% emulsion of cephalin as determined by the decrease in spectral absorption at 575 mu. and 540 mu.

Time (Minutes)	Adult oxyhaemoglobin				Foetal oxyhaemoglobin			
	Solution 1. (with cephalin)		Solution 2. (Control)		Solution 3. (with cephalin)		Solution 4. (control)	
	575 mu.	540 mu.	575.mu.	540 mu.	575 mu.	540 mu.	575 mu.	540 mu.
0.5	.46	.51	.32	.38	.46	.51	.32	.38
5	.44	.50			.445	.50		
10	.435	.49			.43	.49		
15	.41	.475			.41	.47		
20	.395	.46	.32	.38	.39	.46	.315	.38
25	.38	.45			.38	.45		
30	.365	.44			.37	.44		
40	.355	.43			.355	.43		
60	.325	.41	.315	.38	.33	.42	.32	.38
75	.315	.41			.32	.41		
90	.31	.405	.32	.38	.31	.405	.32	.38

in absorption was occasioned by the diluted cephalin suspension. All experiments followed an almost identical pattern demonstrating that cephalin denatures adult and foetal oxyhaemoglobin with equal facility. In pilot experiments, it was remarkable how a small discrepancy in the adult and foetal oxyhaemoglobin concentrations could lead to erroneous conclusions, the dilute solution being denatured much more quickly according to optical density measurements.

Addition of sodium hyposulphite did not produce the typical ferrohaemochromogen spectrum as with urea, salicylates, and formamide, although this was achieved, as stated by Chargaff et al, by the addition of ammonium sulphide which merely reduced the control oxyhaemoglobin to reduced haemoglobin.

Further studies were carried out consequent on the criticism of Lemberg and Legge that this reaction may not be a denaturation. Ammonium sulphate solution was mixed with the cephalin-oxyhaemoglobin solutions to a concentration of 45% saturated ammonium sulphate and in both adult and foetal haemoglobin solutions a floccular precipitate appeared (pH 5.95 at this point). It was impossible to separate this precipitate by centrifugation or filtration

and so the suspension was allowed to stand 24 hours, by which time the solid material had settled to the top of the solution. The clear substrate was removed by a Pasteur pipette. The precipitate was found to be insoluble in distilled water (i.e. near its iso-electric point) but soluble in either a boric buffer (pH 8.92) or a Sorenson phosphate buffer pH 8.2. Addition of a pinch of sodium hyposulphite to this solution produced the typical ferrohaemochromogen spectrum, a dark band at 558mu and a weaker band around 530mu.

Control experiments were undertaken. The earlier substrate removed by Pasteur pipette was examined spectroscopically, and no bands were noted. Addition of pyridine and sodium hyposulphite even after alkalinisation produced no spectral absorption in the first removed specimens. The cephalin suspension was examined spectroscopically and tested for the presence of extraneous blood pigment by addition of pyridine and sodium hyposulphite, but again even in alkaline suspension there was no specific spectral absorption. Adult and foetal haemoglobin solutions were treated by addition of ammonium sulphate to the same concentration and no denaturation was apparent in these specimens over 24 hours. Cephalin suspensions were also treated

with ammonium sulphate and no precipitation occurred.

These results would thus appear to substantiate a conception that denaturation does indeed take place on treating oxyhaemoglobin solutions with a suspension of cephalin. The fact that sodium hyposulphite was necessary to obtain a ferrohaemochromogen spectrum may be explained by the occurrence of autoxidation which is always a rapidly occurring phenomenon with ferrihaemochromogen, especially in the presence of oxygen.

Cephalin suspension was noted to have no action on reduced haemoglobin as found by Chargaff, Ziff and Hogg.

V. Experiment with Sodium Taurocholate:

All solutions contained 0.12g. methaemoglobin per 100ml. in distilled water. The concentration of bile salt was 0.06gm. per 100ml. The final volume of each solution was 10ml. In view of the strongly polar but neutral nature of this reagent, it was considered unnecessary to use buffer solutions. The optical density was measured at 630mu and 540mu and readings taken at 0,5, 3, 5, 10, 15, 30, 45, 60 and 90 minutes.

Results: Table VI expressing the mean of six experiments presents the results obtained. All experiments followed the same pattern and as in the experiments with urea, the rate of denaturation of foetal methaemoglobin was seen to be more rapid than that of the adult methaemoglobin. The concentration of bile salt required for denaturation was decidedly lower than that quoted as necessary by Anson and Mirsky, although the quality of the preparation used might provide an explanation. The addition of a pinch of sodium hyposulphite at 90 minutes resulted in a precipitate which was redissolved by slight alkalinisation viz. the addition of a few ml. of Sorinson's phosphate buffer pH 8.2. The typical absorption spectrum of ferrohaemochromogen then presented.

VI. Experiment with Sodium Dodecyl Benzene Sulphonate (NANSA).

All solutions contained 0.12g. oxyhaemoglobin per 100ml. distilled water. The concentration of detergent was 0.05g. per 100ml. The final volume of each solution was 10ml. The solution was again neutral and as with the bile salt no buffer solution was considered necessary, and no pH change occurred during the reaction. Two experiments were carried out per specimen of blood after the first. The optical density was measured at 575mu and 540mu at 0.5, 2, 5,

TABLE VI.

Rate of denaturation of adult and foetal methaemoglobin by sodium tauroocholate (bile salt) as determined by the increase in spectral absorption at 540 mu.

Time (minutes)	Adult haemoglobin				Foetal haemoglobin			
	Solution 1. (with bile salt)		Solution 2. (control)		Solution 3. (with bile salt)		Solution 4. (control)	
	630 mu.	540 mu.	630 mu.	540 mu.	630 mu.	540 mu.	630 mu.	540 mu.
0.5	.31	.60	.40	.68	.30	.605	.40	.68
3	.26	.63	.405	.68	.24	.645	.40	.68
5	.24	.64	.40	.685	.215	.675	.40	.685
10	.18	.68	.40	.68	.16	.70	.395	.675
15	.16	.695	.40	.68	.13	.72	.40	.68
30	.13	.72	.405	.685	.10	.74	.40	.68
45	.10	.74	.40	.68	.09	.745	.405	.68
60	.08	.76	.40	.68	.08	.765	.40	.69
90	.08	.765	.405	.68	.08	.765	.405	.68

15, 45, 75, 120, 240 and 300 minutes.

Results: The results obtained are presented in Table VII, which expresses the mean of five experiments. In this instance no difference in the rate of denaturation of the two pigments by the detergent was noted. The concentration of NANSA required was very similar to that of Nacconal quoted as necessary by Anson and Mirsky. The addition of sodium hyposulphite to the mixtures at 120 minutes produced a precipitate, which redissolved in Sorenson phosphate buffer pH 8.2 to give the typical absorption spectrum of ferrohaemochromogen. In a pilot experiment, sodium dodecyl benzene sulphonate was also found to denature the foetal and adult methaemoglobin with again no evident difference in rate. The necessity for the addition of sodium hyposulphite to produce the ferrohaemochromogen spectrum was attributable to the rapid autoxidation almost coincidental with the denaturation to ferrihaemochromogen, the bands of which are weak, and so masked by the remaining oxyhaemoglobin in solution.

VII. Experiment with Alcohol:

All solutions contained 0.107g. oxyhaemoglobin per 100ml. A borate buffer solution pH 8.92 was prepared,

TABLE VII.

Rate of denaturation of adult and foetal oxyhaemoglobin by sodium dodecyl benzene sulphonate as determined by the decrease in spectral absorption at 575 mu. and 540 mu.

Time (minutes)	Adult haemoglobin				Foetal haemoglobin.			
	Solution 1. (with detergent)		Solution 2. (control)		Solution 3. (with detergent)		Solution 4. (control)	
	575 mu.	540 mu.	575 mu.	540 mu.	575 mu.	540 mu.	575 mu.	540 mu.
0.5	.58	.97	.6	.98	.58	.97	.6	.98
2	.56	.95			.56	.95		
5	.54	.95	.6	.98	.54	.94	.61	.98
15	.53	.93			.53	.93		
45	.51	.90	.61	.98	.51	.90	.61	.98
75	.50	.88			.505	.885		
120	.485	.88	.61	.99	.485	.875	.615	.98
240	.48	.375	.61	.98	.48	.88	.61	.98
300	.48	.88	.61	.99	.48	.88	.61	.99

and used to dilute oxyhaemoglobin solutions from 8gm. per 100ml. Solution I of each series contained 5ml. absolute ethyl alcohol. Solutions 2 were diluted with distilled water. The optical density was measured at 575mu and 540mu at 0.5, 2, 4, 8, 12, 16, 20, 30 and 40 minutes.

Results: Table VIII expressing the mean of 5 experiments presents the results obtained. Alcohol was seen to denature foetal haemoglobin more quickly than adult haemoglobin. The addition of sodium hyposulphite at 30 minutes (necessary because of the rapid autoxidation) produced the typical ferrohaemochromogen spectrum.

Preliminary experiments on the denaturation of the oxyhaemoglobins in the presence of alcohol produced interesting observations. In the absence of alkaline buffers, the addition of 5ml. ethyl alcohol to the same quantity and concentration of oxyhaemoglobins in distilled water as above, resulted in a rapid precipitation of denatured foetal pigment (5-10 minutes), while the denatured adult pigment remained in solution for two hours. The rapidity of the precipitation appeared to be more due to a difference in the solubility of the denatured forms of the two pigments, than to varying rates of denaturation, as the variance in precipitation was most marked, while that of the

TABLE VIII.

Rate of denaturation of adult and foetal oxyhaemoglobin by alcohol as determined by the decrease in spectral absorption at 575 mu. and 540 mu.

Time (minutes)	Adult haemoglobin				Foetal haemoglobin			
	Solution 1. (with alcohol)		Solution 2. (control)		Solution 3. (with alcohol)		Solution 4. (control)	
	575 mu.	540 mu.	575 mu.	540 mu.	575 mu.	540 mu.	575 mu.	540 mu.
0.5	.35	.6	.44	.74	.345	.59	.44	.74
2	.33	.56			.31	.54		
4	.31	.56	.45	.74	.27	.54	.44	.745
8	.3	.55			.26	.54		
12	.28	.54			.25	.52		
16	.27	.53	.45	.74	.24	.52	.44	.74
20	.25	.54			.23	.51		
30	.23	.52	.45	.74	.23	.52	.44	.74
40	.23	.52	.45	.74	.23	.52	.44	.745

denaturation as shown in Table VIII is not so. It was this distinction which led to the necessity for an alkaline buffer solution. No phosphate buffer proved sufficiently alkaline; the borate buffer was tested against oxyhaemoglobin and found to have no denaturing effect on its own.

VIII. Experiment with Acetone:

All solutions contained 0.1g. oxyhaemoglobin per 100ml. The dilution of a stock haemoglobin solution was carried out with the borate buffer pH 8.92 before full oxygenation was assured. Solution 1 of each series contained 4cc. acetone and Soln. 2, 4cc. distilled water. The optical density was measured at 575mu and 540mu at 0.5, 2, 5, 10, 15, 30, 60 and 90 minutes.

Results: Table IX, expressing the mean of five experiments, presents the results obtained. No difference in the denaturation rates of the foetal and adult pigments with acetone could be demonstrated. The addition of a pinch of sodium hyposulphite at 30 minutes led to the appearance of typical haemochromogen spectrum in both specimens.

Again like alcohol, a solubility difference between the two forms of denatured pigment was evident, and here it was more evidently a solubility effect as no evidence

TABLE IX.

Rate of denaturation of adult and foetal oxyhaemoglobin by acetone as determined by the decrease in spectral absorption at 575 mu. and 540 mu.

Time (minutes)	Adult haemoglobin				Foetal haemoglobin			
	Solution 1. (with acetone)		Solution 2. (control)		Solution 3. (with acetone)		Solution 4. (control)	
	575 mu.	540 mu.	575 mu.	540 mu.	575 mu.	540 mu.	575 mu.	540 mu.
0.5	.46	.7	.48	.74	.455	.7	.48	.74
2	.39	.64			.39	.645		
5	.37	.62	.48	.74	.375	.62	.48	.74
10	.35	.62			.355	.61		
15	.34	.60	.475	.735	.34	.60	.48	.74
30	.32	.60			.32	.60		
60	.3	.585	.48	.74	.305	.58	.48	.74
90	.3	.58	.48	.74	.31	.58	.48	.74

of a difference in the rate of denaturation was observed. These solubility effects with alcohol and acetone on the two pigments have been utilised with effect in a later differential study, where the presence of a foetal haemoglobin was suspected in an adult blood condition.

Discussion: The above experiments show that foetal and adult haemoglobins, besides differing in stability to alkaline denaturation as already known, differ also in their resistance to denaturation by urea, sodium salicylate, sodium taurocholate and alcohol, while reacting similarly to denaturation by formamide (in spite of supposed similarity in action to urea), cephalin, sodium dodecyl benzene sulphonate and acetone.

The fact that the two types of blood pigment may behave differently according to the denaturing agent viz. the higher resistance of foetal haemoglobin to denaturation by alkali or salicylate and lower resistance towards urea, sodium taurocholate or alcohol, can be understood in the light of the modern concept that denaturation is not a single phenomenon; that there are various kinds of denaturation, and that in each one of them the changes may involve various groups of the protein molecule.

The structural basis of a protein is the poly-

peptide chain which consists of α amino acid residues linked by peptide bonds uniting the α carboxyl group of one α amino acid residue to the α amino group of the next. Some 23 of these α amino acid (or imino) residues have been established,[173.] each varying according to the side chain attached to the α carbon atom. In the crystalline proteins like haemoglobin, polypeptide chains are folded together into units of roughly globular dimensions, and are usually soluble in aqueous solution. Fibrous proteins on the other hand seem to be arranged in strands parallel to the fibre axis, and, in general, have a lower solubility than the globular type.

Complete denaturation of a globular protein would appear to result in a state intermediate between the globular and the fibrous forms; viz. the liberation of the polypeptide chains leads to reassociation in a random manner to form a disorderly tangle and to produce in aqueous media a floccular precipitate. It has been possible to align and spin this disorderly tangle into fibres, and it is thus that we now have the new industrial fibres like "Ardil", which is prepared from the denatured globulin of monkey nuts.

It can be well seen however, without drawing any

attention to physical methods of denaturation, that in addition to various kinds of denaturation (evidenced by the variety of chemical agents above with no obvious common denominator), there are various degrees of denaturation, and it is lack of appreciation of this which has led to so much confusion as to the reversibility of the process. The modern approach is simply that minor degrees of denaturation are indeed reversible, but once the whole protein structure is completely tangled or stretched out, reversion to the original form cannot be achieved.

The differences between the two types of haemoglobin described in the present chapter, in addition to those described in Chapter I. leave little doubt that there are definite ontogenetic variations of haemoglobin. Since the prosthetic haemin is identical in various haemoglobins, the distinction between the two types must be attributed to the protein moieties, and this concept is now generally accepted.

It has appeared reasonable to subscribe to the hypothesis of Haurowitz[86]. and Drabkin[60]., that a structurally identical haemin combines with the particular and suitable protein available at the site of chromoprotein fabrication, this "liver" globin was held responsible for

the properties of foetal haemoglobin, and "red bone marrow" globin for those of adult haemoglobin, but evidence against this conception will be presented in Chapter IV.

B. Evaluation of the Alkaline Denaturation Technique for Quantitative Measurement.

That adult haemoglobin is much more susceptible to alkaline denaturation than foetal haemoglobin has been an accepted distinction for almost 90 years. Credit for the introduction of a spectrophotometric method for observing this denaturation goes to Haurowitz (1929, 1930),[83,84] who by means of an expensive apparatus was able to follow the kinetics of the reaction and determine the proportions of the resistant and "labile" pigments. Brinkman and Jonxis (1935)[40] also introduced a photo-electric method but this too required special apparatus. Baar and Lloyd[16] in 1943 preferred to modify the original technique of Haurowitz for their work, but again they required a photometer more sensitive than is usually available. Ponder and Levine (1949) next proposed a technique less dependent on the sensitivity of the photometer, and this method was the obvious inspiration of the most recent technique suggested by Singer, Chernoff and Singer (1951)[199].

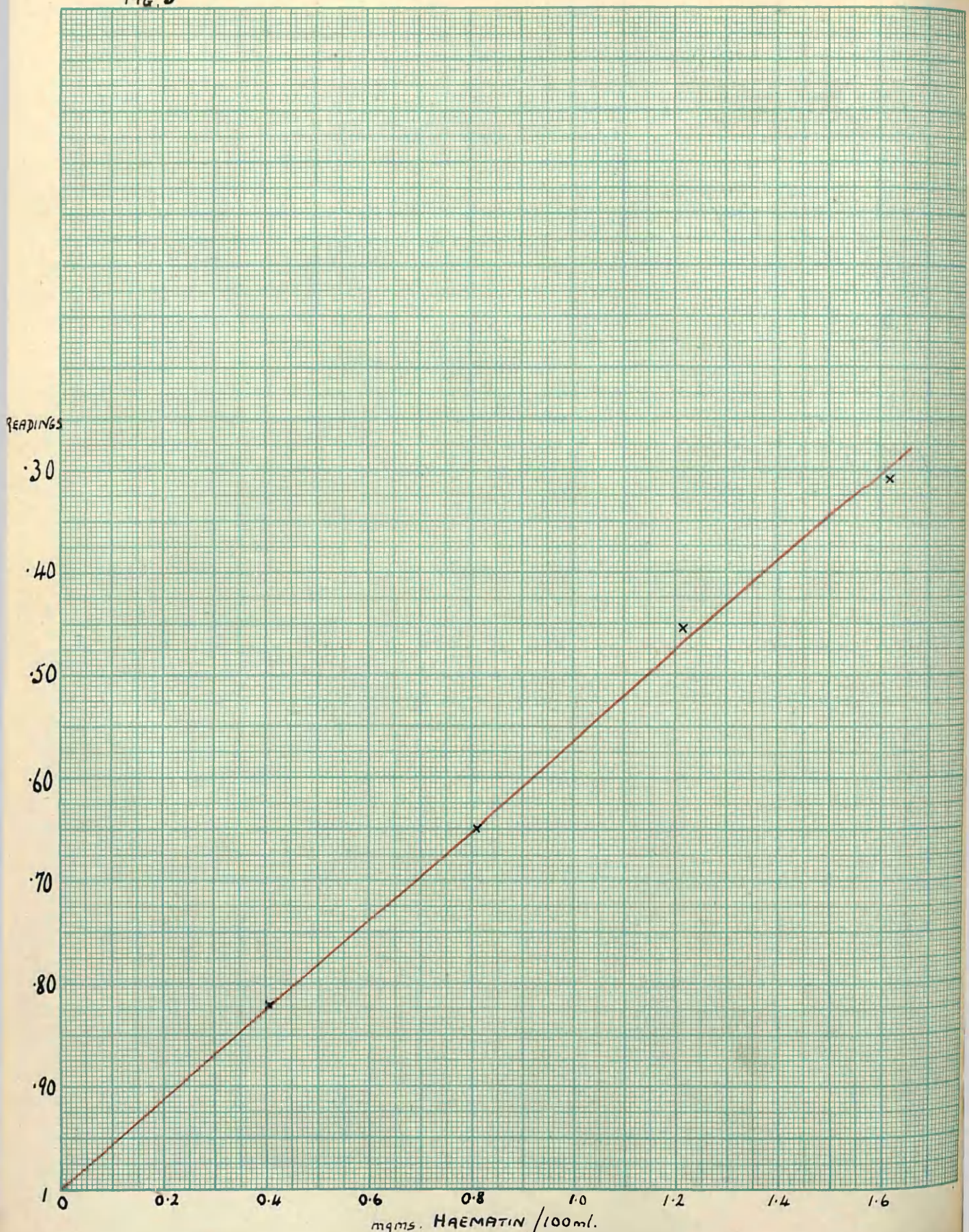
Until this technique of Singer et al, the percentage of resistant haemoglobin was calculated from a graph constructed by plotting the logarithm of the concentration of undenatured pigment against time. Two main

slopes were encountered usually, an initial steep fall and a more gradual line representing the normal adult and foetal pigments respectively. By extrapolating the latter line to zero time, the concentration of the resistant pigment could be estimated to a fair degree of accuracy, especially if a reasonable proportion of the resistant pigment was present. Below 5% considerable difficulty was encountered.

The technique of Singer et al has the advantage of relative simplicity, direct reading and determination from a previously calibrated curve plus reasonable accuracy. By utilising a higher pH than Brinkman and Jonxis, the subdivision of the adult pigment into two groups is thus bypassed, as denaturation of the adult pigment is virtually complete within the one minute, and any higher reading may be presumed due to resistant haemoglobin of the foetal (F) type.

A comparison of results obtained on selected specimens of cord blood by the techniques of Ponder et Levine and Singer et al disclosed that the latter's value for foetal haemoglobin were of a rather higher order (0-5%) but on the whole the values obtained were strikingly similar. From the results to be reported below, it will be

FIG. 3


READINGS
·30
·40
·50
·60
·70
·80
·90
1
0
0·2
0·4
0·6
0·8
1·0
1·2
1·4
1·6
mgms. HAEMATIN /100ml.

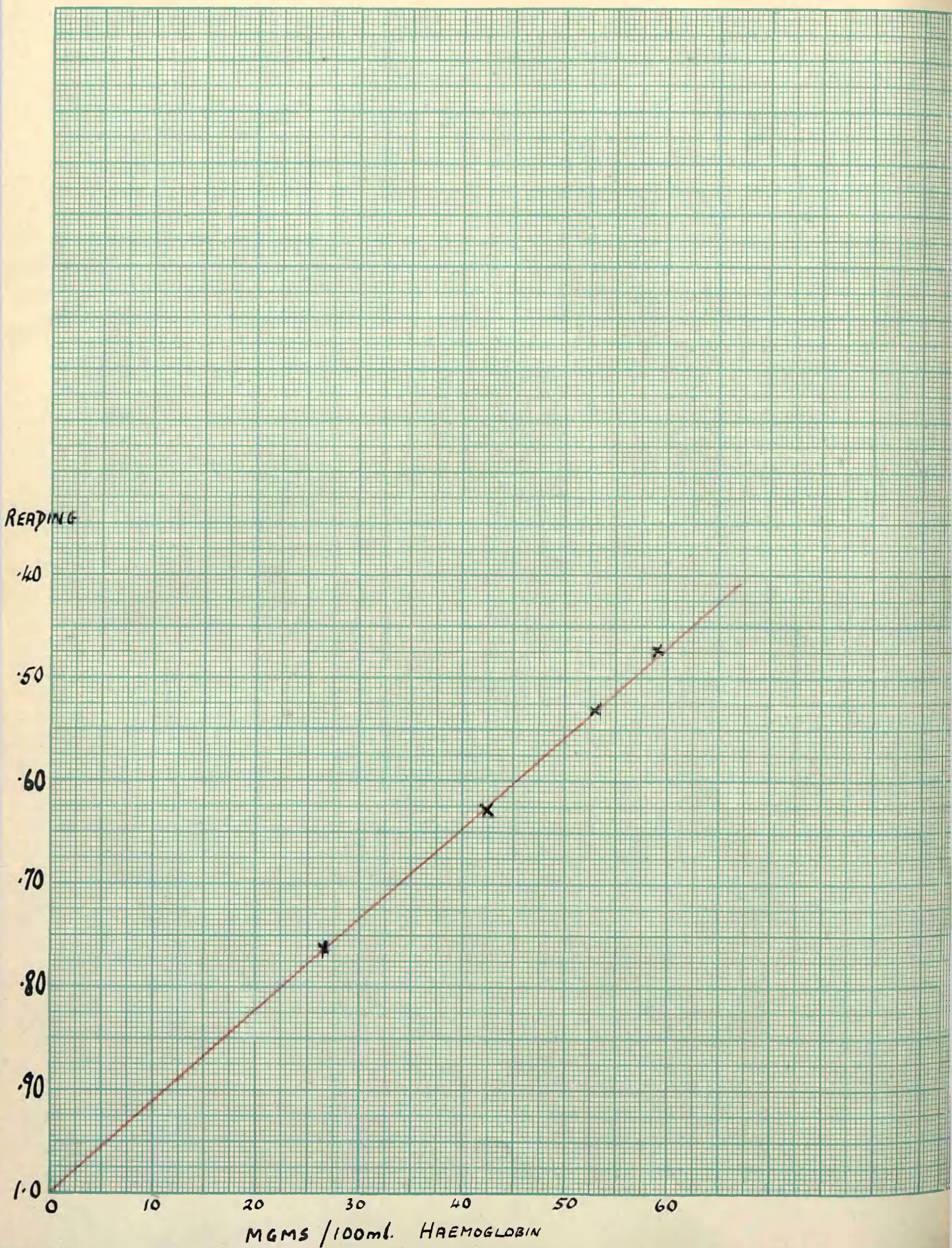
seen that the slightly higher values would actually be the more accurate.

The technique used throughout this work was that of Singer et al very slightly modified, and essentially consisted of exposing a measured quantity of haemoglobin to the alkaline reagent for an exact period of time, interrupting the reaction by means of a solution which simultaneously lowers the pH and precipitates the haemochromogens. After filtration, the unaltered haemoglobin was determined in a Spekker photoelectric absorptiometer. The necessary calibration curve from which to read the value for quantity of haemoglobin was obtained according to the pyridine haemochromogen method of Rimington (1942)[187]. which proved more accurate than constructing a curve from known quantities of haemoglobin in the experimental salt solution.

Calibration Curve for Absorptiometer.

Method: Known quantities of crystalline haemin (e.g. 10mgm) were dissolved in 500ml. of a 0.1 N sodium hydroxide solution. To 10ml. of this alkaline solution, 2ml. of pyrydene were added plus a pinch of pure sodium hyposulphite. The contents of the flask were swirled for a few moments until the solid had dissolved. From this a calibration curve was obtained for crystalline haemin as pyridene

FIG. 4


READING
·40
·50
·60
·70
·80
·90
1·0
0
10
20
30
40
50
60
MGMS / 100ml. HAEMOGLOBIN

haemochromogen using the Spekker photoelectric absorptiometer with an Ilford yellow green filter 605 (Fig. 3). Unknown haemoglobin solutions obtained as pyridene haemochromogen by the same procedure were estimated using the haemin pyridene-haemochromogen curve and the corresponding values of haemin thus determined were multiplied by 25.66 according to the calculations of Rimington

$$\frac{\text{Molecular Weight of Hb}}{\text{Molecular Weight of haemin}} = \frac{66,890}{4 \times 651.4} = 25.66$$

From these results a calibration curve for haemoglobin was constructed, but for the haemoglobin readings, the filter was altered to the Ilford green No.604 (Fig. 4).

The Denaturation Technique.

Materials: Exactly N/12 potassium hydroxide (pH 12.7) was prepared freshly every four weeks and kept in waxed bottles in the refrigerator.

For the precipitating reagent, 2ml. 10N hydrochloric acid were added to 800ml. 50% saturated ammonium sulphate.

Pure haemoglobin solutions were prepared as already described from freshly taken oxalated specimens, and the final content of the haemoglobin was adjusted to be within the range 9 - 11gm. per 100ml.

Method: One and six-tenths ml. of the alkaline reagent is placed in a small test tube and left in the rack for some minutes before use. One or two tenths ml. of the haemoglobin solution is then added, the pipette rinsed thoroughly and the tube gently shaken for ten seconds. After exactly one minute from the introduction of the haemoglobin solution, three and four-tenths ml. of the precipitating solution is added, and the mixture rapidly poured from one tube to the other to mix thoroughly before filtering through a double layer of filter paper (No. 1). The haemoglobin content of the filtrate was then determined from a reading on the absorptiometer.

Discussion: It was discovered in the initial pilot experiments that waterbaths were unnecessary for the temperature control. Bench temperature in the centrally heated laboratory was 18°C - 20°C, and there was no discrepancy in the results with or without the waterbath, provided time was allowed for the KOH to achieve bench temperature.

Singer et al declared that the readings obtained after denaturing normal haemoglobin solutions for one minute were not due to the presence of haemoglobin, and this would appear to be so for most normal specimens, as prolonged exposure to NaOH did not significantly reduce these values.

A series was run from several samples to test the constancy of the final readings, and this was present to a remarkable degree if the readings were below 95 on the absorptiometer (e.g. 66, 66, 65, 66). Between 95 and 100, the readings were still constant to within 1 or 2 (e.g. 99, 97, 99, 98). In this range, however, even a most modern ultraviolet spectrophotometer will not give constant results, but it does emphasize that in the lower ranges, a high degree of accuracy cannot be attained, thus readings of 99 and 97 give readings of 1.5mgm.% and 3.5 mgm.% respectively, which when multiplied by the appropriate factors shows a gross degree of variation, and detracts considerably from the prestige of the technique.

Consequently it was decided to test whether 0.2ml. or 0.3ml. haemoglobin solutions could be added to the KOH instead of 0.1ml., in order to minimise error in these lower ranges. It was quickly found necessary to discard the 0.3ml. idea, as readings were inaccurate.

For Experiment I, the blood used for normal adult haemoglobin was that of the writer. For Experiments II and III, the adult specimens were obtained from patients with polycythaemia vera, venesected in the course of treatment. Absence of resistant pigment in these three specimens

was, of course, confirmed.

Three specimens of cord blood were obtained and the percentage foetal haemoglobin content in the prepared solutions estimated.

Specimen 1: Total haemoglobin 10.4gms.% of which 8.84gms.% were foetal haemoglobin.

Specimen 2: Total haemoglobin 10gms.% of which 9.5gms.% were foetal haemoglobin.

Specimen 3: Total Haemoglobin 10.1gms.% of which 8.07gms.% were foetal haemoglobin.

Various admixtures were then prepared and the denaturation technique applied.

The results of the three experiments are presented in Table X. The validity of the experiments may appear to suffer from the fact that the original value of foetal haemoglobin content of the cord blood specimens was determined by the same denaturation technique, but this was considered reasonable, as this method gives very similar values for the foetal haemoglobin percentage as that of Ponder et Levine, which has been compared to others in the past with reasonably consistent results.

A few conclusions may be drawn from the figures tabulated. In the middle ranges, the technique gives reasonably accurate and consistent results; in the lower

TABLE X.

Experiments to determine whether foetal haemoglobin is fully recovered after admixture with adult haemoglobin.

Solution	Adult Hb. Solution	+	Cord Hb. Solution	Expected recovery of foetal Hb. in mgm%. 0.1 ml.	0.2 ml.	Recovery achieved of foetal Hb. in mgm%. 0.1 ml.	0.2 ml.
Exp. I.							
A.	30 cc.	+	1cc.	5.91	10.97	2.5	7
B.	20 cc.	+	1 cc.	8.25	16.19	7	14
C.	10 cc.	+	1 cc.	15.76	30.91	16	31
D.	6 cc.	+	1 cc.	24.76	48.57	21.5	50
E.	4 cc.	+	2 cc.	57.78	113.33	47	102
F.	2 cc.	+	2 cc.	86.67	170	88.5	156
G.	1 cc.	+	2 cc.	115.56	226.67	102.5	215
Exp. II.							
A.	30 cc.	+	1 cc.	6.01	12.08	5.5	11.5
B.	25 cc.	+	1 cc.	7.1	14.05	5.5	12.5
C.	20 cc.	+	1 cc.	8.87	17.4	9.5	15
D.	15 cc.	+	1 cc.	11.64	22.84	11.5	22.5
E.	10 cc.	+	1 cc.	16.93	33.22	15	32.5
F.	5 cc.	+	1 cc.	30.46	60.9	29.5	63
Exp. III.							
A.	50 cc.	+	1 cc.	3.1	6.09	1.5	5.5
B.	25 cc.	+	1 cc.	6.09	11.94	4.5	10.5
C.	20 cc.	+	1 cc.	7.53	14.78	7	13.25
D.	2 cc.	+	2 cc.	79.12	155. 19	71.5	150.0
E.	1 cc.	+	2 cc.	105.49	206. 9	97.5	197.0

ranges of foetal haemoglobin the technique is not very accurate but never overestimates the resistant proportion, while 0.2ml. does appear to give consistently the more accurate value; and finally that in all ranges the 0.2ml. haemoglobin solution gives at least as satisfactory results as 0.1ml. pigment solution.

Following these results it was decided that in the majority of experiments 0.2ml. haemoglobin solution would be added to the KOH in favour of the smaller quantity especially when the resistant pigment was likely to be in small proportion, and thus the multiplication of the error would by 26 rather than 51. In any case of doubt, both amounts would be used, the experiments repeated four times and a mean taken.

CHAPTER III.

Foetal Haemoglobin in Newborn Infants.

In two recent papers, Jonxis[115,116] postulated that in the blood of newborn suffering from erythroblastosis foetalis, the adult form of haemoglobin was present up to 4gm. per 100ml., and that this constituted such a divergence from the normal to justify an assertion that the Rhesus antibodies have been attacking the cells containing foetal haemoglobin selectively. He reports that serial estimations of the proportions of adult and foetal types of haemoglobin, using the technique of Brinkman and Jonxis (1935), showed that the cells with foetal haemoglobin are broken down very rapidly, and that in severe cases it has practically disappeared from the blood within about 5 days; while in less severe cases the selective breakdown may cease on the 6th day. In neither paper however is the data presented for critical analysis, by the reader.

Baar[14] was quick to challenge these views on principle, and claimed that the destruction of erythrocytes in erythroblastosis foetalis is based upon their sensitisation to RH antibodies which is unrelated to the chemical constitution of the globin component in the cell within. He quoted from his own experience[15,16] and the observations of Mollison[149] that the destructive process attacks trans-

-fused cells as rapidly as the infants own cells and declared furthermore that foetal haemoglobin is constantly formed in the regenerative process in this condition.

Ponder and Levine,[178.] after fresh experimental work using their newly developed technique, concluded that no significant difference in the percentage of foetal haemoglobin in the cord blood was found on comparing fifteen cord blood samples from normal newborn infants with the cord blood samples of fifteen infants affected with haemolytic disease of the newborn.

As the work of Jonxis is still quoted some years later, it was decided that a limited reinvestigation would be of interest in view of the more accurate quantitative technique of Singer et al analysed in the previous section. To submit the neonates to venesection on succeeding days as Jonxis has purported to have done, was not considered in the best interests of the infants - quite apart from its technical difficulty!

. . . . . .

Method: Cord blood from 10 newborn infants suffering from varying degrees of erythroblastosis foetalis was obtained at time of birth for analysis. A similar consecutive series was obtained of 12 normal full term infants at time of birth.

Haemoglobin solutions were prepared from these fresh oxalated blood samples as already detailed, and the experiments were carried out according to the technique of Singer et al using 0.1cc. haemoglobin solution. Dilution of the final solution for estimation was achieved by an appropriate mixture of the sodium hydroxide and ammonium sulphate reagents used in the denaturation technique, freshly prepared and filtered.

Results: In order to give a clear picture of the possible significance of the results, a brief summary of the mother's past obstetric history and of the child's history in hospital is presented, and the infants are classified according to grades of severity of the erythroblastosis foetalis. The bloods of all the affected infants produced a positive reaction to the Coombs test.

Hydrops Foetalis.

1. Mrs. H. Age 28. Healthy. Group O Rhesus -ve. Strong antibodies.

Previous Obstetric History:

1940 NFTD Infant: Normal, alive and well. 8 lbs.12ozs.weight.

1948 NFTD Infant: Jaundiced but A & W. 8 lbs.1 oz. weight.

1949 N.D.-36 wks. Stillbirth - hydrops foetalis.

1950 24 weeks miscarriage.

Infant: Full term infant. Weight 8 lbs.
Fresh stillborn male - hydrops foetalis.

Cord blood: Total haemoglobin not estimated - specimen clotted, but foetal haemoglobin = 91.5% total.

Icterus Gravis.

2. Mrs. C. Age 29. Healthy. Group AB Rhesus -ve
Strong antibodies.

Previous Obstetric History:

1950 NFTD Infant: normal, alive and well. 6lbs.13ozs. weight.

Infant: Full term infant. Weight 9lbs.7ozs. Female.
Slight jaundice at birth.
Exchange transfusion at 3½ hours and 2 later transfusions.

Cord blood: Total haemoglobin: 13.3gms./100ml.
Foetal haemoglobin: 77.4% total.

3. Mrs. C. Age 28. Healthy. Group B Rhesus -ve.
Abtibodies oresent.

Previous Obstetric History:

1951 NFTD Infant: Normal, alive and well. 8 lbs. weight.

Infant: Full term infant. Weight 7lbs. 11ozs. Female.
Slight tinge of jaundice at birth.
Liver and spleen both enlarged.

Exchange transfusion at 5½ hours (206ml. in, and 187cc. out).
Further transfusion at 6th day.
Cord blood: Total haemoglobin: 12.4gms./100ml.
Foetal haemoglobin: 84.3% total.

4. Mrs. L. Age 26. Healthy. Group O Rhesus -ve
Antibodies present.

Previous Obstetric History:
1951 NFTD. Infant: normal, alive and well. Weight 7lbs.13ozs.
Infant: Full term infant. Weight 8lbs.7ozs. Female.
Light icterus at birth.
Exchange transfusion at 5 hours.
Cord blood: Total haemoglobin 11.4gms./100ml.
Foetal haemoglobin 75.5% total.

Acute Haemolytic Anaemia.

5. Mrs. R. Age 31. Anaemia (Hb66%) Otherwise healthy.
Antibodies present.
Given course of I.V. iron antenatally.

Previous Obstetric History:
1940 NFTD Infant: normal, alive and well. Weight 8lbs.12ozs.
1941 NFTD Infant: normal, alive and well. Weight 7lbs.12ozs.
1949 NFTD Infant: normal, alive and well. Weight 7lbs.2ozs.

1951 36wks ND - alive and well. Jaundiced 6 weeks.
Weight 6lbs.5ozs.
(Twins)
36wks ND - Alive and well. Jaundiced 6 weeks.
Weight 6lbs. 3ozs.

Infant: Full term infant. Weight 8lbs. 14ozs. Male.

Icterus 1st.day, disappeared 7th day. Pale looking thereafter.

Cord blood: Total haemoglobin: 13.9gms./100 ml.

Foetal haemoglobin: 92.1% total.

6. Mrs. G. Age 40. Healthy. Group O Rhesus -ve.
Antibodies present.

Previous Obstetric History:

1949 L.S.C.S. Infant: unaffected alive and well.
Weight 8lbs. 1oz.

Infant: Full term infant. Weight 8lbs. 2ozs. Male.

Icterus noted at 24 hours and persisted 11 days.

Cord blood: Total haemoglobin: 16.9gms./100ml.

Foetal haemoglobin: 69.7% total.

7. Mrs. T. Age 29. Healthy. Group O Rhesus -ve.
Antibodies present.

Previous Obstetric History:

1949 NFTD. Infant: lived 2 days, cause of death unknown.

Infant: Full term infant. Weight 9lbs. Female.

Icterus noted second day - never marked.

Cord blood: Total haemoglobin: 17.9gms./100ml.

Foetal haemoglobin: 82.8% total.

8. Mrs. F. Age 25. Healthy. Group A Rhesus -ve.

Antibodies present.

Previous Obstetric History:

1946 NFTD. Infant: Unaffected, alive and well. Weight 5 lbs. 7 ozs.

1949 NFTD. Infant: Unaffected, alive and well. Weight 5 lbs. 13 ozs.

1949 - 14 weeks abortion.

Infant: Full term. Weight 6 lbs.7ozs. Male.

Icterus slight second day.

Cord blood: Total haemoglobin: 20.1gms./100ml.

Foetal haemoglobin: 97.4% total.

9. Mrs. O. Age 32. Healthy. Group O Rhesus -ve.

Antibodies very strong.

Previous Obstetric History:

1944 ND - 36 weeks Infant: normal, alive and well. Weight 4 lbs. 8 ozs.

1948 NFTD Infant: normal, alive and well. Weight 5 lbs.

Infant: Full term. Weight 5lbs. 7ozs. Male.

Icterus noted 3rd day only.

Cord blood: Total haemoglobin: 19.5gms./100 ml.

Foetal haemoglobin: 95.7% total.

10. Mrs. H. Age 23. Healthy. Group O Rhesus -ve.
Antibodies present.

Previous Obstetric History:

1950 FT Mid forceps. Infant: normal, alive and well.
Weight: 9 lbs. 4 ozs.

Infant: Full term. Weight 7lbs. 9ozs. Female.
"Cerebral" for 3 days following mid-forceps delivery.

Cord blood: Total haemoglobin: 17.6gms./100ml.
Foetal haemoglobin: 74% total.

Mean percentage of foetal haemoglobin in cord blood of infants affected with erythroblastosis foetalis is thus 84.04. Standard Deviation ± 9.4.

Normal Infants: The samples quoted are a consecutive series of normal full term infants with normal haemoglobin levels (17 - 20.8gms./100ml.).

Cord bloods 1) 71.2% foetal haemoglobin
2) 71.1% foetal haemoglobin
3) 79.1% foetal haemoglobin
4) 89.4% foetal haemoglobin
5) 95.5% foetal haemoglobin
6) 88.2% foetal haemoglobin
7) 78.5% foetal haemoglobin
8) 88.4% foetal haemoglobin

Cord bloods 9) 82.4% foetal haemoglobin

10) 76% foetal haemoglobin

11) 86.8% foetal haemoglobin

12) 82.1% foetal haemoglobin.

Mean percentage of foetal haemoglobin in cord blood of the normal infants is thus 82.4. Standard Deviation $\pm$7.14.

Discussion: From these observations it was calculated that the Mean of the percentage of foetal haemoglobin in newborn infants with erythroblastosis foetalis was 84.04 with a Standard Deviation of $\pm$9.4. The corresponding Mean in normal newborn infants was 82.4 with a Standard Deviation of $\pm$7.14. The Standard Error of the Difference of these Means was 3.62. As the difference between the means was only 1.56, this difference was consequently proven not statistically significant.

Thus it is evident that the first assertion of Jonxis is incorrect, and that there is no statistical difference in the relative proportions of foetal and adult haemoglobin when newly born normal infants and newly born infants suffering from erythroblastosis foetalis are compared.

In one instance it was possible to take up the other point made by Jonxis that the foetal haemoglobin level

dropped rapidly in severe cases owing to selective destruction. Samples of blood were obtained from Case 3 on three separate occasions and each time the blood was analysed as to the proportion of adult and foetal haemoglobins.

Table XI.

Sample of blood.	Hb level.	%age. foetal haemoglobin.
Cord blood at birth	12.4gms.	84.3%
After Exchange transfusion ($5\frac{1}{2}$ hours).	13 gms.	36.4%
Before "top-up" transfision (6 days).	9.9gms.	35%

These results, while but an isolated series from which few conclusions dare be drawn, suggest strongly that Jonxis erred even in his second conception. There is obviously no selective destruction occurring here as the percentage of foetal haemoglooin is approximately the same at days 1 and 6 in spite of a considerable fall in the total haemoglobin. Owing to the haemopoetic reserve in an infant being small, the production of haemoglobin and cells cannot be greatly increased as compared to normal, and so it is unlikely the new cell formation has much masking effect on

the respective destruction rates. No correlation between erythroblastosis foetalis and foetal haemoglobin is thus conceivable.

The usefulness of this alkaline denaturation technique in determining the degree of success attained by exsanguination (i.e. 56.8% in this instance) is amply demonstrated, and its more frequent application might give valuable information to the Paediatrician in assessing the clinical value of exchange transfusion. At least one more of the unknown variables (i.e. degree of success of the replacement) could be eliminated.

The possibility of a relationship between physiological jaundice and the two forms of haemoglobin was the subject of a very limited uncontrolled but interesting survey. Of the first eight normal newborn infants quoted above, Case 1 (foetal haemoglobin: 71.2%), Case 3 (foetal haemoglobin: 79.1%) and Case 6 (foetal haemoglobin: 95.5%) showed a physiological jaundice worthy of note. In view of the wide range in the proportions of the two pigments in this small series, it would appear that any such correlation is most unlikely.

C H A P T E R IV.

Foetal Haemoglobin in Adult Haematological Conditions.

To Schenck (1930)[193.] must go the credit for the first recognition that foetal haemoglobin may be present in adult blood, as he observed that the blood of several patients with pernicious anaemia exhibited a pigment which behaved towards alkali like foetal rather than normal adult haemoglobin. Haurowitz (1931)[85.] was unable to confirm this observation, and the matter lay dormant until Singer et al examined the blood of two patients with untreated pernicious anaemia, and was able to demonstrate an alkaline resistant pigment in one of them (2.2%). In four patients, treated successfully for pernicious anaemia, no abnormal haemoglobin was detected on analysis.

The interest in the presence of abnormal haemoglobins in adult haematological conditions has been reawakened since Pauling et al[164.] followed up two observations which suggested that the haemoglobin and not the stroma was responsible for the sickling phenomenon in sickle cell anaemia viz. that sickled cells are birefringent[195.] and that sickle cell anaemia cells when lysed show discoidal ghosts;[177.] as a result he discovered that the carriers of the sickle cell trait showed a mixture of adult and sickle cell anaemia haemoglobin, and that the electrophoretic mobilities of sickle cell anaemia haemoglobin and

normal haemoglobin differ. This was demonstrated most easily with the carboxyhaemoglobins in a phosphate buffer of 0.1 ionic strength, the sickle cell anaemia carboxy-haemoglobins moved as a positive ion (isoelectric point 7.09) while the normal compound moved as a negative ion (isoelectric point 6.87). In addition[165.] they could find no significant differences between the acidic and basic amino-acid contents of the two globin portions, sickle cell and normal, and once the haem portions were removed there was strangely no variance in their electrophoretic mobilities. The haems were yet identical! Similarly X-ray crystallography by Perutz et al[175.] revealed no obvious difference between sickle cell and normal oxyhaemoglobins, although the former could be crystallised in a form not assumed by normal oxyhaemoglobin.

Perutz and Mitchison,[176.] using a polarizing microscope of high sensitivity found that while the haemoglobin in normal red cells and oxygenated sickle cells is almost certainly not crystalline, the reduced haemoglobin in sickle cells is in a crystalline state. Observations on solubility in phosphate buffer pH 6.8 revealed that while the normal adult reduced haemoglobin had a solubility only one half of the normal oxyhaemoglobin, the solubility

of the sickle cell anaemia reduced haemoglobin was actually only one hundredth that of the sickle oxyhaemoglobin. These observations thus supported the earlier theoretical conception of Granick[70]. that the reversible changes occurring in sickle cell anaemia cells were due to the simple mechanism of crystallisation within the erythrayte. A third type of abnormal haemoglobin, designated type III[111]. or type C[109,110]. was identified as a hereditary feature in certain sickle cell anaemia families, but while other cases have been reported, not much data has been gathered about this particular haemoglobin.

As a result particularly of Pauling's work, Singer et al (1951)[199]. sought to investigate sickle cell anaemia by recording the reaction of the haemoglobin of sickle cell anaemia to alkaline denaturation. The new technique, already referred to in Chapter IIB was developed, and the study was extended to include many other haematological conditions. Alkali resistant haemoglobins were encountered in fully developed Mediterranean anaemia syndromes (since confirmed by Chloremis),[48]. certain cases of congenital spherocytic anaemia (1 of 4 cases analysed), chronic aregenerative anaemia (3 cases), untreated pernicious anaemia (1 of 2 cases), acute leukaemia (2 cases),

chronic leukaemia (6 of 11 cases) and malignant disease of the marrow (2 of 38). The number of patients with pure haematological conditions thus investigated was very small and inspired further study.

The main aspect taken up by Singer et al subsequently was the variety of pigments discovered in sickle cell anaemia and in the trait. For purposes of simplicity, the haemoglobins were designated thus: Normal haemoglobin - type N; sickle cell anaemia haemoglobin - type S; alkali resistant, foetal or foetal-like haemoglobin - type F. With the denaturation procedure, haemoglobin solutions prepared from sickle cell anaemia cells reveal a resistant pigment from 2 - 24% while the trait erythrocytes exhibited no abnormally denaturing compounds. Wells and Itano[219]. reported around the same time tnat up to 20% of an electrophoretically normal compound may be encountered in haemoglobin solutions prepared from sickle cell anaemia erythrocytes. In view of the fact that slight differences between adult and foetal haemoglobins may be exhibited in electrophoretic mobility, the correlation between these two separate observations would appear to merit further parallel investigation. This is all the more indicated by the very recent preliminary communication by Itano[110]. stating

that electrophoretic studies do now suggest the presence of foetal haemoglobin in sickle cell anaemia.

The alkaline resistant pigments were isolated and concentrated by Singer et al, and then subjected to fractional alkaline denaturation. This revealed that the pigment in practically all instances behaved like foetal haemoglobin; the exceptions were in sickle cell anaemia (7 of 11 cases) and in two members of one family with congenital spherocytic anaemia, where the pigment could but be described as foetal-like. The biochemical basis for these findings is quite obscure, but the data led Singer et al to support the earlier conceptions of Schenck, Drabkin and others, that there is a persistence of, or reversion to, the embryonic form of haemopoeisis, where the globin moiety differed, perhaps in relation to the site of fabrication.

Further work demonstrated that tactoid and gel formation in concentrated solutions was possible only with type S and not with normal adult or foetal haemoglobins. After concentration in purified form the alkaline resistant haemoglobin fraction was not able to produce tactoid or gel formation, thus strengthening the hypothesis that types S and F are unrelated, yet co-exist in sickle cell

anaemia blood. Survival time studies were performed with sickle cell anaemia erythrocytes into normal recipients and various studies (viz. reaction to alkaline denaturation and trauma) revealed that the erythrocyte population in sickle cell anaemia possibly consists of 3 fractions (1) red cells containing S haemoglobin and at most minimal amounts of F (2) "intermediate" cells with S and F pigment and (3) a small proportion of erythrocytes carrying mostly F and little or no S pigment. No direct correlation between the relative proportions of the pigments and the severity of the disease could be established.

A similar study would be of interest on the blood of patients with Mediterranean anaemia especially following the report by Kaplan and Zuelzer[121]. that 25% - 50% of transfused leptocytes disappear within 20 - 30 days, with a subsequent survival time curve resembling that of normal. Any correlation between this finding and the foetal haemoglobin present in the fully established condition would indeed be significant.

As a result of the interest aroused by this existence of F haemoglobin in many adult haematological conditions, it was decided to establish its presence or absence in the common blood disorders encountered in this

country, thus augmenting and advancing the work of Singer et al; and to observe the time of persistence of the pigment, if present, in the blood of patients with pernicious anaemia, who are in the course of treatment.

Method: The alkaline denaturation technique already described in Chapter IIB was employed, and in most instances 0.2ml. haemoglobin solution was utilised, although 0.1ml. was used in any cases of doubt. The mean of 2-6 results is presented.

Results: Normal Controls. Oxalated blood samples from 30 of the students, patients' relatives and staff were analysed in a consecutive series and the following percentages for F haemoglobin were determined from these normal controlls. Age of subject is given in brackets.

Males.				Females.			
%age F.	Age.	%age F.	Age.	%age F.	Age.	%age F.	Age.
1.36	(17)	0.68	(26)	0.78	(23)	1.04	(57)
0.40	(28)	0.36	(19)	1.45	(19)	0.38	(67)
0.87	(27)	1.40	(19)	1.38	(33)	0.88	(20)
1.03	(37)	1.22	(68)	0.84	(64)	0.79	(23)
0.59	(21)	0.43	(53)	0.59	(57)	0.41	(35)
0.79	(29)	0.64	(45)	1.16	(54)	0.30	(19)
0.86	(49)	1.30	(44)	0	(48)	0.93	(17)
0.38	(37)			0.64	(54)		

Mean %age "F haemoglobin" in Normal Males = 0.82%

Standard Deviation = $\pm$0.36

Mean %age "F haemoglobin" in Normal Females = 0.77%

Standard Deviation = $\pm$0.29

Standard Error of the Difference of these Means = 0.1146.

From a short perusal of these results it is evident that no significant difference pertains to sex and no obvious difference pertains to age. All estimations are therefore combined to give:

Mean percentage "F haemoglobin" in normal adults = 0.795%

Standard Deviation = $\pm$ 0.327.

For statistical purposes it may be presumed that any reading over Mean + 3x Standard Deviation is signifi-

-cant. Thus by the technique applied here any value over 1.88% is significant, which is very close to the figure obtained by Singer et al, 1.96%.

Untreated Pernicious Anaemia:

The bloods of 28 consecutive patients with untreated pernicious anaemia were subjected to the alkaline denaturation technique and the results obtained are tabulated in Table XII. It can be seen that 14 of these patients show evidence of abnormal pigment production varying from 2.06% - 5.49% on the first examination. The age and blood counts of the patients are included in the table to give a composite picture. The other 14 patients gave results varying from 0.35% - 1.65% which are within the bounds of experimental error and chance.

The time of persistence of this pigment was considered worthy of continued investigation and eleven of the fourteen patients presenting the resistant F haemoglobin were followed up over periods from 4 - 8 months, by which time only one patient still demonstrated the abnormal pigment. Two patients with no significant readings before treatment were also checked over periods of 3 and 8 months respectively.

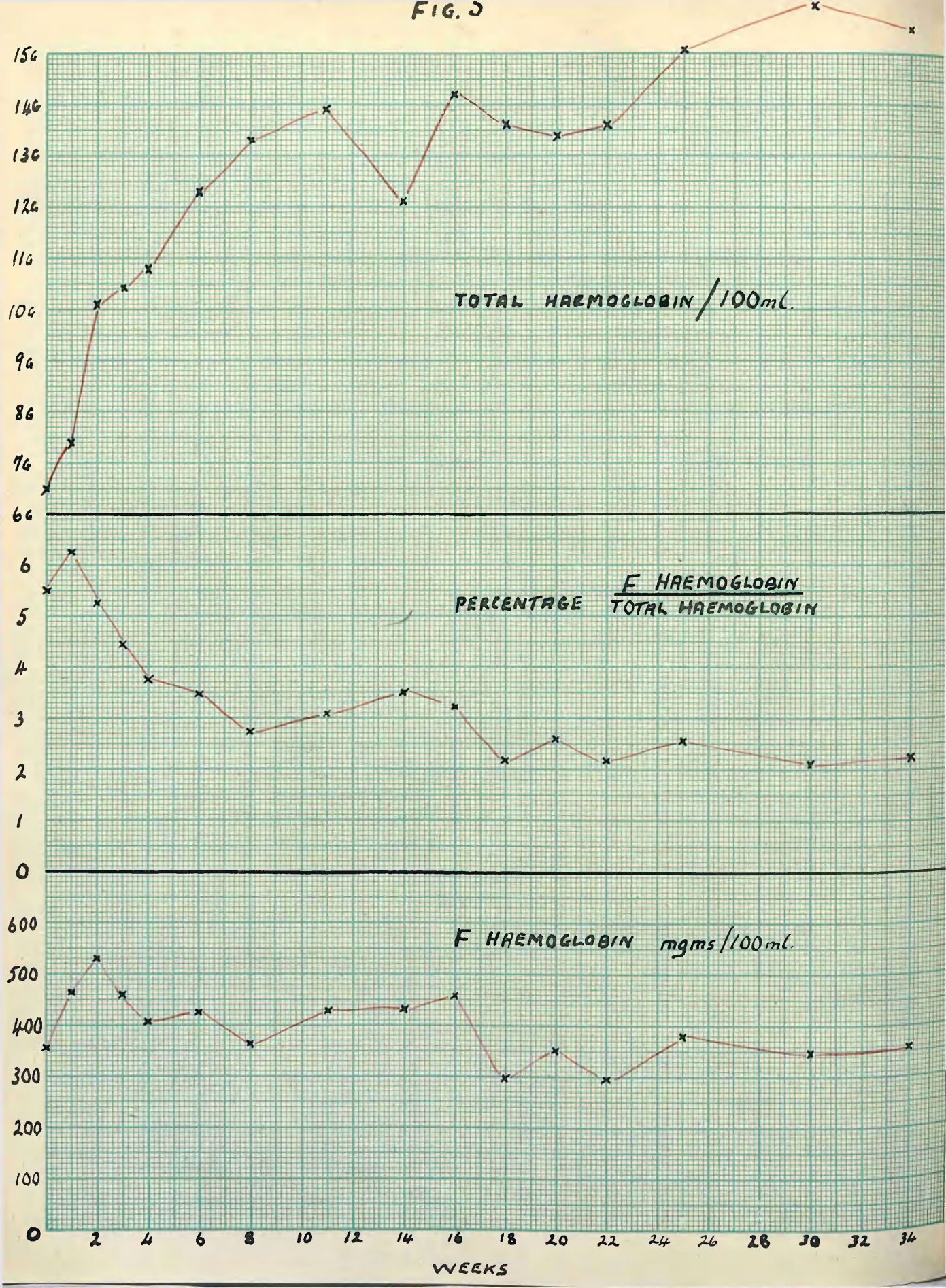
The results are presented in the following series

TABLE XII.

Pernicious anaemia - Untreated

No.	Sympton Onset	Age	Sex	R.B.Cs./c.mm.	Hb. gms/ 100 ml.	%age foetal Hb.
1	3 months	60	M	1,400,000	6.5	5.49
2	1 year	55	F	1,170,000	4.7	3.83
3	9 months	71	F	1,560,000	6.8	3.72
4	2 months	33	F	1,320,000	3.5	3.57
5	3 years	47	F	1,950,000	9.3	3.33
6	1 year	47	F	1,260,000	6.1	3.31
7	1 year	38	F	1,100,000	3.7	3.1
8	1 year	56	M	1,090,000	5.2	3.0
9	1 year	44	F	2,560,000	8.7	2.97
10	8 months	66	F	1,920,000	8.3	2.7
11	9 months	65	M	1,100,000	5.0	2.5
12	9 months	70	F	1,650,000	8.7	2.42
13	1 month	58	F	1,770,000	7.7	2.22
14	7 months	41	M	1,930,000	7.7	2.06
15	5 months	76	M	1,360,000	5.9	1,65
16	9 months	64	F	1,370,000	5.3	1.45
17	1 month	54	F	1,310,000	4.1	1.42
18	2 months	52	F	1,950,000	7.1	1.40
19	1 year	63	M	2,310,000	9.9	1.36
20	9 months	46	F	2,260,000	9.8	0.87
21	2 years	50	F	1,970,000	8.3	0.78
22	3 months	56	F	1,850,000	8.3	0.75
23	3 years	49	F	2,280,000	8.3	0.75
24	20 years	66	F	1,660,000	7.7	0.61
25	6 months	50	F	1,580,000	4.7	0.56
26	9 months	44	M	2,950,000	12.0	0.36
27	3 months	30	F	1,580,000	5.3	0.36
28	5 months	54	F	1,770,000	6.8	0.35

FIG. 3


15G
14G
13G
12G
11G
10G
9G
8G
7G
6G
TOTAL HAEMOGLOBIN / 100mL.
6
5
4
3
2
1
0
PERCENTAGE F HAEMOGLOBIN / TOTAL HAEMOGLOBIN
600
500
400
300
200
100
0
F HAEMOGLOBIN mgms / 100mL.
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
WEEKS

of charts, and each chart is accompanied by a very short résumé of the patient's general condition to present the criteria of diagnosis, and to allow alignment of the clinical with the biochemical states.

Case 1. Occupation: Labourer. Age 60. Sex M.

Diagnosed as pernicious anaemia in 1937 - treated with peps.ac.

No treatment for past 2 years.

Symptoms of anaemia - 3 months. Numerous faints - 1 month.

O/E Pale, icteric, sclerotic and very old for age.

No cord changes.

RBC 1,400,000 per c.mm. C.I. 1.57

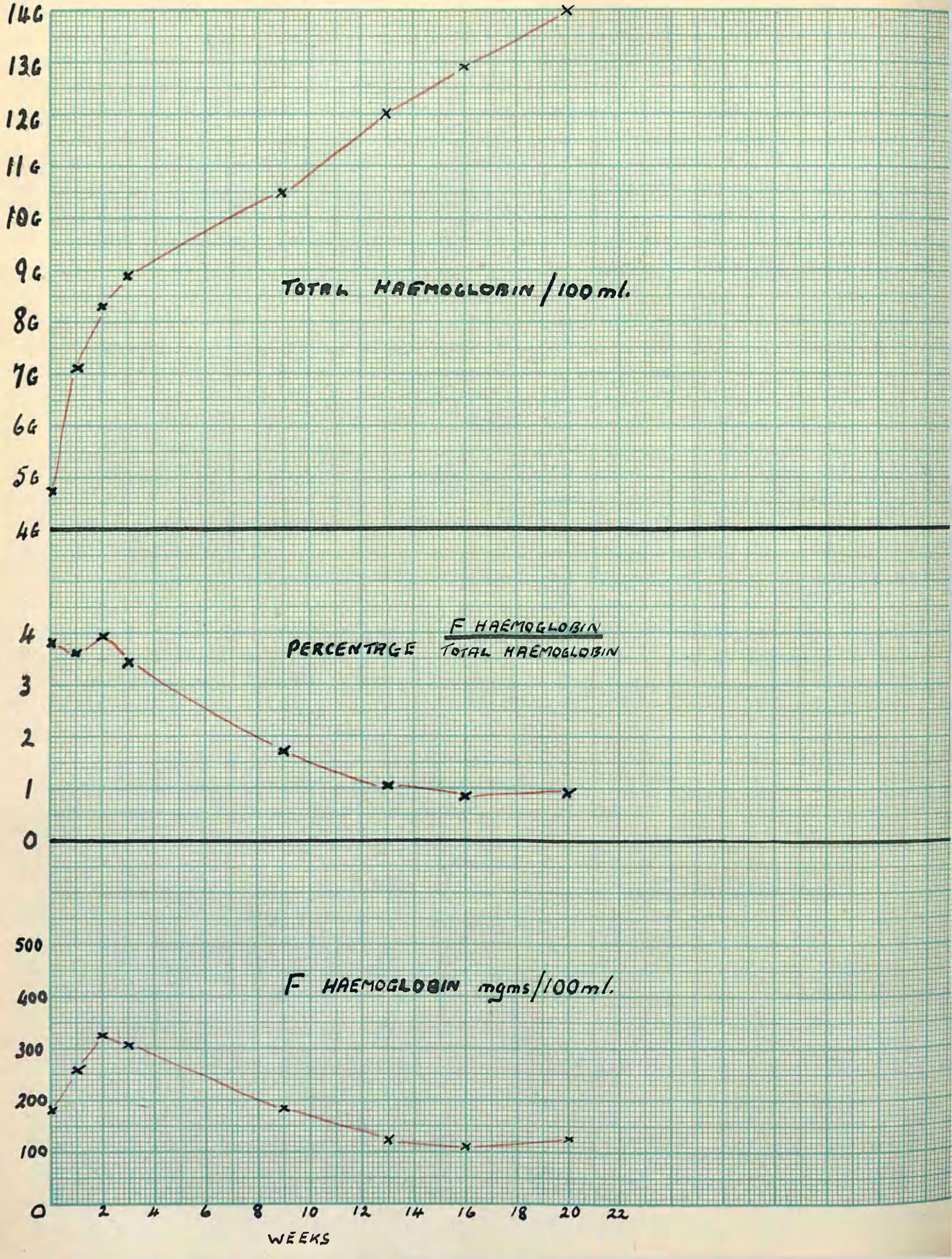
Hb 6,5 gms. % M.C.V. 126 mu.

Achlorhydria Marrow : megaloblastic.

Treatment: Cytamen in full doses.

Fig. 5 demonstrates that the F haemoglobin persisted in this patient even after 8 months; there was a relative diminution in the proportions of normal and F haemoglobin from the first week until about 18 weeks when the percentage stabilised. Until this stage too, there was a definite diminution of production of the pigment, after an initial increase for the two weeks following commencement of treatment.

FIG. 6.


14G
13G
12G
11G
10G
9G
8G
7G
6G
5G
4G
TOTAL HAEMOGLOBIN / 100 ml.
4
3
2
1
0
PERCENTAGE F HAEMOGLOBIN / TOTAL HAEMOGLOBIN
500
400
300
200
100
0
F HAEMOGLOBIN mgms / 100 ml.
2
4
6
8
10
12
14
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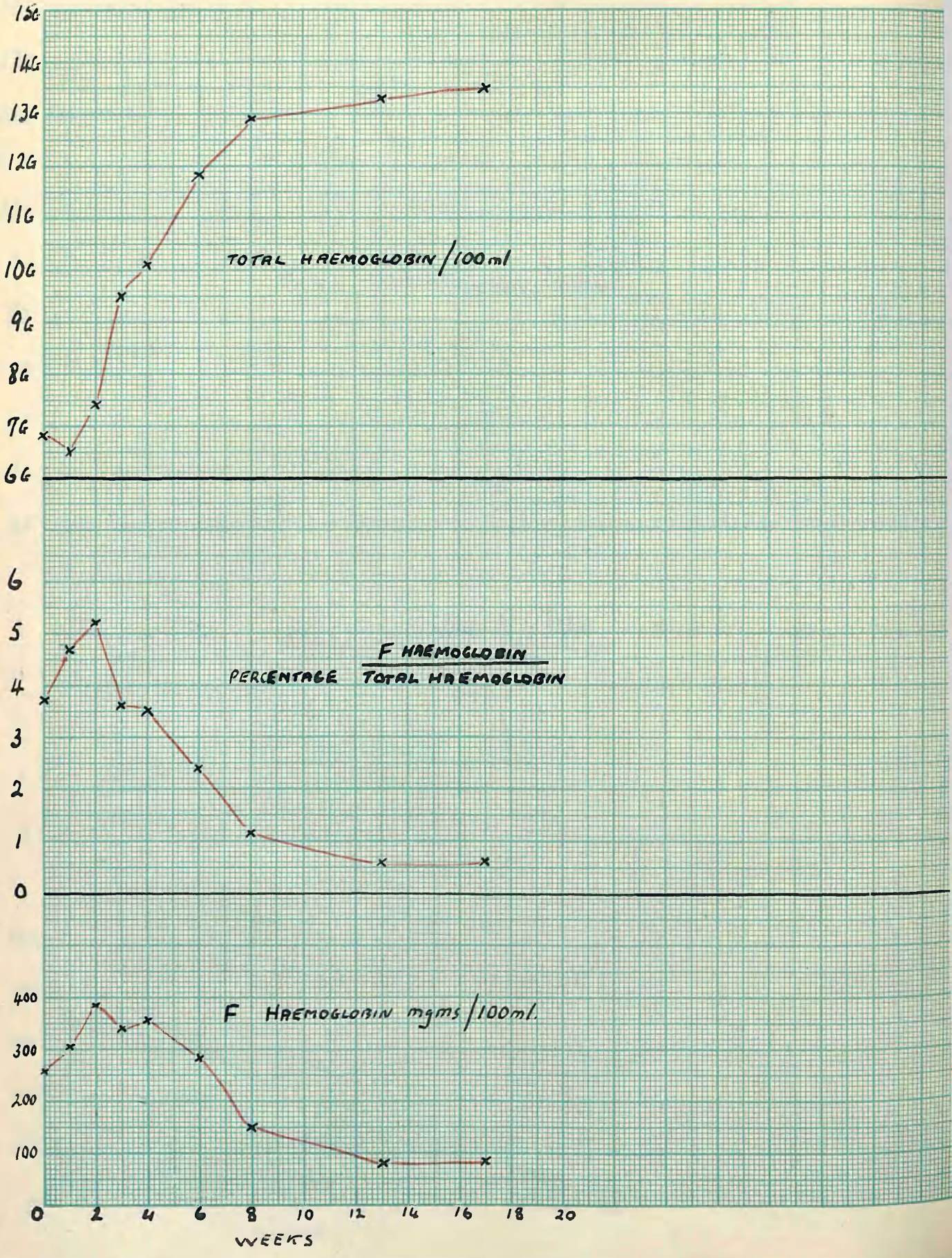
18
20
22
WEEKS

FIG. 7


15G
14G
13G
12G
11G
10G
9G
8G
7G
6G
TOTAL HAEMOGLOBIN/100ml
6
5
4
3
2
1
0
PERCENTAGE F HAEMOGLOBIN / TOTAL HAEMOGLOBIN
400
300
200
100
F HAEMOGLOBIN mgms/100ml.
0
2
4
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8
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12
14
16
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20
WEEKS

Case 2. Occupation: Housewife. Age 55. Sex F.

1 year: increasing breathlessness and anaemic symptoms.

Diagnosed as pernicious anaemia before and treated in hospital.

No treatment since.

P.H. Epilepsy since childhood.

O/E Pale, icteric woman. WR +ve. Diminished vibration sense.

R.BC 1,700,000 per c.mm. C.1. 1.39.

Hb 4.7 gms. % W.B.C. 3,400 per c.mm.

Achlorhydria Marrow: megaloblastic.

Treatment: Cytamen in full doses.

Fig. 6 shows that the resistant pigment again increased over the first two weeks of treatment before steadily decreasing. The level stabilised within 13 weeks.

Case 3. Occupation: Housewife. Age 71. Sex F.

9 months: increasing fatigue, lassitude and lack of energy.

3-4 weeks: cramps in legs.

O/E. Pale, icteric, sclerotic old lady.

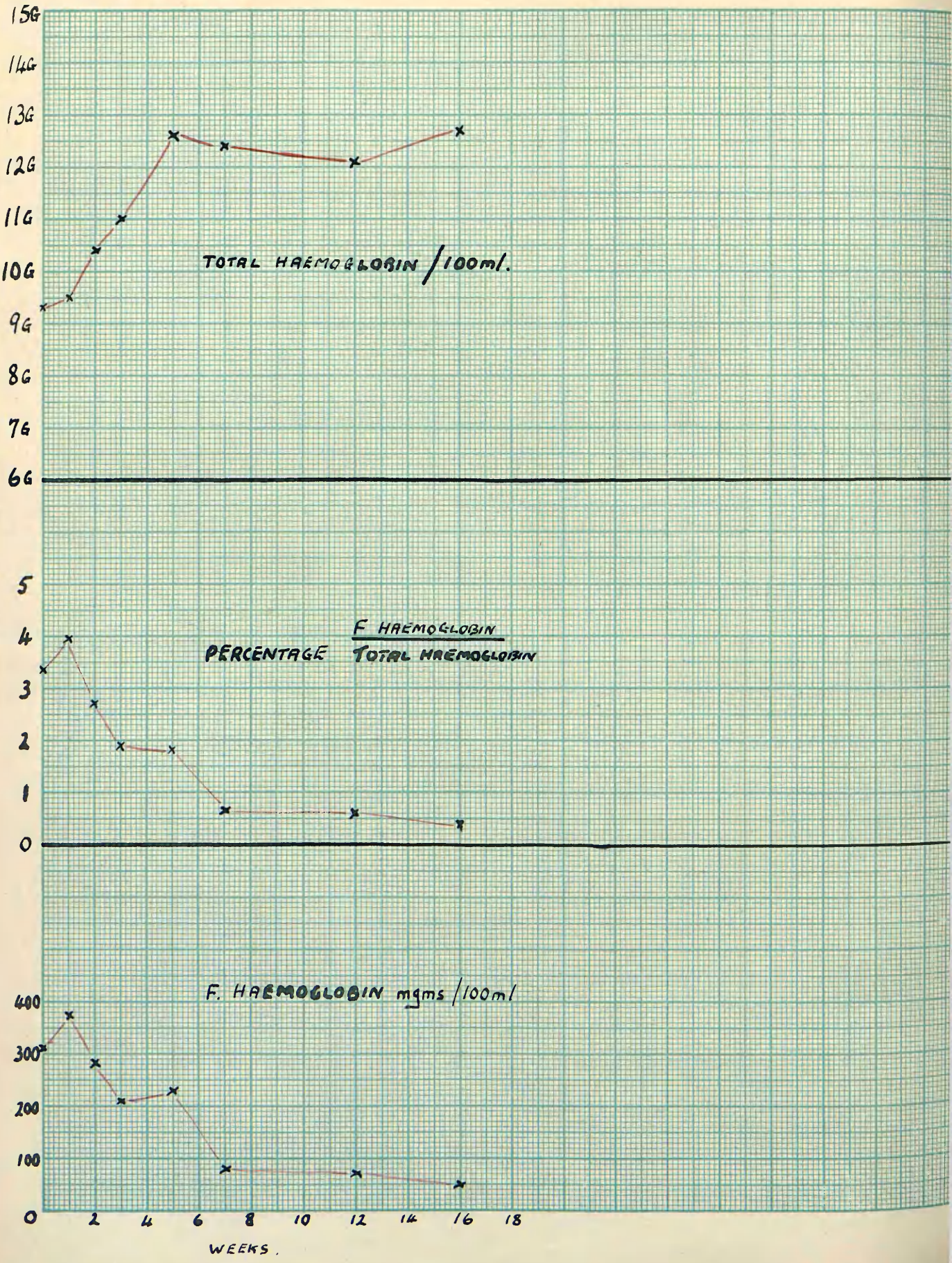
R.B.C. 1,560,000 per c.mm.

Hb 6.8 gms. %. C.1. 1.43.

Achlorhydria Marrow: megaloblastic.

Treatment: After 1 week, treated with American liver preparation (30 U.S.P. units/cc.) 4cc 1.M. for

FIG. 8.


15G
14G
13G
12G
11G
10G
9G
8G
7G
6G
TOTAL HAEMOGLOBIN /100ml.
5
4
3
2
1
0
PERCENTAGE F HAEMOGLOBIN / TOTAL HAEMOGLOBIN
400
300
200
100
0
F. HAEMOGLOBIN mgms /100ml
2
4
6
8
10
12
14
16
18
WEEKS.

4 days. Then routine cytamen.

Fig. 7. shows that, in this instance, the foetal pigment increased for one week after commencement of treatment, and fell very rapidly to a stable normal level within a further 6 - 11 weeks.

Case 5. Occupation: Housewife. Age 47. Sex F.

3 years: increasing lethargy and anaemia - attributed to bleeding haemorrhoids. Referred by Surgeon as pre-operative precaution.

O/E Pale. No icterus. Tongue: smooth.

RBC: 1,900,000 per c.mm. C.I. 1.57

Hb: -9.3 gms. % W.B.C. 2,600.

Achlorhydria Marrow: megaloblastic.

Treatment: Cytamen in full doses.

From Fig. 8. it can be seen that the F haemoglobin increased for the first week of treatment, and then rapidly disappeared over the following 6 weeks.

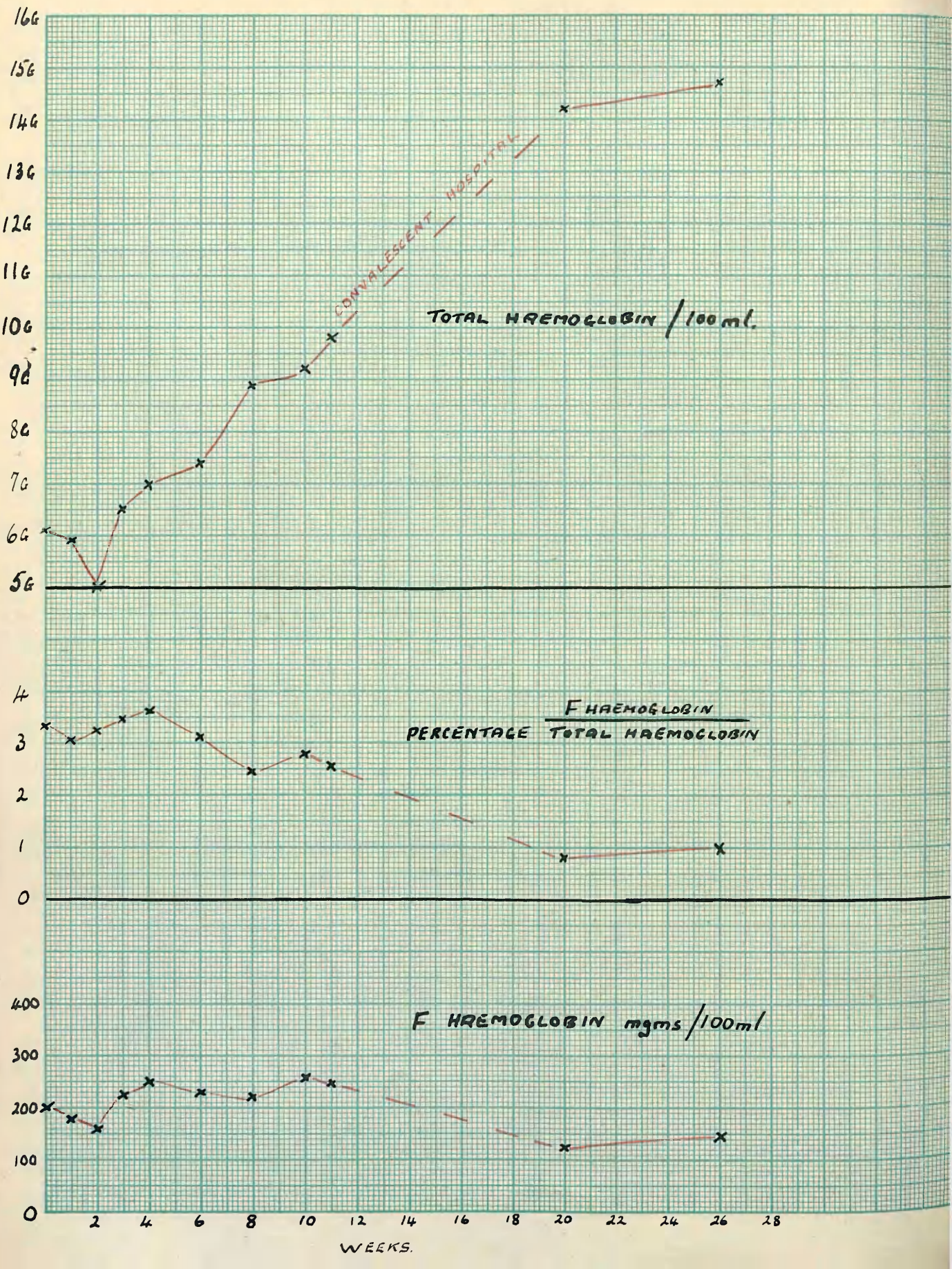
Case 6. Occupation: Examiner in hat factory. Age 47. Sex F.

1 year: increasing fatigue dizziness, angina of effort.

2 months: paraesthesiae, numbness of hands and feet.

O/E Pale, icteric with mild S.C.D. : K.J., A.J., and vibration sense legs: absent.

FIG. 4.


16G
15G
14G
13G
12G
11G
10G
9G
8G
7G
6G
5G
CONVALESCENT
HOSPITAL
TOTAL HAEMOGLOBIN / 100 ml.
4
3
2
1
0
PERCENTAGE F HAEMOGLOBIN / TOTAL HAEMOGLOBIN
400
300
200
100
0
F HAEMOGLOBIN mgms / 100 ml
2
4
6
8
10
12
14
16
18
20
22
24
26
28
WEEKS.

R.B.C. : 1,260,000 per c.mm. C.I. 1.57

Hb : 6.1 gms. % W.B.C. 1,600

Achlorhydria Marrow: megaloblastic

Treatment: 8 days: trial on Penicillin 200,000 units b.i.d. orally

3 weeks: trial on Folic acid 1.67) Cytamen 25 ug) tabs ii daily orally.

Then Cytamen in large doses because of sudden acute onset of subacute combined degeneration of the cord which cleared up after 3 months treatment.

Fig. 9. illustrates the haematological progress in this patient. The F haemoglobin is seen to increase somewhat for about 4 weeks before settling some time in the period 7 - 16 weeks later while the patient was in a convalescent hospital.

Case 8: Occupation: Cobbler. Age 56. Sex M.

12 months: increasing dyspnoea, fatigue, anginal pain on exertion.

F.H. Mother died of pernicious anaemia 25 years ago.

O/E Thin, pale, icteric man.

R.B.C.: 1,090,000 per c.mm. C.I. 1.59

Hb : 5.2 gms. % M.C.V. 100c.u.

Achlorhydria Marrow: megaloblastic.

Treatment: 10 days: trial on penicillin 200,000 units b.i.d. orally

FIG. 10

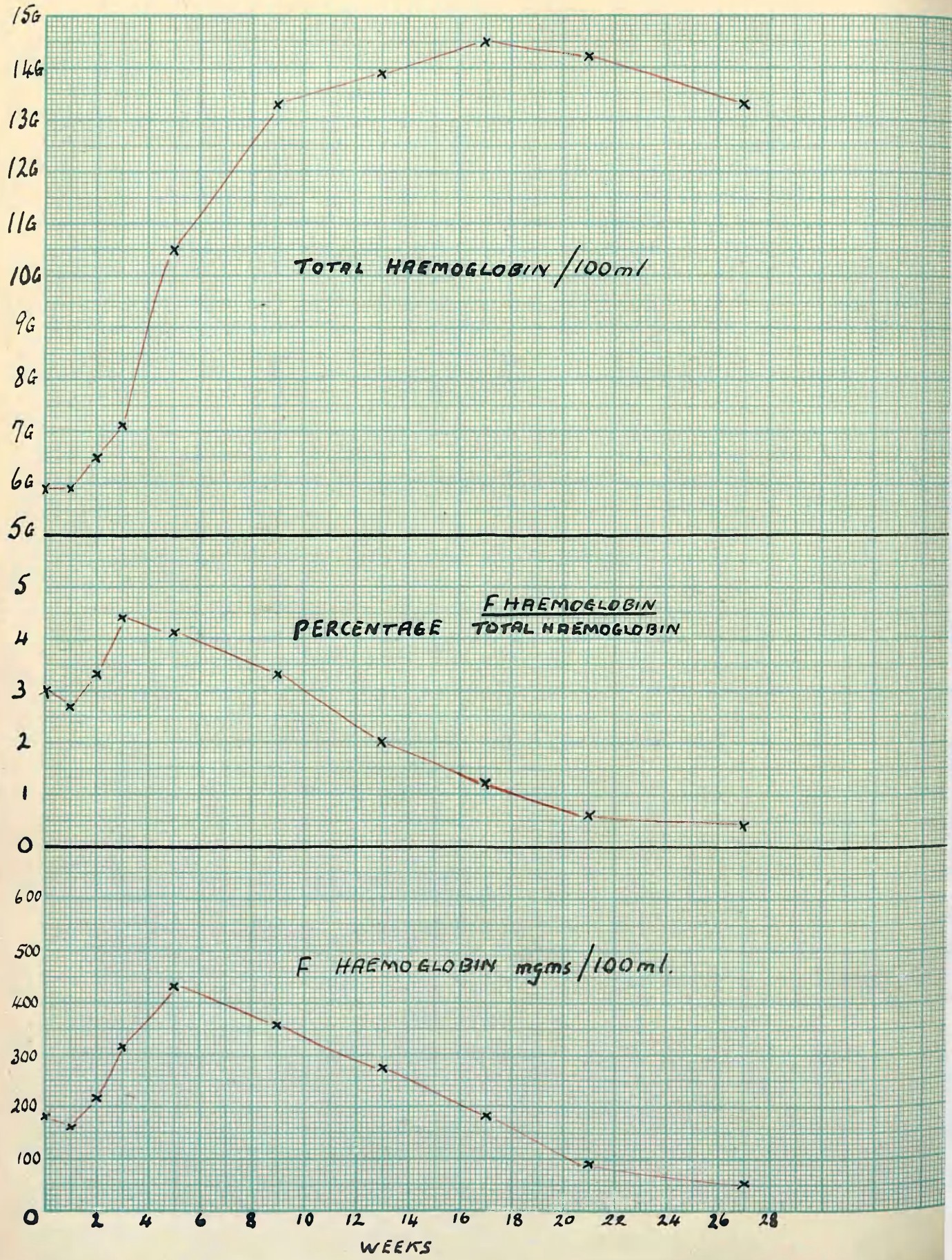
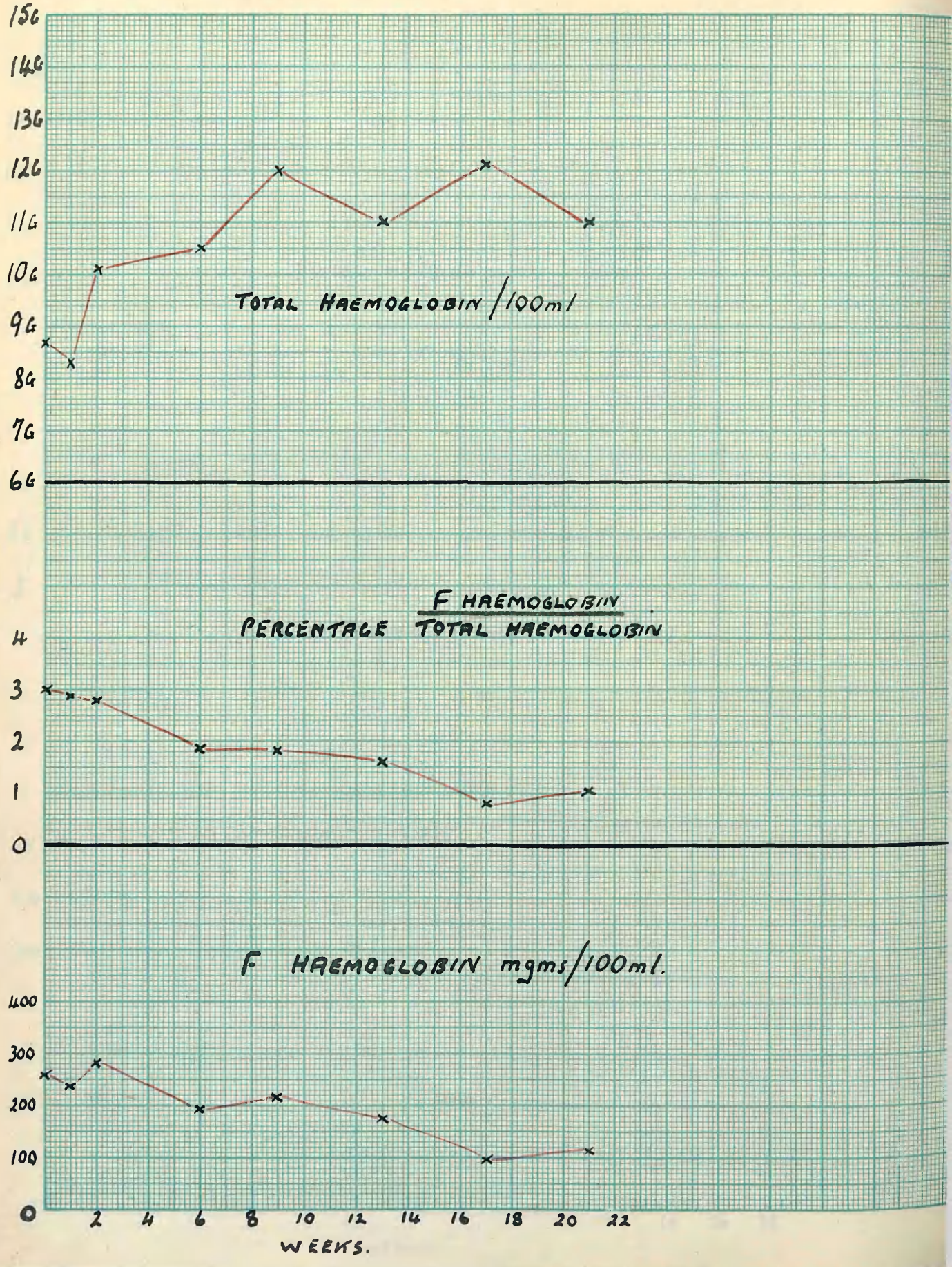

15G
14G
13G
12G
11G
10G
9G
8G
7G
6G
5G
TOTAL HAEMOGLOBIN /100ml
5
4
3
2
1
0
PERCENTAGE
F HAEMOGLOBIN
TOTAL HAEMOGLOBIN
600
500
400
300
200
100
0
F HAEMOGLOBIN mgms /100ml.
2
4
6
8
10
12
14
16
18
20
22
24
26
28
WEEKS

FIG. 11


15G
14G
13G
12G
11G
10G
9G
8G
7G
6G
TOTAL HAEMOGLOBIN /100ml
PERCENTAGE F HAEMOGLOBIN / TOTAL HAEMOGLOBIN
4
3
2
1
0
F HAEMOGLOBIN mgms/100ml.
400
300
200
100
0
2
4
6
8
10
12
14
16
18
20
22
WEEKS.

10 days: trial on Sanatogen liver 28gm. t.d.s. orally
Then Cytamin in full doses.

From Fig. 10, it can be seen that the F haemoglobin increased both relatively and quantitatively for 3 weeks, then quantitatively for 2 weeks after institution of proper therapy, before the readings fell steadily within the next 12 - 16 weeks to a normal stable level.

Case 9. Occupation: Housewife. Age 44. Sex F.

12 months: off-colour, fatigue, headache, nausea and vomiting.

Diagnosed as aplastic anaemia in another hospital and given a liver injection 1 week before referring here.

Reticulocytes on arrival 8.92%.

R.B.C. 2,560,000 per c.mm. C.I. 1.18

Hb 8.7 gms. % W.B.C. 6,900 per c.mm.

Achlorhydria Marrow: intensely normoblastic with a few Reticulocytes: 2% after 1 week. Transitional megaloblasts.

Treatment: After one week's observation: Cytamen in full doses.

In this instance, Fig. 11, it can be seen that from the time of institution of therapy here, the readings levelled off within 12 - 16 weeks.

FIG. 12.

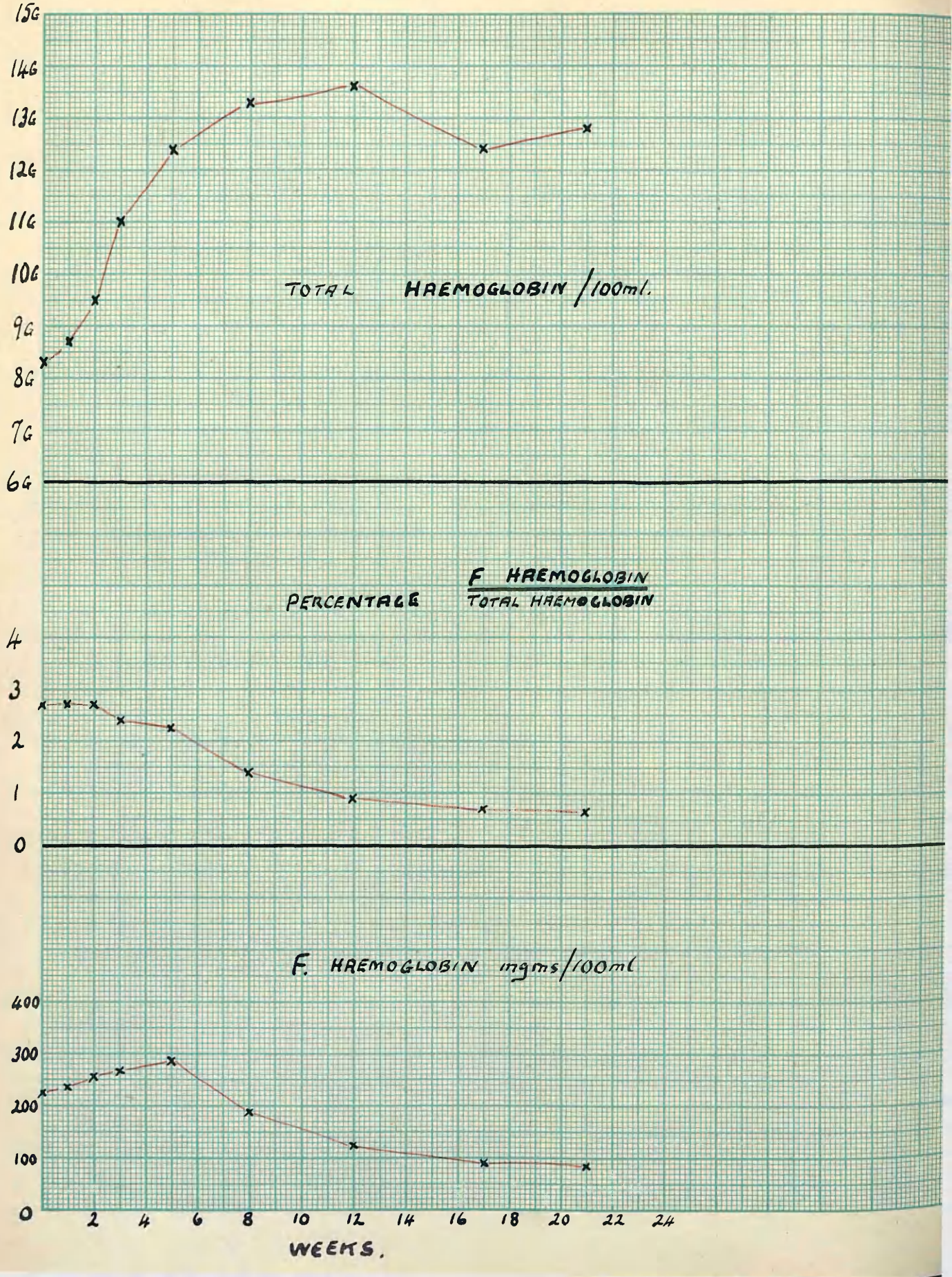
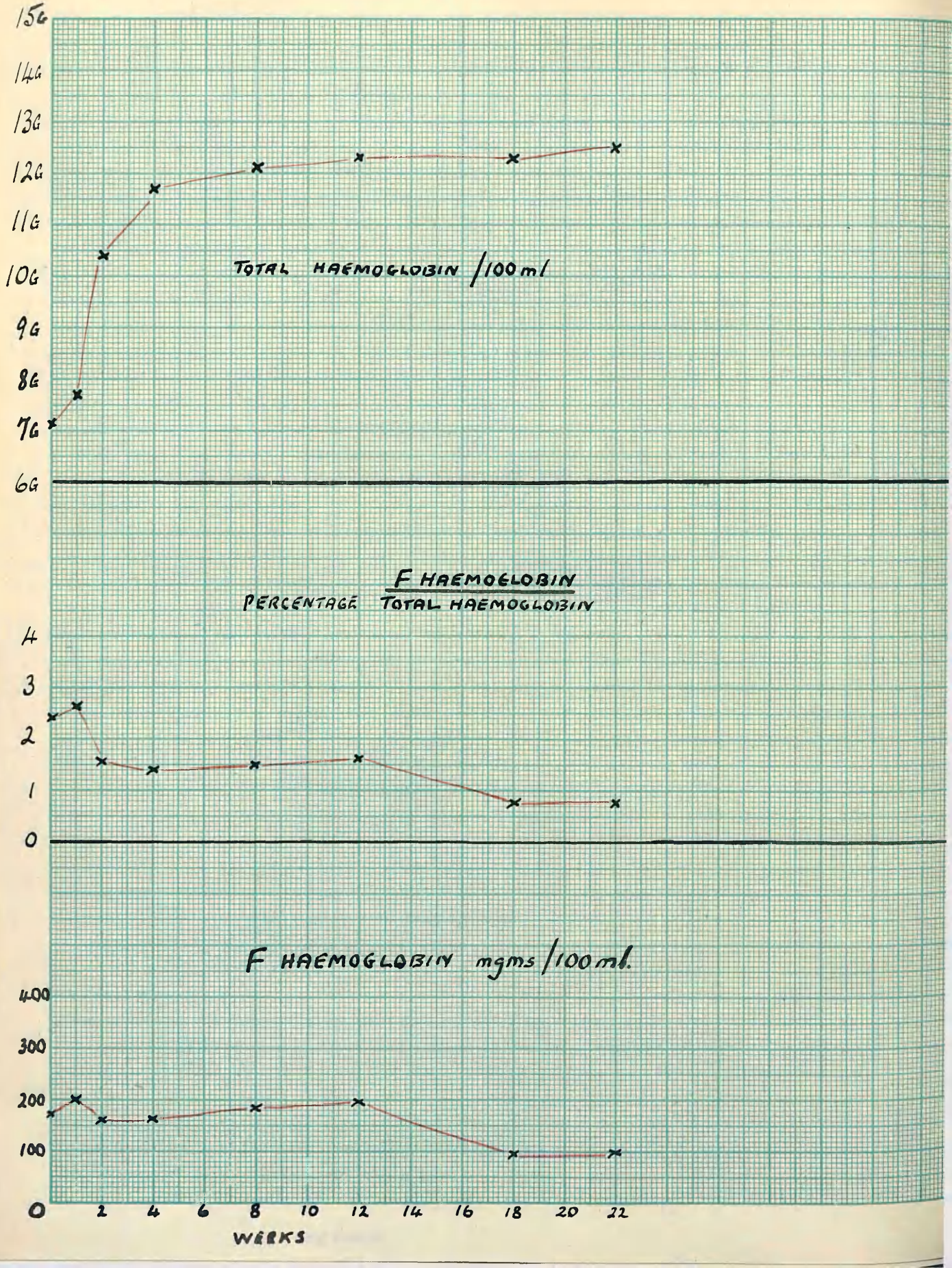

15G
14G
13G
12G
11G
10G
9G
8G
7G
6G
TOTAL HAEMOGLOBIN /100ml.
PERCENTAGE F HAEMOGLOBIN / TOTAL HAEMOGLOBIN
4
3
2
1
0
F. HAEMOGLOBIN ingms/100ml
400
300
200
100
0
2
4
6
8
10
12
14
16
18
20
22
24
WEEKS.

FIG. 13


15G
14G
13G
12G
11G
10G
9G
8G
7G
6G
TOTAL HAEMOGLOBIN /100 ml
F HAEMOGLOBIN
PERCENTAGE TOTAL HAEMOGLOBIN
4
3
2
1
0
F HAEMOGLOBIN mgms /100 ml.
400
300
200
100
0
2
4
6
8
10
12
14
16
18
20
22
WEEKS

Case 10. Occupation: Housewife. Age 66. Sex F.

8 months: lethargy, off colour, breathlessness.

Many years: diverticulitis and mild myxoedema.

O/E. Pale, muddy complexion. Always cold.

R.B.C. 1,920,000 per c.mm.

Hb 8.3 gms. % C.I. 1.47.

Achlorhydria Marrow: megaloblastic

Treatment: Cytamen in full doses.

Fig. 12, demonstrates the pattern of F haemoglobin disappearance in this instance. The percentage of the resistant pigment fell steadily and at between 8 - 12 weeks, the readings must have become quite constant.

Case 12. Occupation: Housewife. Age 70. Sex F.

9 months: off-colour, dyspnoea, angina of effort, slight paraesthesiae and glossitis.

R.B.C. : 1,650,000 per c.mm.

Hb : 8.7 gms. % C.I. 1.53

Achlorhydria Marrow: megaloblastic

Treatment: Cytamen in full doses.

Fig. 13, presents the results of the follow-up in this instance, and it can be seen that when the initial value for F haemoglobin, is low, it is difficult to decide at what point disappearance takes place but it is conjec-

FIG. 14

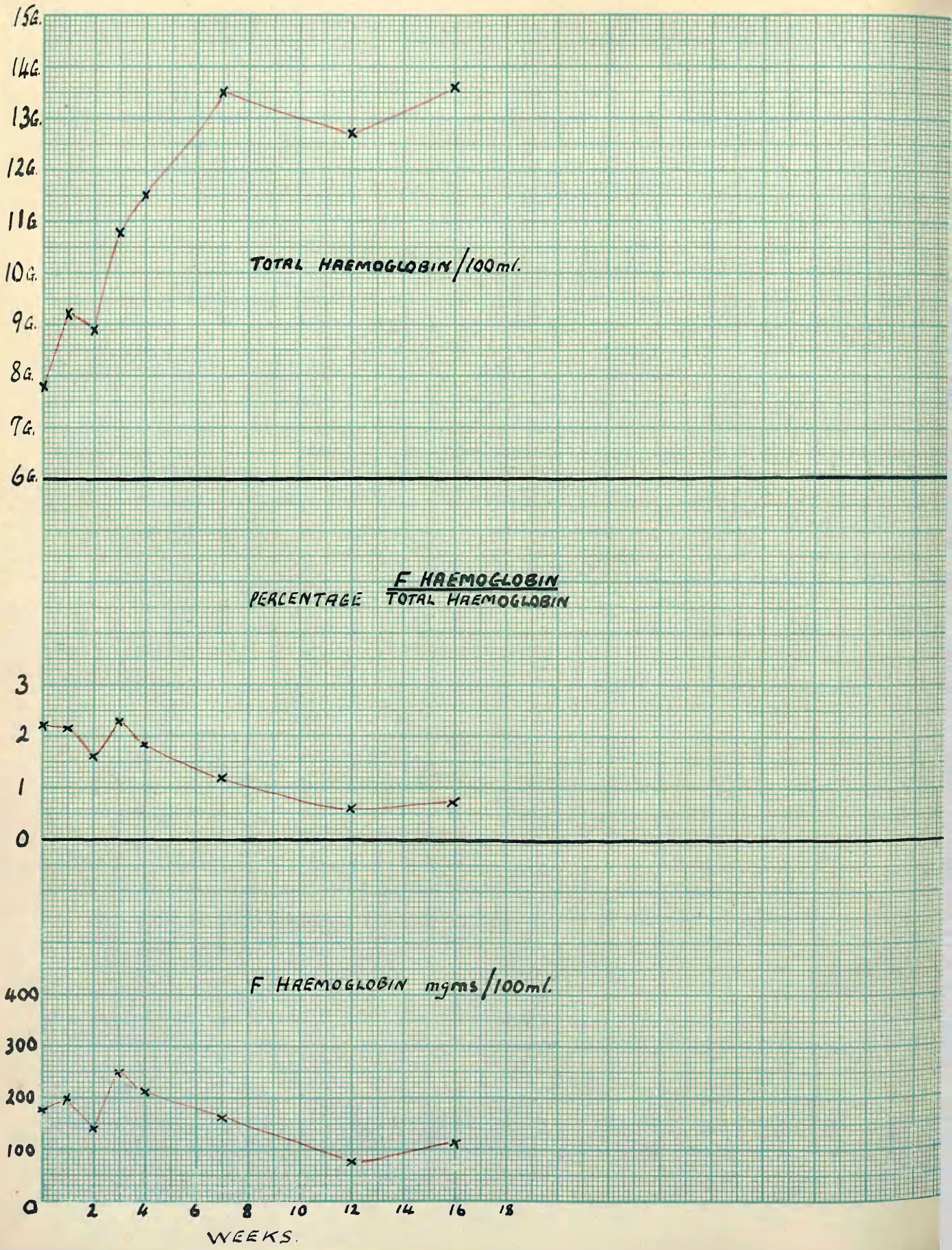
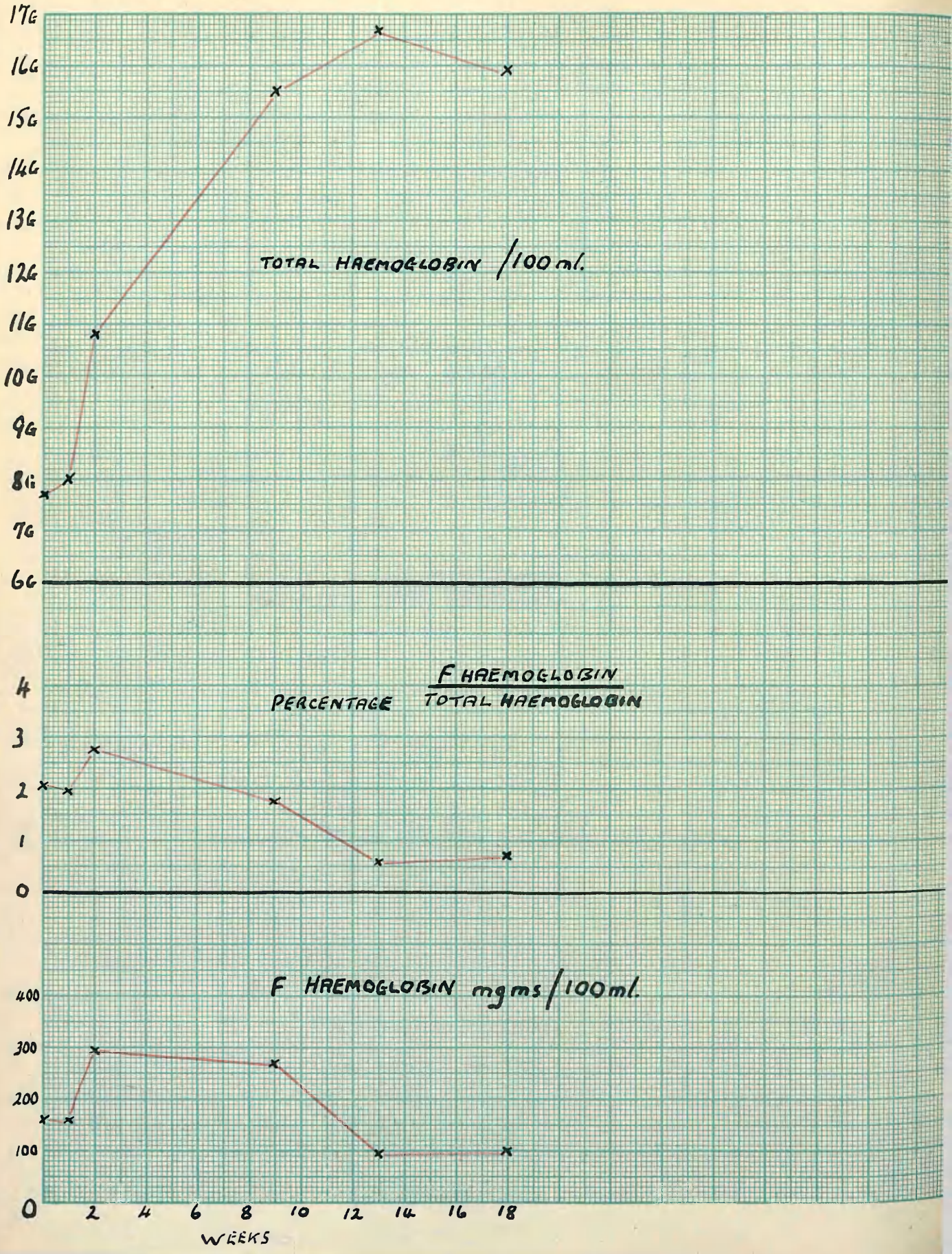

15G.
14G.
13G.
12G.
11G.
10G.
9G.
8G.
7G.
6G.
TOTAL HAEMOGLOBIN/100ml.
PERCENTAGE F HAEMOGLOBIN / TOTAL HAEMOGLOBIN
3
2
1
0
F HAEMOGLOBIN mgms/100ml.
400
300
200
100
0
2
4
6
8
10
12
14
16
18
WEEKS.

FIG. 15


17G
16G
15G
14G
13G
12G
11G
10G
9G
8G
7G
6G
TOTAL HAEMOGLOBIN /100 ml.
4
3
2
1
0
PERCENTAGE
F HAEMOGLOBIN
TOTAL HAEMOGLOBIN
400
300
200
100
0
F HAEMOGLOBIN mgms /100ml.
2
4
6
8
10
12
14
16
18
WEEKS

-tured here to be at most between 12 - 18 weeks.

Case 13. Occupation: Weaver. Age 58. Sex F.

1 month: palpitations and easily tired.

O/E Pale, slight icterus, smooth tongue.

R.B.C.: 1,770,000 per c.mm. C.I. 1.52

Hb 7.7 gms. % W.B.C. 5,050

Achlorhydria Marrow: Intensely normoblastic with occasional transitional megaloblasts.

Natural remission occurred, and treatment postponed 2 weeks. Then cytamen was given in routine doses.

Again from Fig. 14, a levelling off of readings 5 weeks - 10 weeks after treatment can be postulated as being the maximum end-point of foetal haemoglobin persistence.

Case 14. Occupation: Minister of Church. Age 41. Sex M.

7 months: off colour, easily tired.

F.H.: Father had pernicious anaemia.

O/E Pale, sallow with papillated tongue.

R.B.C. : 1,930,000 per c.mm.

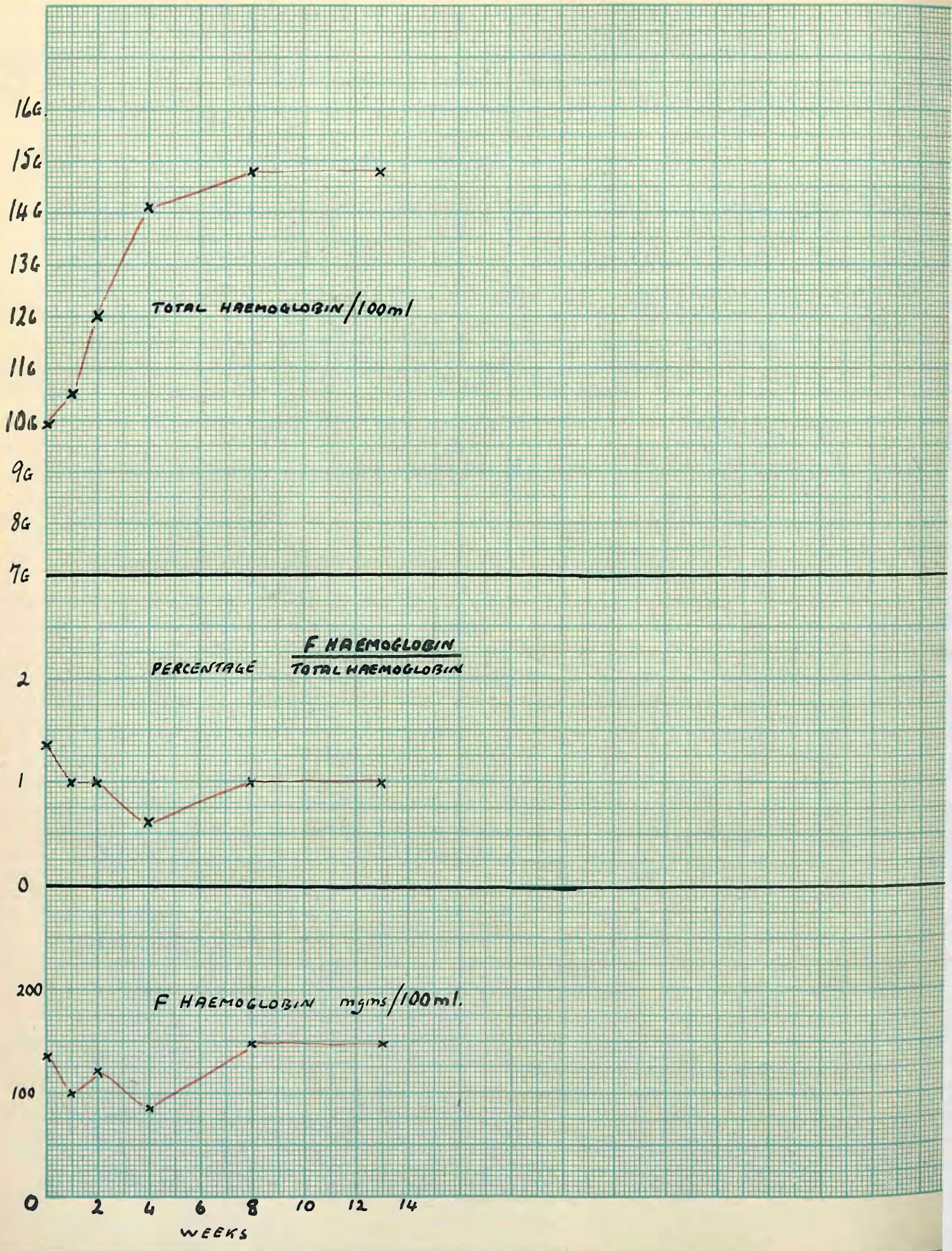
Hb : 7.7 gms. % C.I. 1.35

Achlorhydria Marrow: megaloblastic.

Treatment: Cytamen in full doses.

From Fig. 15, it would appear that the F haemo-

Fig. 16


16G.
15G
14G
13G
12G
11G
10G
9G
8G
7G
TOTAL HAEMOGLOBIN/100ml
PERCENTAGE
F HAEMOGLOBIN
TOTAL HAEMOGLOBIN
2
1
0
200
100
0
F HAEMOGLOBIN mgms/100ml.
2
4
6
8
10
12
14
WEEKS

-globin increased somewhat for 2 weeks and then disappeared between 9 and 13 weeks.

. . . . . .

No constant disappearance curve, logarithmic or otherwise, was thus obtained for the foetal haemoglobin in these 11 cases.

Two Patients exhibiting no F Haemoglobin.

Case 19. Occupation: Maintenance Engineer. Age 63. Sex M.

1 year: increasing breathlessness on exertion, lack of concentration.

O/E Well built, pale, icteric man.

Diminution of vibration sense.

R.B.C.: 2,310,000 per c.mm.

Hb.: 9.9 gms. % C.I. 1.45.

Achlorhydria Marrow: megaloblastic.

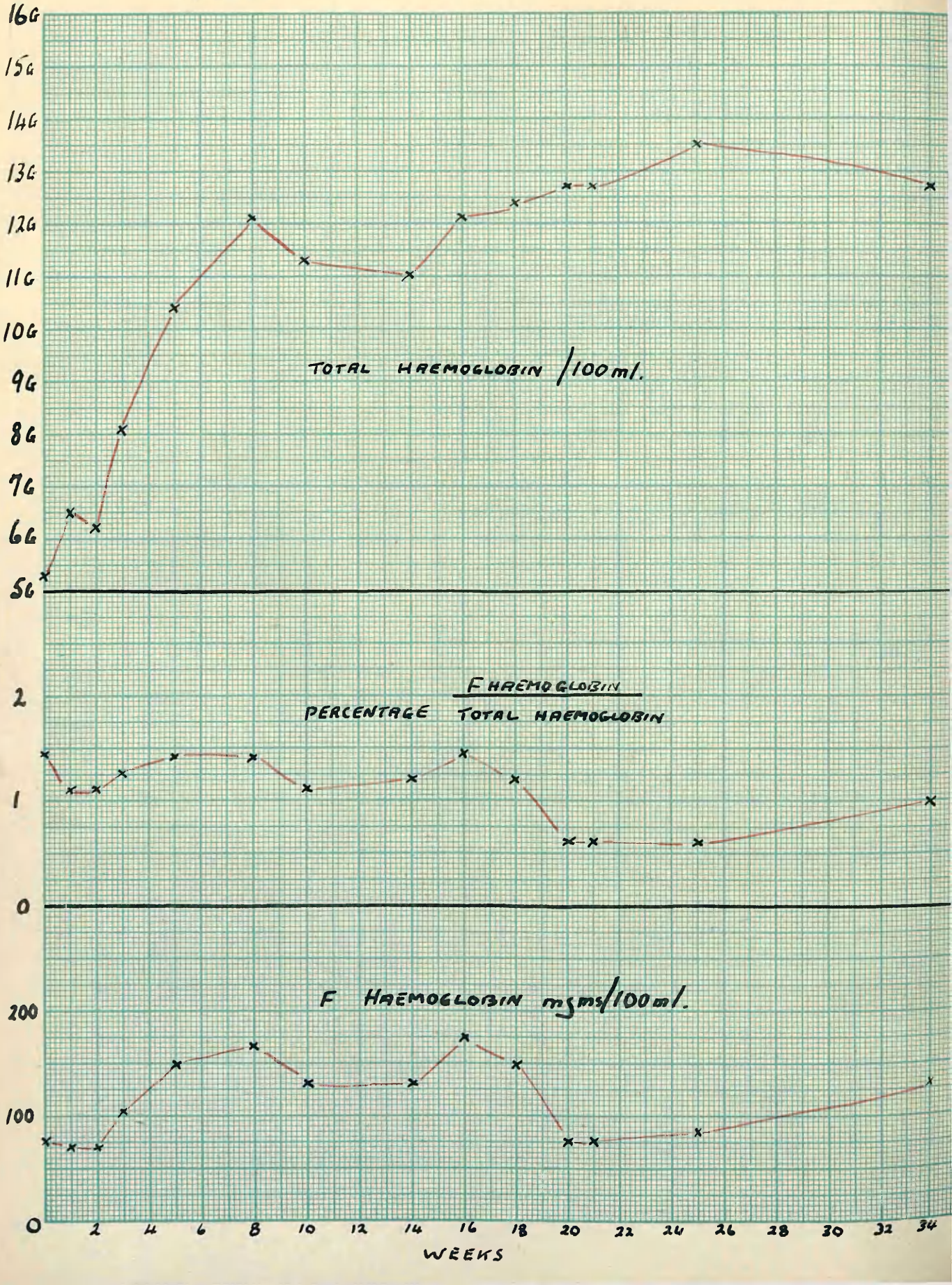
Treatment: Proteolysed liver 1 oz. daily.

A remarkably constant set of readings can be observed from Fig. 16, where no evidence of F haemoglobin existence ever presented.

Case 16. Occupation: Housewife. Age 64. Sex F.

9 months: breathlessmess, lassitude, lack of energy.

FIG. 17


16G
15G
14G
13G
12G
11G
10G
9G
8G
7G
6G
5G
TOTAL HAEMOGLOBIN /100ml.
2
1
0
F HAEMOGLOBIN
PERCENTAGE TOTAL HAEMOGLOBIN
200
100
0
F HAEMOGLOBIN mgms/100ml.
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
WEEKS

O/E Pale, icteric with diminution of vibration sense.

R.B.C.: 1,370,000 per c.mm. C.I. 1.38

Hb. : 5.3 gms. % M.C.V. 98 cu.

Achlorhydria Marrow: megaloblastic

Treatment: 2 weeks: trial on penicillin 200,000 units b.i.d. orally

: trial on Sanatogen liver 2 ozs daily for 2 weeks

1 oz. daily for 15 weeks

Then cytamen 100ug. weekly.

Fig. 17, demonstrates again the relative stability encountered in the 12 estimations of alkaline resistant haemoglobin over a period of 8 months. No evidence of F haemoglobin existence ever presented.

Treated Pernicious Anaemia.

Table XIII presents the results obtained with the blood of 13 patients treated for periods of 12 - 24 months, 5 patients treated 25 - 30 months and 12 patients from 30 months to 20 years. There was no premeditated selection of cases, the sample consisting of patients who attended at a time suitable for the expeditious carrying out of the experimental work excepting that patients of under $2\frac{1}{2}$ years standing were given preference. The sex,

TABLE XIII.

Pernicious anaemia - Treated.

Time treated	Age	Sex	R.B.Cs/c.mm.	Hb.gms/100 ml.	%age foetal Hb.
12 months	76	M	4,520,000	14.7	1.53
12 months	67	M	4,950,000	11.8	0.56
12 months	68	F	4,160,000	12.4	0.60
14 months	73	F	5,310,000	15.4	0.91
14 months	64	F	4,510,000	12.7	0.95
15 months	61	M	5,220,000	12.7	2.34
18 months	41	M	5,160,000	14.5	0.56
18 months	61	F	4,820,000	14.2	0.30
20 months	51	M	4,880,000	13.9	1.29
21 months	68	M	4,860,000	17.2	0.50
24 months	77	M	5,010,000	14.8	0.64
24 months	55	M	4,500,000	13.0	0.34
24 months	59	M	4,800,000	13.6	0.84
25 months	38	F	5,520,000	14.5	0.89
26 months	56	M	4,070,000	12.6	1.34
26 months	66	F	4,980,000	14.5	0.80
28 months	64	F	4,550,000	13.3	0.72
30 months	66	F	4,520,000	13.0	0.75
Over 30 months	60	F	4,900,000	14.8	1.38
"	61	F	4,660,000	13.6	0.72
"	53	F	4,990,000	14.8	0.34
"	59	M	4,880,000	14.5	0.68
"	56	F	5,020,000	14.8	0.38
"	79	F	4,300,000	14.7	1.08
"	67	M	5,300,000	15.7	1.59
"	62	M	6,320,000	16.9	0.74
"	60	F	5,210,000	14.5	0.67
"	68	F	4,620,000	13.0	0.93
"	63	F	4,110,000	12.5	0.72
"	53	F	3,980,000	14.2	0.87

age, and relevant blood state are details included to give a composite picture. The original haemoglobin levels before treatment varied from 22% - 59%.

It can be seen that only one patient was shown to have a significant amount of F haemoglobin in his blood, and this at 15 months from inception of treatment with cytamen. Four months later when this patient reattended, the resistant pigment had disappeared (0.6%).

. . . . . .

From the latter results and those reported graphically, it can be surmised that the foetal haemoglobin disappears at any time between 5 weeks and 19 months after the inception of treatment and that any persistence beyond this time will be exceptional.

. . . . . .

Idiopathic Steatorrhoea: The blood of 3 patients with this condition, who presented with obvious anaemia, megaloblastic marrow and incomplete fat absorption were examined and initial results presented in Table XIV. Only one patient presented any foetal haemoglobin and she exhibited the largest percentage of F haemoglobin in this whole work, rising after 14 days therapy with folic acid to 11.2%.

TABLE XIV. Idiopathic Steatorrhoea.

Age	Sex	R.B.Cs/c.mm.	Hb.gms/100 ml.	%age foetal Hb.
42	M	1,860,000	5.6	1.36
30	F	2,210,000	6.5	1.27
38	F	1,330,000	2.9	6.64

TABLE XV. Pernicious Anaemia of Pregnancy.

Age	R.B.CS/c.mm.	Hb.gms/100 ml.	%age foetal Hb.
30	1,930,000	6.1	1.16
28	2,010,000	7.4	0.38
24	1,490,000	6.5	0.84

TABLE XVI. Erythraemic Myelosis.

Age	Sex	R.B.Cs/c.mm.	Hb.gms/100 ml.	%age foetal Hb.
14	F	1,030,000	3.5	0.45
47	M	1,350,000	3.8	0.87

TABLE XVII. Polycythaemia Vera.

Age	Sex	R.B.Cs/c.mm.	Hb.gms/100 ml.	%age foetal Hb.
61	M	6,735,000	14.7	1.08
68	F	6,550,000	18.8	1.61
62	F	6,760,000	15.5	0.88
42	M	7,410,000	18.4	0.98
66	F	6,750,000	19.8	0.68
65	M	6,430,000	16.0	0.34
45	F	6,110,000	18.9	0.42
45	M	6,150,000	17.9	0.72
55	M	6,500,000	18.6	0.87
61	M	7,050,000	15.5	0.94

A long term follow-up was not obtained as the patient also suffered from an advanced and crippling rheumatoid arthritis, and living a distance away would not attend the follow-up clinic, preferring naturally to attend a local hospital.

Pernicious Anaemia of Pregnancy: The results of the blood analysis of 3 patients with this condition are shown in Table XV. No abnormal levels of resistant pigment were discovered in this small series. Two other patients treated for 4 months and 14 months respectively also showed no abnormal levels.

Erythraemic Myelosis: Table XVI presents the results obtained in the initial experiments with the blood of 2 patients suffering from this incurable condition. Serial experiments were carried out in both instances with never any sign of abnormal pigment production.

Polycythaemia Vera: Samples of blood from ten patients, suffering from this condition, were submitted to the alkaline denaturation technique with results tabulated in Table XVII. No significant levels of a resistant haemoglobin were demonstrated.

Iron Deficiency Anaemia: Samples of blood from 31 patients suffering from iron deficiency anaemia (18 ideopathic and

TABLE XVIII.

Iron Deficiciency Anaemia

Type	Age	Sex	R.B.Cs/c.mm.	Hb.gms/100 ml.	%age foetal Hb.
Primary Achlorhydric	31	F	2,520,000	4.3	0.30
"	49	F	3,590,000	5.9	0.68
"	43	F	2,380,000	4.6	1.21
"	37	F	3,310,000	6.8	0.94
"	26	F	2,860,000	5.3	0.34
"	63	F	2,600,000	8.3	0.76
"	50	F	3,990,000	7.3	0.37
"	63	F	3,200,000	8.7	0.71
"	29	F	4,160,000	7.6	0.49
"	31	F	3,280,000	8.3	0.98
"	45	F	3,460,000	7.1	0.68
"	22	F	2,940,000	4.7	0.48
"	40	F	2,980,000	5.8	0.89
"	45	F	3,100,000	5.0	0.74
Nutritional	58	M	2,790,000	4.1	1.06
"	58	M	3,290,000	7.8	1.10
"	16	M	4,010,000	6.5	0.94
SECONDARY TO:					
Pregnancy	23	F	2,520,000	4.0	0.32
"	31	F	3,350,000	6.5	0.67
Menorrhagia	43	F	2,850,000	5.3	0.96
"	48	F	4,110,000	7.6	0.68
Meckel's Diverticulum	8	F	2,350,000	4.7	0.74
Diaphragmatic Hernia	56	F	1,670,000	3.7	0.70
" "	61	F	3,740,000	7.1	0.57
Gastrectomy	52	M	4,600,000	8.6	0.68
Gastric Ca.	54	M	2,680,000	5.6	1.12
"	68	M	3,190,000	5.3	0.84
Megacolon	50	F	2,950,000	5.9	1.04
Cirrhosis	48	M	4,510,000	7.4	0.68
Duodenal Ulcer & haemorrhoids	60	M	3,890,000	9.3	0.95
Duodenal Ulcer	44	M	3,680,000	6.7	1.08

13 secondary to haemorrhage) were examined and the results tabulated in Table XVIII. In no instance was there any evidence of foetal haemoglobin production even in a child of 8 with chronic blood loss of many years standing from ectopic gastric mucosa in a Meckels diverticulum.

Haemolytic Anaemia: Table XIX presents the results obtained with samples of blood from patients with this condition. The 17 patients tested included 7 cases of congenital spherocytic anaemia, 3 cases of acquired haemolytic anaemia, cause unknown, 5 cases of reticulosis manifested by hypersplenism and 2 cases of paroxysmal haemoglobin urea. Each patient was tested on more than one occasion and the results were always similar. The only patients showing a significant appearance of F haemoglobin were 2 belonging to the congenital spherocytic group, who showed 2.23% and 3.01%. The first was a lady of 58 years, who give a long history of jaundice attacks and a gallstone operation age 20, the second was a lady of 43 years who give a similar history of many attacks of jaundice and a gallstone operation age 23 years. The first lady refused operation and the second has not yet attended the follow-up clinic after a satisfactory splenectomy. An estimation of her F haemoglobin just before discharge showed 3.13%.

TABLE XIX. Haemolytic Anaemia.

Type	Age	Sex	R.B.Cs/c.mm.	Hb.gms/100 ml.	%age foetal Hb.
Congenital Spherocytic	40	M	1,630,000	5.3	0.59
"	3	M	2,470,000	8.3	0.83
"	43	F	2,210,000	6.5	3,01
"	8	M	3,070,000	6.8	0.39
"	18	M	4,220,000	12.9	0.72
"	23	M	3,340,000	10.7	1.17
"	58	M	3,210,000	9.5	2.23
"	54	M	4,030,000	10.8	0.68
Reticulosis (with hypersplenism)	61	M	2,500,000	6.2	1.27
"	60	F	990,000	2.9	1.1
"	32	F	1,980,000	6.1	0.99
"	52	F	3,030,000	9.2	0.34
Secondary	58	F	2,110,000	6.5	0.38
"	47	F	3,040,000	9.4	1.15
"	30	F	2,710,000	8.7	0.43
Paroxysmal Haemoglobinuria	58	M	2,090,000	6.2	0.72
"	65	M	1,320,000	6.2	1.67

TABLE XX. Aplastic Anaemia.

Type	Age	Sex	R.B.Cs/c.mm.	Hb.gms/100 ml.	%age foetal Hb.
Primary	53	M	1,860,000	5.8	0.47
"	22	M	1,820,000	5.6	0.36
"	36	M	1,440,000	4.6	1.38
"	47	M	1,000,000	3.5	1.41
"	59	F	1,570,000	4.4	1.36
"	62	M	1,720,000	4.9	0.37
Hodgkins	50	F	1,010,000	2.8	0.68
(.T.E.M.)	47	F	1,920,000	5.3	1.32
Chronic Myel.Leuk. (Myleran)	47	F	1,810,000	5.9	1.15
Chronic Lymph.Leuk. (Myleran)	50	M	2,450,000	7.7	0.72
Myeloid reticulosis (X-radiation)	52	M	2,010,000	6.4	0.43

A third patient in this group, a myeloid reticulosis with hypersplenism showed a normal F haemoglobin (1.1%) until the 2 - 3 weeks before demise when this rose to 3.22% gradually. Her total haemoglobin level at this point was 1.8gms.%.

Aplastic Anaemia: The results obtained in patients with this condition are presented in Table XX. The 11 patients comprised 6 primary or ideopathic types, and 5 secondary (to T.E.M.:2; to myleran:2; and to X-radiation:1). In no instance was there a significant concentration of F haemoglobin encountered. All patients had serial examinations with no relevant variance, and no appearance of the pigment occurred in two cases who improved either spontaneously or with the help of cortisone, over a period of 6 - 12 weeks.

Miscellaneous Anaemias: Table XXI presents the results obtained from a miscellany of blood conditions.

(a) Anaemia associated with Hepatic Disease: Samples of blood from 12 patients with liver failure of varying degree (due to conditions varying from cirrhosis, to I.V.C. block, to acute infection) were submitted to analysis and all results were within normal levels.

(b) Anaemia associated with Renal Disease: Samples of blood from 3 patients who were dying from uraemia were tested, and found to be within the normal range.

(c) Anaemia associated with Endocrine Disorder: Samples of blood from 2 patients suffering from Addison's Disease, and 2 patients with Simmonds disease were examined for the presence of the F pigment, and again no significant percentage was encountered.

(d) Anaemia associated with Carcinomatosis: 3 patients with anaemia consequent on haemorrhage from a carcinoma in the Gl tract, and 1 with bone marrow involvement, had their blood examined and no case exhibited a significant level of resistant pigment.

(e) Haemophilia: 3 patients who had suffered from severe bleedings due to their haemophilic constitution were observed immediately and during the period of recovery. No evidence of F haemoglobin production was obtained.

(f) Ovalocytosis: A mother and son with this condition which had previously been diagnosed as ?haemolytic anaemia ?sickle cell anaemia, showed no evidence of F pigment production.

(g) Achrestic Anaemia: One patient with this diagnosis who

TABLE XXI. Miscellaneous Anaemias.

Type	Age	Sex	R.B.Cs/c.mm.	Hb.gms/100 ml.	% age foetal Hb.
Associated with:					
Hepatic disease	48	F	3,270,000	9.3	0.62
"	8	M	3,010,000	8.0	0.96
"	42	F	1,860,000	5.0	0.85
"	33	M	4,710,000	11.0	0.33
"	13	M	3,010,000	6.5	0.76
"	15	M	4,360,000	11.0	1.13
"	36	F	3,110,000	10.1	0.83
"	19	F	3,370,000	13.0	0.74
"	9	M	3,780,000	9.8	1.47
"	39	F	4,420,000	12.0	1.02
"	38	M	4,140,000	12.0	0.64
"	37	M	2,520,000	6.5	1.13
Renal disease	48	M	2,580,000	7.1	0.32
"	41	M	3,210,000	10.9	0.74
"	46	F	2,610,000	6.8	1.48
Addison's disease	33	F	4,220,000	12.6	0.78
Addison's disease	52	M	4,220,000	13.0	0.62
Simmond's disease	50	F	2,990,000	9.6	1.13
Simmond's disease	58	F	4,030,000	13.3	0.68
Carcinomatosis	68	M	3,280,000	6.4	0.84
"	72	M	3,800,000	10.2	0.96
"	48	M	2,900,000	8.6	1.42
"	56	F	3,150,000	7.8	0.78
Haemophilia	3	M	1,740,000	5.0	0.34
"	24	F	3,950,000	11.7	0.72
"	12	M	2,910,000	8.9	0.68
Ovalocytosis	3	M	4,010,000	12.4	0.36
"	27	F	3,710,000	11.0	0.34
Achrestic Anaemia	56	M	1,610,000	5.0	1.22

attends for regular transfusions showed no evidence of F haemoglobin in his blood.

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Acute Leukaemia: Table XXII presents some results obtained on the examination of blood samples from 19 patients suffering from various forms of acute leukaemia. It can be seen that in only 3 cases was the presence of an alkaline resistant haemoglobin established. These 3 patients only presented the pigment in the terminal stages of the condition, and although serial examinations were performed, the proportion of F haemoglobin never rose over 2.74% of the total haemoglobin.

Reticulosarcoma: The results of the blood examination of 3 patients with this condition are presented in Table XXIII. In no instance was there evidence of an alkaline resistant haemoglobin.

Multiple Myelomatosis: Five patients with this condition were investigated with the results shown in Table XXIV. No significant percentage of resistant pigment was demonstrated even on serial examinations until demise in three of the patients.

Chronic Leukaemia: The results of the blood examinations

TABLE XXII. Acute Leukaemia.

Type	Age	Sex	R.B.Cs/c.mm.	Hb.gms/100 ml.	%age foetal Hb.
Lymphatic	19	M	2,010,000	5.6	2.34
"	18	M	2,480,000	7.6	0.96
"	3	F	2,460,000	8.3	0.68
"	4	M	2,310,000	8.6	0.78
"	6	M	2,130,000	7.1	1.21
"	5	M	2,360,000	7.6	0.87
"	5	M	900,000	2.3	0.78
Myeloblastic	11	M	1,590,000	5.2	2.17
"	21	M	1,510,000	4.4	1.16
"	15	M	1,520,000	4.6	2.74
"	5	F	2,360,000	7.1	0.49
"	25	M	2,310,000	6.5	0.84
"	37	M	1,610,000	4.4	1.34
"	6	F	1,560,000	5.0	1.11
"	56	F	2,330,000	7.6	1.4
Myeloid	41	F	2,490,000	7.1	0.68
Stem Cell	34	F	3,020,000	8.0	1.72
Monocytic	47	M	1,410,000	4.3	0.71
"	10	M	2,110,000	6.1	0.36

TABLE XXIII. Reticulosarcoma.

Stage	Age	Sex	R.B.Cs/c.mm.	Hb.gms/100 ml.	%age foetal Hb.
Severe	5½	M	2,310,000	6.8	1.21
Moderate	76	M	3,430,000	12.0	0.76
Severe	36	F	1,020,000	3.2	0.48

TABLE XXIV Multiple Myelomatosis.

Severity	Age	Sex	R.B.Cs/c.mm.	Hb.gms/100 ml.	%age foetal Hb.
Moderate	61	F	3,820,000	14.9	0.86
Severe	41	M	3,530,000	11.2	0.72
"	46	M	2,810,000	7.4	1.0
"	60	F	3,720,000	9.5	1.41
"	46	F	2,310,000	6.3	1.44

of 33 patients with chronic leukaemia are tabulated in Table XXV.

Of 15 patients with the myeloid form only one patient, and he almost in the terminal stages, showed a significant proportion of F haemoglobin (2.16%). Three months earlier he had none (0.36%).

Of 15 patients with the lymphatic form, none exhibited any foetal haemoglobin.

Of 3 patients with the monocytic form, again one only showed an abnormal proportion of F haemoglobin (2.34%) and again this patient was near unto death, and had not exhibited the pigment 3 months before.

In all the negative cases, serial examinations have given similar results, even in two who have died.

Hodgkins Disease: Samples of blood from 13 patients with this condition have been examined with the results presented in Table XXVI. In only one instance was the presence of F haemoglobin demonstrated, again in a patient within a few weeks of death.

Mycosis Fungoides: Finally, an unusual series of 3 patients with this condition presented within a few months and estimations were carried out on samples of their blood (Table XXVII). No evidence of F pigment was discovered

TABLE XXV.

Chronic Leukaemia.

Type	Severity	Age	Sex	R.B.Cs.	Hb. gms/ 100 ml.	% foetal Hb.
Myeloid	Severe	57	F	2,330,000	6.2	1.49
"	Moderate	49	F	3,210,000	9.5	0.90
"	Severe	60	M	1,640,000	4.7	2.16
"	Moderate	50	F	4,140,000	13.0	0.68
"	"	54	M	4,660,000	13.6	0.84
"	"	54	M	4,710,000	11.5	1.54
"	Severe	58	F	2,630,000	8.7	1.08
"	Moderate	20	M	4,080,000	11.5	1.56
"	"	53	F	3,340,000	10.2	0.86
"	"	65	F	4,700,000	15.1	1.32
"	"	34	F	3,950,000	11.7	0.65
"	"	40	F	4,310,000	13.3	0.79
"	"	68	F	2,710,000	8.3	0.58
"	"	44	F	4,060,000	12.4	0.87
"	"	47	F	4,420,000	12.4	0.64
Lymphatic	Severe	63	M	4,180,000	9.6	1.36
"	Mild	39	M	4,660,000	13.6	1.12
"	Moderate	45	M	4,010,000	13.2	1.36
"	"	56	M	4,210,000	13.2	1.08
"	"	61	M	4,720,000	13.9	0.73
"	"	63	F	4,620,000	12.1	0.48
"	"	63	M	3,680,000	12.7	0.77
"	"	45	M	4,280,000	12.7	0.57
"	"	55	M	3,780,000	11.0	1.49
"	"	51	F	4,010,000	12.1	1.26
"	"	71	F	4,360,000	12.4	0.87
"	"	50	M	4,150,000	12.1	0.76
"	"	67	M	4,620,000	14.9	0.48
"	"	57	M	3,630,000	10.8	0.54
"	"	50	M	4,640,000	12.2	0.98
Monocytic	Severe	71	F	2,010,000	5.3	2.34
"	"	51	F	3,880,000	10.1	0.95
"	"	66	M	4,010,000	9.8	1.41

TABLE XXVI. Hodgkin's Disease.

Stage	Age	Sex	R.B.Cs/c.mm.	Hb.gms/100 ml.	%age foetal Hb.
Mild	43	M	4,660,000	14.9	1.08
"	29	M	5,130,000	16.3	0.72
"	40	M	5,010,000	13.9	0.82
Moderate	64	M	3,710,000	10.1	0.68
"	45	F	3,800,000	13,5	0.44
"	33	F	4,420,000	13.5	0.48
"	23	M	4,980,000	14.2	0.97
"	36	F	3,850,000	10.5	0.68
Severe	57	F	2,040,000	6.2	2.5
"	27	F	2,660,000	7.1	0.84
"	31	M	3,710,000	13.6	1.12
"	40	M	1,310,000	3.8	0.34
"	22	M	3,220,000	8.1	0.76

TABLE XXVII. Mycosis Fungoides. (Reticulum cell Reticulosis).

Age	Sex	R.B.Cs/c.mm.	Hb.gms/100 ml.	%age foetal Hb.
69	F	3,520,000	0.95	1.34
70	M	3,010,000	8.9	0.83
76	M	1,610,000	4.4	1.72

in any of them.

Discussion: From the results just reported it may be gleaned that an alkaline resistant haemoglobin was detected in a significant proportion in 14 out of 28 patients with untreated pernicious anaemia; 1 out of 3 patients with ideopathic steatorrhoea; 2 out of 7 patients with congenital spherocytic anaemia, both longstanding cases; 1 out of 5 patients with a reticulosis manifested by hypersplenism, a terminal case; 3 out of 19 patients with acute leukaemia, all three practically terminal; 1 terminal case of Hodgkins Disease out of 13 such patients; and finally in 2 terminal cases out of 33 with chronic leukaemia.

Apart from patients with pernicious anaemia, ideopathic steatorrhoea and congenital spherocytic anaemia, those cases exhibiting the resistant pigment were in the terminal stages of leukaemia or another reticulosis, and the amount found was always small. In a shorter series, Singer et al found evidence of this pigment production more constantly, and it may be that all his estimations were carried out on terminal cases, who were hospitalised. The majority of the estimations in this series were carried out on patients who were attending the Out-patients Clinic of the Department of Haematology, and were thus in varying

stages of the conditions.

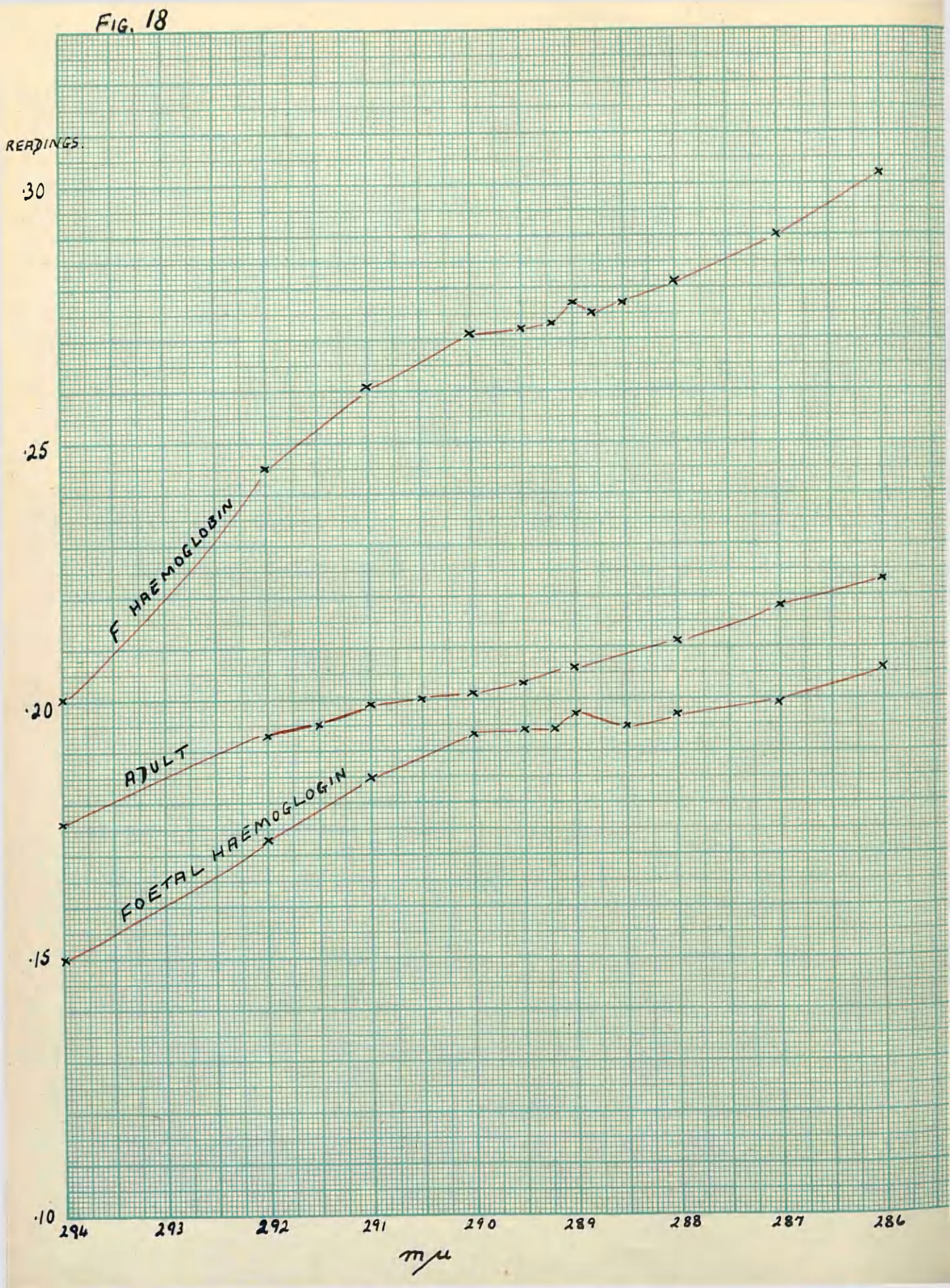
Evaluation of the follow-up on the resistant haemoglobin production in pernicious anaemia yields perhaps only one definite conclusion viz. that the F pigment does disappear with treatment, even although it may take 19 months. That the pigment disappeared in periods varying from 5 weeks to 19 months (except the one still persistent at 8 months) does not lend itself to a discourse on red cell survival time although some of the results suggest that the red cell life in the early stages of treatment might be shorter than the normal (120-140 days),[196.] perhaps intermediate between this and the untreated pernicious anaemia cell (up to 20 days).[157.] The above grounds for this concept, however, are admittedly flimsy, and bear little scrutiny.

Singer et al (1951) established that the resistant pigments behaved towards fractional alkaline denaturation in a manner at least similar to foetal haemoglobin, and so other techniques for the presence of the foetal pigment were deemed worthy of study, in order to establish whether the resistant pigment was indeed a foetal haemoglobin.

A. Ultraviolet spectroscopy.

The work of Jope (1948)[118.] and of Beavan et al

FIG. 18


READINGS.
·30
·25
·20
·15
·10
F HAEMOGLOBIN
ADULT
FOETAL HAEMOGLOGIN
294
293
292
291
290
289
288
287
286
mμ

(1952)[26]. in this field of study has already been referred to in Chapter I, and this inspired study into the relative absorptions of the resistant pigment of pernicious anaemia, an approximately similar concentration of foetal haemoglobin from cord blood, and an approximately similar concentration of adult haemoglobin, all in the same media viz. the reagents used in the Singer technique. Jope's note that salts do not interfere with absorption served as encouragement.

Fig. 18. presents the readings observed on a Beckman Ultraviolet Spectrophotometer, a most accurate instrument, but not so finely sensitive by any means as that used by the above workers. Nevertheless the slope of the two curves, foetal haemoglobin and P.A. resistant haemoglobin, are remarkably similar, viz. a steep slope levelling sharply at 290A, in contrast to the gentle slope of adult haemoglobin which levelled slightly only at 291A. The small peaks observed with the foetal and F haemoglobins can hardly claim significance, but the general contour of the curves do appear to lend credence to the hypothesis that the two pigments are identical.

B. Denaturation Experiment with Alcohol.

That alcohol results in a rapid precipitation of

the foetal pigment, while retaining the adult pigment in solution has drawn comment in Chapter II. Application of this finding was entertained.

A specimen of blood from a patient with pernicious anaemia was estimated to contain 4.6% alkaline resistant haemoglobin. A specimen of cord blood was estimated to contain 90% foetal haemoglobin and a mixture of foetal and adult haemoglobin was prepared to give the same proportions as of the alkaline resistant and adult haemoglobins respectively in the pernicious anaemia sample.

Four samples of haemoglobin solution in concentration 120mgm. Hb per 100ml. were then prepared viz. (a) Foetal (cord), (b) Adult, (c) Pernicious anaemia, (d) Adult and Foetal mixture, and 2ml. absolute ethyl alcohol was rapidly mixed with 4ml. of each sample.

The following results were obtained:-

1. Foetal haemoglobin + alcohol: turbid in 3 minutes and rapidly floccular (2 hours).
2. Adult haemoglobin + alcohol: clear solution at 2 hours.
3. P.A. sample + alcohol: turbid at 8 minutes, more turbid only at 2 hours.
4. Adult + foetal sample + alcoholic: turbid at 9 minutes, more turbid only at 2 hours.

The pH in each sample was constant in range 6.81 - 6.83, and the experiment was carried out at the steady bench temperature of 19°C.

These results indicate another correlation between foetal haemoglobin and the alkaline resistant haemoglobin in pernicious anaemia viz. a precipitation effect by alcohol in the course of denaturation. Similar results were obtained with two further specimens of blood from new cases of pernicious anaemia, but it was not possible to utilise this reaction with the alkaline resistant haemoglobin found in certain cases of congenital spheroytic anaemia etc. as no such patients were found to exhibit this pigment in the past 3 months.

C. Denaturation Experiment with Acetone.

A similar precipitation phenomenon on the addition of acetone to foetal and adult haemoglobin has also drawn comment in Chapter II and identical experimental conditions were applied as in B, excepting that 2ml. acetone (analar) was rapidly mixed with 5ml. haemoglobin solution.

The results were as follows :-

Foetal haemoglobin + acetone: At 30 minutes, turbid +++

Adult haemoglobin + acetone: At 30 minutes, clear

Pernicious anaemia haemoglobin + acetone: At 30 minutes, turbid.

Adult + foetal haemoglobin + acetone: At 30 minutes, turbid.

The pH (6.92 - 6.94) and temperature(19°C) were standard, and two fresh experiments with new cases of pernicious anaemia gave similar results.

So again the alkaline resistant haemoglobin in pernicious anaemia was found to behave as foetal haemoglobin.

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Although it is conceded that none of these results would on its own establish as fact that the alkaline resistant pigment found in pernicious anaemia is indeed foetal haemoglobin, nevertheless all three experiments, especially when taken in conjunction with Singer's work on fractional alkaline denaturation, would appear to make this supposition reasonably substantiative. The same supposition will possibly hold good for the alkaline resistant haemoglobin in the other conditions, and this aspect will be the subject of investigations when possible.

The source of this foetal pigment has also been a subject of conjecture, and the hypothesis currently accepted is that the pigment is the fruit of extramedullary haemopoesis and more specifically hepatic haemopoesis. Thus the differences between foetal and adult haemoglobin have been generally ascribed to the site of fabrication;

PLATE 1 (I)

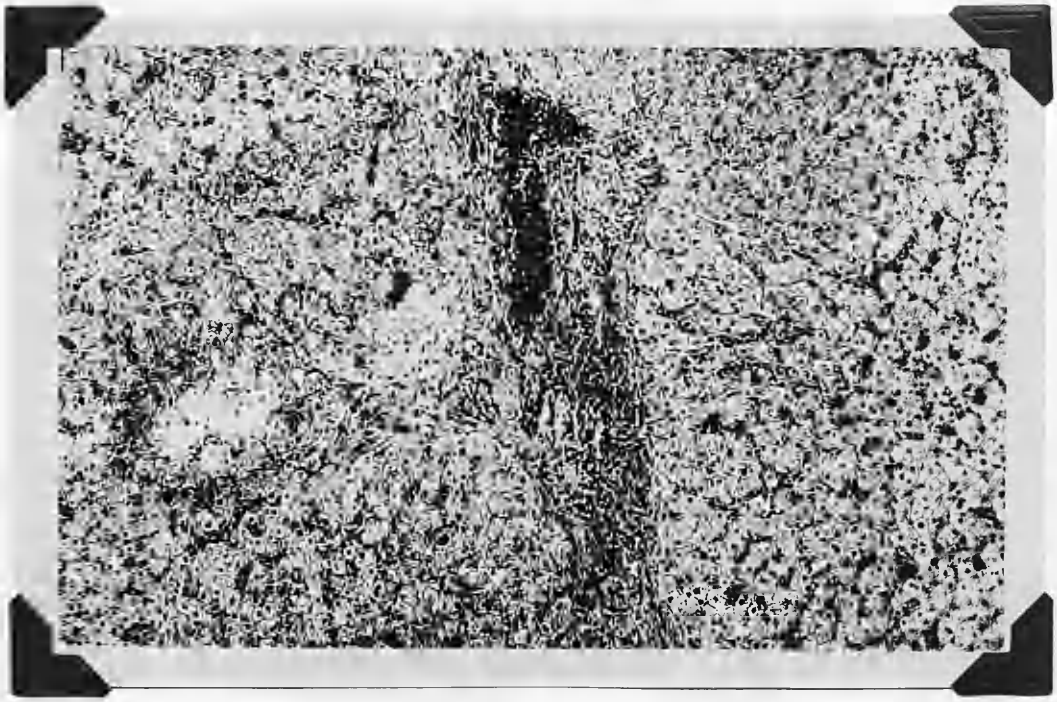
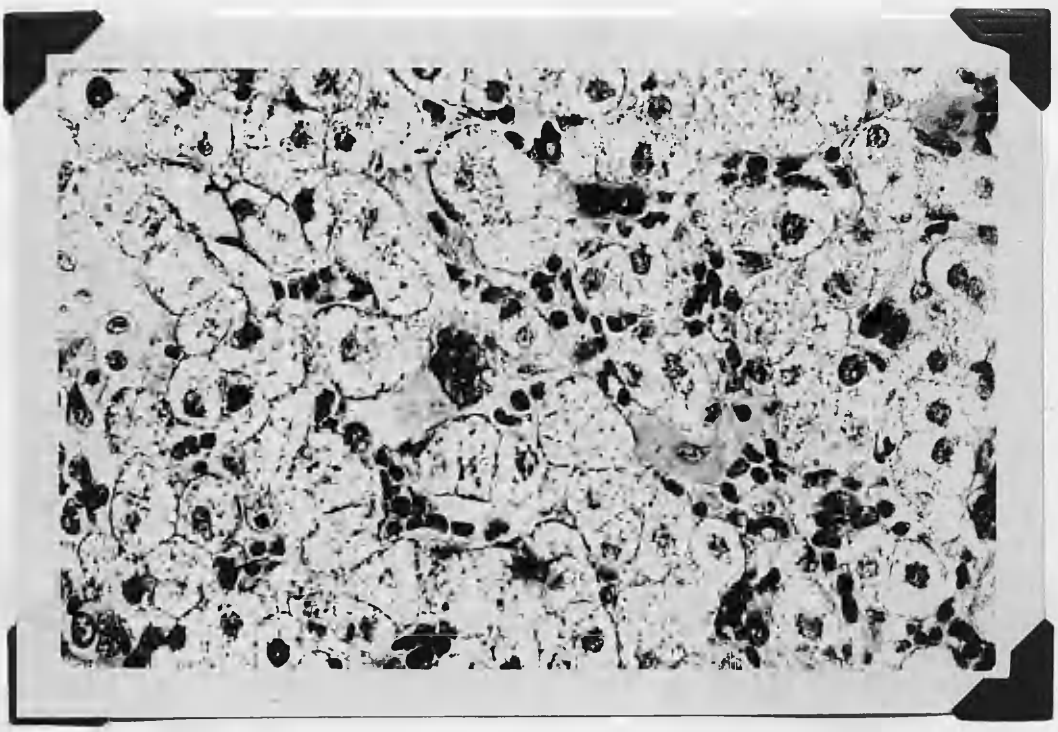


PLATE 2 (II)



the "liver" globin has been held responsible for the properties of foetal haemoglobin and "red bone marrow" globin for those of adult haemoglobin.

It was thus of great interest when a patient with an unknown type of reticulosis manifested by hypersplenism was operated on. From a splenic puncture preoperatively it had been surmised that both blood formation and blood destruction was occurring in the spleen, and so a sample of blood was subjected to the alkaline denaturation technique. No evidence of resistant haemoglobin was found. At operation, it was noted that the liver was enlarged and showed many reddish-plum coloured nodules, which, on section, were found to be nodes of hepatic haemopoeisis. Histological preparations were stained with Mayer's haemalum, and Plate I shows a low power view which demonstrates the gross infiltration of the liver structure by small cells. Plate II shows an oil-immersion view which exhibits numerous large megakaryocytes amongst the liver cells with a general infiltration of small cells which are mainly normoblasts. Serial studies of her blood were undertaken over a period of 4 months, and no alkaline resistant haemoglobin was ever found.

Although this is but a single case, it does make

the current plausible theory quite untenable. If indeed "liver" globin was responsible for the different properties of alkaline resistant haemoglobin in adult conditions, an ample amount would be expected in this instance. This finding also lays very open to doubt the concept that the different properties of foetal haemoglobin in the infant as compared to the haemoglobin of the adult are simply due to the former's hepatic origin.

Considering again the foetal haemoglobin in pernicious anaemia, attention was now directed to the marrow. Two untreated patients had specimens of their blood and their sternal marrow submitted to the alkaline denaturation technique with these results.

Table XXVIII.

Patient.	Blood.	Sternal Marrow.
Case I	3.92% resistant haemoglobin	4% resistant haemoglobin
Case III	3.89% resistant haemoglobin	3.76% resistant haemoglobin

It is not intended to be too dogmatic about the evaluation of these percentages. However, if the foetal

pigment production was extramedullary in pernicious anaemia, it would be expected that the blood from the marrow, which will contain a large proportion of immature cells and mature macrocytes formed therein, would exhibit a substantially lower proportion of the resistant pigment. This is contrary to our observations. The fact that there is no experimental difference in the foetal/adult proportions would accord with the production of the resistant pigment in the marrow, the accepted normal centre of haemoglobin formation.

Studies of the cytology of the sternal marrow were next undertaken to ascertain any common factor for the foetal haemoglobin production. It is well known that in pernicious anaemia there is early haemoglobinisation in the megaloblasts and well appreciated following the work of Israels,[108.] that early haemoglobinisation occurs in haemolytic anaemia. This suggested itself as a possible factor, but was discounted following the finding of this phenomenon in three patients with haemolytic anaemia and without abnormal pigment production.

The relative activity of the marrow was studied for a clue, but apparent hyperplasticity or hypoplasticity did not appear to be of significance, nor was the relative

proportion of immature cells in the marrow a connecting link.

Larson[135] has suggested that it is the macrocytes in pernicious anaemia which carry the foetal haemoglobin, and that only liver preparations cause disappearance of these large cells. That this is not so was amply demonstrated in Case I (Table XII, Fig. 5), who continued to exhibit foetal haemoglobin after the disappearance of macrocytosis. In several blood smears taken at the time of his last estimation, no large erythrocytes could be found.

Pernicious anaemia has received, as a clinical condition, extensive study which, from the clinician's standpoint, has culminated in complete success, Nevertheless it is very evident that much more work in the biochemical field is still hampered by the lack of knowledge concerning the normal processes of syntheses of proteins in vivo, and of the evolution and maturation of cells.

The above observations strongly suggest that foetal haemoglobin production in the adult is consequent on a biochemical disturbance in the marrow, as it is encountered only in conditions where there is some upset in the metabolism or enzymatic control of the immature red cells viz. pernicious anaemia idiopathic steatorrhoea and

the terminal stages of the reticuloses, where changes in their morphology are evident on marrow examination, and congenital spherocytic anaemia, where the morphology is apparently normal yet spherocytes are produced instead of erythrocytes.

It is this lack of understanding of the normal process of synthesis, which leaves inexplicable the infant switch to the adult type of haemoglobin, and the adult reversion to the foetal type under certain conditions of stress. The immature red cell is apparently responsible for these changes, and is thus not only worthy of study from the view point of the cytologist or morphologist but merits equal attention and study by biochemists into its property of globin synthesis. Until more is known about this physiological process, the nature of pathological variations can only be guessed at.

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