STUDIES ON THE DEGRADATION OF HAEMOGLOBIN.

Thesis presented for the degree of
Master of Science in Biochemistry
of the University of Cape Town
November, 1966.

by

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ACKNOWLEDGEMENTS.

I wish to thank

Prof. J.E. Kench, my supervisor, for his encouragement and stimulating guidance during the course of this work;

Drs. G.M. Potgieter and M.C. Berman and other colleagues in the Department of Chemical Pathology and the C.S.I.R. Protein Research Unit for their interest and discussion;

Mr. C. Edwards and Mr. E.G. Duncan of the Department of Chemical Pathology who kindly collected a number of specimens;

Dr. P.L.M. le Roux, Department of Medical Physics, Groote Schuur Hospital, who was helpful at all times in giving advice about the use of radioisotopes;

Prof. J.F. Brock, for allowing us the facilities of the Department of Medicine and Dr. R. Hoffenberg and Miss E. Black who were generous with advice;

Prof. A. Kipps who made it possible for us to carry out the animal experiments by providing facilities in the animal house;

Dr. W. du T. Naude for giving us bacteria and valuable advice on culture methods; also, other members of the Department of Bacteriology, for providing culture media.

Dr. J.A.H. Campbell who was responsible for the microphotograph and Dr. A.H. Timme who made a histological examination of the goose liver;

Mr. A. Kooy, who was in charge of the experimental animals;

Mr. K. Kaufmann who was most helpful in the construction of equipment.

Professors F.J. Ford and J.T. Louw and their staffs, Drs. L.S. Smith and M.J. Greeff of the Government Laboratory, Cape Town, and the nursing staff of the Maternity wards in Groote Schuur Hospital and the New Somerset Hospital for collecting clinical materials and

Miss M. Manne who drew most of the figures.

I am most grateful to Mrs. P. de Gouveia who carried out all the secretarial work with her customary efficiency.

PREFACE.

Much of the quantitative data assembled during the course of this work, is too voluminous to present in this thesis, but all of it is available to interested persons, on application to Prof. J.E. Kench, Director of the C.S.I.R./U.C.T. Protein Research Unit, Department of Chemical Pathology, University of Cape Town.

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PART 1.

INTRODUCTION

PART I. INTRODUCTION.

1.1 LITERATURE REVIEW.

The chemical relationship which exists between haemoglobin and bile pigments was first established in 1933, when, after monumental studies covering a period of 24 years, Hans Fischer and his colleagues succeeded in synthesizing bile pigments and protoporphyrin. He thereby demonstrated the fundamental tetrapyrrolic structure which is common to both haem and bilirubin[1].

Prior to this, in vivo experiments on dogs performed by Mann, Sheard, Bollman and Blades[2, 3] showed that haemoglobin, injected into the arterial blood of the spleen or bone-marrow, produced a rise in the bilirubin content of the venous blood. Haematin was also evident in both arterial and venous blood.

At that time, there was a good deal of controversy as to which organ was the major site of haemoglobin degradation and bile pigment production. Early experiments by Minkowski and Naunyn in 1886 had shown that the promotion of haemolysis in hepatectomised geese did not produce jaundice[4]. Mann, Bollman and Magath, however, found that bile pigment was present in the plasma of hepatectomised dogs, 3 - 6 hrs. after removal of the liver[5]. The reason for the conflicting evidence is due to differential distribution of the reticulo-endothelial system in birds and mammals (McNee[6]; Rich[7]). In birds, most of the reticulo-endothelial elements are concentrated in the liver whereas in the dog and man, reticulo-endothelial cells are

widespread in liver, spleen and bone marrow. Nevertheless, the liver capacity for bile pigment production in dogs and rats has been calculated to be at least 75 - 80% of the total body capacity (Mann et al.[5]; Wise[8]).

In mammals, bilirubin, the final product of haemoglobin catabolism, is conjugated to glucuronic acid and excreted by the liver. Under certain circumstances, the capacity of the liver to excrete bilirubin is exceeded and this results in hyperbilirubinaemia of the unconjugated form. This occurs when there is an accelerated rate of red cell destruction, e.g. in haemolytic jaundice (Billing and Lathe[9]; Schmid[10]) or in disorders of red cell formation associated with high degrees of "ineffective erythropoiesis", e.g. thalassemia minor (Robinson, Vanier, Desforges and Schmid[11]; Israels, Suderman and Ritzman[12]).

The situation pertaining in haemolytic jaundice in man, illustrates an important feature of haemoglobin breakdown; namely, that the body's capacity to degrade haemoglobin to bilirubin, exceeds that of the liver to conjugate it. The apparent absence of any rate-limiting step in the catabolic pathway explains why accumulation of intermediates has never been observed and is also, therefore, the reason for the uncertainty regarding the individual steps whereby haemoglobin is degraded.

At present, 2 theories have been put forward to explain how haemoglobin might be biochemically broken down to biliverdin, the

oxidized form of bilirubin. (The enzymic conversion of biliverdin to bilirubin has been demonstrated by Singleton and Laster using a biliverdin reductase isolated from guinea-pig liver[13]).

1. The haem prosthetic group is split off from the globin apoprotein and is subsequently converted to biliverdin by oxidation at the α-methene bridge of the pyrrole nucleus with the production of CO, followed by removal of the iron atom.

Although, intermediates in the conversion of haematin to biliverdin have not been isolated, this theory is supported indirectly by considerable evidence derived from various sources.

In vitro, Kench showed that haematin was as good a source of biliverdin as haemoglobin, when undergoing coupled peroxidation with ascorbic acid[14]. Similarly, the cell-free enzyme system of the haemophagous organ of dog placenta, produced higher yields of biliverdin-C^{14} when haemin-C^{14} was used as the substrate than when haemoglobin-C^{14} was used (Wise[8]). In a recent experiment, Robinson, Owen, Flock and Schmid have shown that when δ-amino laevulinic acid-4-C^{14} was perfused, together with reticulocyte poor blood or plasma, through an isolated rat liver, it was significantly and rapidly incorporated into bilirubin[15]. Glycine was also incorporated but with decreased specific activity. Since, both glycine and ALA are synthetic precursors of haem, this suggests that they were incorporated in the liver into haem or an immediate precursor, which was then directly degraded to bilirubin.

Evidence that haematin promoted increased production of bile pigments in vivo was obtained from experiments on dogs (Mann et al.[2][3]) and man (Pass, Schwartz and Watson[16]). Its actual conversion to bilirubin was shown in the dog by radioactive labelling of the pyrrole nucleus (London[17]) and Snyder and Schmid have shown that labelled haematin injected into rats is quantitatively converted to bile pigment[18].

In the last 2 experiments, methaemalbumin was probably formed in the serum as an intermediate.

There is considerable evidence that not all the bilirubin produced in vivo is derived from the haemoglobin of senescent erythrocytes.

The "early-labelled" stercobilin excreted in the first 4 - 5 days after injection of glycine-N^{15} into a normal man comprises 10 - 20% of the excreted label. Most of the rest (about 70%) is excreted 130 days later, when the labelled haemoglobin of the erythrocytes is degraded (London, West, Shemin and Rittenberg[19]; Gray, Neuberger and Sneath[20]).

This "early-labelled" bile pigment fraction is slightly raised in neonatal infants (Vest, Strebel and Hauenstein[21]) and markedly increased in certain disease states, e.g. (a) congenital erythropoietic porphyria where "early-labelled" stercobilin comprises 31 - 80% of the total excreted label (Gray, Kulczycka, Nicholson, Magnus

and Rimington[22]). (b) "shunt" hyperbilirubinaemia, a congenital abnormality described by Israels et al.[12] in which there is primary overproduction of bilirubin. The investigations on this disease have suggested that there are 2 components of the "early-labelled" bile pigment (Israels, Yamamoto, Skanderbeg and Zipursky[23]). The one is derived from marrow erythrocytes or their precursors, either by their destruction or their synthesis of bilirubin directly from haem ot its tetrapyrrole antecedents. The other bile pigment source is probably hepatic in which bilirubin may be synthesized from a common precursor pool (Israels, Skanderbeg, Guyda, Zingg and Zipursky[24]). The liver perfusion experiment of Robinson et al.[15] adds weight to this suggestion.

None of these experiments, however, provide conclusive evidence that haematin is a normal intermediate in the degradation of haemoglobin; they only show that enzyme systems exist for the conversion of both haematin and haemoglobin to bile pigments. Some of the labelled stercobilin must be derived from haem-containing proteins other than haemoglobin, e.g. liver catalase which has a rapid turnover rate (Price, Sterling, Tarantola, Hartley and Rechcigl[25]), myoglobin, peroxidases etc. It is thought that these proteins are the probable source of the stercobilin excreted (less than 10%) between the 30th and 80th day after injection of isotopically labelled glycine (Schmid[26]).

Certainly, another haemoprotein, namely methaemalbumin, is known to be a source of bile pigment in vitro, both in chemical

systems (Kench[14]) and, when the protein is denatured, in the isolated perfused rat liver (Wise[8]).

The formation of methaemalbumin from extracorpuscular haemoglobin in vivo, albeit under pathological conditions (Fairley[27]) is the only conclusive evidence that haematin can be an intermediate in the degradation of haemoglobin. Whether methaemalbumin is formed under normal circumstances is however questionable.

2. The second theory is based on the work of Lemberg and Legge in which they studied the degradation of haemoglobin by coupled peroxidation with a variety of chemical systems[28]. They postulated the following sequence of events: The α-methene bridge of the haem prosthetic group is oxidized by molecular oxygen to produce cholehaem, which attached to globin or part of the globin molecule, gives choleglobin. The α-methene bridge is then broken, with the production of CO, to give verdoglobin. Finally the apoprotein is split off and the iron of the biliverdin ferrichloride thus produced, is removed, giving rise to the final product, biliverdin.

The evidence for this degradative pathway is purely spectral in character. Until fairly recently the formation of choleglobin had only been demonstrated in in vitro chemical systems in which the degradation of haemoglobin is effected by its coupling to a reversible oxidation-reduction system such as ascorbic acid and oxygen (Lemberg[29]; Kench[14]; Kaziro, Kikuchi and Hanaoka[30]). Choleglobin has never been isolated although it has been shown to be a precursor of bili-

verdin ferrichloride, which was purified by Lemberg[29]. Furthermore, it has never been conclusively shown to occur in vivo. At one time, it was thought to be present in small quantities in normal circulating erythrocytes (Lemberg and Legge[31]; Lemberg[32]) and was shown by Kiese and Seipelt[33] to be present in the red blood cells of rabbits after phenyl hydrazine administration. Kench, Gardikas and Wilkinson in a series of 3papers demonstrated, however, that the presence of biliverdin precursors in circulating erythrocytes was minimal and that the small quantities that were found were probably due to subsequent oxidation in vitro.[34, 35, 36]

An important finding as regards these peroxidative systems was that of Petryka, Nicholson and Gray who were able to show that in these systems, haem undergoes non-specific degradation, all the methene bridges being equally susceptible to attack, wereas, in vivo, all bile pigments are derived from the IX α isomer of biliverdin, i.e. only the α-methene bridge undergoes oxidative cleavage.[37]

Unfortunately, at the time of writing, in none of the biological liver systems, shown by spectral absorption to have produced choleglobin, (Mills and Randall[38]; Kench and Varma[39]; Nakajima, Takemura, Nakajima and Yamaoka[40]) has the biliverdin produced been shown to consist only of the IX α isomer.

Rather more definitive evidence of the existence of bile pigment-protein complexes comes from plants; namely, the finding by Virtanen and Laine of choleglobin derived from leghaemoglobin in the root

nodules of leguminous plants[41] and the discovery of the light-sensitive protein, phytochrome, the chromophoric group of which has been shown to be a linear tetrapyrrole (Siegelman and Hendricks)[42].

Both the above theories postulate the production of one molecule of carbon monoxide for each haem group degraded. Sjöstrand in a series of publications demonstrated the formation of CO under conditions of haemoglobin degradation both in vivo and in vitro[43 - 47]. Ludwig, Blakemore and Drabkin, using isotopically labelled pyridine haemochromogen coupled with hydrazine or ascorbic acid, showed that the peak of CO production lags far behind that of green pigment formation[48].

Coburn, Williams and Forster have been able to quantitatively estimate the in vivo CO production under a variety of circumstances using a "rebreathing method". Intravenous injection of damaged erythrocytes caused an increase in CO production over the control level in man. All the increased CO was produced within 3 hrs. after injection with an average half-time of CO production following cell injection, of 87 minutes. The average molar ratio of the "extra" CO to haem was 0.97[49]. This represents an almost quantitative yield of CO from haemoglobin which is not duplicated in experiments in which bile pigment yields from injected haemoglobin or erythrocytes were measured (Ostrow, Jandl and Schmid[50]; Schmid[26]). From their results, Coburn et al. calculated that in normal man, 23% of the total average rate of CO production, arises from sources other than circulating haemoglobin[49]. In their experiments on patients with haemolytic anaemia (Coburn, Williams and Kahn), in which the level of CO production is elevated above normal values, this fraction was

increased[51]. They suggest that it originates from the same sources as the "early-labelled" bile pigment (London et al.[19]; Gray et al.[20]).

Recently, a carbon-monoxide binding pigment has been isolated from liver microsomes (Omura and Sato) and was shown to be a haemoprotein[52, 53]. Its relationship to haemoglobin catabolism has not, however, been investigated.

3. In order to explain the lower than theoretical recoveries of labelled bile pigments produced as a result of injection of labelled haemoglobin or sensitized red cells (Ostrow et al.[50]), Schmid[26] has suggested that alternative catabolic pathways may exist, which, although minor, might explain this phenomenon. Dipyrrolic compounds (bilifuscin, mesobilifuscin and pentdyopent) have been demonstrated in the faeces of normal individuals.

Haem compounds are converted to colourless oxidized products when they catalyze peroxidation of unsaturated fatty acids (Haurowitz, Schwerin and Yenson[54]). Although there is no evidence for this process occurring in biological systems, the close proximity of most of the haemoproteins with structures of a lipoprotein nature (e.g. haemoglobin in erythrocytes is surrounded by stromal lipoprotein; the cytochromes are associated with lipoproteins of mitochondrial membranes), suggests that co-oxidation of haem and unsaturated fatty acids of the lipoproteins may occur under special circumstances. Haemolysis under hyperoxic conditions is associated with the formation of lipid peroxides in the stroma of erythrocytes (Mengel and

Kann[55]).

In vertebrates, haemoglobin catabolism is complicated by being associated with erythrocyte destruction. In those animals possessing nucleated red blood cells in their circulation, haemoglobin may still be synthesized after the release of the cells from erythropoietic tissues (Hammel and Bessman[56]). This implies that, at the time of sequestration, unless these circulating cells contain the necessary apparatus for haemoglobin catabolism, each erythrocyte carries a population of haemoglobin molecules of varying age. Pertinent to this is the evidence that the life span of the nucleated erythrocytes of vertebrate poikilotherms, e.g. amphibians and reptiles, is as long as 2 - 3 years (Grisolia[57]).

In the case of the non-nucleated red blood cells of mammals, the evidence suggests that haemoglobin is not catabolized unless the erythrocyte has "aged" sufficiently to undergo normal breakdown after approximately 120 days (Shemin and Rittenberg[58]; London et al.[19]; Gray et al.[20]), or is haemolysed intravascularly for some reason. In the erythrocyte, haemoglobin is protected from peroxidative degradation by the action of glutathione peroxidase (Mills and Randall[38]; Jacob and Jandl[59]), α-tocopherol (Nitowsky, Cornblath and Gordon[60]; Pokrovskii and Abrarov[61]; Mengel and Kann[55]) and possibly catalase (Nishimura, Hamilton, Kobara, Takahara, Ogura and Doi[62]; Jacob, Ingbar and Jandl[63]).

At present, there is no evidence for the existence of any bile

pigment precursors in circulating mammalian erythrocytes (Kench et al.[36]); although, if free haematin is an intermediate in the degradation of haemoglobin, the possibility of it being present has not been excluded. Nucleated erythrocytes of other vertebrates have not been investigated in this regard.

Considerable doubt exists concerning the cellular site of haemoglobin degradation. It is commonly assumed to be the reticulo-endothelial system, in the cells of which engulfed red blood cells and iron-containing compounds have been microscopically observed (Rich[7, 64]). The assumption is that the normal destruction of senescent red blood cells is followed, at the same cellular site, by haemoglobin degradation. However, even if the phagocytic cells of the reticulo-endothelial system are responsible for sequestration of erythrocytes, it is still not certain whether they are also capable of producing bile pigment (Wise[8]). The question arises chiefly because the tissues containing reticulo-endothelial elements, i.e., spleen, liver and bone marrow also have parenchymal cells and it has been difficult to distinguish between these as regards bile pigment production. Differences have also been found in the organ uptake of labelled haemoglobin as compared with labelled, injured red cells. Jandl, Jones and Castle found that Fe^{59}-labelled, sensitized erythrocytes were sequestered preferentially in the spleen, whereas most of the radioactivity derived from Fe^{59}-labelled haemoglobin injected into human subjects, accumulated in the liver[65]. Subsequent studies on rats (Garby and Obara[66]) and rabbits, (Murray, Connell and Pert[67]) confirmed the role of the liver as the principal site of haemoglobin breakdown.

It is evident, therefore, that the metabolic disposition of haemoglobin derived ffom intravascular haemolysis, is different from that of the intracorpuscular haemoglobin of senescent red cells, although the biochemical pathway by which it is degraded to bile pigments is probably similar. Evidence from isotope studies on normal subjects, suggests that 10% (Garby and Noyes[68]) and even as much as 30% (Freeman[69]) of the haemoglobin from senescent red cells passes through a free plasma compartment.

Haemoglobin released into the plasma during intravascular haemolysis is bound to haptoglobin (Laurell and Nyman[70]) and removed as such from the circulation. If the haemoglobin binding capacity of the haptoglobins is exceeded, free haemoglobin is filtered and excreted by the kidneys or the haem is split off and bound to albumin, or the β_1 globulin, haemopexin (Aber and Rowe[71]).

Recently, Keene and Jandl have shown that apart from its effect in preventing renal uptake and excretion of haemoglobin, haptoglobin binding in the plasma of rats, had no effect on the relative distribution of haemoglobin at the principal catabolic sites, i.e. liver (70%), spleen (8%), and bone marrow (22%) although the rate of uptake was decreased[72]. Nakajima et al.[40] have isolated an enzyme, α-methenyl oxygenase, from guinea-pig liver. It appeared that this enzyme exhibited its greatest activity when a haemoglobin-haptoglobin complex was used as the substrate and, on the basis of this result, these workers have suggested that haemoglobin is degraded in the form of this complex.

However, the studies of Keene and Jandl[72] have substantiated

the finding by Wise[8], that haptoglobin binding was not required for haemoglobin degradation by the isolated perfused rat liver. Wise, however, found that the globin had to be denatured before haemoglobin uptake and bile pigment production, in the isolated rat liver, approached the rate and values observed in the intact rat[8].

It has been shown that short periods of incubation at 37°C, of free haemoglobin mixed with serum, results not only in binding of haemoglobin to the α_2 (haptoglobin) fraction but also in the production of methaemalbumin (Liang[73]; Korinek[74]). It is possible, therefore, that even under normal circumstances of intravascular haemolysis, methaemalbumin may be formed.

1.2 OBJECTIVES OF THE PRESENT STUDY.

In the light of the evidence presented here we considered the following aspects of haemoglobin degradation worthy of further investigation:

(1) The preferred route of breakdown of haem in biological systems.

(2) The factors controlling this preference, i.e. enzymes, co-factors, conditions of pH and temperature, and species differences.

(3) The presence of recognizable intermediates, their isolation and whether they have general metabolic importance in living cells.

1.3 THE RESULTS ACHIEVED IN THIS RESEARCH.

(1) Pure albumin can facilitate or bring about the removal of haem from native haemoglobin when only these 2 proteins are brought into apposition with one another.

(2) The rate of haem transfer and binding is highly dependent on pH and temperature - being maximal at alkaline pH's and temperatures above 25°C.

(3) The transfer is expedited by oxidation of ferrohaemoglobin to ferrihaemoglobin.

(4) The liberated globin moiety is simultaneously denatured when haem is removed from its attachment to it.

(5) The presence of unsaturated fatty acids both accelerated the rate and increased the quantity of methaemalbumin formed.

(6) The presence of albumin protected ferrihaem from coupled peroxidation with unsaturated fatty acids.

(7) Haemoglobin bound to serum haptoglobins, in contrast to reports of Nakajima et al.[40], was found not to be a better precursor of bile pigments than free haemoglobin, and we have been unable to demonstrate the presence in mammalian liver tissue of an enzyme system specifically active in the degradation of haptoglobin-haemoglobin complexes to bile pigments.

(8) A technical method has been devised for measurement of serum haptoglobin concentration depending on separation of complexes on dextran gels.

(9) Heat-labile components were demonstrable in guinea-pig and human foetal liver tissues which gave rise to products from haemoglobin, which absorb light in the red region of the spectrum.

(10) A series of haem-containing molecules of different sizes have been separated as degradation products of haemoglobin in the presence of foetal liver homogenate, ascorbic acid and $NADPH_2$.

(11) In vivo experiments on geese have been performed to trace the sequence of the steps involved in the degradation of Fe^{59}-labelled haemoglobin.

The half-clearance time of the injected label was found to be approximately 3 hours. The fate of most of this fraction was unaccounted for. The Fe^{59} that was present in the liver tissue, was found to be associated with the non-particulate cell fraction. Subsequently (after 26 hr.), the liver was found to contain 40% of the injected dose, which was widely dispersed throughout the organelles and soluble fraction of the cells. In the latter, the greatest radioactivity was associated with non-haem containing proteins of large molecular size, i.e. >70,000. At

least 30% of the Fe^{59}, however, was eluted in conjunction with small molecules of molecular weight less than 30,000.

(12) An experiment was performed in which haemolysis of Fe^{59}-labelled erythrocytes was induced by administration of acetyl-phenyl hydrazine to a goose. 48 hours later the label was found to be present in all fractions of liver cells, the highest activities being associated with the microsomal and mitochondrial fractions.

PART 2.

EXPERIMENTAL METHODS AND RESULTS.

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2.1 IN VIVO EXPERIMENTS.

2.11 Introduction: CHOICE OF THE GOOSE AS EXPERIMENTAL ANIMAL.

The goose was chosen as the experimental animal because it excretes biliverdin, thus eliminating one degradative step present in the normal mammalian catabolism of haemoglobin to bilirubin, and because early experiments showed that removal of the liver shortly after inducing red cell haemolysis did not produce jaundice in geese, suggesting that haemoglobin is broken down chiefly in the liver of these animals (Minkowski and Naunyn 1886[4]; McNee 1913[6]). This simplifies the search for intermediates by confining their site of production to a single organ.

The aim of the experiments was to prepare Fe^{59}-labelled goose haemoglobin which would be administered to a normal goose. After a time interval calculated to have produced maximal accumulation of labelled intermediates in the liver, the goose was to be killed, the liver removed and fractionated, and the radioactivity in each fraction determined. In this way, we hoped to demonstrate the presence of iron-containing intermediates of haemoglobin catabolism in the goose liver and to determine with which particular cell fraction they are associated.

2.12 NORMAL VALUES.

2.121 Bile pigment excretion.

The excretory products of the goose were collected daily in a

tray covered with firm wiring on which the goose was supported and allowed to move about (Colour plate 1). In birds, faeces and urine are excreted via a common cloaca so it was not possible to distinguish between the two as regards their capacity for bile pigment excretion.

The 24-hour collections of the excreta, which appear greenish in colour, were weighed, and aliquots were taken for the estimation of urobilinogen and biliverdin.

The urobilinogen excretion was determined by the method of Watson[75] as modified by Maclagan[76] and found to be less than 0.1 mg./day.

Biliverdin was extracted from a known weight of faeces by grinding with an equal amount (w/v) of water and then shaking up the suspension several times with separate additions of acetic acid and ether as described by Gardikas, Kench and Wilkinson (1950)[35]. The pooled ether extracts were washed with sodium acetate and water and the biliverdin was then extracted into hydrochloric acid of increasing concentration. Ethanol was added to give a final volume of 40% ethanol in HCl and the concentration of biliverdin was determined in the Beckman DU spectrophotometer at 675 mμ using the specific extinction coefficient ($E^{1\%}_{1\ cm} = 46.5$) determined by Lemberg, Lockwood and Legge (1941)[77]. Biliverdin excretion in the normal young goose (weighing approximately 4 - 6 kg.) ranged between 2 and 12 mg./day with a mean daily excretion of 6 mg. Assuming that biliverdin excretion in the faeces and urine is the only means whereby the goose eliminates the products of haemoglobin degradation, these daily

COLOUR PLATE 1

The domestic goose

excretion values correspond to the breakdown of 50 - 350 mg. of haemoglobin per day.

2.122 Serum bilirubin.

Levels of total serum bilirubin determined by the method of Nosslin[78] using caffeine benzoate as modified by Michaëlsson[79, 80] were less than 0.5 mg./100 ml.

2.13 EFFECT OF PHENYL HYDRAZINE ADMINISTRATION.

50 - 80 mg. phenyl hydrazine/kg. body weight were injected subcutaneously, in the pectoral region, to anaesthetized young geese in the form of a 2% neutralized solution of phenyl hydrazine hydrochloride. It was usually given in 2 doses, the second being administered 48 hours after the initial injection.

In all four experiments, the haemolysis, so caused, produced immediate methaemoglobinaemia, followed by a marked rise in daily biliverdin excretion (up to 52 mg./day in one case) accompanied by a rapid drop (up to 15% in 24 hours) in the red cell haematocrit value. One of these experiments is illustrated graphically (Fig. 1). A dosage of 94 mg. phenyl hydrazine/kg. body weight proved fatal (one animal). The effect of phenyl hydrazine on haematocrit levels is shown in Table 1.

In one of the experiments, 40 hours after a dose of phenyl hydrazine was administered to a goose, the animal was killed and the liver removed and examined histologically. There was evidence

FIG.1.

EFFECT OF PHENYL HYDRAZINE ON RED CELL H'CRIT VALUES AND BILIVERDIN EXCRETION.

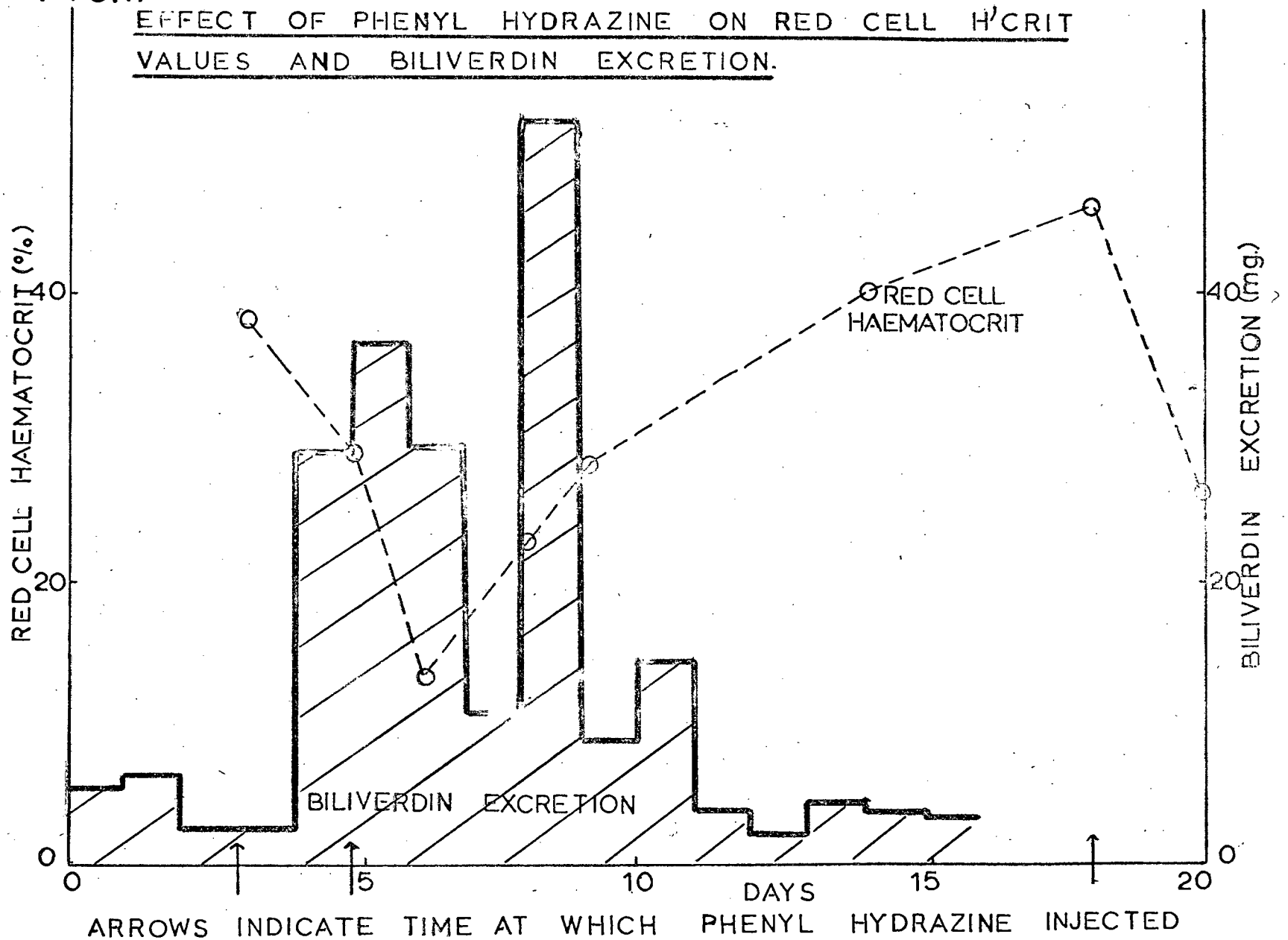


TABLE 1.

Effect of phenyl hydrazine administration on the red cell haematocrit.

Goose	Duration of expt.		Phenyl hydrazine administered	red cell haematocrit
	Days	Hours		
240 (1.0 kg.)	1	0	50 mg./kg. body weight	30%
	2	24		17%
	3	51		15.5%
	4	71		16%
	6	118		23.5%
	8	170		30%
225 (4.2 kg.)	1	0	48 mg./kg.	31.5%
	2	24		24.5%
	3	43		19%
		48	5 mg./kg.	18%
	4	67		20.5%
		69	24 mg./kg.	
		73		16%
	5	91		15%

of marked red cell breakdown with large quantities of Fe present in the Kupffer cells.

2.14 PRODUCTION OF ANAEMIA IN THE GOOSE.

Having found that on phenyl hydrazine administration, the extent of haemolysis in the goose could not always be predicted and owing to the detrimental effects of this substance on the general health of the animal, we decided to employ a different technique for reducing the number of circulating red cells (i.e. for the production of anaemia) and the subsequent stimulation of erythropoiesis, which we required for the incorporation of administered Fe^{59} into the red cell haemoglobin of the goose.

In three separate experiments, adult geese were rendered anaemic by repeated daily bleedings (up to 70 ml. at a time) followed by reinjection of the sterile serum obtained from the previous day's venepuncture. Body weight and red cell haematocrits were determined daily. As can be seen from Table 2, this procedure continued for about a week results in a drop of approximately 10% in the red cell haematocrit value and the loss of roughly 0.25 kg. in the body weight of each goose.

TABLE 2.

Goose	Days	Vol. bled ml.	Serum infused ml.	Body weight kg.	Haematocrit %
240	1	-	-	4.26	37
	3	95	20	4.03	25
	6	150	35	4.03	27.5
223	1	-	-	5.68	32
	3	110	24	5.62	28
	6	185	51	5.56	25.5
	8	275	84	5.42	24.5
891	1	-	-	4.88	34
	3	110	11	4.80	31.5
	6	155	29	4.68	31
	8	240	62	4.74	28
	9	310	87	4.65	23.5

2.15 PREPARATION OF Fe^{59}-LABELLED GOOSE HAEMOGLOBIN.

2.151 Incorporation of Fe^{59} into red cells in vivo.

Fe^{59}-Cl_3 in 0.1 N HCl, with an activity of 2.0 mc and a specific activity of 4.2 μc/μg. Fe, was purchased from the Radiochemical Centre, Amersham, Bucks. The solution was neutralized by adding an equal volume of sterile 3.8% sodium citrate. A small quantity (2.55% of the final injected dose) was removed for use as a standard measure of the decay in gamma emmission of Fe^{59} throughout the experimental period. The rest (approximately 3 ml.) was added to about 35 ml.

sterile goose serum and injected into an anaemic goose (red cell haematocrit was 23.5%) in 3 doses. The first injection was the highest radioactive dose (0.6 mc), the second(0.19 mc) was administered on the following day, with the third being given 48 hours later. The total dose of Fe^{59}-Cl_3 was 0.83 mc. The extent of incorporation of Fe^{59} into circulating red cell haemoglobin during the 2 weeks following administration of the radioactive dose, was ascertained by collecting 4 - 6 ml. venous blood every second day in a heparinized syringe and by counting separately the diluted plasma and laked red cells in a well-type scintillation counter fitted with an iodide crystal. This type of Y counter was used throughout for estimating radioactivities. The rise in the red cell haematocrit was also followed during this period.

The results are shown(Table 3 and Figure 2.). In the Table, all values are corrected for radioactive decay from the day the samples were first counted (i.e. 6 days after Fe^{59} was injected into the goose). All samples with an activity greater than 1000 counts/second were corrected for "dead time" by applying the formula:

$$Rt = \frac{Ro}{1 - (Ro \times 5 \times 10^{-6})}$$

Where Rt is the true counting rate per second, Ro the observed counting rate and 5×10^{-6} seconds, is the predetermined resolving time of the counter (Quimby and Feitelberg(81)).

Each day, the background activity was monitored for 1000

TABLE 3.

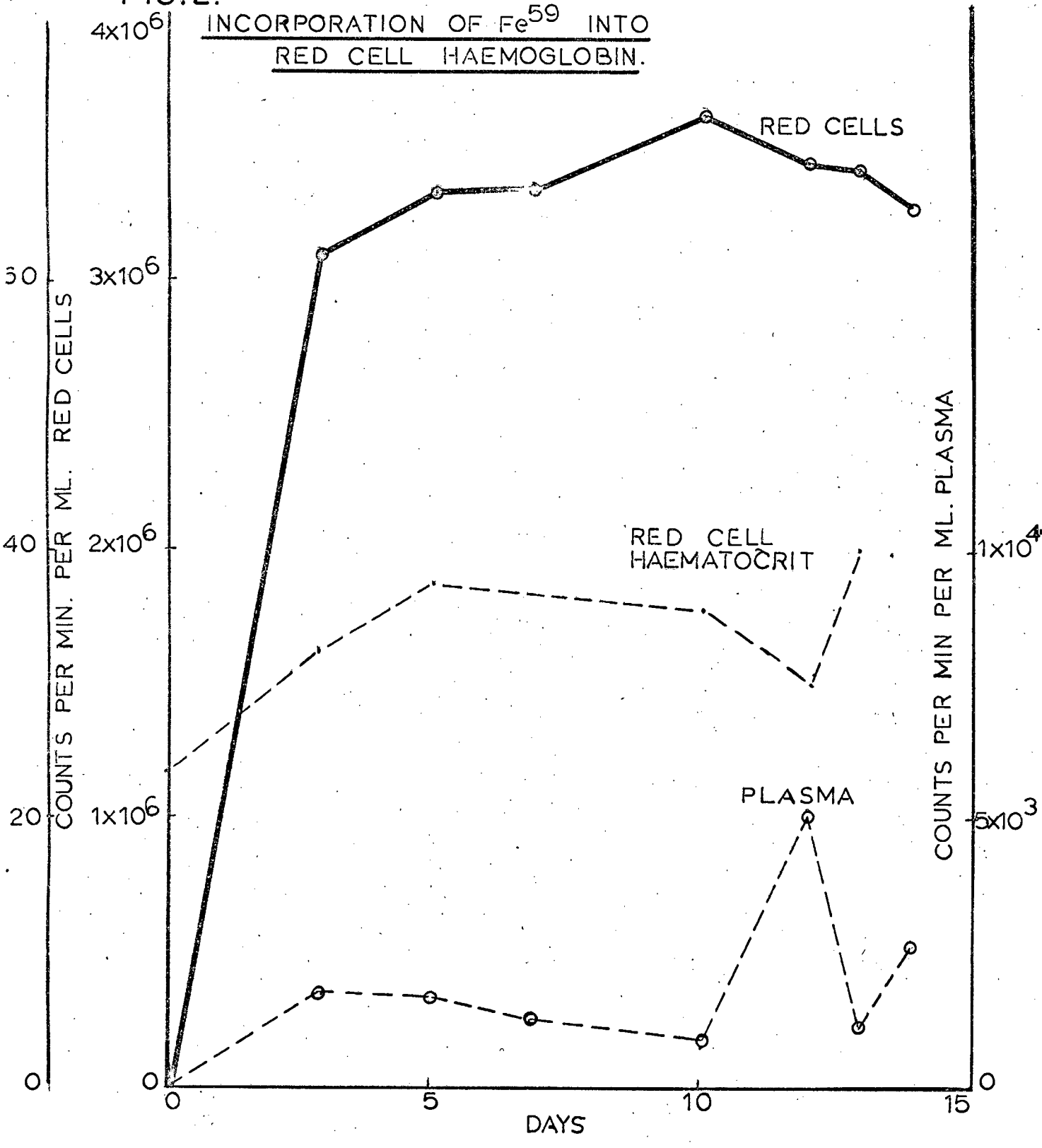
(a) Fe^{59} incorporation into red cells.

After 1st dose		Dilution of sample	Background activity c/1000 secs.	Observed counting rate c/100 secs.	True* counting rate c/100 secs.	Activity of sample cpm/ml.	Decay** factor	Corrected activity cpm/ml.
Day	Hrs.							
3	69	0.5/5ml H_2O	5,786	2,286,865	2,581,373	3,097,648	-	3.098×10^6
5	120	1/5ml H_2O	5,786	4,345,269	5,550,436	3,330,262	-	3.330×10^6
7	164	0.1/5ml H_2O	6,794	518,230	531,300	3,187,800	1.04916	3.345×10^6
10	240	0.1/5ml H_2O	6,794	557,222	572,473	3,434,838	1.04916	3.604×10^6
12	288	0.1/5ml H_2O	5,548	506,968	519,569	3,117,414	1.10284	3.438×10^6
13	309	0.1/5ml H_2O	5,548	468,552	514,784	3,088,704	1.10284	3.406×10^6
14	332	0.1/5ml H_2O	5,324	457,201	467,340	2,804,040	1.16712	3.273×10^6
17	405	0.1/5ml H_2O	5,324	424,973	433,644	2,601,864	1.16712	3.037×10^6
24	573	0.1/5ml H_2O	7,118	282,942	286,270	1,717,620	1.25775	2.160×10^6
			(b) Fe^{59} in Plasma.					
3	69	1/5ml saline	5,786	3,474	2,895	1,737	-	1.74×10^3
5	120	1/5ml "	5,786	3,431	2,852	1,711	-	1.71×10^3
7	164	1/5ml "	6,794	2,771	2,092	1,255	1.04916	1.32×10^3
10	240	1/5ml "	6,794	2,206	1,527	916	1.04916	0.96×10^3
12	288	1/5ml "	5,548	8,214	7,659	4,595	1.10284	5.07×10^3
13	309	1/5ml "	5,548	2,356	1,801	1,081	1.10284	1.19×10^3
14	332	1/5ml "	5,324	4,337	3,805	2,283	1.16712	2.66×10^3
17	405	1/5ml "	5,324	6,104	5,572	3,343	1.16712	3.90×10^3
24	573	1/5ml "	7,118	1,794	1,082	649	1.25775	0.82×10^3

* Corrected for background activity at low counting rates and for background and "dead-time" at counting rates over 1,000/sec.

** Calculated from standard values counted each day.

FIG. 2.


INCORPORATION OF Fe^{59} INTO RED CELL HAEMOGLOBIN.
RED CELLS
RED CELL HAEMATOCRIT
PLASMA
COUNTS PER MIN. PER ML. RED CELLS
COUNTS PER MIN PER ML. PLASMA
4x10^6
3x10^6
2x10^6
1x10^6
0
1x10^4
5x10^3
0
60
40
20
0
5
10
15
DAYS

seconds after the samples were counted.

14 days after administration of the first radioactive dose, 90 ml. whde blood was collected by venepuncture from the goose in 20 ml. 3.2% sodium citrate dihydrate in a centrifuge tube and the haemoglobin was isolated using the method of Drabkin(82).

2.152 Preparation of Fe^{59}-labelled goose haemoglobin.

From the 90 ml. whole blood obtained from the goose, the packed red cells (activity = 3.27×10^6 cpm/ml.) were lysed with distilled water and shaken with toluene as in Drabkin's method(82). After centrifugation, 30 millilitres haemoglobin solution was pipetted off and the stroma was washed with a further 10 ml. distilled water, which, after another centrifugation, was added to the haemoglobin solution. This was centrifuged at 40,000 rpm for 1 hour in a Beckman Model L Ultracentrifuge to remove the last traces of stroma and the clear solution was then dialysed against isotonic saline at $0^{\circ}C$ overnight.

After dialysis, the haemoglobin concentration, estimated by the cyanmethaemoglobin method was 12.3 g.% and the activity was 1.11×10^6 cpm/ml. haemoglobin solution, i.e. 9×10^3 cpm/mg. haemoglobin.

Just prior to injection, the isotonic haemoglobin solution was Seitz-filtered to remove micro-organisms and traces of toluene, which forms a thin film on the surface of the aqueous solution.

2.16 RATE OF REMOVAL OF LABELLED HAEMOGLOBIN FROM THE CIRCULATION.

Fe^{59}-labelled haemoglobin uptake from the plasma was followed in 2 experiments. In the first, (goose No. 858) haemoglobin uptake was assessed over a 26-hour period after which the goose was killed by exsanguination and the liver and other viscera removed for further study. In the second (goose No. 861), the animal was killed three hours after injection of the label.

2.161 Techniques.

The geese, after injection of the labelled haemoglobin into a wing vein, were placed in a small cage in which movement was limited and which was fitted with a tray for the collection of faeces and urine. Confined in this way, it was possible to obtain serial blood samples by puncturing the veins in the feet and letting the blood flow into a pipette from the wound.

(i) Serial blood samples were collected as recommended by Jandl and Kaplan[83] but with certain modifications; (a) the pipettes themselves were rinsed with heparin just prior to use as any slight delay in the collection of blood samples due to the difficulties associated with venipuncture of the foot of the goose (tough skin, veins not easily visible), resulted in blood clotting in the pipettes. It was assumed that the slight alteration in the volume of the blood samples caused by this procedure, would be similar for the whole series, thus making the values at different times comparable.
(b) 0.1 ml. blood was drawn up into the heparinized pipette and

washed into 1 ml. saline. The sample was later centrifuged and 0.6 ml. plasma taken off for estimating plasma radioactivity. (c) Directly afterwards, the same saline-washed pipette was used to withdraw a further 0.1 ml. blood, which was washed into 1 ml. distilled water for whole blood radioactivity.

In these experiments, we were really only concerned with the level of plasma radioactivity, either as free Fe^{59}-haemoglobin or transferrin-bound Fe^{59}, for the period tested. Furthermore, since the total activity of the saline samples before centrifuging paralleled the activity in the whole blood samples (Fig. 3), for the second experiment, only 0.1 ml. blood was taken at each time interval, diluted in saline and the total activity of the uncentrifuged sample (i.e. whole blood radioactivity) was determined before removing 0.6 ml. plasma for counting plasma radioactivity.

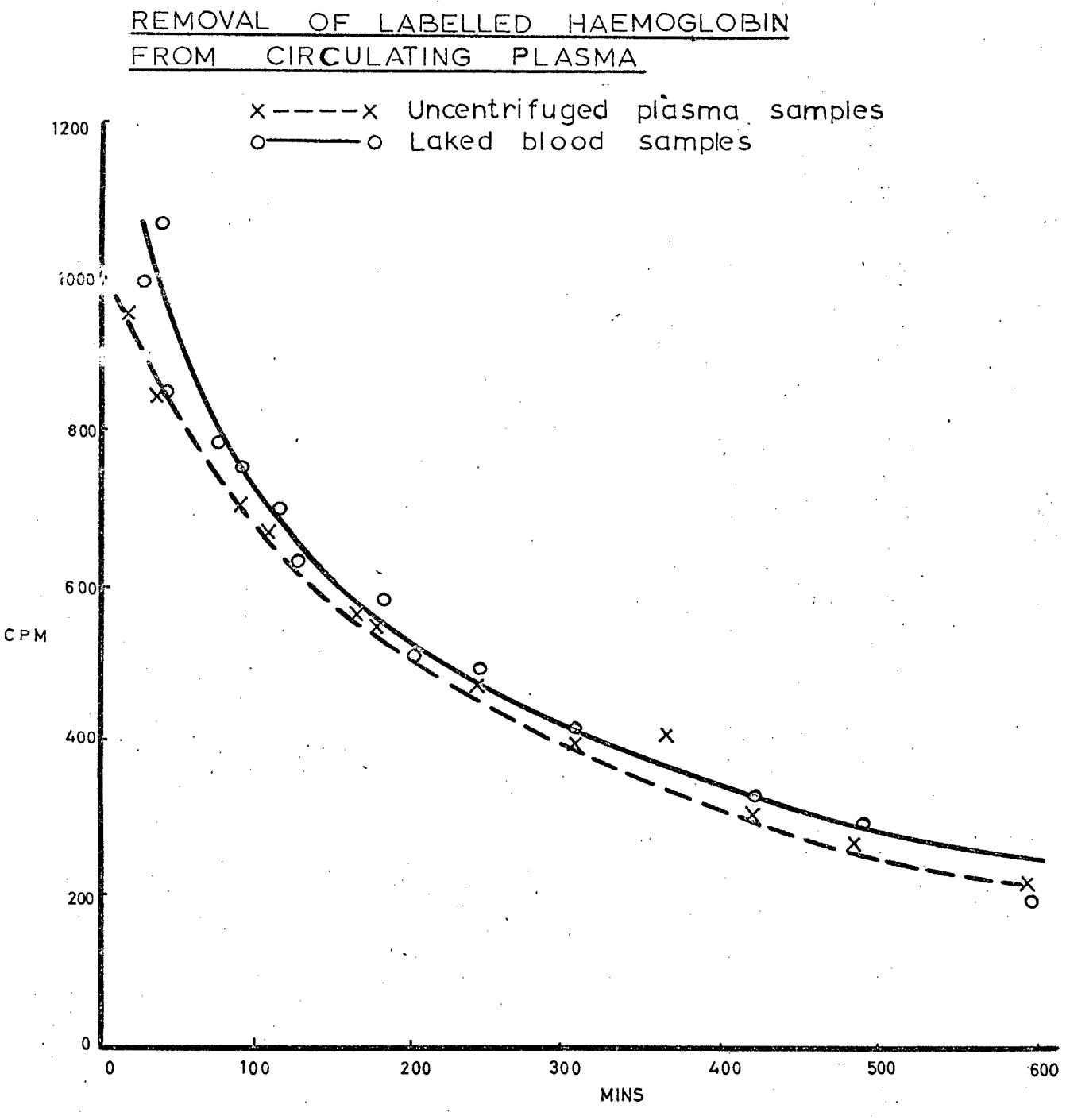
The blood was sampled every 15 minutes for the first 2 hours, half-hourly up to 4 hours and then hourly for the next four hours. In the 24 hour experiment further aliquots were taken at 10 and 25 hours.

(ii) Collection of urine and faeces.

(a) 26 hour experiment - collected every 2 hours up to 10 hours followed by one collection in the 10 - 26 hour period.

(b) 3 hour experiment - faeces and urine were collected when excreted - roughly half-hourly.

FIG. 3.


REMOVAL OF LABELLED HAEMOGLOBIN FROM CIRCULATING PLASMA
x----x Uncentrifuged plasma samples
o——o Laked blood samples
1200
1000
800
600
400
200
0
CPM
0
100
200
300
400
500
600
MINS

Each sample was weighed and a weighed aliquot of each was taken for counting.

2.162 26 hour experiment.

(i) Removal of labelled haemoglobin from the circulation.

3 ml. Fe^{59}-labelled goose haemoglobin was injected into the wing vein of a goose (No. 858) weighing 4.7 kg. This was a dose of 79 mg. haemoglobin/kg. body weight (Ostrow, Jandl and Schmid,(50)) with a total activity of 3.33×10^6 cpm. Blood was sampled at various time intervals as described above. Each sample was counted for 300 seconds and activities of the serial blood and plasma samples are given in Table 4 and plotted in Figs. 3 and 4.

The plasma activities are corrected to 0.1 ml. of plasma, i.e. the activity in the plasma should be 40% greater than in the whole blood sample taken at the same time if the red cell haematocrit is 40%. The plasma values shown are only about 20% higher than the corresponding whole blood activities for the first 3 hours and the difference diminishes thereafter. This is probably due chiefly to inaccuracies in sampling and to haemolysis and dilution of the plasma with non-radioactive red cell haemoglobin in vitro. The lessening in difference after 3 hours may be due to incorporation of Fe^{59} into newly-formed red cells which would cause a relative decrease in plasma activity. It is rather doubtful that the amounts formed would make an appreciable difference at this early stage. However, at 25 hours, when the plasma activity is only 20 - 30% of

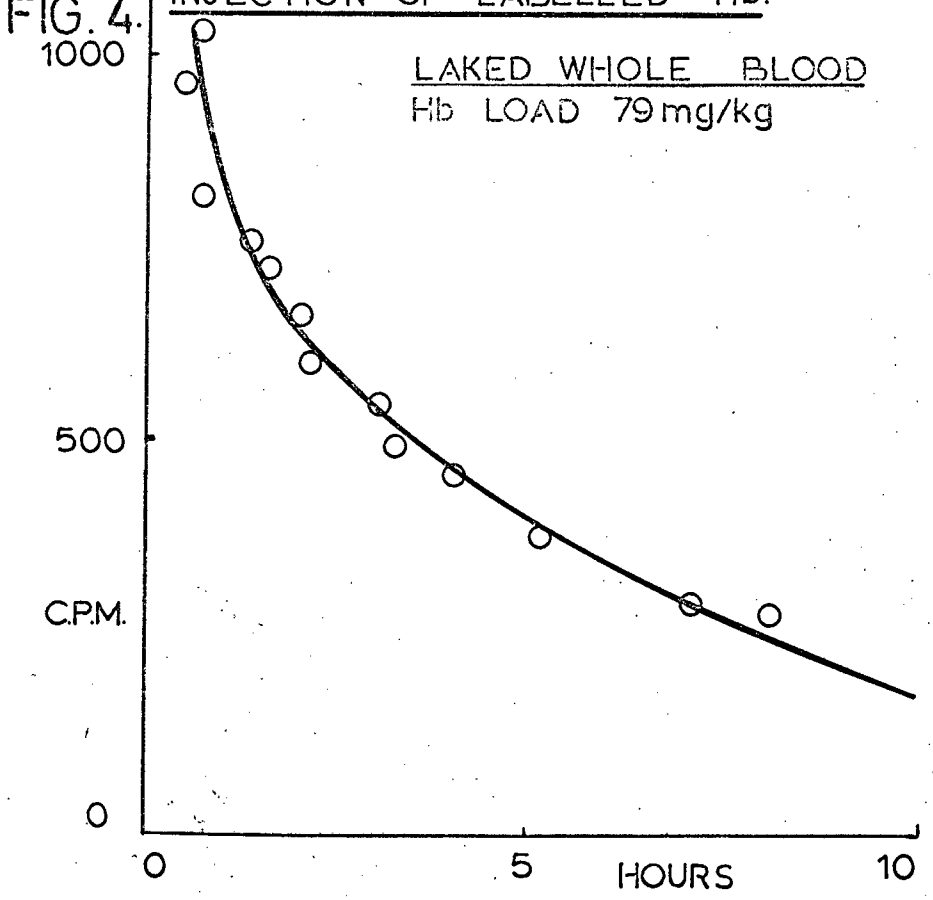
TABLE 4.

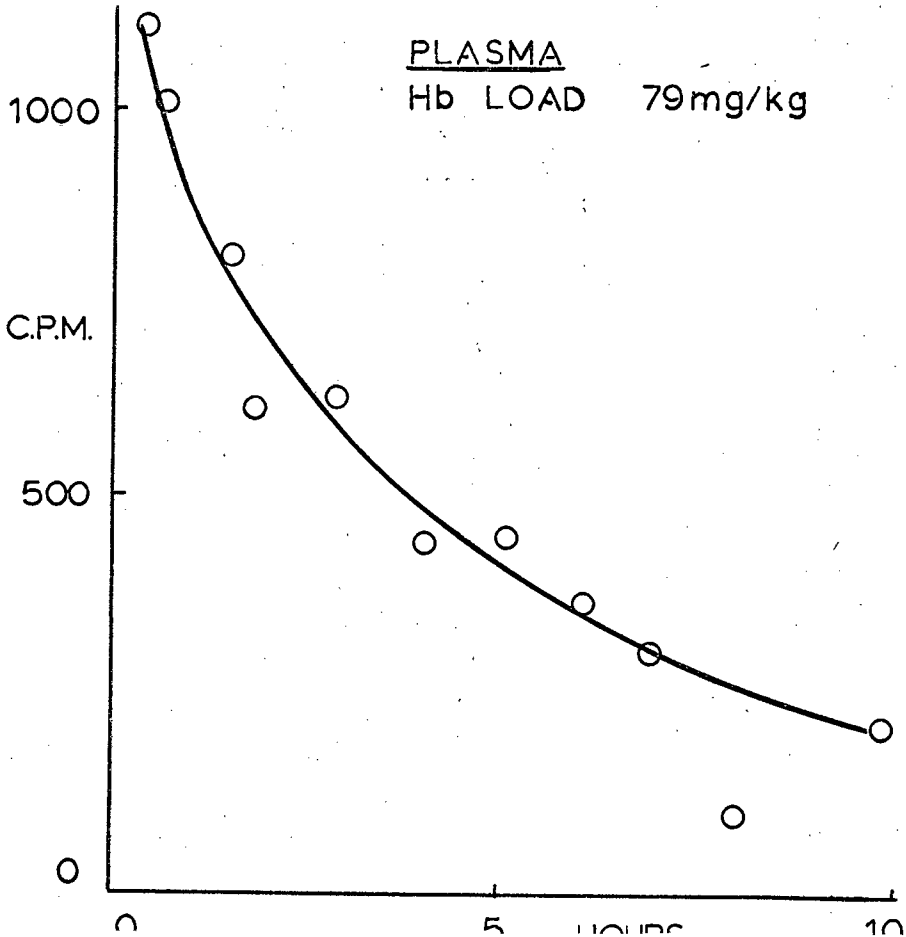
Time (hr.)	Activity of whole blood samples*		Activity of** plasma
	Laked blood	(Plasma samples)	
	cpm/0.1 ml blood	cpm/0.1 ml blood	cpm/0.1 ml plasma
0.3		945	1147
0.4	993		
0.6	1065	852	1044
0.7	847		
1.3	785		
1.5	757	695	844
1.8		656	635
1.9	696		
2.1	630		
2.8		550	654
3.0	578	533	608
3.3	512		
4.0	485	474	466
5.1	408	400	465
6.1		401	383
7.0	320	305	324
8.1	294	259	110
10.0	190	215	220
25.0	46	64	15

* Background activity = 5381 counts/1000 secs.
Samples were counted for 300 secs.

** Background activity = 7118 counts/1000 secs.
Cpm of plasma samples was multiplied by $\frac{1.1}{0.6}$ to give cpm/0.1 ml. plasma.
Samples were corrected for the decay in activity since the counting of the whole blood samples.

FIG. 4. DECREASE IN RADIOACTIVITY AFTER INJECTION OF LABELLED Hb.





that in whole blood, this is almost certainly due to radioiron incorporation into newly-synthesized red cell haemoglobin (Murray, Connell and Pert[67]).

Plotting log cpm against time (Fig. 5) provides a linear relationship, the graph of which can be extrapolated to give a value of the activity in 0.1 ml. whole blood at zero time. This is approximately 940 cpm. Since the total radioactive dose was 3.33 x 10^6 cpm, one can calculate the approximate blood volume of the goose, i.e. 350 ml. or 74 ml./kg. body weight. This is more than the 4.5% of body weight (45 ml./kg.) calculated for rats (Jandl and Kaplan[83]) and less than the value of 11 ml./100 g. (100 ml./kg.) given for Pilgrim geese (Spector[84]).

The 50% clearance time of the label from whole blood is roughly 3 hours (Fig. 7A). The clearance of Fe^{59}-labelled haemoglobin is probably slightly faster but the entry of iron from haemoglobin degradation into the plasma as transferrin-bound-Fe^{59}, obscures the true clearance rate. The low plasma activity at 25 hours (1.4% injected dose) suggests, however, that Fe^{59} is not recycled in appreciable quantities. This agrees with findings of other workers (Garby and Noyes[68]). The 50% clearance time of 3 hours for plasma haemoglobin is slow compared with values determined by others for mammals, i.e. 25 minutes for rabbits (Murray et al.[67]), 15 minutes for the rat (Ostrow et al.[50]).

At 26 hours, the goose was killed by exsanguination and 220 ml.

whole blood was collected by filtering through glass wool into a bottle containing 30 ml. Na citrate. Red cells and plasma were separated and 5 ml. of each taken for counting. The radioactivity in the red cells was twice that in the plasma and the approximate number of circulating counts was calculated as 2.08×10^5 cpm, i.e. 6% of the total injected dose.

	ml.	cpm/ml.	cpm/sample
Plasma	122	484	59,048
Red cells (not packed)	85	965	82,025

(ii) Excretion of Fe^{59} in faeces and urine.

Results of the 2-hourly collections of faeces and urine are shown in Table 5 and plotted in Fig. 6.

TABLE 5.

Excretion of Fe^{59} in faeces and urine in 26 hours .

Period of sampling	Wt. of sample	Specific activity *	Activity of total sample
hr.	g.	cpm/g.	cpm.
0 - 2	11.6	6,518	75,609
2 - 4	0.99	11,055	10,944
6 - 8	3.7	1,344	4,972
9 - 10	1.3	220	286
10 - 26	40	665	26,600(Average for 2 hours = 3,325)
			118,411 **

* Background activity has been subtracted.
** Total x 1.07765 (Decay correction factor) = 1.28×10^5, i.e., 4% of injected dose.

FIG. 5

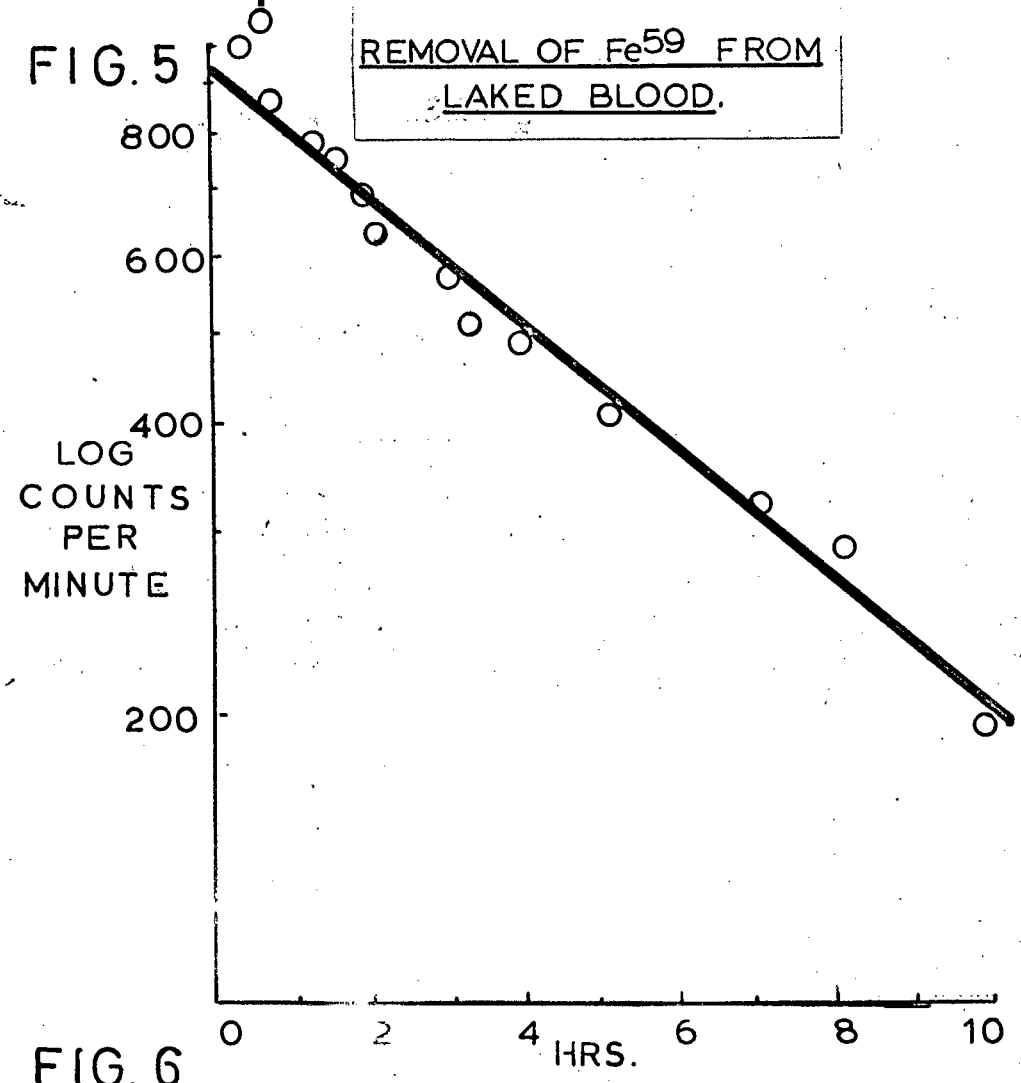
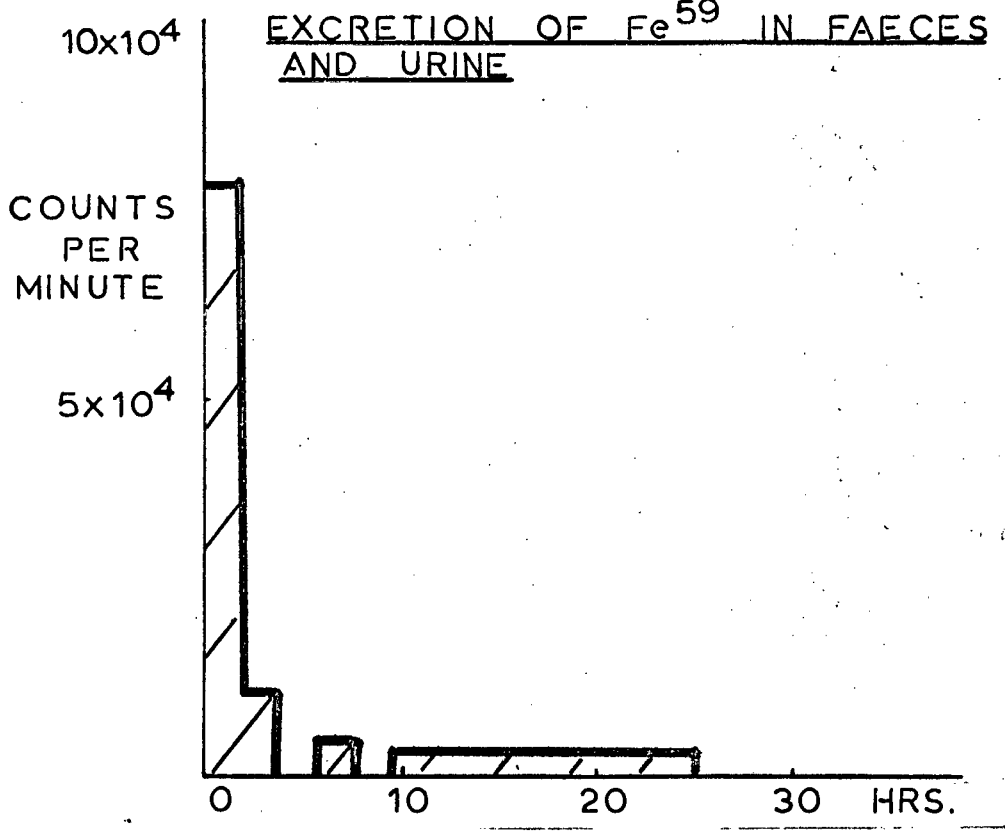

REMOVAL OF Fe59 FROM LAKED BLOOD.
800
600
400
200
LOG COUNTS PER MINUTE
0
2
4
6
8
10
HRS.

FIG. 6


EXCRETION OF Fe59 IN FAECES AND URINE
10x10^4
5x10^4
COUNTS PER MINUTE
0
10
20
30
HRS.

The value given for 26 hours is that calculated from the average 2-hourly excretion observed during the last 16 hours of the experiment. The total activity recovered in the urine and faeces constitutes 4% of the initial injected dose. It can be seen that the excretion of Fe^{59} is greatest in the first 2 hours and then diminishes rapidly. This suggests an initial haemoglobinuria due to the injected haemoglobin load exceeding the haemoglobin binding capacity of the plasma, and not an excretion of haemoglobin degradation products (e.g. biliverdin ferrichloride) which one would expect to be produced continuously throughout the period of haemoglobin removal from the plasma.

The labelled constituents of these samples were further investigated. The sample collected during the 0 - 2 hour period (i.e. the one containing the most Fe^{59}) was centrifuged and the supernatant fractionated on a molecular weight basis on Sephadex G-50 (Pharmacia, Uppsala, Sweden).

Aliquots of the effluent fractions were counted, their spectra determined on a Beckman DB Recording Spectrophotometer and the protein concentration estimated by the method of Lowry, Rosebrough, Farr and Randall[85]. The following results were obtained:

TABLE 6.

Fraction	Absorption maxima	Protein Content	Specific Activity
	mμ	mg.	cpm/mg. protein
(1)	275	0.084	560
(2)	575, 535, 503, 410, 275	2.1	49
(3)	640, 595, 575, 492, 410, 270.	6.61	45
(4)	635, 585, 475 Intense UV absorption.	12.5	9
(5)	610, 510, 464 Intense UV absorption.	0.4	58

The fractions are numbered according to the order in which they were eluted from the column, i.e. in order of decreasing molecular size. The proteins of the first two fractions were excluded from diffusion into the gel and have molecular weights over 50,000. Fraction (3) is roughly in the molecular weight range 15,000 - 50,000 and (4) and (5) are small molecular weight molecules (<15,000).

It is clear that the highest specific activity is associated with a small amount of non-haem-containing protein or large molecular weight.

Goose haemoglobin has absorption maxima at 578 mμ, 542 mμ, 414 mμ, 340 mμ, and at 273 mμ. Therefore, fraction (2) is probably mainly methaemoglobin.

Intermediate fraction (3) contains the most radioactivity although the specific activity is not high. It is tempting in view

of the absorption at 640 mμ, the similar specific activity to the haemoglobin fraction and the smaller molecular weight of the protein in fraction (3), to suggest that this fraction may contain intermediates of haemoglobin degradation which by some means have been excreted by the kidneys. It should be pointed out that the haemoproteins were present in low concentration and the absorption maxima were very diffuse. Also, protein estimations were performed some weeks after the other parameters had been determined and cannot be considered very reliable.

The specific activity associated with the diffusible small molecular weight proteins is low. The low protein content of fraction (5) suggests the presence of some Fe-containing bile pigment, not associated with an apoprotein moiety.

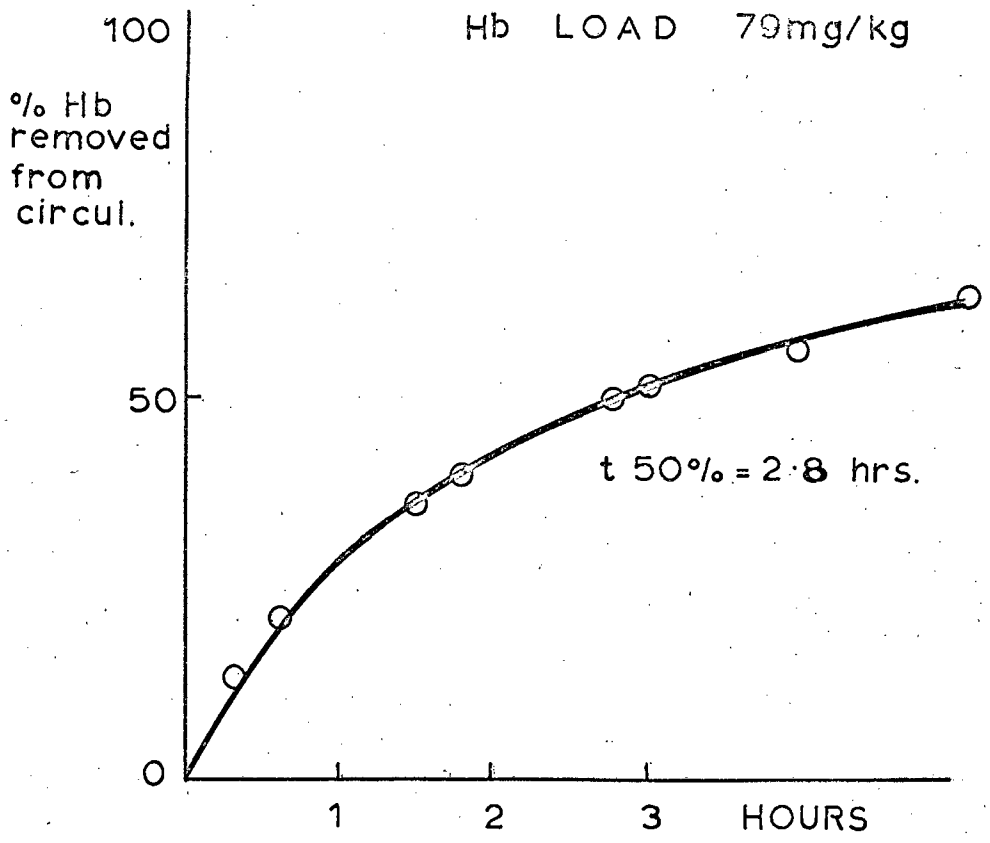
2.163 3-hour experiment.

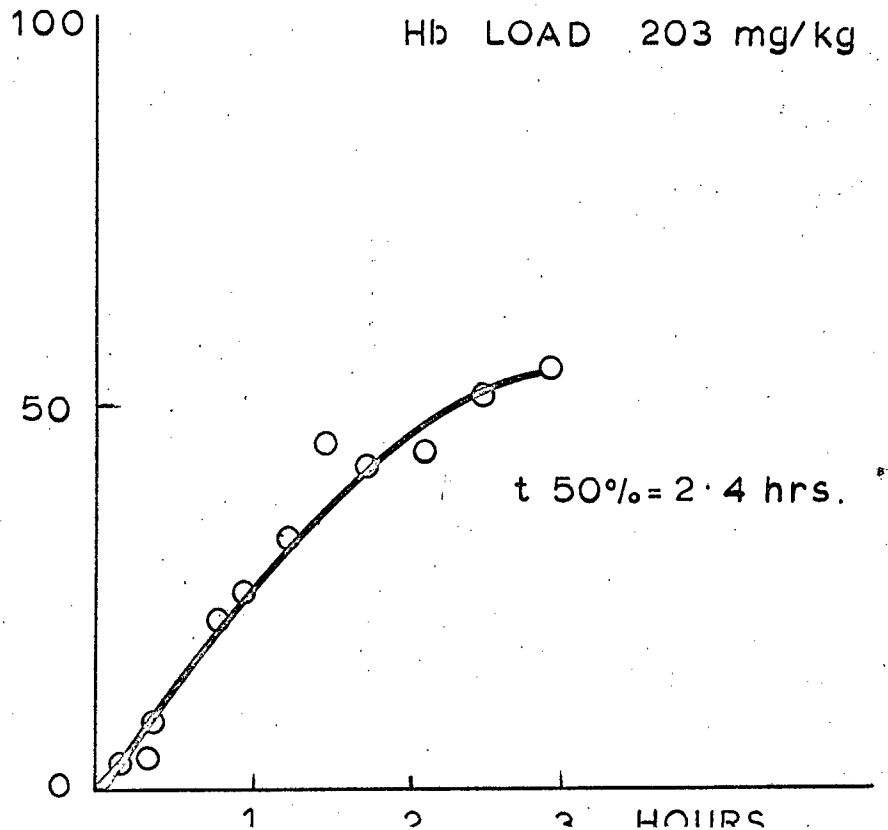
(i) Removal of labelled haemoglobin from the circulation.

7.5 ml. Fe^{59}-labelled goose haemoglobin (923 mg.) was injected into the wing vein of goose No. 861 weighing 4.5 kg. This dose was equivalent to 203 mg./kg. body weight and was approximately 3 times the haemoglobin load administered in the previous experiment. The total radioactive dose was 5.42×10^6 cpm.

Serial whole blood samples were taken as described previously and the rate of haemoglobin clearance from the circulation is shown in Fig. 7B. The rate is slightly faster with higher loading - 50%

FIG. 7. RATE OF REMOVAL OF Fe 59- Hb FROM CIRCULATION.





haemoglobin removed in 2.4 hours as compared with 2.8 hours for the 79 mg./kg. load (Fig. 7A).

Plotting log cpm against time (Fig. 8) gives a value of 16×10^3 cpm/ml. whole blood at zero time. From this, the estimated total blood volume of the goose is 340 ml., i.e. 76 ml./kg. body weight (cf. goose 858 - 74 ml./kg. body weight).

At $3\frac{1}{4}$ hours, when the goose was killed, 45% of the injected dose was still present in the circulation.

(ii) Excretion of Fe^{59} in faeces and urine.

Since in the previous experiment, the haemoglobin load administered resulted in slight haemoglobinuria it was to be expected that in spite of individual variations in haemoglobin binding capacity, the 3 times increased load in this experiment, would produce marked haemoglobinuria. It was evident to the naked eye that the three watery samples passed by the goose during the 90 minute period after injection of the haemoglobin load, contained haemoglobin. The first green faecal excretion was at 2 hours (Fig. 9).

REMOVAL OF Fe^{59} FROM CIRCULATING BLOOD.

FIG.8

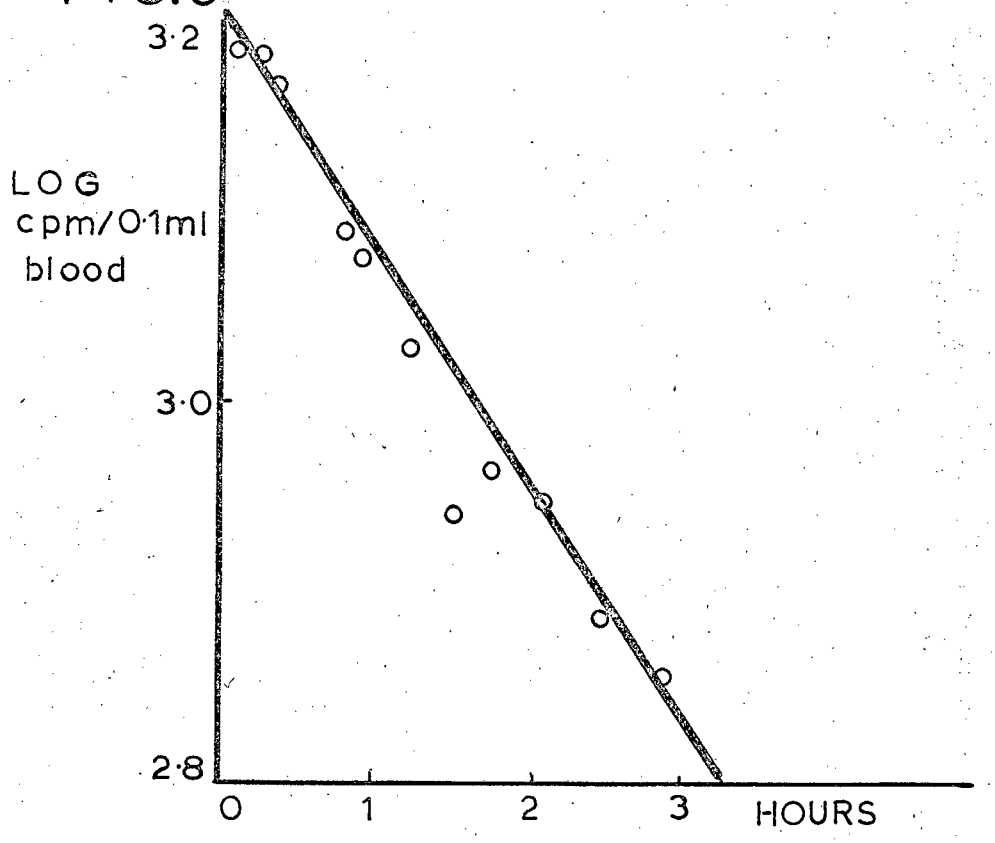


TABLE 7.

Excretion of Fe^{59} in faeces and urine after a haemoglobin load.

(5.42×10^6 cpm)

Time of sampling	Weight of sample	Total activity* of sample	
mins.	g.	cpm.	
20	31	257,364	haemoglobinuria
60	19	91,206	
90	11	90,805	
120	23	53,394	green faecal excretion.
196	7	42,313	
		535,082	

* Corrected for background contribution to activity and for radioactive decay in the time period between administration of load, and counting of samples.

The total radioactivity excreted was 10% of the injected dose as compared with less than 3% of the injected dose excreted in 3 hours in the previous experiment (Fig. 10). The rapid elimination of excess haemoglobin probably accounts for the slightly shorter 50% clearance time obtained in this second experiment.

2.17 FRACTIONATION OF GOOSE LIVER.

3 different preliminary experiments were undertaken to obtain

FIG. 9

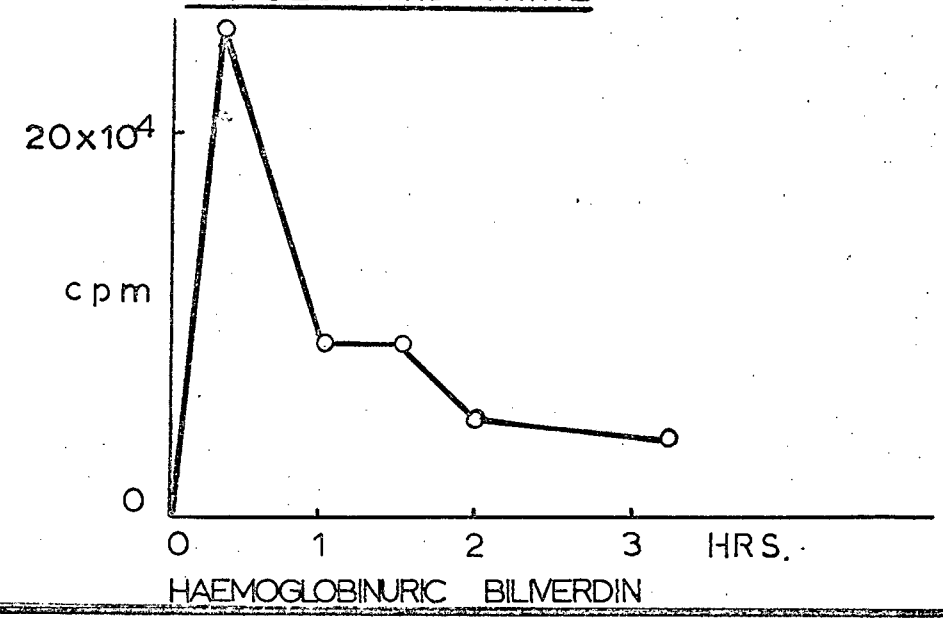
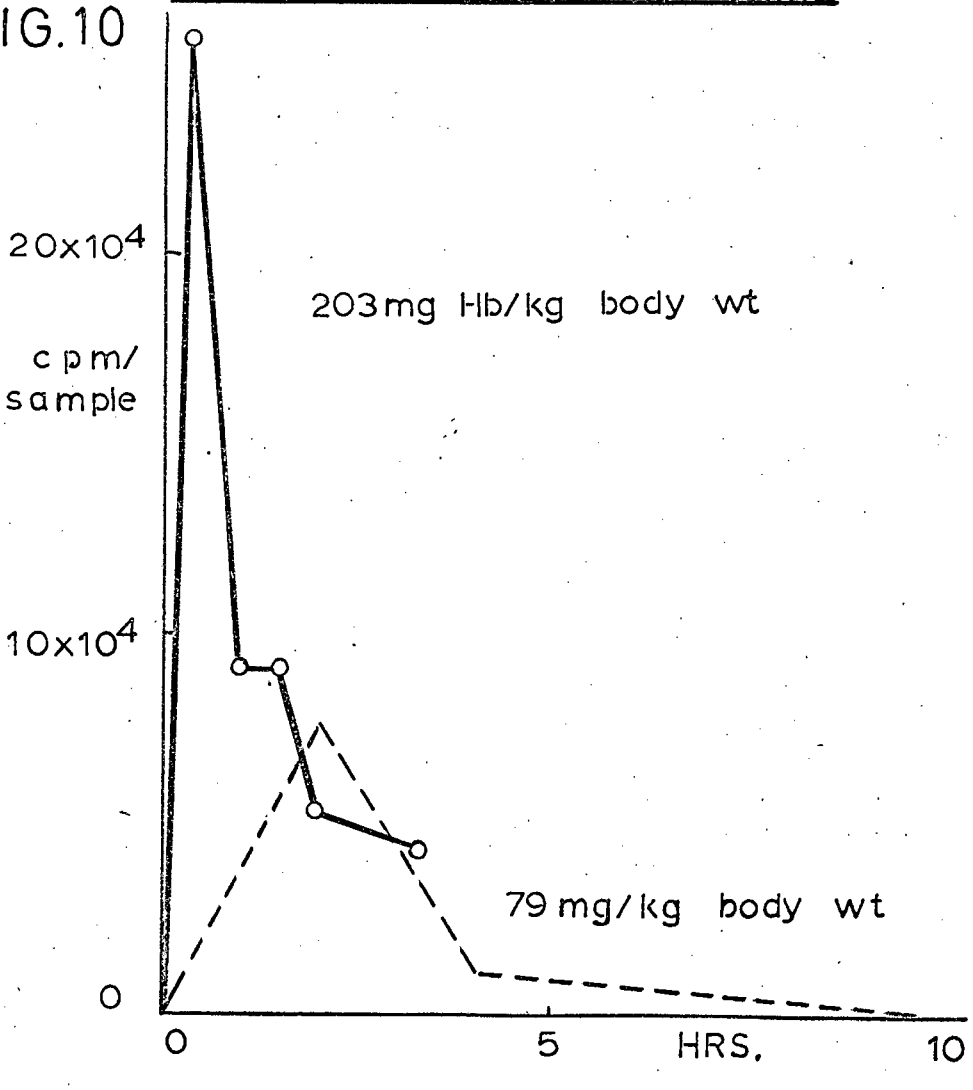

TOTAL ACTIVITY EXCRETED IN FAECES AND URINE
20x10⁴
cpm
0
0
1
2
3
HRS.
HAEMOGLOBINURIC
BILIVERDIN

FIG. 10


EFFECT OF Hb LOAD ON EXCRETION OF Fe59 IN FAECES AND URINE
20x10⁴
cpm/ sample
10x10⁴
0
203mg Hb/kg body wt
79 mg/kg body wt
0
5
HRS.
10

goose liver at a time when haemoglobin degradation would be maximal in the hope that under these conditions bile pigment precursors would accumulate.

In the first, a dose of phenyl hydrazine was given to a goose whose circulating erythrocytes had been labelled in vivo with Fe^{59}. Forty-eight hours after this stimulation of red cell destruction, the goose was killed and the liver removed.

The second liver was removed 26 hours after the administration of an Fe^{59}-labelled haemoglobin load of 78 mg./kg. body weight, the radioactive dosage totalling 3.33×10^6 cpm.

In the third experiment, the goose was killed, and the liver removed, 3 hours after 203 mg. haemoglobin/kg. body weight was injected. The radioactive dose was 5.42×10^6 cpm.

2.171 METHODS.

(i) Ultracentrifugation of liver homogenate.

The techniques used for subcellular fractionation of goose livers are based on those of Löw and Vallin[86] for the isolation of mitochondria, and Wust and Novelli's method of preparing microsomes[87].

The procedure adopted was as follows: Immediately after removal, the goose liver was placed in ice-cold sucrose solution

(0.25 M) and washed 3 or more times with fresh, cold sucrose solution. All further procedures were carried out at 0 - 4°C. The liver was cut up into small pieces, blotted, and the total volume measured by displacement in a known volume of sucrose solution. The liver was then minced with 0.5 volume sucrose in a Waring Blendor for 5 - 10 minutes. Aliquots of the liver mince were homogenized by hand with 3 strokes in a glass homogenizer fitted with a Teflon pestle. During homogenization a further 2 volumes of sucrose solution were added. The homogenate was filtered through fibre-glass mesh to remove fibrous connective tissue strands, and then centrifuged in a Beckman Spinco Model L Ultracentrifuge at 2,500 rpm (600 x g.) for 10 minutes.

The supernatant obtained was kept at 0°C and the sediment, containing intact cells and nuclei, was rehomogenized in 1.5 volumes sucrose solution and spun again at 2,500 rpm for 10 minutes. This step was repeated once more with 1 volume of sucrose. Thus, the liver was homogenized 3 times in all in a total volume of 0.25 M sucrose, equal to five times the volume of liver used. The final sediment was suspended in one volume of sucrose and stored.

Separation of mitochondria.

The combined supernatants (from 3 centrifugations) were spun at 7,500 rpm (5,000 x g) for 10 minutes and the mitochondrial fraction which sediments, was suspended in 1 volume 0.25 sucrose solution and stored. A second mitochondrial fraction was obtained by spinning the supernatant at 11,500 rpm (10,000xg) for 10 minutes

(iii) Gel filtration of the soluble fraction of liver cells.

In 2 of the three experiments, an attempt was made to fractionate the protein constituents of the cell supernatant on the basis of molecular size by gel filtration through columns of dextran gel (Sephadex) using types with varying degrees of cross-linkage, e.g. G-100, G-75, G-50 and G-25. The absorption of the column effluent at either 260 mμ or 415 mμ was recorded continuously during elution byusing a Uvicord apparatus or a flow cell fitted into a Beckman DB recording spectrophotometer. Effluent fractions were collected manually or in a fraction collector and assayed for radioactivity.

2.172 Stimulation of Fe^{59}-labelled red cell destruction with phenyl hydrazine - Experiment I.

18 days after administration of a dose of Fe^{59} to a goose, during which time the iron was incorporated into red cell haemoglobin (para. 2.151), 36 mg. acetyl-phenyl hydrazine/kg. body weight was injected subcutaneously in the pectoral region. Forty-eight hours later, when the red cell haematocrit dropped to 13.4%, the goose was killed by exsanguination, the liver excised and immediately transferred to ice-cold 0.25 M sucrose solution.

(i) Fractionation of liver.

The liver was then fractionated by the procedures described above. Since the stimulation of red cell destruction by phenyl hydrazine can hardly be regarded as physiologically normal, the

results were viewed solely as an indication of what might occur under conditions of normal red cell turnover. Furthermore, as this was simply a pilot experiment, no attempt was made to estimate the protein concentrations in the different fractions nor their specific activities.

(ii) Counting of fractions.

The results that were obtained (Table 8) seem to suggest that most of the Fe^{59} was associated with the mitochondrial fraction of liver (about one-third of the total counts in the liver homogenate) although the microsomes had the highest activity per unit weight.

TABLE 8.

Fe^{59} in liver fractions after labelled-red cell breakdown.

Fraction	% of liver homogenate.	Specific activity*	Activity of whole fraction
		cpm/g.	cpm.
Liver tissue		369,292	23.63×10^6
Liver homogenate		104,453	23.40×10^6
Intact cells & nuclei	~ 1	63,650	2.55×10^6
Washed mitochondria	~30	13,607	5.58×10^6
Washed microsomes	< 1	236,207	3.31×10^6
Soluble fraction	~70	18,443	4.52×10^6
			16×10^6

* All values are corrected for "dead time" and background activity. The liver fractions, which were counted some time after the whole tissue, were corrected for decay over that period.

It is probable that even if most of the radioactive Fe^{59} associated with the liver fractions was present due to the phenyl hydrazine-stimulated destruction of red cells, a large porportion of the Fe^{59} would have been removed from haem and bound to storage compounds in the 48-hour period prior to death, and such compounds could be associated with any of the particulate cell fractions. In other words, in view of the experimental conditions, the activity associated with the mitochondrial and microsomal fractions, does not necessarily indicate that these organelles are the sites of haemoglobin destruction or intermediate accumulation.

(iii) Gel filtration of soluble fraction.

Gel filtration of the liver supernatant on G-100 which can fractionate compounds ranging from 5,000 - 60,000 in molecular weight and excludes those over 300,000 from diffusion into the gel particles (Andrews[88]), separates three 260 mμ-absorbing fractions. (Fig. 11, Table 9). The first, containing proteins of molecular weight over 150,000 and with only slight absorption in the Soret region, has the highest activity. The second peak (M.W. 60,000 - 150,000) consisted mainly of haemoglobin and contained less of the Fe^{59} label. The small-molecular weight fraction (i.e. $<60,000$) contained very little radioactivity.

On Sephadex G-75 (M.W. range 3,000 - 35,000; exclusion weight = 110,000 (Andrews[88])) similar results were obtained. (Fig. 12).

TABLE 9.

Fractionation of liver supernatant on Sephadex.

	G-50		G-75		G-100	
	Activity[a]	Absorption max.[c]	Activity	Absorption max.	Activity	Absorption max.
	cpm/fraction	mμ	cpm/fraction	mμ	cpm/fraction	mμ
Liver supernatant[b]	73,766		73,766		73,766	
Void volume	556					
First Peak (i)	76,515	540, 410, 280	67,217	410, 280	64,211	410, 280
(ii)			12,408	414, 280	15,388	630, 414 U.V.
Interval	710	U.V.	256	410 U.V.		
Second Peak	6,076	U.V.	2,593	U.V.	1,950	U.V.
Total activity	83,857		82,474		81,549	
% Recovery	114%		112%		111%	

(a) All activities have been corrected for background activity and differences in radioactive decay.

(b) Activities have all been related back to 5 ml. of supernatant, although this was not always the volume applied to the respective columns.

(c) Under "Absorption Maxima", wavelengths not underlined indicate low extinction values. The letters U.V. indicate that maximal absorption of the constituents is between 260 and 270 mμ and not at the tyrosine maximum, 280 mμ.

FIG. 11. GOOSE LIVER SUPERNATANT FRACTION ON SEPHADEX G-100

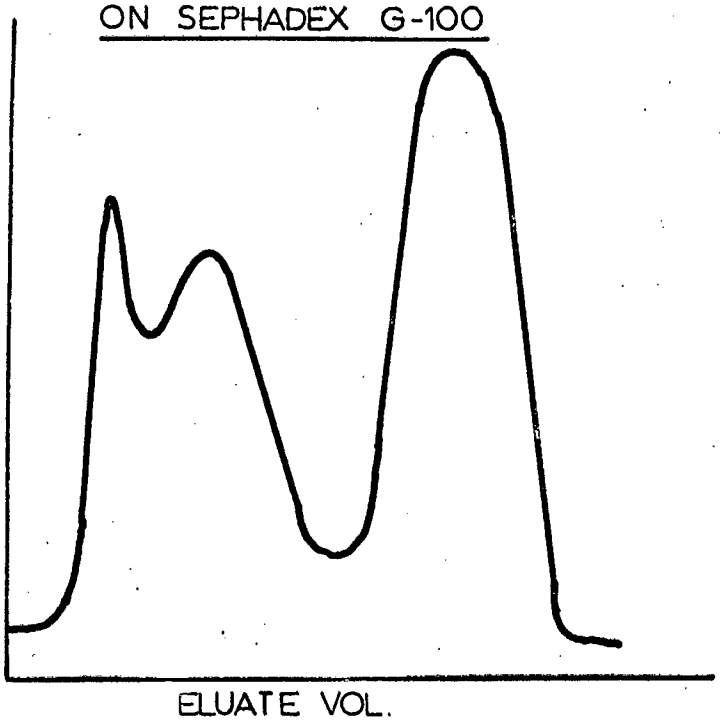
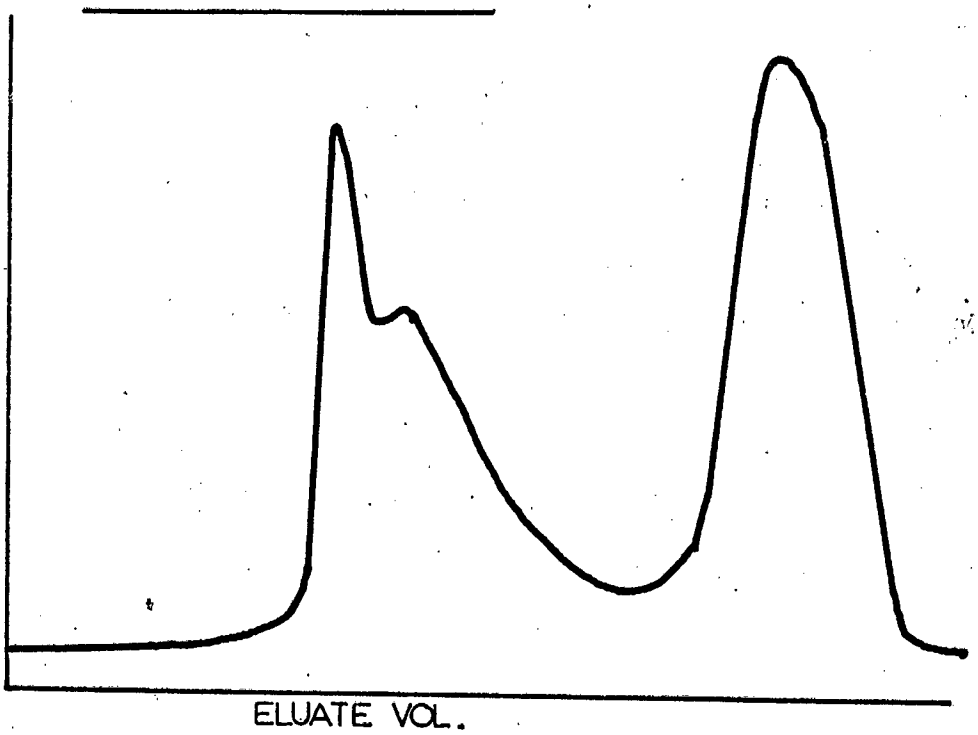


FIG. 12. ON SEPHADEX G-75



Proteins of large molecular size (M.W. over 70,000) had the highest activity. The fraction containing haemoglobin (M.W. 35,000 - 70,000) had the same ratio of activity to the first peak as on G-100 (1:4) and the small molecules were once again low in activity.

Fractionation on gels with a greater number of cross-links (i.e. Sephadex G-50 and G-25) which separate substances of molecular weight less than about 30,000 did not produce any clear-cut results other than that the non-diffusible, large molecular weight, first peak had the highest activity. (In these gels the first peak includes haemoglobin).

2.173 Liver fractionation 26 hours after haemoglobin load - Experiment II.

Fe^{59}-labelled goose haemoglobin, 78 mg./kg. body weight was injected intravenously into a goose. Clearance of the label from the plasma was followed, and the excretion in urine and faeces determined. 26 hours later, when approximately 95% of the Fe^{59} had been removed from the circulation and 4% of the injected dose had been eliminated in faeces and urine (para 2.162 (ii)), the goose was killed by exsanguination and the liver, gall-bladder and spleen were excised.

Radioactivity in tissues.

The gamma emission of a small piece of the goose liver, the total weight of which was 41 g., was 32×10^3 cpm/g. i.e. 1.32×10^6 cpm

were taken up by the liver in 26 hours. This figure represented 40% of the injected dose. The uptake of Fe^{59} by the spleen, and secretion of the isotope into the bile was negligible (about 0.05% of the administered dose in each case). Blood, trapped in the extracellular and interstitial fluid spaces of the tissues did not contribute significantly to the activity in the tissues as only about 5% of the injected Fe^{59} was detectable in the circulation at 26 hours (para 2.162 (i)).

Half the injected radioactive haemoglobin was thus accounted for; 40% was present in the liver and of the other 10%, some was excreted in the urine and the rest degraded to provide Fe^{59} for incorporation into newly-synthesized red cell haemoglobin and for transport in the plasma, bound to transferrin.

(i) Fractionation of the liver.

In this experiment, we decided to omit the complete fractionation procedure and only to isolate the supernatant fraction in which we hoped to find labelled intermediates of haemoglobin degradation (Nakajima et al.(40)).

The liver homogenate prepared as before was centrifuged at 40,000 rpm for 1 hour. The supernatant was recentrifuged and the cell supernatant fraction was diluted with a further 1 volume 0.25 M sucrose solution (original liver was diluted as previously with 5 volumes sucrose) and spun again to remove lipids.

(ii) Counting.

21% of the radioactivity in the liver homogenate is attributable to the soluble fraction. Since approximately 60% of the volume of the liver consists of non-particulate matter, it is evident that particulate protein has a higher specific activity, 26 hours after administration of Fe^{59}-labelled haemoglobin, than the proteins of the soluble fraction.

(iii) Gel filtration of soluble fraction.

The liver supernatant was fractionated on columns of Sephadex G-75 and G-50. The optical density of the effluent at either 415 mμ or 280 mμ was recorded by allowing it to pass, after coming off the column, through a flow cell placed in a Beckman DB spectrophotometer.

At selected stages in the elution of the liver fraction, the flow from the column was halted by closing off the outflow tap at the bottom, a second recording apparatus was attached to the spectrophotometer and the spectrum of the substances in the flow cell at that particular moment were scanned automatically.

There are numerous disadvantages encountered by intermittently halting the flow of fluid in the column; the most important being that mixing occurs on the column and good separation is not achieved; also, one cannot obtain a continuous flow recording of the effluent optical density. However, as we only expected to find very small amounts of bile pigment precursors of different types (ranging from bilihaemoglobins, through haems attached to single chains of globin,

to choleglobin and biliverdin), we wondered if fraction collecting was obscuring these proteins by diluting them with ones having similar spectral characteristics. Thus, by this method any small maxima or shoulders appearing on the recording of the effluent optical density could be investigated immediately by halting the flow and recording the spectrum.

The results obtained for fractionations of the liver supernatant on Sephadex G-75 and G-50 are shown in Table 10.

In view of the recoveries of radioactivity indicated on the Table, which suggest some contamination on the columns or other pieces of apparatus, one can only attempt a tentative interpretation of the results.

It is clear that in general the characteristics shown by the liver supernatant in the previous experiment, involving phenyl hydrazine administration, are repeated here.

(a) In both cases the predominant haem pigment is haemoglobin, presumably occurring mainly as a contaminant from the extracellular fluid space. 26 hours after a dose of labelled-haemoglobin, this does not significantly affect the activity of the soluble fraction of the liver and on Sephadex G-75, which separates haemoglobin from proteins of higher molecular weight, this is evident both in the total activity of the fraction (2nd peak) and its specific activity. In the first experiment, the circulating haemoglobin which has not been degraded as a result of phenyl-hydrazine-promoted red cell

TABLE 10.

Fraction	Approx. molecular weight.	Effluent volume at which flow was stopped.	Absorption Maxima	Protein content	Activity of fraction	Specific Activity
SEPARATION ON SEPHADEX G-75. (Recovery 158%)						
		ml.	mμ	mg.	cpm.	cpm/mg. protein
1st Peak 47-63 ml.	>70,000	52	Diffuse absorption peaks at 605, 580, 485, 405. Intense UV absorption	16.5	1,947	118
		62	Peaks more defined - 603, 575, 490, 406. Intense UV absorption			
2nd Peak 63-90 ml.	<70,000	78	610, 575, 535, 483, 414, 270.	23.8	537	23
Interval 90-115 ml.	30,000-70,000	96	608, 578, 540, 478 (low) 414, 260.	7.9	412	52
		113	613, 536, 495, 450, 412, 260.			
3rd Peak 115-170 ml.	<30,000	132	General absorption in visible region; 600 - 475. 260.	21.0	1,795	85
SEPARATION ON SEPHADEX G-50. (Recovery 240%)						
1st Peak 25-49 ml.	>50,000	30	614, 575, 537, 510, 413. Intense UV absorption	33.6	3,856	115
		39	612, 577, 540, 414. Intense UV absorption			
		46	615, 578, 530, 414. Intense UV absorption			
Interval 49-58 ml.	10,000-50,000	50	615, 570, 550, 490, 455, 413, 260.	12	120	10
		56	605, 475, 410, 260.			
2nd Peak 58-96 ml.	<10,000	62	610, 475, 263.	9.4	1,709	182
		70	613, 500, 450, 265.			
		81	613, 580, 493, 450. Intense UV absorption.			

destruction is still highly labelled and would be expected, if contaminating the liver supernatant, to obscure the true activity. On G-75 the activity of the second peak (i.e. haemoglobin) is 50 times that of the haemoglobin fraction in Experiment II.

(b) In both experiments, the greatest activity is associated with proteins of molecular weight greater than haemoglobin (i.e. more than 70,000). This is particularly the case after the 48 hours of red cell destruction (Experiment I) in which some Fe^{59} must have been incorporated into liver protein prior to phenyl hydrazine administration. Although the total activity of this fraction is much higher than its counterpart in Experiment II, the ratios of first to second G-75 fractions are comparable.

	48 hrs.(I)	26 hrs.(II)
$\frac{\text{Activity of 1st peak (M.W. > 70,000)}}{\text{Activity of 2nd peak (Hb)}}$ =	5.5	5.0

It appears from these results, that during the time periods studied, Fe^{59} had already been removed from the haemoglobin delivered to the liver for degradation, and was present attached to storage proteins of high molecular weight, e.g. apoferritin.

This only applies to the Fe^{59} of the supernatant. In microsomes and mitochondria, which we have shown to be highly active, the fate of Fe^{59} was not investigated.

(c) The presence of a Soret band, albeit small, in the high-molecular weight protein fractions at 405 - 410 mμ suggests that

this is not due to contamination of the fraction with haemoglobin (absorption maximum 414 mμ) but to the presence of some large haemoproteins, e.g. liver catalase (Soret maximum = 406 mμ), which, in addition to the non-haem iron-containing proteins, and in view of its rapid turnover rate, may be labelled.

(d) The small molecular weight constituents of the liver supernatant contain a larger proportion of the supernatant activity in the liver 26 hours after a haemoglobin load than in the first liver, removed 48 hours after phenyl hydrazine was administered.

	Activity in fractions of small molecular weight.	
	26 hours.	48 hours.
G-50	30%	7%
G-75	38%	3%
G-100	-	2%

The possible reasons for these findings are as follows:

(i) The high recoveries encountered in the column fractionations of the 26-hour liver supernatant may be chiefly due to contamination of the small molecular weight fraction, a constituent of which could adsorb or bind iron left on the column; or else a substance of low molecular weight, previously adsorbed on the columns, was eluted during the current fractionations. These possibilities seem unlikely in view of the similarity in the relative activities of this fraction on both G-50 and G-75 (30% and 38%) and the large discrepancy in the total recoveries from the columns - 240% and

158% respectively.

(ii) Phenyl hydrazine (Experiment I) may stimulate a particular haemoglobin degradative pathway preferentially, favouring removal of iron or haem and its binding to large protein molecules.

(iii) The Fe^{59}-haemoglobin administered as a single intravenous load is bound to plasma haptoglobin (Laurell and Nyman[70]). Any haemoglobin present in excess of the binding capacity of the plasma haptoglobin is usually excretion in the urine. Administration of phenyl hydrazine, on the other hand, causes extensive breakdown of red cells in the liver and large-scale intravascular haemolysis, resulting in the release of large quantities of free haemoglobin into the circulation over a longer period than is the case with a single haemoglobin dose. The capacity of the plasma haptoglobin is rapidly exceeded and it is removed from the circulation. Some of the remaining,unbound haemoglobin is excreted,but large quantities are made available to the liver for degradation. Since the haemoglobin-laden animal has had only half the time of the phenylhydrazine treated goose to degrade the haemoglobin, it is possible that the Fe^{59} or Fe^{59}-haem is associated with small molecules (M.W. $< 20,000$) prior to being bound to the larger proteins, i.e. that small molecular-weight intermediates are present.

Although this possibility exists, it is more likely that the differences in the two experiments are a result of different degradative pathways rather than the length of time allowed for degradation. In addition, quite a number of workers have suggested

that, in mammals, intracorpuscular and unbound plasma haemoglobin may differ in metabolic disposition from haptoglobin-bound haemoglobin (Ostrow et al.[50]; Murray et al.[67]; Nakajima et al.[40]).

(e) However, when one considers the absorption spectra of the fractions of smaller molecular weight, the situation becomes much more complicated. They appear to consist mainly of polynucleotides, e.g. sRNA and nucleotide coenzymes, with U.V. absorption at 260 mμ. Some of the constituents do have absorption maxima in the visible region of the spectrum but no one pigment predominates and, in view of the multiplicity of constituents of liver cells, it is very difficult to interpret these results.

Thus, although the tentative findings on the labelling of the supernatant fractions appeared promising as regards our search for labelled bile pigment intermediates of low molecular weight, the spectral properties of the relevant fractions defied analysis and so further characterization by gel filtration was abandoned.

We assumed that 26 hours after an intravenous dose of haemoglobin, a large proportion of it had been degraded. Garby and Noyes found that 40% of a dose of Fe^{59}-labelled haemoglobin reappeared in the plasma of man as Fe^{59}-transferrin within 8 hours[68]. The rest is incorporated into slow-turnover storage compounds. Ostrow and colleagues found that 22 hours after injection of haemoglobin-C^{14} into rats, the excretion of bilirubin-C^{14} ceased[50]. Most of it was excreted in the first 6 hours, although even after 22 hours

only 50 - 70% of the label was recovered if large haemoglobin loads were administered. Recoveries were higher with low haemoglobin loads.

We decided to investigate the possibility that the time allowed for degradation of haemoglobin was responsible for the differences in the results of the experiments described above; and a shorter experiment on goose liver was performed. We also hoped that a more definitive accumulation of small molecular weight labelled intermediates would occur before the transfer of Fe^{59} onto protein of large molecular size.

2.174 3-hour liver experiment - Experiment III.

The liver was excised from an exsanguinated goose $3\frac{1}{4}$ hours after the intravenous injection of labelled haemoglobin (203 mg./kg. body weight; 5.42×10^6 cpm).

At $3\frac{1}{4}$ hours, 45% of the radioactivity present in the plasma 5 minutes after the injection, was still circulating (see 2.163 (i)). Contamination of the liver fractions with circulating blood would thus markedly increase their respective activities. An attempt was made, therefore, to remove the extracellular haemoglobin prior to homogenizing the tissue.

(i) Liver fractionation.

The excised liver was transferred immediately to ice-cold 0.25 M

sucrose solution. It was thoroughly washed and an attempt was made to perfuse it with sucrose solution. After cutting up the tissue into fairly large pieces, it was washed again and then minced in sucrose solution in a Waring Blendor for about 2 minutes.

The mince was transferred to a weighed centrifuge bottle and centrifuged at 2,300 rpm for 1 hour at 0°C in an MSE refrigerated centrifuge. The supernatant fluid ("liver wash") was kept for counting. The sediment was stirred up with sucrose solution, recentrifuged and the supernatant was added to the previous "liver wash". The bottle containing the liver sediment was weighed and the value obtained after subtraction of the original weight of the bottle was taken as the total liver mass. The washed liver sediment was minced again in a Waring Blendor for about 5 minutes, filtered through terylene net to remove connective tissue and then centrifuged as before. The liver was washed once more with sucrose, all the washings were pooled and the minced sediment (being creamy in colour) was homogenized in 2 volumes of sucrose solution with 3 strokes in an all-glass hand homogenizer.

The homogenate was centrifuged 3 times at 3,500 rpm for 10 minutes in the Beckman Spinco Model L Ultracentrifuge as described under "Methods" (2.171) and the pooled supernatant was processed as before except that the 2 mitochondrial fractions (heavy and light) were not pooled but counted separately.

(ii) Counting of fractions.

The results shown in Table 11 are rather puzzling. Of the

TABLE 11.

Fate of injected Fe^{59}-labelled haemoglobin in 3 hours.

Fraction	Specific Activity* cpm/ml.	Total vol. of fraction ml.	Total Activity** cpm.	% of injected dose
Plasma	8,656	~184 (i)	1.92×10^6	
Red cells	764	~156 (i)	0.14×10^6	
			2.06×10^6	38%
Liver washings	1,317	380	0.42×10^6	
Liver homogenate	758 (cpm/g.)	237g.	198,404	3.7%
Intact cells and nuclei (by calculation)		5ml.	14,247	
Heavy mitochondria (unwashed)	838	70	66,436	
Heavy mitochondria (washed)	672	57	43,402	
Light mitochondria (unwashed)	450	33	16,827	
Microsomes (unwashed)	792	50	41,811	
Soluble fraction	826	186	174,085	
Faeces and urine	-	-	535,082	10%
Bile	-	-	4,152	0.1%

(i) Approximate volumes calculated from apparent blood volume and the red cell haematocrit.

* Corrected for background activity.

** Corrected for radioactive decay.

approximately 50% Fe^{59} cleared from the plasma, 10% of which is excreted, only about 4% of the rest is in the liver. The activity of the final supernatant fraction constitutes 88% of the liver homogenate. However, the unwashed particulate fractions, when their activities are added, total 63% of the homogenate activity. This is obviously due to contamination of the particles with supernatant. Calculation of what the total particulate fraction might be if washed, still gives a value for the activity which is 129% of the homogenate activity. Since a certain amount of the material is also lost during the fractionation process, it is evident that either some contamination has occurred or else, even the washed mitochondrial fraction still contains some of the supernatant.

The results indicate that at 3 hours, the soluble fraction has the highest specific activity.

Unfortunately, before it was possible to do any further analysis on the liver supernatant, the proteins were denatured and precipitated out of solution.

Although certain aspects of this work appeared promising in terms of isolating and characterizing intermediate compounds of haemoglobin degradation, we did not feel justified in continuing this line of approach. Possibly, although the expense involved would be even greater, the only fruitful method of investigation is one in which both the iron and the haem nucleus (labelled with C^{14}) are traced after administering a haemoglobin load. This was

the approach used by Ostrow, Jandl and Schmid in their in vivo studies on bilirubin production in the rat[50].

The complexity of the results which were obtained in these in vivo studies prompted us to investigate simpler, in vitro systems for mechanisms of bile pigment production.

2.2 IN VITRO EXPERIMENTS.

2.21 BACTERIAL SYSTEMS.

2.211 Introduction.

Hart and Anderson showed in 1933 that when laked blood was added to a broth culture of the bacterium Diplococcus pneumoniae at pH 8 and incubated in air, the oxyhaemoglobin was reduced within half an hour and this was followed by formation of a brown pigment. After 24 - 48 hrs., a dark green precipitate had formed while the colour of the culture fluid was green or greenish-brown[89]. They found that oxyhaemoglobin, methaemoglobin, and to a lesser extent, carboxy-haemoglobin could replace laked blood as a source of the green pigment[90]. Alkaline haematin was inactive. Lemberg subjected the pigments produced in this system to spectral analysis[29]. He found that the mixture contained (a) mainly unaltered proto-haemochromogen (b) a haemochromogen with an absorption band at 620 mμ belonging to the type produced by autoxidation of alkaline haematin and (c) a small quantity of verdohaemochromogen.

Fuller and Maxted[91] demonstrated that the green variants of β-haemolytic streptococci produce peroxide prior to formation of haemolysin, and that if peroxide formation was prevented by anaerobic incubation or by the action of catalase, the variants regained their β-haemolytic action. They thus showed that peroxide was required for the production of α-haemolysis (i.e. greenish discolouration with partial lysis of red blood cells around the colonies on blood agar plates) and Isaacs[92] found that the depressed production of haemolysin in these variants (i.e. Streptococcus viridans) was due to the presence of peroxide.

Herbert and Todd[93] purified the oxygen-labile streptolysin O produced by the β-haemolytic streptococci and were able to show that it was protein in nature and required a reducing agent such as cysteine, thiolacetic acid or reduced GSH, as a co-factor. It was inhibited by peroxide or oxidized GSH or dehydroascorbic acid.

It appears, therefore, that Streptococcus viridans causes the green discolouration, characteristic of α haemolysis, by means of its potent capacity to produce peroxide. Since Lemberg claimed that choleglobin was produced in this system[29], we thought it might be possible to control the degradative action on haemoglobin of the micro-organism, by adjusting the experimental conditions, so that choleglobin-like intermediates would be produced in sufficient quantities to enable us to isolate and characterize them.

2.212 Materials and Methods.

Culture methods.

A strain of Streptococcus viridans, showing α-haemolysis on blood agar plates, was subcultured in tryptose-phosphate broth containing 20 g. bacto-tryptose (Difco), 2 g. bacto-dextrose (Difco), 5 g. NaCl and 2.5 g. Na_2PO_4 in 1 l. of water and having a pH of 7.3.

Before experimentation, 1 ml. of the subculture (checked by Gram stain and subculture on blood agar plates) was added to 50 ml. broth and incubated for 16 hrs. The broth culture was then transferred to a centrifuge tube and centrifuged at 2,000 rpm for 10 mins. The white precipitate was washed three times with phosphate buffer, pH 7.8, and the organisms were then suspended in approximately 15 ml. of the same buffer.

Laked blood.

15 ml. human blood was collected in a sterile bottle containing 4 ml. 5% sodium citrate in 0.9% w/v NaCl solution. 75 ml. sterile distilled water was added and the mixture shaken to haemolyse the red cells.

2.213 Experimental.

2 ml. 10% w/v K_2HPO_4 solution (boiled and cooled) was added to 2 ml. of the buffer mixture containing the micro-organism. A control in which the culture had been boiled was also prepared.

0.5 ml. laked blood was added to each sample, which was then well shaken and incubated at 37°C.

When, after 25 hrs. incubation, no change had been observed in either preparation, the mixtures were frozen and thawed in order to lyse the organism and 0.5 ml. glucose solution (20% w/v in water) was added to each. The samples were then reincubated. After a further 16 hrs. incubation with added glucose, the colour of the test mixture had become brown and there was a greenish sediment at the bottom of the tube. The colour of the control remained unchanged, i.e. red.

The preparations were centrifuged and the optical densities at different wavelengths determined in a Beckman DU Spectrophotometer.

2.214 Results.

The spectroscopic changes which had taken place in the lysed bacterial mixture as compared with the boiled control were as follows:

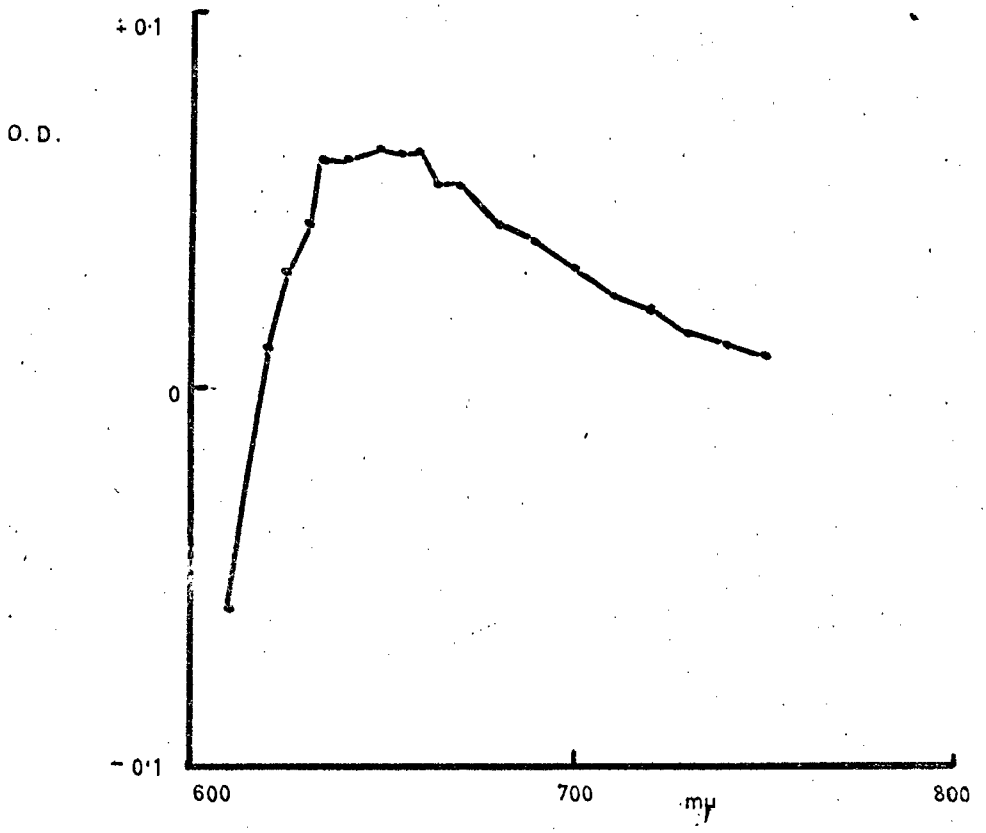
(a) There was a marked decrease in Soret absorption accompanied by a shift in the wavelength of maximal absorption from 415 mμ to 405 mμ.

(b) The bands at 575 and 540 mμ had almost completely disappeared.

(c) There were slight increases throughout the red region of the spectrum, these being maximal at 650 - 655 mμ and at 635 mμ (Fig. 13).

FIG. 13

DIFFERENCE IN OPTICAL DENSITY BETWEEN BACTERIAL PREPARATION AND A BOILED CONTROL



(d) The absorption at 380 mμ was higher in the test than the control. The optical densities in the ultraviolet region were not determined.

Apparently, only small quantities of bile pigments were formed in the presence of large amounts of haemoglobin; glucose was required for the reaction and the rate of formation was slow. The main effect of the organisms seemed to be to convert oxyhaemoglobin to methaemoglobin. Presumably, since there was serum present in the laked blood preparation, some methaemalbumin was also formed.

We decided to investigate other biological systems in the hope of finding tissues which would be more active in the production of bile pigments from haemoglobin.

2.2 IN VITRO EXPERIMENTS.

2.22 LIVER TISSUE.

2.221 Introduction.

Kench and Varma in 1962 studied the effect of different tissue preparations on the degradation of haemoglobin to biliverdin[39].

They showed that erythrocyte cell stroma had no effect on haemoglobin peroxidation by the ascorbic acid-oxygen system. Leucocytes had a marked inhibitory action while adult human liver and splenic tissue appeared to have little or no effect on bile pigment production.

Splenic pulp from a patient suffering from haemolytic anaemia, and neonatal tissues containing reticulo-endothelial elements, i.e. bone-marrow, liver and spleen, markedly enhanced the production of bile pigment precursors. Part of the increased yield obtained by adding these tissues was due to the action of a heat-labile component. In the foetal liver, diffusible and non-diffusible fractions contributed equally to the production of bile pigment.

Mills has demonstrated the conversion of haemoglobin to "choleglobin" by rat liver homogenates[94]. He and Randall showed that the enzyme chiefly responsible for protecting the haemoglobin of circulating erythrocytes from peroxidative breakdown, was glutathione peroxidase[38]. This enzyme is also active in liver and inhibition of the activity of this enzyme and that of catalase enhanced "choleglobin" production. After the liver homogenate had been dialysed, haemoglobin was not degraded. These findings led Mills to postulate that haemoglobin was degraded in rat liver by hydrogen peroxide generated during the catabolism of dialyzable purine nucleotides[94].

Nakajima[95] has partially isolated an enzyme system present in the non-particulate fraction of cells from the liver and kidney of the guinea-pig, which he and his colleagues (Nakajima, Takemura, Nakajima and Yamaoka[40]) claim to be responsible for the conversion of pyridine haemochromogen and haemoglobin-haptoglobin complex to a bile pigment precursor with absorption at 656 mμ. They have

called this enzyme haem α-methenyl oxygenase, and have demonstrated a requirement for ferrous ions and reduced nicotinamide adenine dinucleotide phosphate as co-factors. Meanwhile, in more recent work, Yamaoka is reported to have isolated an α-methenyl oxygenase from pig livers and to have found a second enzyme responsible for catalysing the next step in the formation of bile pigments, i.e. haem α-methenyl formylase which has a co-factor requirement for tetrahydropteroyl glutamate(96).

Wise has confirmed the finding that guinea-pig liver homogenates convert pyridine haemochromogen to a pigment which absorbs at 656 mμ but failed to produce this substance from pyridine haemochromogen using rat liver homogenates(8). On the other hand, his experiments on the isolated perfused rat liver showed that it was active in producing bilirubin from haemin and from denatured haemoglobin and methaemalbumin - all poor substrates in Nakajima's guinea-pig liver system(40).

Furthermore, Wise and Drabkin have succeeded in isolating an enzyme from haemophagous tissue of the dog placenta, which catalyses the degradation of haemoglobin-C^{14} and haem-C^{14} to biliverdin-C^{14}, only the IX α isomer of which, was formed(97). This has been shown by Petryka, Nicholson and Gray(37) to be the only bile pigment isomer found in biological systems. The isolated enzyme was associated with a fast-sedimenting nuclear fraction and required ATP, NAD, NADP, nicotinamide and boiled cell sap for maximum activity.

It thus exhibits major differences from the α-methenyl oxygenase of guinea pig livers.

Our experiments on cell-free liver systems using different substrates were completed in October, 1963 (Kench, du Toit and Green[98]) prior to the discovery by Wise and Drabkin of the enzyme system present in the placenta of the dog.[97].

2.222 Materials and Methods.

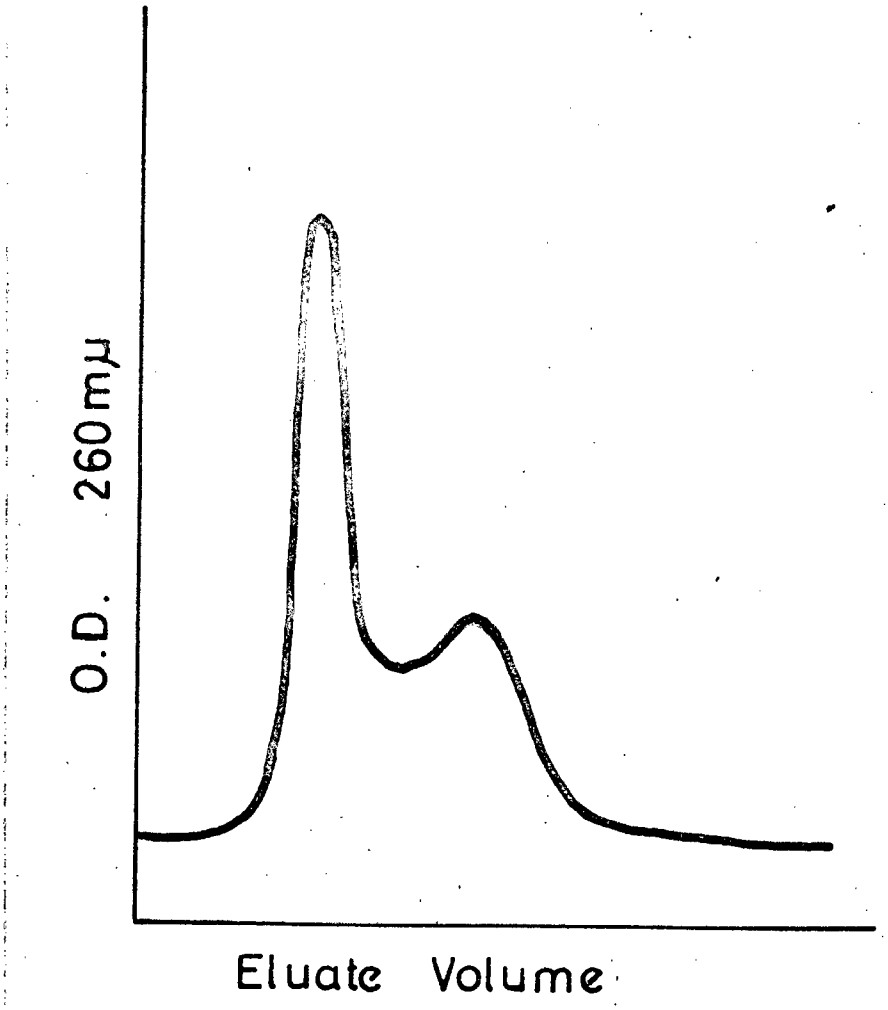
(i) Haemoglobins - foetal and adult human haemoglobins and bovine haemoglobin were prepared according to the method of Drabkin[82].

(ii) Haemoglobin-haptoglobin complexes - a crude haptoglobin fraction was prepared from pooled human serum or bovine serum. The serum protein fraction which was soluble in 33% saturated ammonium sulphate solution but insoluble at 50% saturation was dialysed against distilled water. An excess of haemoglobin was added to the crude haptoglobin preparation and the haemoglobin-haptoglobin complex was separated from the excess free haemoglobin by passing the mixture through a column of dextran gel (Sephadex G-100) (Green and Kench[99]). The first peak which contains the complex (Fig. 14) was collected and appropriately diluted or concentrated for use in the incubation experiments.

(iii) Liver preparations - Foetal and adult human and guinea-pig livers were obtained just after death and prepared by homogenizing

FIG. 14.

SEPARATION OF FREE HAEMOGLOBIN FROM HAEMOGLOBIN-HAPTOGLOBIN COMPLEX ON DEXTRAN GEL.



1:1 w/v in 0.1 M phosphate buffer pH 7.3.

(iv) Incubation procedure.

The mixtures and controls, containing substrates and liver homogenates with or without added co-factors, were incubated in air at 37°C in an automatic shaking apparatus. At regular intervals during the experiment, the volumes of the mixtures were measured, the decrease due to evaporation was made up by adding distilled water, the samples were centrifuged and the optically clear supernatants were diluted and their optical densities at various wavelengths determined in a Beckman DU Spectrophotometer.

2.223 Results.

(a) Guinea-pig liver.

We were able to detect the presence of a heat-labile component of liver which gave rise to compounds with increased spectral absorption at 630 mμ (Fig. 15). In general, haemoglobin in the form of a haemoglobin-haptoglobin complex was a better source of green pigment than haemoglobin alone. Although we were able to show small increments in absorption in the region of 630 - 660 mμ (Fig. 16), the yields of biliverdin never exceeded those obtained in the in vitro chemical experiments using ascorbic acid (Kench and Varma[39]). They never approached those of Nakajima and his colleagues when using pyridine haemochromogen[40].

The addition of a number of different co-factors made no

FIG. 15.

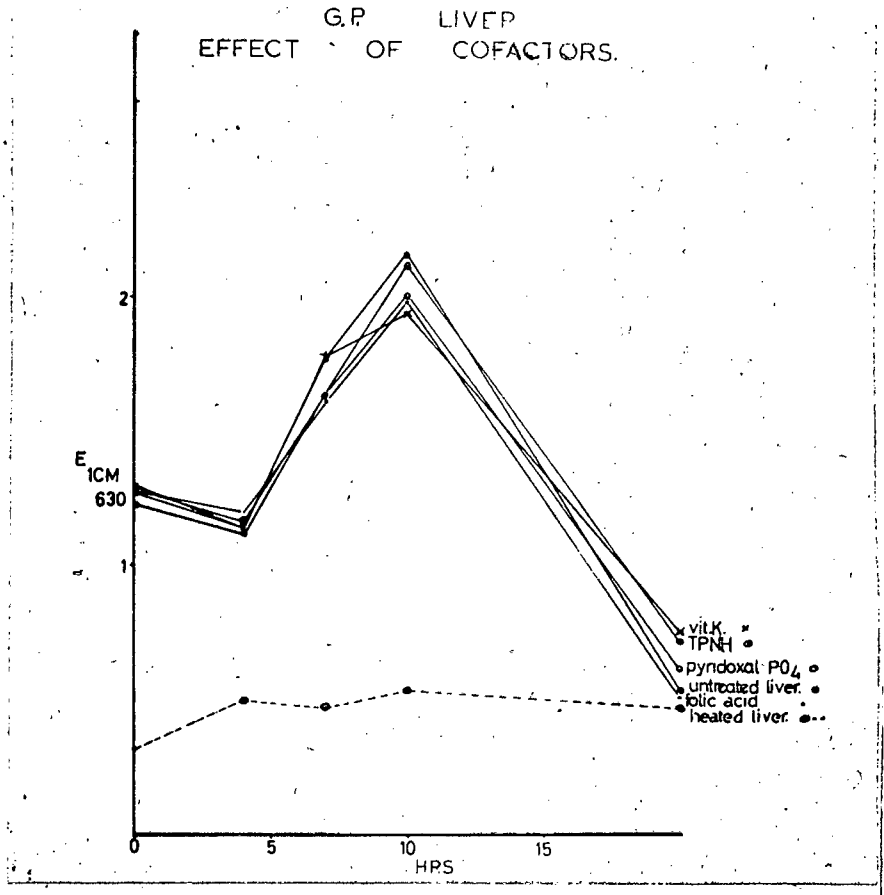
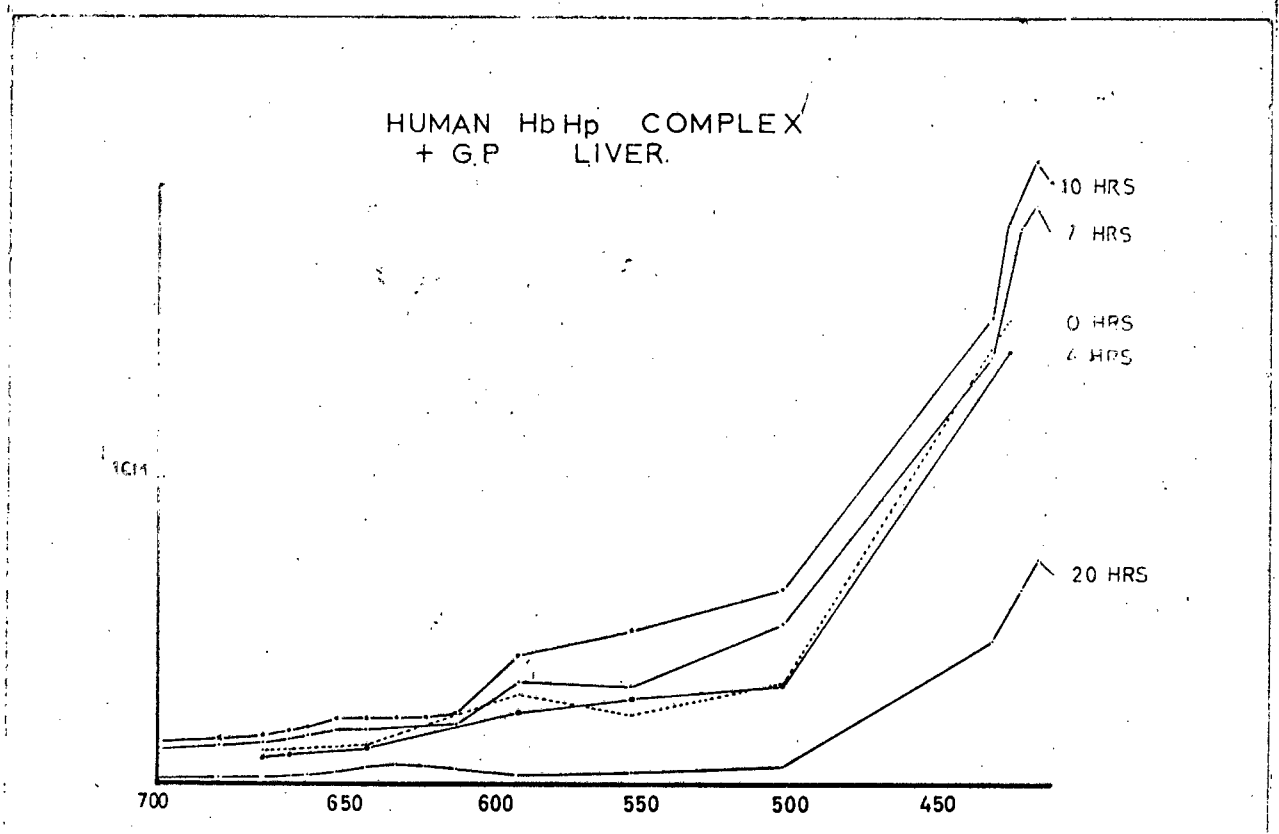

G.P LIVER
EFFECT OF COFACTORS.
E 1CM 630
2
1
0
5
10
15
HRS
vit K.
TPNH
pyridoxal PO4
untreated liver.
folic acid
heated liver.

FIG. 16.


HUMAN Hb Hp COMPLEX
+ G.P LIVER.
10 HRS
7 HRS
0 HRS
4 HRS
20 HRS
700
650
600
550
500
450

appreciable difference to the production of bile pigment precursors by guinea pig liver homogenate using human haemoglobin-haptoglobin complex as the substrate.

(b) Human foetal liver.

It had previously been shown by Kench and Varma[39] that foetal tissues had a stimulatory effect on bile pigment production from haemoglobin in ascorbic acid-oxygen mixtures.

Using different substrates, i.e. foetal or human haemoglobin complexed with haptoglobins of normal or bilious adult serum, had no effect on the bile pigment production by foetal liver homogenates which, without added co-factors, was rather low (Fig. 17).

Formation of products which exhibit spectral absorption at 630 mμ was greatly enhanced, however, by the addition of ascorbic acid and $NADPH_2$ to the incubation mixtures.

In one experiment, human foetal haemoglobin, complexed with human haptoglobin obtained from pooled sera (6 ml. containing 0.18 μmoles of haemoglobin) was incubated with foetal liver homogenate (1.8 ml.). Ascorbic acid and $NADPH_2$ were added as co-factors. After incubation, the mixture was centrifuged and the reaction products in the supernatant were passed through a column of dextran gel (Sephadex G-100). The effluent scan at 260 mμ is shown in Fig. 18. The emergent fractions were examined for optical density at selected wavelengths. The presence of haem-derivatives

FIG. 17. FOETAL LIVER HOMOGENATE. DIFFERENT SUBSTRATES.

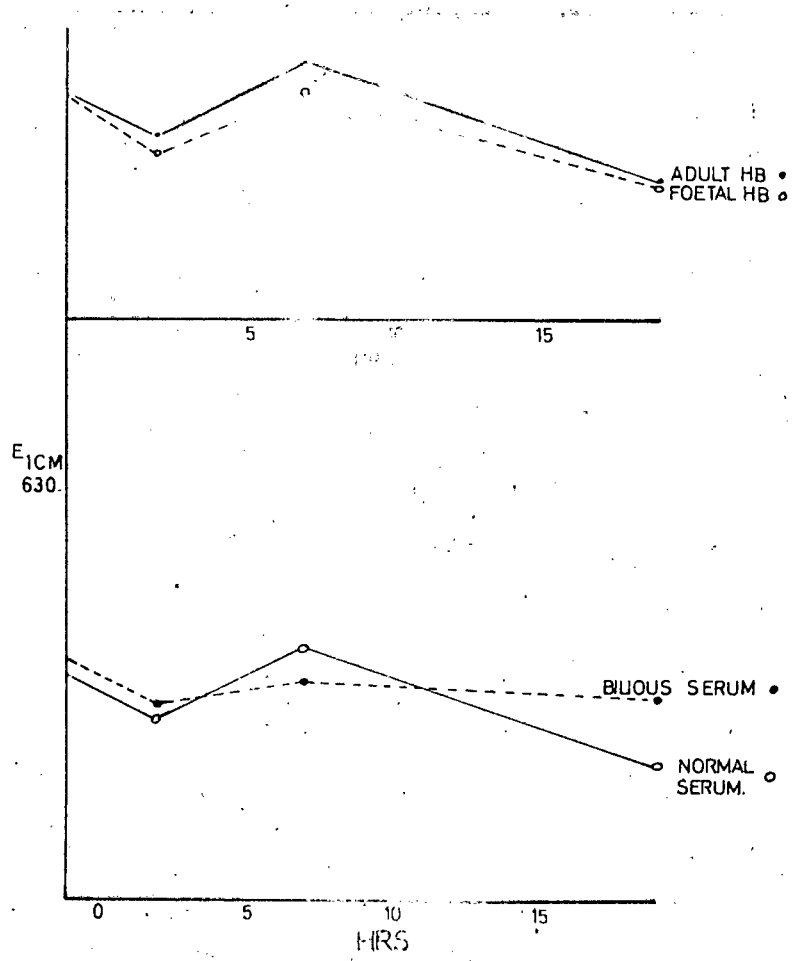
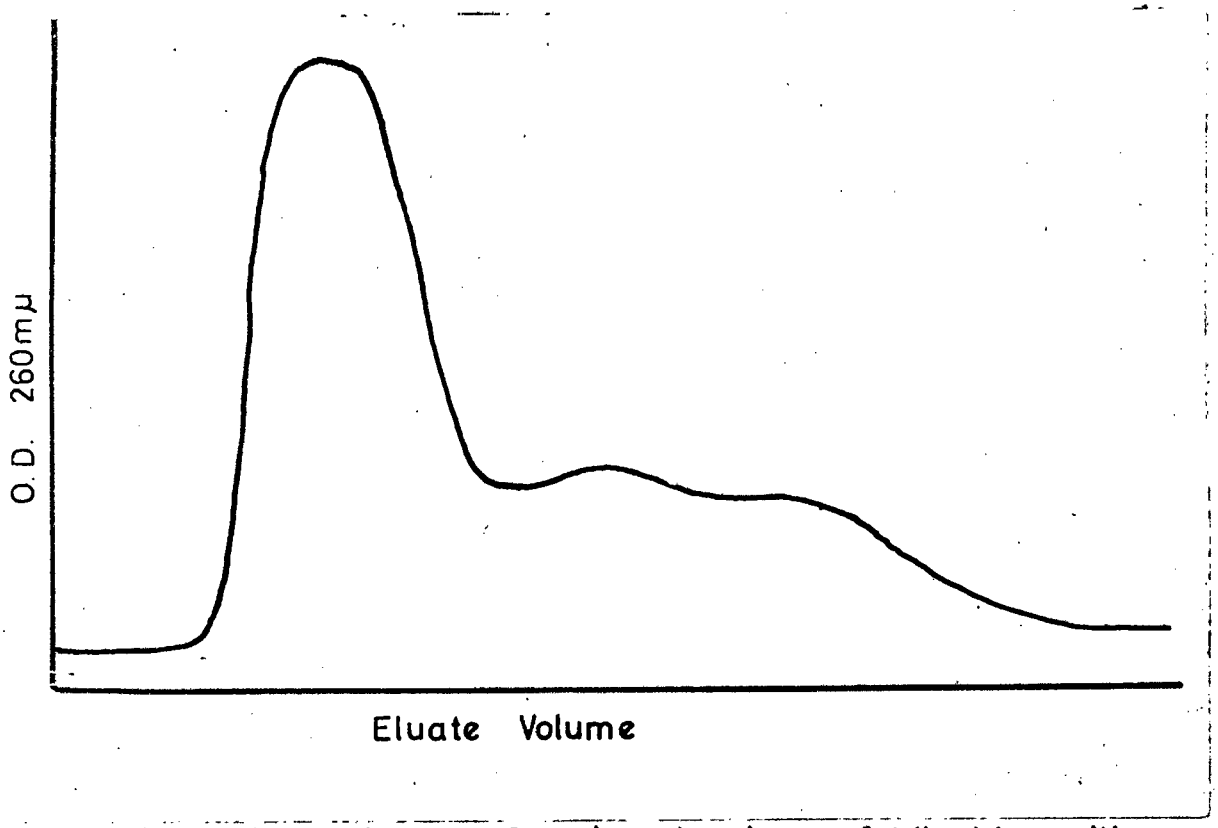
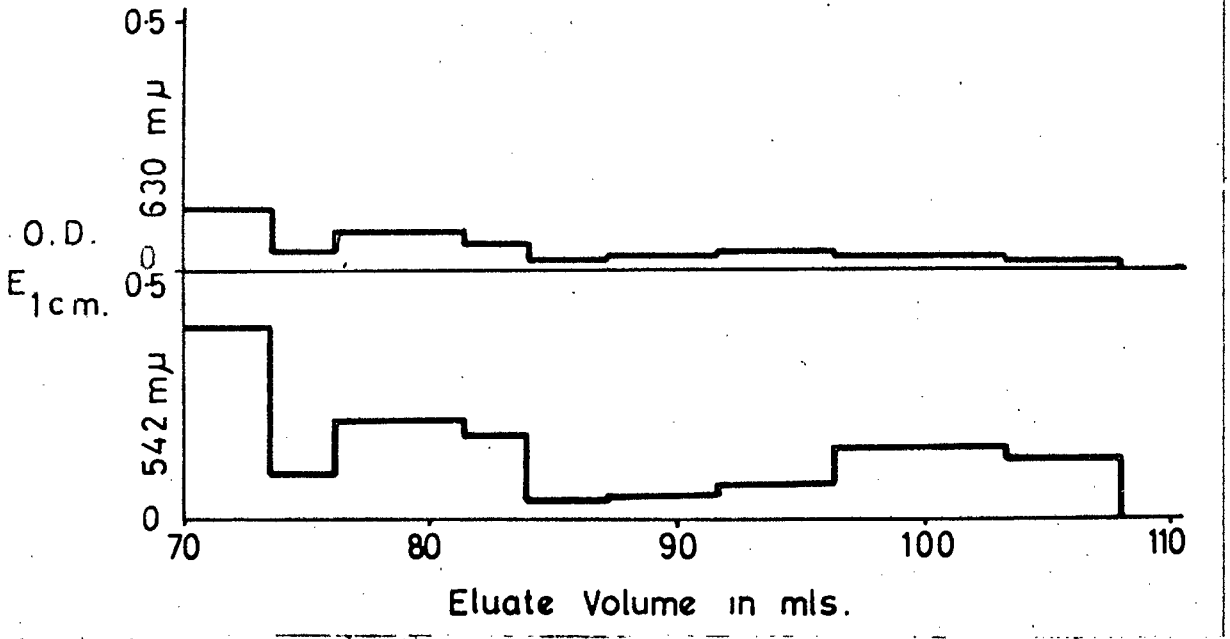


FIG. 18.



Fractionation of products after incubation of Hb-Hp with foetal liver, ascorbic acid and $NADPH_2$ on dextran gel.

FIG. 19.



Optical densities of eluted fractions containing products of Hb breakdown.

(absorption at 543 mμ) of relatively small molecular size which was eluted in the fractions from 96 - 108 ml. (Fig. 19) is of particular interest, as this suggests enzymic or oxidative degradation of the globin molecule while haem is still intact.

Although both foetal and guinea-pig liver preparations were found to be active in biliverdin production, they did not improve on the yields obtained in the chemical system in which haem pigments are degraded by coupled oxidation with ascorbic acid and oxygen.

2.2 IN VITRO EXPERIMENTS.

2.23 HAEM TRANSFER SYSTEMS.

2.231 INTRODUCTION: METABOLIC SIGNIFICANCE OF METHAEMALBUMIN.

Since N. Hamilton Fairley's discovery, in 1938,[27, 100 - 103] that methaemalbumin is present in the sera of patients suffering from blackwater fever, methaemalbuminaemia has become associated with a few pathological states, occurring mainly as a result of excessive intravascular haemolysis (Neale, Aber and Northam[104]), or severe haemorrhage. The high incidence of raised serum levels of methaemalbumin in acute haemorrhagic pancreatitis is well-known (Northam, Winstone and Banwell[105]) and has been used diagnostically to distinguish this disease from oedematous pancreatitis (Northam, Rowe and Winstone[106]).

In the case of haemorrhagic pancreatitis, it has been postulated (Northam et al.[105]), that the apohaemoglobin is broken down by pancreatic enzymes, and, as they are unable to degrade the prosthetic

group, i.e. haematin, it diffuses into the plasma where it is bound to albumin.

The binding of haematin by purified albumin preparations was demonstrated in vitro by Fairley[27] and the chemistry of the haem-protein interaction which occurs, has been studied fairly extensively by Keilin in England[107], Rosenfeld and Surgenor in the United States[108], and O'Hagan in Australia[109, 110].

When intravascular haemolysis is the cause of methaemalbumin formation in the plasma, the process is more complicated because the haem is initially bound to haemoglobin, which, on rupture of the erythrocyte, is set free. Free plasma haemoglobin is rapidly bound to haptoglobin (Laurell and Nyman[70]) and removed from the circulation (Nyman[111]). In the case of severe intravascular haemolysis, the binding capacity of the haptoglobins is exceeded and the remaining free haemoglobin passes through the renal glomerulus; some is reabsorbed by the tubules (Laurell and Nyman[70]) and the rest is excreted. Simultaneously, haem is removed, or dissociated from some of this circulating haemoglobin and bound to albumin. Whether the removal of haem from its globin apoprotein in the plasma is a spontaneous or enzymic process is not known.

In vitro electrophoretic experiments on human serum or plasma to which haemoglobin has been added, reveal that methaemalbumin is formed in the presence of free haemoglobin added in excess of the binding capacity of the haptoglobins (Nyman[111]; Liang[73]; Aber and Rowe[112]). Korinek discovered that methaemalbumin formation,

under these conditions, increases with time and with incubation at higher temperatures[74]. He and others (Valeri, Bond, Fowler and Sobucki[113]) claim to have shown that the haem bound to methaemalbumin in these in vitro experiments, is derived from excess "free" haemoglobin and that the formation of a haemoglobin-haptoglobin complex protects the haemoglobin from this type of breakdown. Recently, Rossi-Fanelli and Antonini have shown that haematin can dissociate from ferrihaemoproteins at neutral pH in the presence of another apohaemoprotein with a higher affinity for the haematin[114].

It is clear from the above evidence that under certain abnormal conditions, methaemalbumin could be an intermediate in haemoglobin degradation. The influence of temperature on its formation in serum, suggests that a haem transfer enzyme may be involved in the reaction.

At present, the evidence for a physiological role of methaemalbumin is extremely tenuous. However, it is more than likely that small quantities of methaemalbumin are formed in the course of normal daily red cell turnover. Hanks and Chaplin, using a modified benzidine method, have found that the normal value for plasma haemoglobin concentration is 0.42 mg.%, ranging from 0.25 - 0.58 mg.%[115]. This means that roughly 0.8 g. of haemoglobin flows in and out of the plasma compartment each day and this value corresponds to slightly more than 10% of the overall red cell haemoglobin breakdown, i.e. 10% of the daily red cell destruction

occurs intravascularly (Garby and Noyes[68]). Of course, most of the haemoglobin liberated would be bound to haptoglobin. However, Shinowara and Walters, while developing a spectroscopic method for the determination of methaemalbumin and haemopexin (haematin binding β_1-globulin), found that normal serum could contain up to 5 mg. haematin/100 ml. serum[116].

The fate of methaemalbumin in vivo is unknown. In pathological conditions, it persists for longer periods in the plasma than either the haemoglobin-haptoglobin complex or haemopexin (Northam et al.[106]). In vitro, methaemalbumin was a better source of bile pigment than either ferrihaem or methaemoglobin in the ascorbic acid-O_2 system (Kench[14]) and altered methaemalbumin was as good a source as haematin and alkali-denatured haemoglobin in Wise's liver perfusion experiments[8].

Even if the role of albumin as a physiological intermediary of haemoglobin degradation appears relatively insignificant in the circulation, its possible role intracellularly at the main sites of haemoglobin degradation has never been investigated. Small quantities of albumin (approximately 0.4 mg./g. wet weight) are known to persist in liver parenchymal cells, in association with particulate fractions, for some time after synthesis (Gordon and Humphrey[117]). Albumin destined for breakdown is probably ingested by both Kupffer and parenchymal cells of the liver (Freeman, Gordon and Humphrey[118]). After ingestion, its presence in these

cells as an intact molecule, is, however, short-lived (Humphrey and McFarlane[119]) and it is rapidly degraded. Accumulated evidence from many workers, starting with Rich in 1925[7], suggests that under normal conditions, the liver is the chief site of haemoglobin breakdown. In addition, although it was thought for many years that the reticulo-endothelial system was responsible for red cell destruction and bile pigment formation, recent studies (Wise[8]; Kosaka[120]) suggest an important role for parenchymal cells in haemoglobin degradation.

Our interest in studying haemoglobin breakdown was chiefly concerned with finding intermediates, the production of which would affect the rate of haemoglobin turnover directly, or indirectly by controlling certain aspects of cellular respiration. Furthermore, the "choleglobin" theory of haemoglobin catabolism differs mainly from the "haem-splitting" theory in that the haem remains bound to the apoprotein while undergoing the initial degradative steps. If choleglobin is an intermediate, it might be expected to play a key-role in general metabolism and persistence of the globin part of the molecule would be essential to this role.

Thus, methaemalbumin is a convenient substrate for studying the effect of an apoprotein on the permutations and combinations of the haem prosthetic group and for assessing, by comparison, the importance of globin in these reactions.

Our aim in the following experiments was to study the factors affecting the mechanism and rate of methaemalbumin formation in model chemical systems resembling as nearly as possible the physiological situation.

2.232 EXPERIMENTS ON HUMAN AND ANIMAL SERA.

Some preliminary experiments were performed on human and animal (chiefly goose) sera. Our aims in performing these investigations were as follows:

(a) We wanted to ascertain whether methaemalbumin formation is a common phenomenon among vertebrates. Liang has only encountered methaemalbumin, after incubation with excess haemoglobin, in sera from man, the frog and the turtle[73].

(b) We wished to study the effect of temperature and length of incubation time on in vitro methaemalbumin formation somewhat along the lines of work recently reported by Valeri et al.[113] and by Korinek[74].

(c) All the electrophoretic studies on haemoglobin binding by the proteins of human plasma in vitro, indicate that methaemalbumin is formed only if excess "free" haemoglobin is present. (Nyman[111]; Liang[73]; Aber and Rowe[112]; Allison and Rees[121]; Korinek[74]; Valeri et al.[113]). Since the mechanism by which haem is transferred from globin to albumin in serum is unknown,

we hoped, by further study, to discover an enzymic mechanism for haem transfer. It is not inconceivable that such an enzyme would catalyse haem transfers between all three haem-binding plasma proteins, i.e. haptoglobin, to which haemoglobin is bound by means of its protein moiety (Nyman[111]); haemopexin (the β_1 haem-binding protein) which is thought to be able to combine with both haemoglobin and haematin, (Neale et al.[104]) and albumin, which reacts with the propionic acid side chains of the haematin molecule (Keilin[107]; O'Hagan[109]), and, as far as we are aware, such transfers have not yet been described.

A. Materials and Methods.

(i) Sera - Human serum samples were obtained from the routine investigation laboratory of the Department of Chemical Pathology. They were selected from among sera submitted for protein electrophoresis, the only criterion of choice being a defined band in the α_2 globulin region, i.e. the fraction in which haptoglobin is present.

Goose serum. Blood was withdrawn from the wing vein of a goose, using a syringe. The blood was allowed to clot in a test-tube, which was then centrifuged, and the serum pipetted off.

(ii) Plasma was obtained from various vertebrate species, i.e. goose, monkey, rabbit, guinea-pig and rat, by collecting venous blood in 3.2% w/v sodium citrate and, thereafter, centrifuging at 3,500 rpm for 30 minutes to separate the plasma from the red blood cells.

(iii) Haemoglobin - Human haemoglobin was prepared according to Drabkin[82] from citrated whole blood obtained from a donor. After haemolysing the erythrocytes and removing the stroma by centrifugation at 30,000 rpm for 1 hour, the clear haemoglobin solution obtained was used as such for the experiments, without crystallizing the protein.

Goose haemoglobin was similarly prepared except that the stroma-free haemoglobin was crystallised by dialysing at 0°C against 3 M phosphate buffer pH 6.8, followed by dialysis against distilled water.

The concentration of haemoglobin was measured by the cyanmet-haemoglobin method using Acuglobin (Ortho Pharmaceutical Corp., New Jersey) as the standard.

(iv) Gel filtration.

A technique was devised for estimating the haemoglobin-binding capacity of serum haptoglobins by molecular sieve chromatography on cross-linked dextran gel (Green and Kench[99]; Kench, Green and Hines[122]). It was possible, by this method, not only to obtain a value for the serum haptoglobin level, but also to isolate the fraction containing the haemoglobin-haptoglobin complex from the other main haem-containing proteins, i.e. excess "free" haemoglobin and methaemalbumin.

The technique used was as follows:

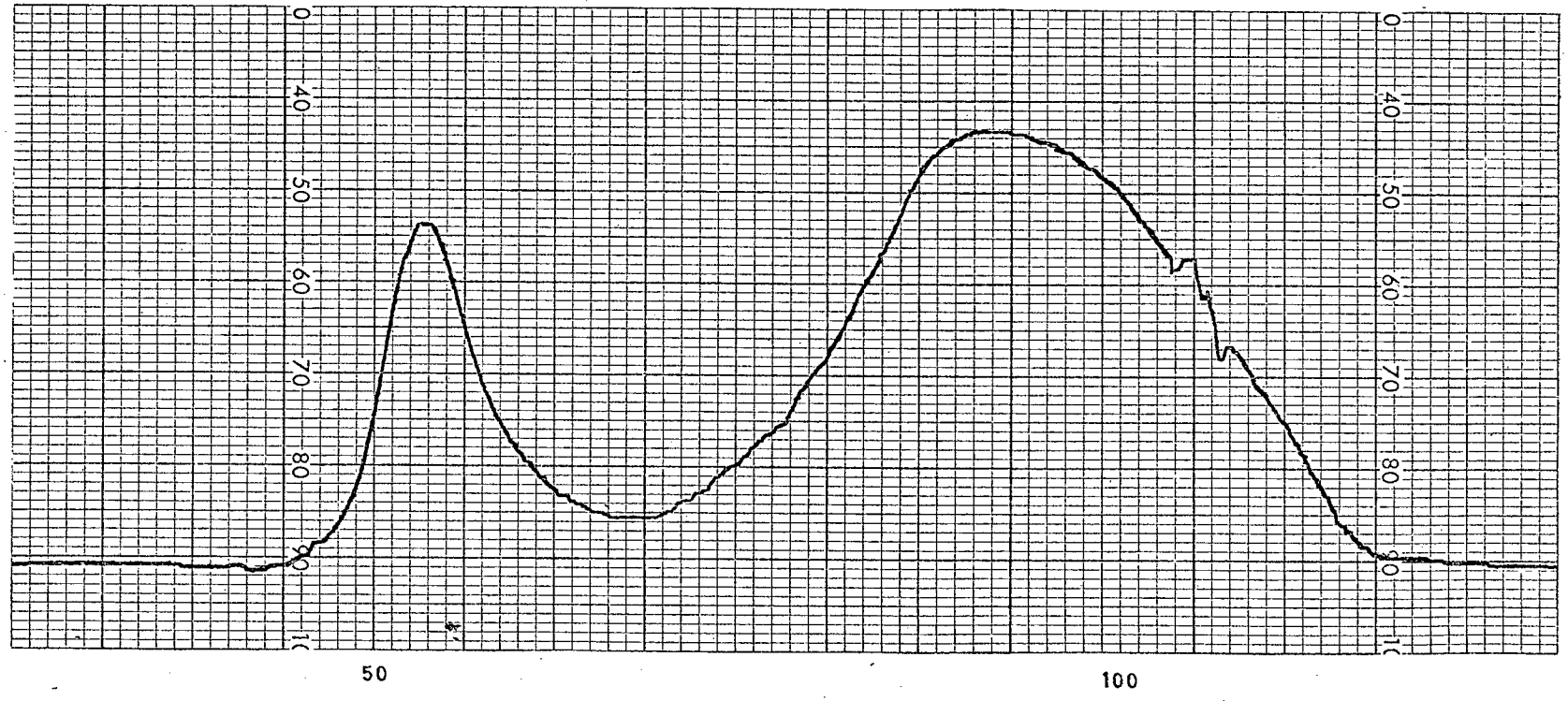
A known concentration of haemoglobin (usually in the region of 400 mg./100 ml.) was added to 1 ml. serum and, after 30 minutes at room temperature, the mixture was quantitatively applied onto the top of a column of Sephadex G-100 (53 cm. x 2 cm.). The column was developed with M/15 phosphate buffer pH 7.4 in 0.5 M NaCl, at a flow rate of 16 - 18 ml. per hour. The eluate was monitored at 400 - 410 mμ. With normal sera, 2 peaks, absorbing in the Soret region, were obtained. These were collected, centrifuged, and their optical densities at 415 mμ and 540 mμ determined using a Beckman DU Spectrophotometer.

The 2 protein components of the haemoglobin-haptoglobin complex combine stoichiometrically in a 1 to 1 ratio. On Sephadex G-100, which effectively separates compounds of molecular weight between 60,000 and 150,000 (Andrews[88]), the complex, which has a molecular weight of 155,000 (Guinand, Tonnelat, Boussier and Jayle[123]) was eluted in the first peak showing absorption at 410 mμ. The second fraction contained albumin (M.W. 68,000) and haemoglobin (M.W. 67,000). (Fig. 20).

Since the extinction coefficient of the haemoglobin-haptoglobin complex in the Soret region ($E^{mM}_{1\ cm}$ at 408 mμ = 450 (Guinand et al.[123])) is less than that of free haemoglobin ($E^{mM}_{1\ cm}$ at 412 mμ = 540), the quantity of haemoglobin remaining free in the second fraction was determined spectrophotometrically and the haemoglobin binding capacity, i.e. the haptoglobin concentration, was calculated by

FIG. 20.

SEPARATION OF HAEMOGLOBIN - HAPTOGLOBIN COMPLEX FROM EXCESS HAEMOGLOBIN ON A COLUMN OF SEPHADEX G — 100.



subtracting the free haemoglobin recovered, from the amount originally added. Corrections were made to account for the recovery which was always less than the theoretical, namely 94% with high concentrations of haemoglobin and 87% with low haemoglobin loads, i.e. 50 - 100 mg.%.

The haptoglobin concentration was difficult to estimate in jaundiced sera, as high concentrations of bilirubin bound to the albumin in the second fraction, raised the optical density of the haemoglobin at 415 mμ. With these samples, the optical density of the complex, which is not contaminated with bilirubin, was measured directly, and the haemoglobin concentration thus obtained was corrected, using a conversion factor derived from normal sera.

Methaemalbumin formation, which is minimal under these experimental conditions, did not interfere appreciably with the method.

(v) Cellulose acetate electrophoresis.

The technique employed was based on the method described by Valeri et al.[113] to estimate serum haemoglobin-binding capacity.

Cellulose acetate membranes (Oxoid electrophoresis strips, manufactured by Courtaulds, Ltd., Coventry) 18 cm x 5 cm, were floated on phosphate buffer (pH 7.0; ionic strength 0.05), and, when uniformly wetted on the undersurface, were immersed, and left to soak for at least 3 hours. Prior to electrophoresis, the membranes were removed from the buffer, blotted, and the sample applied using a Beckman sample applicator. The strips

were supported on a frame in a horizontal electrophoretic cell, each end dipping into one of the buffer compartments running along the length of the cell on either side, or, if the strips were too short, blotting paper wicks were used. The strips were left in the cell to equilibrate for thirty minutes (in later experiments, equilibration time was reduced to 10 minutes), after which approximately 200 volts were applied for $2\frac{1}{2}$ hours. The voltage applied was varied according to the size of the cell used and the instrument supplying the current.

After separation, the membranes were placed between blotters and dried, either in a 55°C oven or at room temperature, pressed flat under a heavy book. Afterwards, they were stained.

When the membranes were stained for detection of proteins, either with Ponceau S or lissamine green, they were not dried after electrophoresis but placed directly in the dye after removal from the cell. As both these dyes contain a protein precipitant, drying was found to be unnecessary.

Staining.

(a) O-dianisidine (British Drug Houses, Ltd.) was made up freshly just prior to use, according to the method of Valeri et al.[113], as follows:

70 ml. alcoholic o-dianisidine solution,

10 ml. 1.5 M acetic-acetate buffer pH 4.7,

18 ml. distilled water and

2 ml. 3% w/v hydrogen peroxide, freshly prepared.

The dried membranes were floated on the surface of the dye until wet and then immersed until colour development was complete (approximately 10 minutes). Fractions having peroxidase activity, i.e. haemoglobin, haemoglobin-haptoglobin complex and methaemalbumin, were stained orange-brown in colour. The strips were washed in distilled water and dried between pieces of blotting paper.

(b) Ponceau S (Gurr) - 0.2% solution in 3% trichloracetic acid. The wet strips were floated on the surface until the stain had penetrated all the protein bands. They were then immersed for 15 to 20 minutes and the excess stain was washed out with two or three changes of 5% acetic acid.

Protein fractions were stained bright pink against a pale pink background.

(c) Lissamine green - 9.75 g. lissamine green (Gurr) and 15.0 g. salicylsulphonic acid were dissolved together in water which was added up to 1 l. The membranes were stained in the same manner as with Ponceau S and the excess stain was removed by washing with 1% acetic acid. Protein bands were green and the strips showed very little background staining.

Elution.

To quantitate the peroxidase activity associated with each o-dianisidine band and, thus, the distribution of haem between the serum proteins, some of the cellulose acetate strips obtained by electrophoresing goose serum and homologous haemoglobin, were eluted. In our experience, o-dianisidine staining using the phosphate buffer (pH 7.0; ionic strength 0.05) recommended by Valeri et al.[113], does not produce sharply delineated bands of peroxidase activity. There is also a good deal of background staining. However, t he bands were cut out and the width of each measured. Pieces of corresponding width were cut from the unstained portions of the same membrane to serve as blanks.

If large, the pieces to be eluted were folded, and all were placed into test-tubes, to each of which was added 5 ml. of the eluting solvent, consisting of 90% chloroform and 10% ethanol. After adding the solvent, the tubes were shaken vigorously at frequent intervals over a period of roughly 1 hour, at which time the cellulose acetate was usually completely dissolved. The optical density of the eluates at 450 mμ was determined in a Beckman DB Spectrophotometer. In each case the blank reading was subtracted from that of the eluate containing peroxidase. It was assumed that the total absorption of the eluted bands accounted for all the peroxidase activity and the relative percentages of each fraction were calculated. Since the concentration of haemoglobin (mg./100 ml.) was known, it was possible to calculate the actual amounts of haemoglobin or peroxidase-active

groups that were associated with each fraction.

B. Haemoglobin binding by human sera.

The experiments performed on human sera were mainly qualitative. The usual procedure was as follows:

Immediately after the haemoglobin was added to the serum to give concentrations of between 90 and 300 mg. haemoglobin per 100 ml. serum, an aliquot was removed and electrophoresed as described above. The mixture of serum and haemoglobin was then incubated at 37°C and aliquots were withdrawn for electrophoresis at various times up to 24 hours. Each sample was run in duplicate, one strip being stained for protein with Ponceau S and the other with o-dianisidine, which stains the haem-containing proteins.

For gel filtration on Sephadex G-100, 400 mg. haemoglobin was added per 100 ml. serum, 1 - 2 ml. of the mixture applied on to the column, and, after elution, the optical densities of the haem-containing effluent peaks were read in a Beckman DU Spectrophotometer.

The haptoglobin binding capacity in the serum of a normal adult is approximately 100 mg. haemoglobin per 100 ml., although the level ranges widely (Nyman[111]). The values obtained on Sephadex were of the same order. It was to be expected, therefore, that the concentrations of haemoglobin added would invariably exceed the binding capacity of the serum and that there would be excess "free" haemoglobin present in the mixture.

In general, our results agreed with those obtained by Korinek[74] and Valeri et al.[113]. Namely, that added haemoglobin was immediately bound to haptoglobin in the serum and that, although small amounts of methaemalbumin were detectable immediately after a large excess of haemoglobin was added, it was only found in appreciable quantities after considerably longer periods at room temperature. Incubation at 37°C enhanced this effect. A typical experiment is shown in Fig. 21.

Five minutes after haemoglobin (300 mg./100 ml.) was added to the serum sample at room temperature, aliquots of the mixture were applied to 2 strips and electrophoresed at pH 7.0 as described. Two protein bands exhibited peroxidase activity; the one which moves towards the cathode with the γ globulin fraction is free haemoglobin and the band which has migrated slightly towards the anodic end is the haemoglobin-haptoglobin complex. After incubation at 37°C for 1 hour, faint peroxidase activity is detectable in the albumin band. The electrophoretic pattern, after the sample had been incubating for 24 hours at 37°C, showed a marked increase in methaemalbumin, which had become the most peroxidase-active of the three, less intensity in the band of free haemoglobin, and some denatured protein at the point of application.

The patterns shown in Fig. 22 are of interest in view of the current theories propounded by Nyman[111], and Valeri et al.[113], concerning methaemalbumin formation in vitro. The serum used, exhibited a normal protein pattern on routine paper electrophoresis

FIG. 21. CELLULOSE ACETATE ELECTROPHORESIS OF HUMAN SERUM WITH ADDED HAEMOGLOBIN.

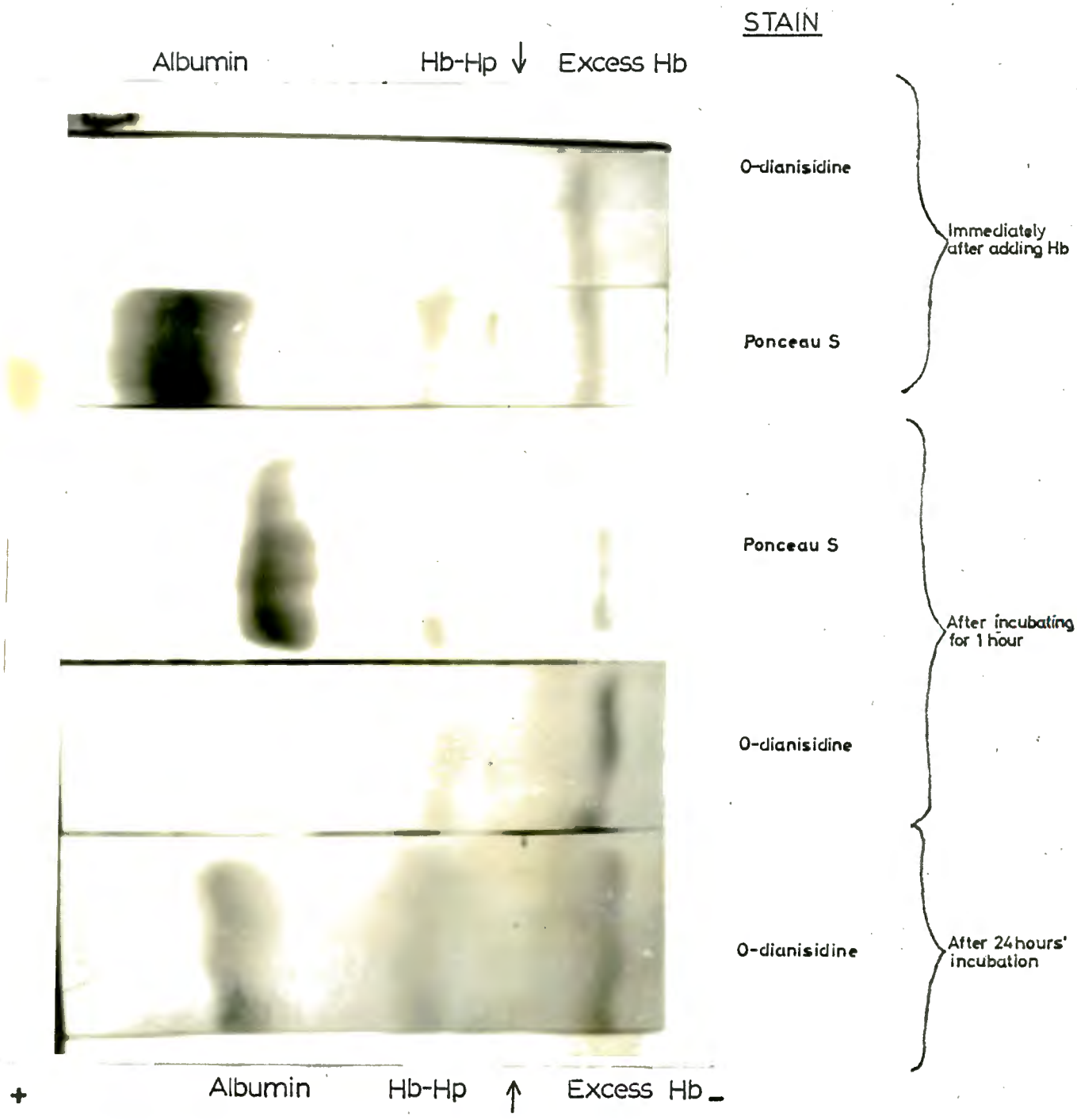


FIG. 22. FORMATION OF MHA IN THE ABSENCE OF EXCESS "FREE" HAEMOGLOBIN.

at pH 8.6, with well-defined α_2 and β globulin areas. The albumin concentration was 4.6 g./100 ml. and the globulins 1.6 g./100 ml. Haemoglobin, equivalent to 300 mg./100 ml. was added and the pattern obtained after 5 minutes showed that all of it was complexed to haptoglobin. After 1 hour at 37°C, the presence of methaemalbumin is just discernible, while there appears to be a second band adjacent to the complex. This could be the haem-binding β_1 globulin, which, at pH 7.0 migrates with the α_2 globulin fraction in which the haptoglobins also travel. After 30 hours of incubation, the methaemalbumin band was clearly visible. This shows that haematin, derived from sources other than excess free haemoglobin, can be bound to albumin. Naturally, because of the prolonged incubation time and the above-normal haemoglobin binding capacity of the serum, this result has to be viewed with reservations, but it certainly adds weight to our proposal that haem transfers occur between all the haem- or haemoglobin-carrying moieties in the plasma.

C. Haemoglobin binding by animal sera.

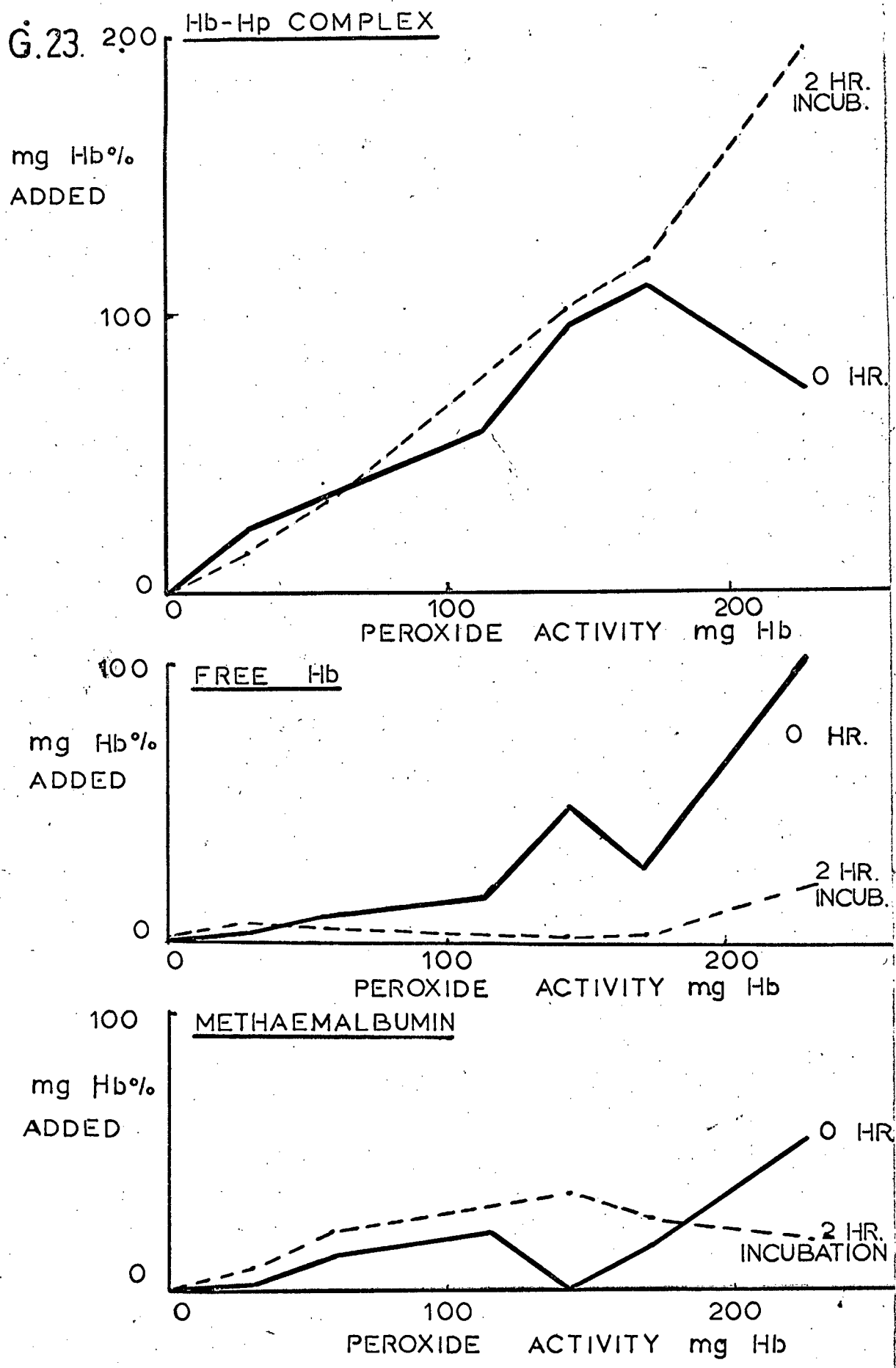
(a) Of the plasma samples obtained from the various animal species mentioned, only guinea-pig plasma formed methaemalbumin at low concentrations of added haemoglobin (approximately 20 mg./100 ml.). The guinea-pig and rabbit plasma tested both had low haptoglobin concentrations as evidenced by a second band of free haemoglobin. The others, goose, monkey and rat, only showed o-dianisidine staining in the α_2 region, i.e. haptoglobins.

Higher concentrations of haemoglobin were not added except to goose serum.

(b) Increasing concentrations of haemoglobin were added to goose serum and the mixtures were subjected to different conditions of time and temperature prior to electrophoresis. However, in spite of altering the buffer pH and increasing its ionic strength, it was difficult to obtain bands showing peroxidase activity which were suitable for eluting. They were almost always diffuse and in addition, as mentioned before, the contrast between the bands and the background was not sufficiently marked to permit accurate quantitation.

Results showed that there was almost always a small percentage of haem attached to albumin and this methaemalbumin fraction increased with increasing concentrations of added haemoglobin. So did the bound haemoglobin until the binding capacity of the haptoglobins was exceeded, after which the relative proportion of bound to other fractions dropped. The actual quantity of haemoglobin complex also decreased, probably due to methaemalbumin formation occurring before all the free haemoglobin could be complexed (Fig. 23). Incubation for two hours, however, seemed to increase the binding capacity of the haptoglobins at the expense of both the free haemoglobin and methaemalbumin fractions. The graphs in Fig. 23 were constructed from a series of incubation experiments in which the individual samples showed wide variations after the 2-hr. incubation

FIG. 23.


Hb-Hp COMPLEX
200
mg Hb%
ADDED
100
0
2 HR.
INCUB.
0 HR.
0
100
200
PEROXIDE ACTIVITY mg Hb
100
FREE Hb
mg Hb%
ADDED
0 HR.
2 HR.
INCUB.
0
0
100
200
PEROXIDE ACTIVITY mg Hb
100
METHAEMALBUMIN
mg Hb%
ADDED
0 HR
2 HR.
INCUBATION
0
0
100
200
PEROXIDE ACTIVITY mg Hb

period, if left at 37°C or stored at 0°C. When the same concentration of haemoglobin was added to goose serum a few days later, the observed changes, although similar in general, were not reproduced quantitatively. This may have been due to aging of the haemoglobin solution, although Nyman claims to have found little effect of age on haemoglobin over periods of up to a year[111]. Her experiments, however, were largely qualitative in character, and may not have reflected small changes. However, the chief source of error probably lies in the technique of quantitation itself. Valeri et al. also used o-dianisidine for their peroxidase stain but quantitation was achieved by using a Beckman Spinco Analytrol at 450 mμ and integrating the curves[113].

Pending the arrival of this instrument in our Department, investigations on sera were discontinued.

The impression we received from doing these experiments though, was that, in vitro, haematin was shuttled back and forth between the three main haem-containing proteins of the plasma and that these transfers were influenced by temperature and other unknown factors. This suggested the presence of a reversible "haem-transferase" enzyme system and much of the remainder of our work described in this thesis was directed towards a search for this system. We decided to isolate some of the known components of the haemoglobin-serum system to test whether any of these interact independently in a purely chemical manner.

2.233 CHEMICAL INTERACTIONS.

A. Methods of preparation.

(i) Albumin.

Albumin was prepared from human plasma by the trichloracetic acid-acetone method described by Vallance-Owen, Dennes and Campbell[124] based on that of Debro, Tanner and Korner[125].

Citrated whole blood, obtained from a donor, was centrifuged, after which the separated plasma was filtered, and an equal volume of freshly diluted 10% (w/v) trichloracetic acid (stored as a 50% solution in a dark bottle at 0°C) was added. After centrifugation, the supernatant fluid was discarded and the precipitate was washed with 5% TCA. It was then suspended in 96% aqueous ethanol (or fresh acetone) containing 1% TCA; the volume used being equivalent to 3 times the original plasma volume. The above procedures were carried out at room temperature. The TCA precipitate suspended in solvent was left overnight at 0°C to allow the albumin to redissolve. After centrifuging off the undissolved plasma protein, the solvent solution was dialysed for about 24 hours at 4°C against several changes of distilled water. The solution was pervaporated to about half the volume and redialysed (Schwert[126]). After further pervaporation, the albumin solution was dialysed against 0.01 M phosphate buffer pH 7.0, followed by exhaustive dialysis against distilled water and finally centrifuged to remove any precipitated or denatured protein.

With this method, applied to human serum, one frequently

encountered some lipid contamination evidenced by cloudiness of the albumin solutions and shown to be an α or β lipoprotein on cellulose acetate- and immuno- electrophoresis (Potgieter[127]). There appears to be a species variation, since this troublesome contaminant is not encountered in monkey serum (Kench and Sutherland[128]).

If the albumin preparation was contaminated it was either centrifuged at high speed (20,000 rpm for 1 hour in a Beckman Model L Ultracentrifuge) in an attempt to flotate the lipid or it was reprecipitated with 10% TCA and isolated as described above. Sometimes, if left to stand at $0^{\circ}C$ for a few days particularly if the pH of the solution was below 5, the contaminant precipitated out and could be removed by centrifugation. (This same technique was used by Foster, Sogami, Petersen and Leonard to defat albumin samples[129]).

Once the preparation appeared homogeneous on the basis of 3 criteria; immunoelectrophoresis vs antihuman serum (Hyland Laboratories) in 1.5% agarose (purchased from Seravac Laboratories, S.A. Ltd., Cape Town) at pH 8.4, cellulose acetate electrophoresis at pH 8.6 (barbitone-acetate buffer) and at pH 7.8 (phosphate buffer) and gel filtration on Sephadex G-100, the albumin was lyophilized, and stored in a vacuum desiccator at $2^{\circ}C$.

(ii) Defatted Albumin.

Long-chain fatty acids normally bound to albumin in plasma, are known to affect the albumin-binding of small molecules in

isolated systems, e.g. McMenamy found that the association of indole analogues to non-defatted albumin was only two-thirds of that to defatted albumin[130].

Fatty-acid-free human serum albumin was prepared using acetic acid and iso-octane as described by Goodman[132] and modified by McMenamy[131]. The procedure was as follows:

Albumin was prepared from human plasma as described above. In order to remove the contaminating β-lipoprotein present in the ethanol-soluble preparation, the proteins were reprecipitated with TCA, and acetone was used as the solvent. After dialysing against distilled water and pervaporating in the usual way, the albumin solution was dialysed against 0.005 M phosphate buffer pH 7.0 overnight, and finally subjected to exhaustive dialysis against deionised water (McMenamy[130]). The solution was centrifuged and the supernatant lyophilized.

The freshly dried albumin powder was placed in a beaker and rapidly covered with a volume of 5% glacial acetic acid in iso-octane (trimethylpentane, British Drug Houses, Ltd.) which had been shaken with sodium sulphate to remove all traces of water. This procedure and all the following extractions were carried out in a cold room at 2°C. After leaving the albumin in the mixture overnight, the solvent was decanted and the protein powder, which had hardened, was washed twice with aliquots of iso-octane (also pretreated with sodium sulphate) and extracted twice more with the

acetic acid-iso-octane mixture for periods from 8 - 16 hours. The albumin was then extracted four times with iso-octane (similarly dried over Na_2SO_4), with intervals of exposure to the solvent of 2 - 4 hours.

The beaker, containing the albumin residue was placed in a desiccator and dried at room temperature, under vacuum (using a water suction pump) for more than 7 hours, to remove the iso-octane (boiling range = 98 - 99.5°C). The dried albumin was dissolved in deionized water and the slightly turbid solution was dialysed at 2°C against 7 changes of deionized water using Visking cellulose casing, previously boiled in sodium carbonate solution to remove traces of glycerol and fatty acids.

The dialysis is supposed to remove the last traces of iso-octane and acetic acid. Some of the albumin precipitated out and, after centrifugation, the denatured protein settled on the surface at the water-iso-octane interface. The protein was pipetted off and the remaining iso-octane removed from the solution by evacuation in a desiccator. The albumin solution was redialysed, freeze-dried, and stored in a vacuum desiccator at 2°C.

An aliquot of the lyophilized albumin was dissolved in deionized water immediately after freeze-drying. After storing this solution for 3 weeks at 2°C, spectroscopy revealed that the ultraviolet absorption maximum was at 265 mμ instead of the usual protein maximum at 279 mμ (Fig. 24). We were at a loss to explain

why this shift had occurred, and the problem was further complicated when 2 months later, the dried defatted albumin, dissolved in distilled water, exhibited the normal absorption maximum at 279 mμ.

However, even this sample seemed to be denatured in some way since addition of fatty acid produced hardly any change in its haem binding properties while addition of extra fatty acid to undefatted albumin resulted in characteristic alterations to the haem-albumin reaction mechanism.

Albumin concentrations at high levels were measured either by the biuret reaction or the protein method of Lowry et al.[85]. Usually, however, the absorption at 280 mμ was read and compared with a standard solution of crystalline bovine serum albumin or calculated from the extinction coefficient at 279 mμ for bovine plasma albumin, i.e. $E^{1\%}_{1\ cm} = 6.67$ (Petersen and Foster[133]).

(iii) Haemoglobin.

Two different methods for the preparation of purified haemoglobin were used. In both cases, the red cells obtained from citrated whole blood were washed repeatedly (roughly 5 times) with 0.85% saline, and then 3 times with 1.2% saline containing 0.0025 M $AlCl_3$ (Drabkin[82]). They were then haemolysed by shaking with one volume of distilled water and left at 2°C overnight. The haemolysate was then treated in one of two ways:

(a) Method of Rossi-Fanelli, Antonini and Caputo[134]:

Distilled water was added to the haemolysate in order to dilute the original red cell volume 3 - 4 times and this solution was brought to 20% saturation with neutralized ammonium sulphate solution. It was left standing for about an hour and then centrifuged at 20,000 x g. (i.e. 16,000 rpm in Beckman Model L Ultracentrifuge) for 1 hour at 2°C. The precipitate was discarded and the supernatant haemoglobin solution was dialysed in the cold against several changes of deionized water for 3 days or more to remove the ammonium sulphate.

The dialysate was centrifuged at 2,500 rpm for 1 hour to remove any denatured protein and converted to carboxyhaemoglobin, before dividing into aliquots of 10 ml. for storage at -15°C.

(b) Ammonium sulphate fractionation method of Gutter, Sober and Peterson[135]:

The stroma was removed from the haemolysate by centrifuging, after predipitation in 12.5% saturated $(NH_4)_2SO_4$. The haemoglobin was precipitated by bringing the concentration to 52% $(NH_4)_2SO_4$ saturation. The precipitate was dissolved in distilled water and dialysed exhaustively to remove salts. The haemoglobin solution was finally dialysed against 2 changes of CO-saturated distilled water and stored at -15°C. Homogeneity of the carboxy-haemoglobin preparations was tested on Sephadex G-75 and by cellulose acetate electrophoresis in phosphate buffer pH 7.8; ionic strength 0.1. Cellulose acetate electrophoresis usually showed some heterogeneity present in the preparation. A smaller quantity of protein, slower moving than the main component, usually formed a "shadow" band

behind it. This probably was haemoglobin A_2 which occurs normally in adult erythrocytes in small quantities (usually 3% of total circulating haemoglobin), (Kunkel and Wallenius[136]; Masri, Josephson and Singer[137]). Immunoelectrophoresis against antihuman serum showed no precipitin bands indicating that the preparations were not contaminated with any plasma protein.

Haemoglobin concentration was measured either by the cyanmet-haemoglobin method using Acuglobin (Ortho Pharmaceutical Corp.) as the standard or by the pyridine haemochromogen method of Rimington[138].

For the spectroscopic studies, however, the optical density of the dilute haemoglobin solutions was read at 540 mμ and the concentrations calculated from the extinction coefficients; i.e. $E^{1\%}_{1\ cm}$ at 540 mμ for HbCO = 8.4 (Antonini, Wyman, Brunori, Bucci, Fronticelli and Rossi-Fanelli[139]) and $E^{1\%}_{1\ cm}$ at 540 mμ for HbO_2 = 8.5 (Rossi-Fanelli et al.[134]).

Methaemoglobin was formed from isolated carboxyhaemoglobin, prepared by the method of Rossi-Fanelli et al.[134]. Oxygen was bubbled through the solution to convert the haemoglobin from the carboxy- to the oxy- form. Addition of a small crystal of potassium ferricyanide resulted in the formation of methaemoglobin. This was checked spectrophotometrically and the solution was dialysed against several changes of O_2-saturated distilled water for 3 days at 2°C, to remove the $K_3Fe(CN)_6$. (Drabkin[140]).

Methaemoglobin concentration was calculated from the molar extinction coefficient as haem at 500 mμ, given by Rossi-Fanelli, Antonini and Caputo[141], which is 9.5×10^3.

(iv) Globin.

Native human globin was isolated from a stroma-free, dialysed haemolysate of human red cells, prepared according to Rossi-Fanelli et al.[142].

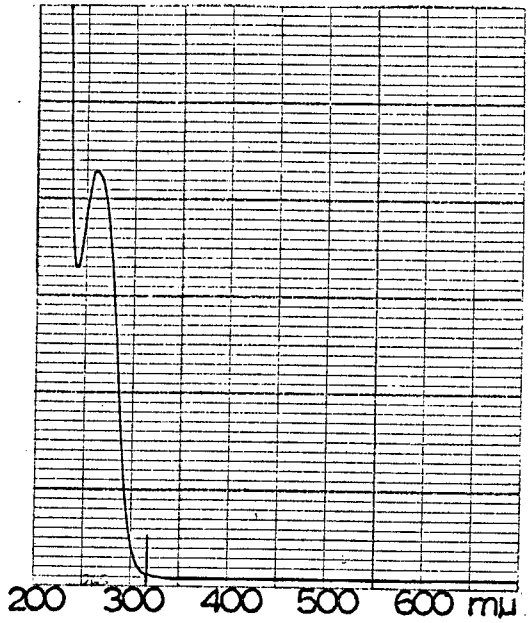
The method used was one described by Rossi-Fanelli and his colleagues in 1958[143] using acetone and hydrochloric acid to which the salt-free haemoglobin solution was added drop by drop at -20°C with occasional stirring. The acetone supernatant, containing haematin, was discarded and the almost colourless protein precipitate left to dissolve in cold deionized water. The solution was dialysed at 2°C against deionized water for a few hours and then against 0.0016 M sodium bicarbonate for about 30 hours. The flocculate of denatured globin was then filtered off, all steps being performed in a cold room. At this stage, the pH of the globin solution was 8.0, the spectrum recorded showed that some haem was still associated with the globin, and cellulose acetate electrophoresis revealed 2 haem-containing proteins both of which had migrated slightly towards the anode at pH 7.8 (at this pH, haemoglobin has very little electrophoretic mobility and usually appears just anodic to the point of application).

The globin solution was left standing at 2°C for 2 days, during which time, more denatured protein precipitated. This was removed by filtration. The 280 mμ to 408 mμ optical density ratio of the supernatant was 5.06 (Fig. 25 A). (The ratio for oxyhaemoglobin prepared according to Rossi-Fanelli et al.[134] is 0.41). Electrophoresis on cellulose acetate separated a split globin fraction with still greater mobility than purified carboxyhaemoglobin, and some denatured protein, which remained at the origin and which, on O-dianisidine staining, proved to be the only fraction still containing appreciable quantities of peroxidase activity.

The solution of globin was then dialysed for a few days against 0.02 M phosphate buffer at pH 7.0, after which it was centrifuged. The ratio of the optical densities at 280 mμ and 408 mμ was 5.68 (i.e. less haem/protein than before).

On dialysing against deionized water, prior to lyophilization, a considerable quantity of the protein was precipitated out of solution and was removed by filtration. This decreased the protein concentration relative to the haem, so that the final tyrosine to Soret ratio was only 4.73 (Fig. 25 B). This last dialysing procedure may have interfered with the stability of the globin rendering it insoluble at very low salt concentrations. The possibility that the freeze-dried preparation contained a large proportion of denatured protein cannot be excluded, when considering the findings of some of the experiments which follow.

FIG. 24.



DEFATTED ALBUMIN

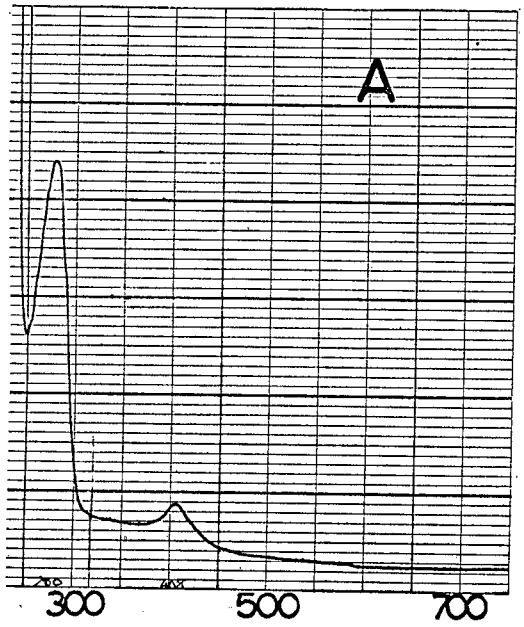
ABSORPTION MAX. 265mμ

FIG. 25.

GLOBIN PREPARATION

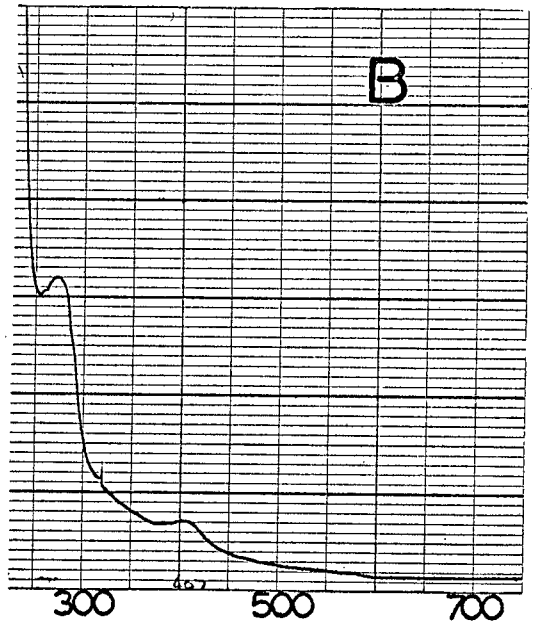
AFTER DIALYSIS vs. Na_2HCO_3

$\frac{O.D.\ 280m\mu}{O.D.\ 408m\mu}$ = 5·06



FINAL PREPARATION

$\frac{O.D.\ 276m\mu}{O.D.\ 407m\mu}$ = 4·73



The concentration of globin solutions was estimated by the optical density measurement at 280 mμ and calculating from the extinction coefficient determined by Gibson and Antonini[144] as 8.5, for a 1% solution of native human globin.

(v) Crystalline Haemin.

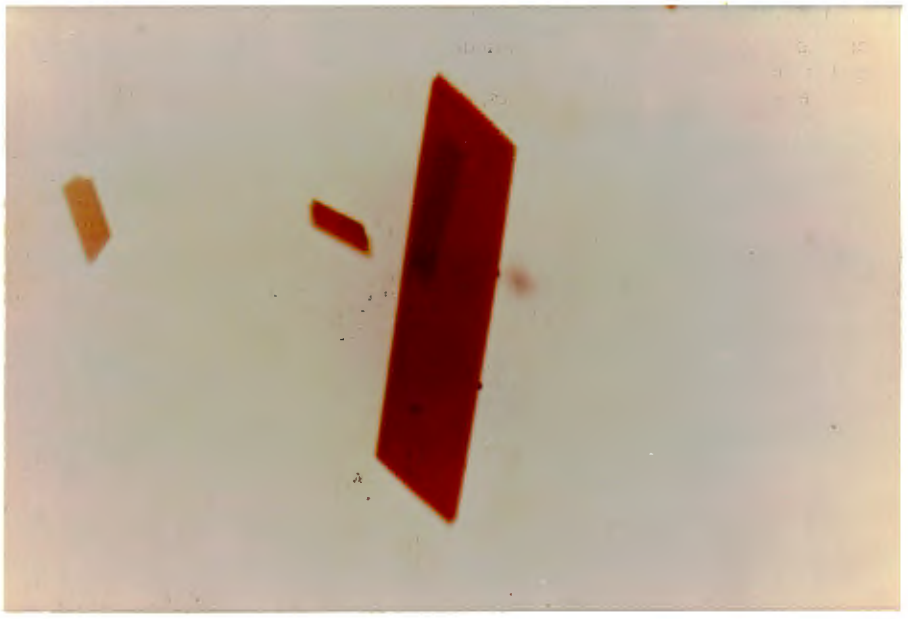
Haemin crystals were prepared from red cell haemolysates by the method of Willstätter and Fischer as described in Koch and Hanke's "Practical Methods in Biochemistry"[145]. The haemoglobin solution was allowed to drop directly into an equal volume of boiling glacial acetic acid, containing 0.5% sodium chloride, with constant stirring, over a period of 10 minutes. Heating was continued for 5 minutes, after which the mixture was left to cool gradually overnight. Crystal formation was checked by examining the liquid under a microscope (Colour Plate 2). The supernatant was decanted and the crystals were washed with 10% acetic acid, followed by 2 washings with distilled water, and then alcohol and ether. The washed crystals were dried in a desiccator.

They were recrystallized by dissolving the haemin in alkali (1% haemin solution in 0.1 N NaOH), pouring this solution into boiling acetic acid and following the method as described above.

The concentration of haematin (M.W. 633.5) in aqueous solution was measured by the pyridine haemochromogen method of Rimington[138], or calculated from the absorption maximum in the Soret region

COLOUR PLATE 2

· Haemin crystals



(E mM Fe at 385 mμ = 49 for haematin solutions dissolved in 5×10^{-4} N aqueous NaOH (Dalziel[146])).

B. Methods of investigation.

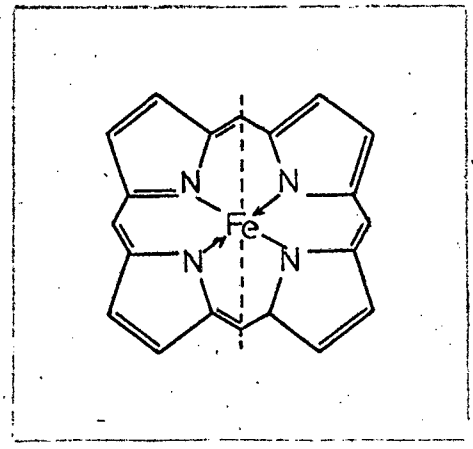
(i) Cellulose acetate electrophoresis was performed as before. A different buffer was used, i.e., phosphate pH 7.8; ionic strength 0.1 and 120 volts were applied for 100 minutes.

(ii) Spectrophotometry.

Results from most of the investigations described below have been obtained from spectral analyses.

The characteristic spectral properties of haem and haemoproteins in the visible and near ultra-violet (Soret) regions of the spectrum are ascribed to the cyclic resonating structure of the tetrapyrollic nucleus and its β substituents. Experiments with other types of porphyrins (having different side chains) have shown that there is a firm correlation between the nature of the side-chains on the different porphyrins, and the positions and order of intensity of their visible bands (Lemberg and Falk[147]). Both the visible and Soret absorption bands are associated with electronic transitions resulting in movement of electrons towards the periphery of the porphyrin nucleus (Phillips[148]). Thus, electron-attracting substituents which facilitate these electron displacements towards the periphery will require less energy for the transitions in the

porphyrin nucleus and the absorption bands will be at longer wavelengths, i.e. shifted towards the red end of the spectrum. (Falk[149] calls these substituents "rhodofying"). The incorporation of a divalent metal ion (e.g. Fe^{2+}) into the porphyrin molecule, where it is bound by 2 co-ordinate and 2 ionic bonds to the 4 nitrogen atoms (see figure below) will tend to attract electrons from the periphery to the centre of the flat molecule. Thus, for metalloporphyrins, shifting of absorption maxima to shorter wavelengths is indicative of strong metal-porphyrin bonding and thermodynamic stability.



Further co-ordination of a metalloporphyrin, e.g. haem, with other ligands produces changes in spectroscopic properties which are characteristic for each type of ligand molecule. In the case of the iron atom of haem, complexing with other molecules occurs by co-ordination with the 2 ligands which project above and below the flat plane of the porphyrin ring, forming an octahedral structure.

Haem is known to form complexes with a large number of ligands, e.g. pyridine, cyanide ions, hydroxide ions, carbon monoxide, oxygen, aliphatic amines, peptides, amino acids, imidazoles, etc.

The highly characteristic spectra obtained for these haem complexes and haemoproteins provides one with a useful tool for studying their interactions with other molecules without having to actually isolate the products of these reactions.

All the spectroscopic determinations were performed using a Beckman DB Recording Spectrophotometer.

Difference spectra were recorded using two or four ground-glass cuvettes fitted with stoppers, each having a light path of 1 cm.

Quantitative data on which all the graphs were based, were read directly on the absorbance scale of the spectrophotometer. All calculations are based on a value of zero at 700 mμ for all blanks and samples.

This measure was adopted to correct for opalescence when using fatty acid emulsions, for any slight turbidities which developed in the samples, and for dust particles which could not always be excluded. In the case of the fatty acids, this procedure was found to be effective down to wavelengths of approximately 400 mμ. At shorter wavelengths and particularly in the ultraviolet region, the effect of turbidity on the optical density often greatly exceeded that at 700 mμ. Under these circumstances, the results were only interpreted qualitatively. Some protein solutions, of albumin for example, exhibit lower absorbance at shorter wavelengths than at 700 mμ. These optical density values were depicted as minus quantities.

The difference spectra obtained using 4 cuvettes in the Beckman DB cell compartment were never quantitated but only used as a qualitative indication of the reactions taking place.

C. Reaction between haemoglobin and albumin - qualitative experiments.

(i) Electrophoresis of haemoglobin-albumin mixtures.

Carboxyhaemoglobin (prepared by the method of Gutter et al.[135]) was added to a solution of albumin (prepared by the TCA-acetone method, but containing a very small quantity of a β-lipoprotein which was only evident on immunoelectrophoresis).

Albumin was measured by the biuret method and the haemoglobin concentration calculated from the O.D. at 540 mμ. The haemoglobin/albumin molecular ratio was 1 to 22. Neither of the preparations had been dialysed against buffer, consequently the pH of the mixture was roughly between 4 and 5.

Blanks of both albumin and haemoglobin appropriately diluted were also investigated. All the samples (the test mixture was run in duplicate) were incubated at 37°C for 1½ hours. At this point, the test samples were brownish in colour whereas the haemoglobin blank appeared to be the same colour as it was prior to incubation, i.e. bright red. Aliquots of each were electrophoresed on Oxoid strips in phosphate buffer, pH 7.8; ionic strength 0.1, for 100

minutes at 120 volts. The results are shown in Fig. 26.

Each blank only contains its original protein constituent. In both test samples, only one protein band, besides the denatured protein at the origin, is evident with lissamine green staining, i.e. albumin; and the same is true for the strips stained with o-dianisidine. In other words, some of the albumin fraction has been transformed into methaemalbumin while the globin and any remaining haemoglobin has become denatured.

An aliquot of the mixture applied to a column of Sephadex G-100, with effluent scanning at 410 mμ, revealed a small quantity of high molecular weight protein, i.e. 300,000 which presumably was the haem-containing denatured constituent, while the methaemalbumin and haemoglobin were eluted in the same protein peak with the albumin coming off first.

Scanning the spectra of the samples, revealed that some methaemoglobin had been formed in the haemoglobin blank (band at 630 mμ) but was otherwise unaltered. The test sample, however, showed a marked rise at about 620 mμ, small maxima at 530 and 500 mμ and a shift in the Soret maximum towards the blue region of the spectrum (Fig. 27).

There was little doubt that methaemalbumin had been formed as a result of some interaction between the haemoglobin and albumin molecules. It is possible that the lipoprotein fraction that was present as a contaminant contained a haem-transferase, although in view of later results this seemed unlikely.

FIG. 26.

ELECTROPHORESIS ON CELLULOSE ACETATE OF A MIXTURE OF CARBOXYHAEMOGLOBIN AND ALBUMIN AFTER INCUBATION AT 37°C.

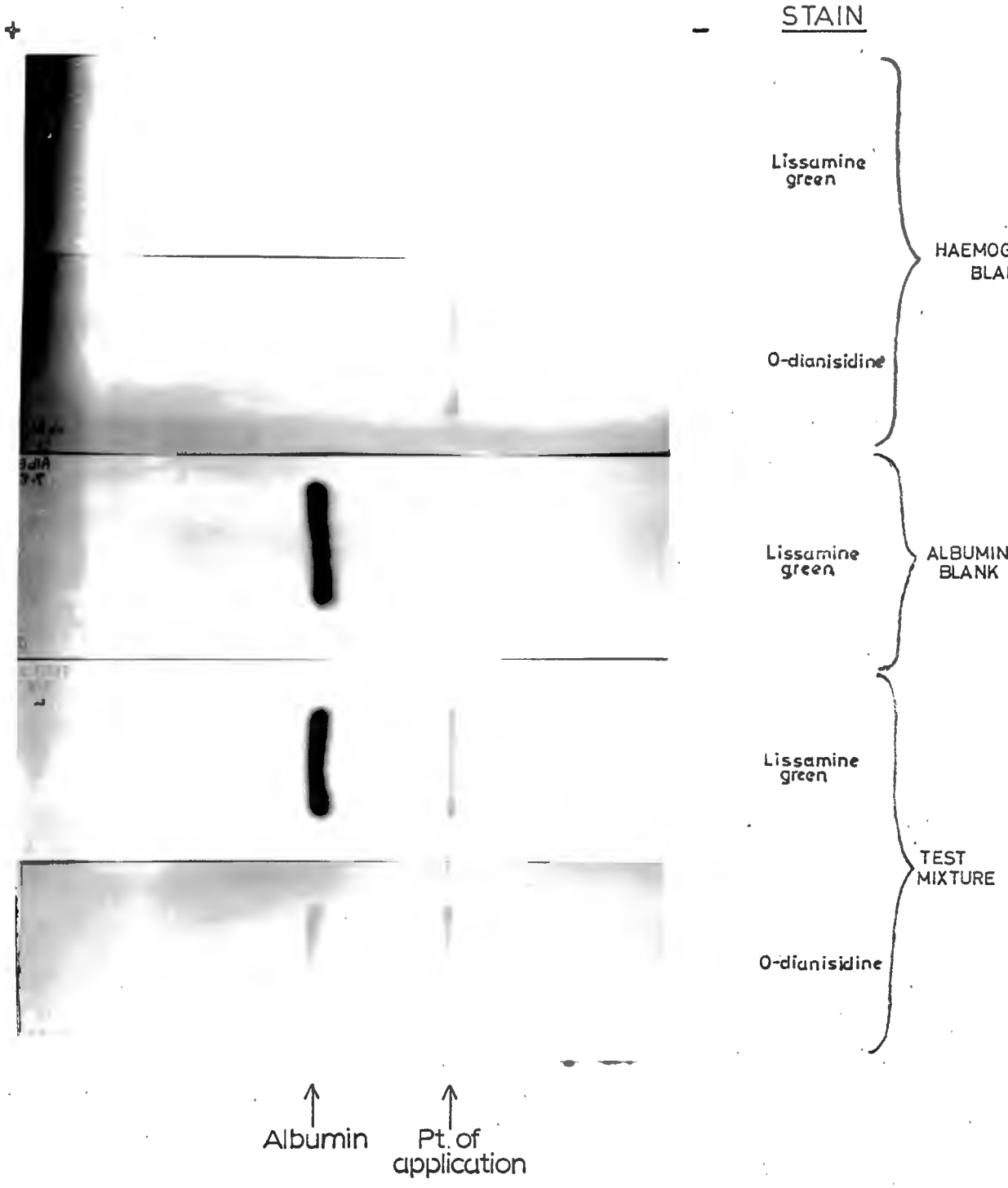
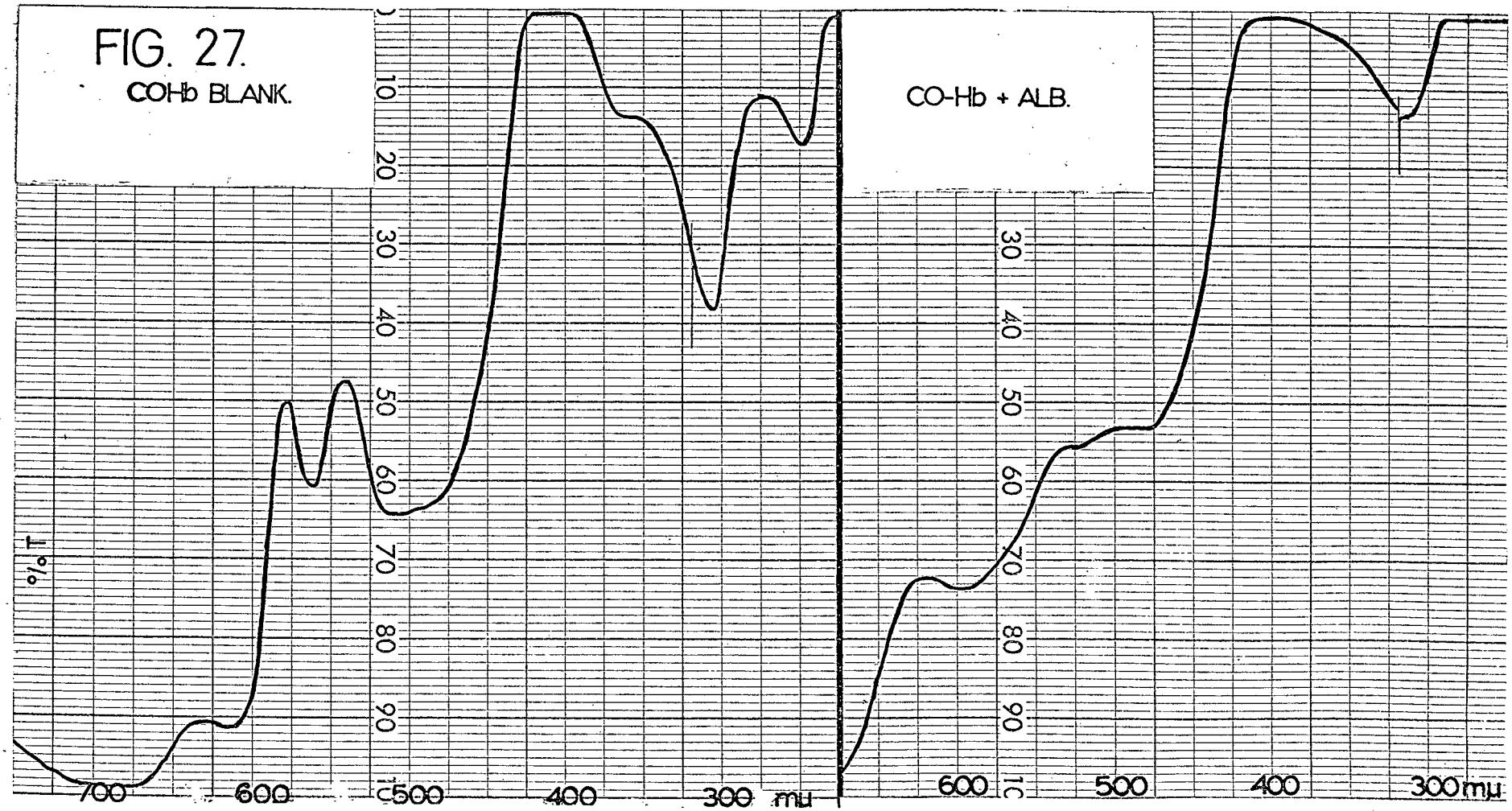


FIG. 27.


COHb BLANK.
CO-Hb + ALB.
% T
10
20
30
40
50
60
70
80
90
100
700
600
500
400
300 mμ
600
500
400
300mμ

The test samples were at a slightly lower pH than the haemoglobin blank (pH of albumin solution = 3.8; pH of carboxyhaemoglobin solution = 5.2) and this may have caused the denaturation not evident in the case of the blank; and the subsequent binding by albumin of the haem moiety which might be more easily dissociated from the resulting haemochromogen molecule.

Electron spin resonance studies by Hollocher and Buckley[150] at high temperatures have shown that methaemoglobin becomes very heat-sensitive at a pH of 4.6 - 4.7 and undergoes reversible denaturation. Thus, any methaemoglobin formed from the carboxyhaemoglobin in the experiment (and the spectrum of the blank indicated that this did occur) might have accounted for the fact that the denatured protein still had peroxidase activity.

The low pH may have changed the normal conformation of the albumin molecule as it is at pH 7.4 imbuing it with a greater affinity for haem than the globin. Other workers have shown that under these circumstances the haem prosthetic group appears to migrate, although all the proteins used were native apohaemoproteins of different types. (Rossi-Fanelli and Antonini[114]; Rosenqvist and Paul[151]).

Korinek and Dvorakova are reported to have found that when methaemoglobin is added to serum, and the mixture then incubated the prosthetic group of the unbound haemoglobin undergoes " trans-hemation" to the albumin and β_1-globulin fractions, with concomitant

aggregation and sedimentation of the globin[152].

Later experiments in our chemical systems showed that precipitation of protein occurred whenever there was marked methaemalbumin formation from haemoglobin, and the denaturation was not in itself due to the hydrogen ion concentration.

This suggests that removal of haem groups from haemoglobin results in denaturation of the globin. Theoretically, this seems feasible; however, in practice, workers have repeatedly been able to reconstitute the haemoglobin molecule from isolated globin and added haematin, shown by the restoration of the spectroscopic properties of native haemoglobin (Winterhalter and Huehns[153]; Gibson and Antonini[144]).

Added to this is the fact that the electrophoresis strips showed that the denatured protein exhibited peroxidase activity and, therefore, still contained haem.

Possibly the haem-transfer was selective, with only one or two of the 4 haem groups from each haemoglobin molecule being bound to albumin.

In suggesting these possible explanations, we have not really considered the question of how the haem transfer is actually accomplished, i.e. whether it is due to dissociation from the haemoglobin molecule or dependent on some sort of interaction with albumin. This point will be considered later.

The facility with which this reaction occurred in the absence

of any enzyme prompted us to investigate what effect the addition of a small amount of serum would have on the haemoglobin-albumin interrelationship.

(ii) Effect of serum on haemoglobin-albumin mixtures.

The experiment was set up with 2 controls both containing haemoglobin; the one having homologous human serum and no added albumin, and the other containing only albumin. The haemoglobin albumin ratio was approximately 1 to 10.

Assuming a serum protein concentration of 6 g.%, the serum protein added to the test mixture comprised roughly a quarter of the protein in the sample.

Unfortunately, the albumin preparation in this experiment was contaminated with what appeared to be an α_2 globulin when electrophoresed in phosphate at pH 7.8. The contaminant was not detectable at pH 8.6.

The samples were incubated at 37°C for 1 hour after which aliquots were taken for electrophoresis. Whatever was left, was kept at room temperature for 2 days and the effect of this prolonged contact on the electrophoretic patterns determined after centrifuging off any denatured proteins.

The results on testing for peroxidase activity were as follows:

TABLE 12.

	CONTROL 1.	CONTROL 2.	TEST.
Haemoglobin	2 mg.	2 mg.	2 mg.
Albumin	-	20 mg.	20 mg.
Serum	0.1 ml.	-	0.1 ml.
1 hr. incubation	(1) Free haemoglobin (2) Hb-Hp complex	(1) Mainly free Hb. (2) Methaemalbumin (heterogeneous). (3) Some denatured haemoprotein.	(1) Mainly free Hb. (2) Haem bound to 3 other serum fractions including Hp. (3) Some denaturation had occurred.
2 days at room temperature.	(1) Mainly Hp complex (2) Some methaem-albumin. (3) No free Hb.	(1) Methaemalbumin. (2) Small quantity of free Hb.	(1) Methaemalbumin. (2) Some free Hb. (3) No other haemo-proteins .

The quantity of free haemoglobin present in relation to the serum is roughly equivalent to 2 g./100 ml. This far exceeds the normal haemoglobin binding capacity of the haptoglobins and this is borne out by the results. In the test sample containing an excess of albumin, it is surprising, therefore, that no methaemalbumin is formed after incubation. Nor is there any formed in Control 1 where the albumin concentration is minimal. In the sample containing no serum, however, a considerable quantity of methaemalbumin is formed in 2 distinct o-dianisidine bands, both corresponding to the albumin fraction stained with lissamine green.

These findings suggest that there is a factor in serum, which,

if present even in small quantities inhibits methaemalbumin formation, possibly by acting to prevent oxidation of haemoglobin to methaemoglobin which is more labile. In the test sample, 3 other serum fractions, present only in low concentration, were utilized for haem-binding in preference to the albumin which was available in much larger quantities. Presumably with the prolonged presence of free haemoglobin in serum, this inhibition is lifted and methaemalbumin can be formed in large quantities.

This finding should prove extremely significant if discovered to be a general property of human serum under conditions of haemolysis. Since this was a single experiment on serum from one individual, it is clear that repetition of the experiment on a number of individuals and under different conditions is indicated before the results can be properly assessed.

(iii) The effect of separating the two protein reactants by means of a dialysis membrane.

We devised an experiment to test whether methaemalbumin formation in carboxyhaemoglobin-albumin mixtures was simply due to dissociation of free haematin from the globin and subsequent binding by albumin.

Carboxyhaemoglobin (approximately 10 ml., containing 4.7 mg./ml.; pH = 5.3) was placed in a dialysis sac, which had been previously boiled in 1% w/v Na_2CO_3 solution and which was closed at both ends

by rubber stoppers connected to each other with a glass rod. The rod was attached to an automatic stirrer and the sac was immersed in 150 ml. albumin solution contained in a cylinder. The albumin concentration was approximately 1 mg./ml. and the pH was 4.3. The arrangement was left thus at 0°C overnight without agitation. The following day when neither haematin nor methaemalbumin was detectable in the dialysing medium, the sac was immersed in the albumin solution at room temperature for 8 hours with intermittent agitation.

There was no evidence of haematin dissociation and subsequent passage through the pores of the dialysing membrane. One drop of haemoglobin was then added to the dilute albumin solution to ascertain whether the volume of the dialysing medium was too large to detect small quantities of haematin which may have diffused out of the sac. The added haemoglobin caused a detectable rise at 405 mμ in the absorption spectrum of the albumin.

From the result of this experiment we deduced, that under the conditions prevailing in this experiment, methaemalbumin production must proceed only slowly by dissociation of haem from the haemoglobin unless albumin molecules are present to provide acceptor sites. However, this experiment was performed at low pH, and subsequent investigations have shown that dissociation at this pH, even in the presence of contiguous albumin, is very small indeed; the formation of ferrihaem, taking place only at higher pH, is a necessary requisite for transfer of the prosthetic group from one protein to the other. This was a preliminary trial only, and it

is possible that haem did not pass freely through the membrane - this point was not checked.

Possibly at 37°C, at higher pH, and in the light, the dissociation of haem from carboxyhaemoglobin could have been demonstrated. Evidence suggests that ferrihaem does dissociate from methaemoglobin under conditions of higher pH and temperature. In methaemoglobin, the ferrihaem metal porphyrin co-ordinates tend towards the low spin (covalent) type of bonding with increasing pH (Hollocher and Buckley[150]) and temperature (Phillips[148]), one of which binds the haematin to the apoprotein. Thus, without repeating this experiment using variously liganded haemoglobins under different conditions of pH and temperature, one cannot be certain that the haem prosthetic group does not dissociate independently of albumin. The readiness with which the haem was transferred to albumin on incubation, when the proteins were mixed together, has suggested a definite role of the albumin molecule in the reaction, but only more detailed studies will allow a firm conclusion to be reached on this question.

(iv) Rate of methaemalbumin formation.

Experiments were performed in which the rate of increase in optical density of haemoglobin-albumin mixtures at 620 - 625 mμ, was compared with that of a haemoglobin blank.

The blank and test samples were compared in stoppered cuvettes of 1 cm. light path, using a Beckman DB Spectrophotometer, and the

increase at 625 mμ of the test, relative to the blank, was recorded. The optical density of the haemoglobin blank was adjusted to zero and the required amount of haemoglobin was not added to the cuvette containing the albumin solution until the recording had been started.

After recording for a number of hours at 624 mμ, the spectra of the blank and test samples were scanned using a water blank and the optical densities of each read at certain wavelengths. The constituents of both samples were then usually separated electrophoretically on cellulose acetate.

Unfortunately, the extinction coefficient of methaemalbumin at 624 mμ is not so much greater than that of haemoglobin and considerable methaemalbumin formation will produce only small increments in optical density. Moreover, the formation in the blank, of methaemoglobin, which has an absorption maximum at 630 mμ, will tend to diminish the relative increase in the red part of the spectrum due to methaemalbumin. This is especially true at high concentrations of haemoglobin, when only a fraction of the haem will be bound to albumin.

One set of experiments was performed using carboxyhaemoglobin and albumin mixtures prepared as before. The albumin preparation (pH 4.5) still contained a small quantity of the lipoprotein contaminant. The pH of the haemoglobin solution was approximately 6. It is clear, that the test samples were all more acidic than the blanks owing to the presence of the albumin and consequently one cannot consider the controls as "true" blanks.

At the time that these experiments were performed we did not realize the significance of our disregarding certain important factors such as pH, methaemoglobin formation, and denaturation.

In spite of their preliminary nature, certain of the findings are still relevant in the light of later investigations.

On the basis of the continuous recording at 624 mμ and the spectra of the different preparations, one could never be certain that the increase at 624 mμ in test samples, relative to the blanks, was not due to enhanced methaemoglobin formation, induced in some manner by the presence of albumin.

In this regard, the ratio of the optical densities at 540 mμ and 625 mμ were found to be useful. Except at very low concentrations of carboxyhaemoglobin, the 540/625 ratio is in the region of 8 to 10. In the blank, this decreases as methaemoglobin is formed, the extent depending on the time of experimentation and the initial concentration. After 24 hours, the ratio was generally between 5 and 6. In the test mixtures, if methaemalbumin was the only pigment present after the incubation period, i.e. there was no free haemoglobin or methaemoglobin; under such conditions the 540/625 ratio was 1.5 - 2.0. The presence of free haemoglobin caused this figure to be raised. This empirical evaluation was checked by electrophoresing the test samples. Those with ratios above 2 usually exhibited, after staining with o-dianisidine, a band of free haemoglobin in addition to the methaemalbumin and denatured protein.

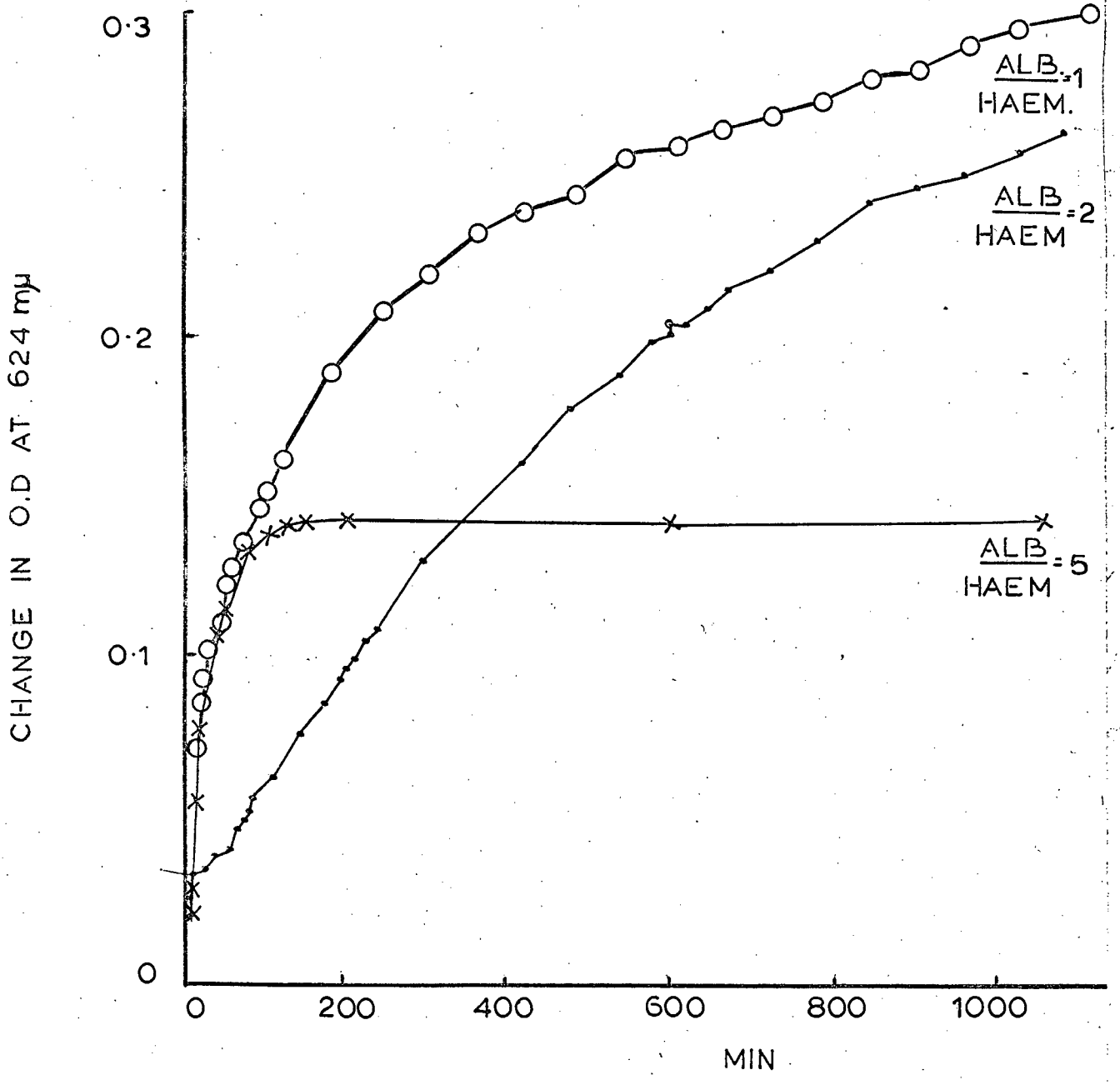
Most of the experiments were performed using excess quantities of albumin, with molar ratios of between 5 and 20 of albumin to 1 of haem. In these cases, methaemalbumin formation was complete (i.e. there was no free haemoglobin present) within 2 hours at room temperature.

With a haem/albumin molecular ratio of 1 to 1, the reaction is much slower and only levels off at about 6 hours although the optical density of the haemoglobin-albumin mixture continues to rise, probably on account of turbidity due to denaturation of the proteins. At higher concentrations of haemoglobin, the reaction appears even slower. Furthermore, the difference in optical density at 624 mμ between blanks and test samples was also less, making comparative evaluations of these results impossible (Fig. 28).

Increasing the temperature from 25°C to 40°C appeared to increase the rate but not the eventual amount of methaemalbumin formed.

A similar experiment was performed at pH 7, in which the relative concentrations of oxyhaemoglobin and albumin provided 1 haem to 8 albumin molecules, and in which complete surrender of all haem molecules for methaemalbumin formation had not occurred even after 48 hours at room temperature, by which time a considerable quantity of the haemoglobin in the blank had been oxidized. There was still protein denaturation but a free haemoglobin band was evident on electrophoresis.

FIG. 28. RATE OF METHAEMALBUMIN FORMATION AT VARIOUS CONCENTRATIONS OF HAEMOGLOBIN



It is unlikely that using oxyhaemoglobin rather than the carboxy-form, could have been responsible for this marked decrease in methaem-albumin production. Clearly then, bringing the pH to neutrality must be responsible. One point should be mentioned however. The albumin contaminant, not visible on any of the cellulose acetate strips from the experiments carried out at low pH, was clearly visible in this case, with both protein and peroxidase stains.

It is difficult to ascertain in this instance whether the change in pH had altered the affinity of the albumin for haem or whether the stability of haemoglobin itself had been changed.

Albumin is known to undergo striking changes at and below pH 4, associated with expansion of the molecule at low ionic strengths and "isomerization", leading to exposure of hydrophobic surfaces previously buried within the molecule (Foster[154]).

Further experiments with different concentrations of carboxy-haemoglobin and albumin in phosphate buffer (pH 7) or after dialysis to remove ions (pH 6) certainly resulted in methaemalbumin formation as evidenced by cellulose acetate electrophoresis, and, removal of phosphate ions enhanced this. The albumin seemed to have far less binding affinity, however, and required long incubation periods for formation of appreciable quantities of methaemalbumin. The free haemoglobin present in many of the experiments after incubation obscured the methaemalbumin formation usually detectable by spectral scanning. One could never be certain except with subsequent

electrophoresis and peroxidase staining which is not quantitative, whether methaemalbumin itself was present in the samples or whether the presence of albumin was greatly increasing the rate of oxidation of the haemoglobin to ferrihaemoglobin.

Difference spectroscopy was introduced at this stage in an attempt to clarify the issue. These were strikingly characteristic (Fig. 29) - the test samples having absorption maxima at 623 mμ, 504 mμ and 372 mμ in relation to the blank, with marked decreases at 575 mμ, 545 mμ and 415 mμ.

This type of difference spectrum could easily reflect methaemoglobin formation as well as methaemalbumin. Both have lower extinction values at their respective Soret maxima than the ferrohaemoglobins. The peak at 623 mμ indicated methaemalbumin formation since the band in the red for methaemoglobin usually tends towards longer wavelengths. The peak at 504 mμ could indicate the presence of either compound. The absorption maximum at 372 mμ is striking and we became increasingly aware of this phenomenon as time went on. Some of the area under the peak is undoubtedly due to the presence of albumin in the test solution.

Examining some haemoglobin spectra, we noted that the γ^1 band of the ferrohaemoglobins at 347 mμ becomes increasingly less evident as oxidation proceeds due to a rise in the ferrohaemoglobin minimum around 370 mμ. However, with albumin, this rise is so dramatic that the γ^1 band disappears.

Whether this absorption maximum in the difference spectrum indicates only increased ferrihaemoglobin or free ferrihaem (Soret maximum: 365 - 385 mμ) or methaemalbumin formation, is not known, nor is this effect described in the literature as far as we know.

The uncertainty surrounding many of these results prompted a study on ferrihaem-albumin reactions.

D. Reaction of ferrihaem with albumin.

(i) Absorption spectrum of ferrihaem.

Ferrihaem in solution, in contrast to the haemoproteins, has a rather diffuse absorption spectrum. This is attributed to the effect of charge transfer, from unreacted ligands to the ferric ion, interacting with the electronic transitions normally associated with the porphyrin nucleus (Falk[149]). Also, in aqueous alkali solution , neither ferro-nor ferrihaem are molecularly dispersed but form associated dimeric units loosely bound together as colloidal aggregates (Shack and Clark[155]) and held in solution by means of their ionized carboxylic acid groups.

Protonation, i.e. neutralization of ferrihaem-hydroxide solutions increases the acid strength of the propionate side chains, thereby increasing the affinity of the ferric ion for ligands such as water molecules and possibly diminishing the tendency of the molecules to dimerize. However, the ferrihaem

also becomes less soluble, and begins precipitating out of solution at a pH of roughly 6.0.

The absorption maxima of ferrihaem-hydroxide solutions were at 590 - 615 mμ, and at 385 - 390 mμ in the Soret region. Neutralized solutions varied considerably even when freshly made up; the band in the red usually shifting to longer wavelengths, i.e. 635 - 640 mμ and the Soret band more towards the ultraviolet region, i.e. 365 mμ - 375 mμ.

We did not make up fresh ferrihaematin solutions prior to each experiment and this may explain the variation in the calculated quantities of unreactive pigment. However, since most of the comparative experiments were performed simultaneously, the effect of non-reacting haem was cancelled out.

Prior to use, the neutral haematin solutions were centrifuged to remove any precipitated haemin and the optical density in the Soret region determined. The millimolar extinction coefficient of 49 at 385mμ calculated on the basis of the Fe atoms present, (Dalziel[146]) was used to estimate the concentration.

(ii) Haematin-binding capacity of albumin.

Our incubation experiments with haemoglobin and albumin had suggested that haem molecules are bound to albumin in a molecular ratio of 2 to 1. This has been the finding of numbers of workers including Rosenfeld and Surgenor[108], Keilin[107] and O'Hagan[110].

Figure 30 shows the effect of addition of increasing quantities of ferrihaem to albumin at room temperature. The firm binding of haematin in the range of 0.6 - 1.8 molecules ferrihaem to 1 of albumin, is evidenced by the sharpened Soret maximum at 404 mμ and the rise at 624 mμ.

The stoichiometric binding of 2 molecules of haem by one of albumin or even complete 1 to 1 association was not a consistent finding in our experience and depended on the experimental conditions.

Difference spectra were frequently observed which suggested a loose association of unbound ferrihaem with albumin, in addition to some methaemalbumin formation. A similar type of association between albumin and fatty acids has been postulated by Björntorp[156] to account for his experimental findings on fatty acid oxidation by isolated rat liver mitochondria in the presence of albumin, i.e. in addition to the fatty acids firmly attached to specific binding sites, the albumin molecule carries loosely associated fatty acids (Goodman[157]) which are more readily oxidized.

There is another explanation for the difference spectra encountered (Fig. 32) and that is that albumin exerts a stabilizing effect on the unbound ferrihaem molecules in solution (also noted by Rosenfeld and Surgenor[108]), protecting them from oxidation by molecular oxygen, resulting in a higher concentration of free ferrihaem in the haem-albumin mixtures than in the ferrihaem blank and consequently producing the rise at roughly 370 mμ encountered in the difference spectra.

FIG. 30.

FERRIHAEM + ALBUMIN.

pH 7·0; ROOM TEMP.

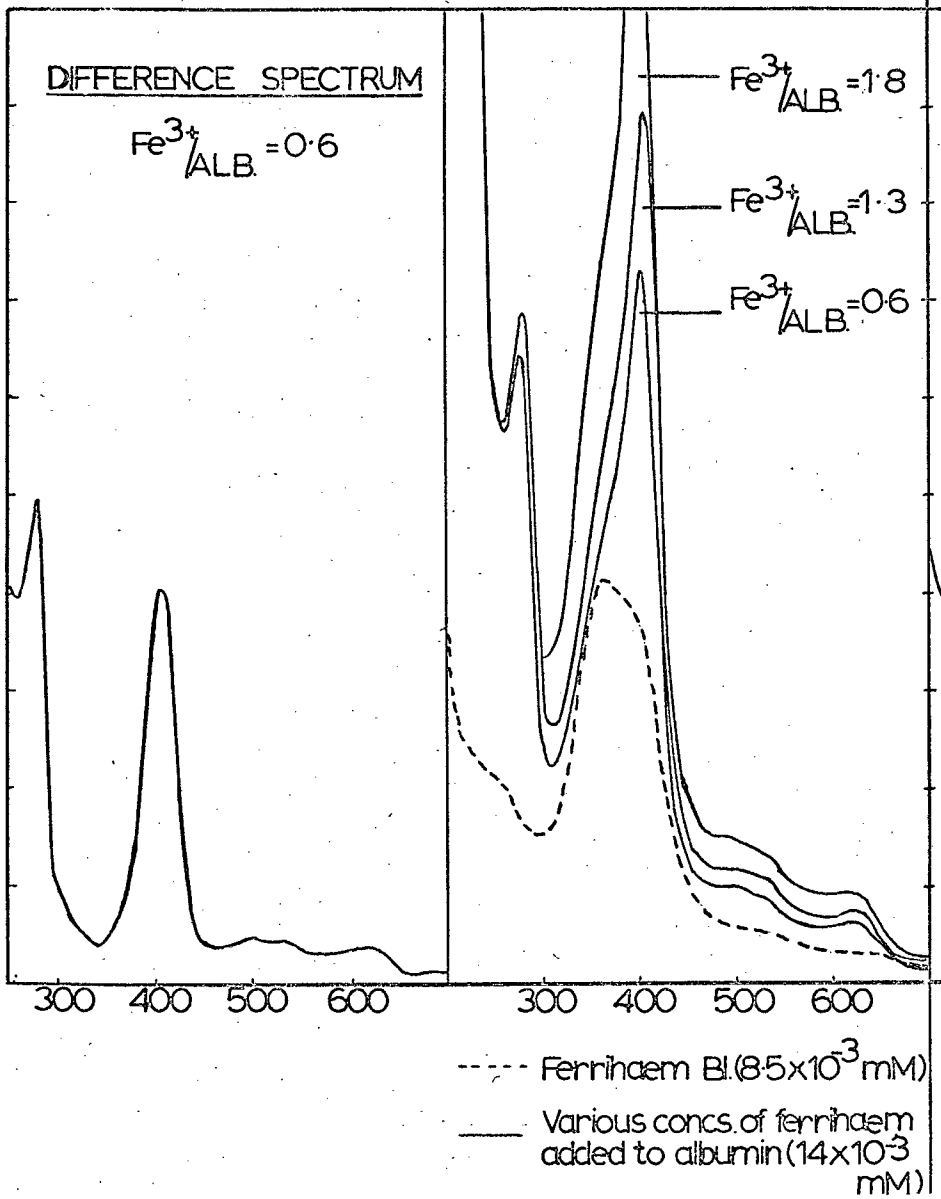
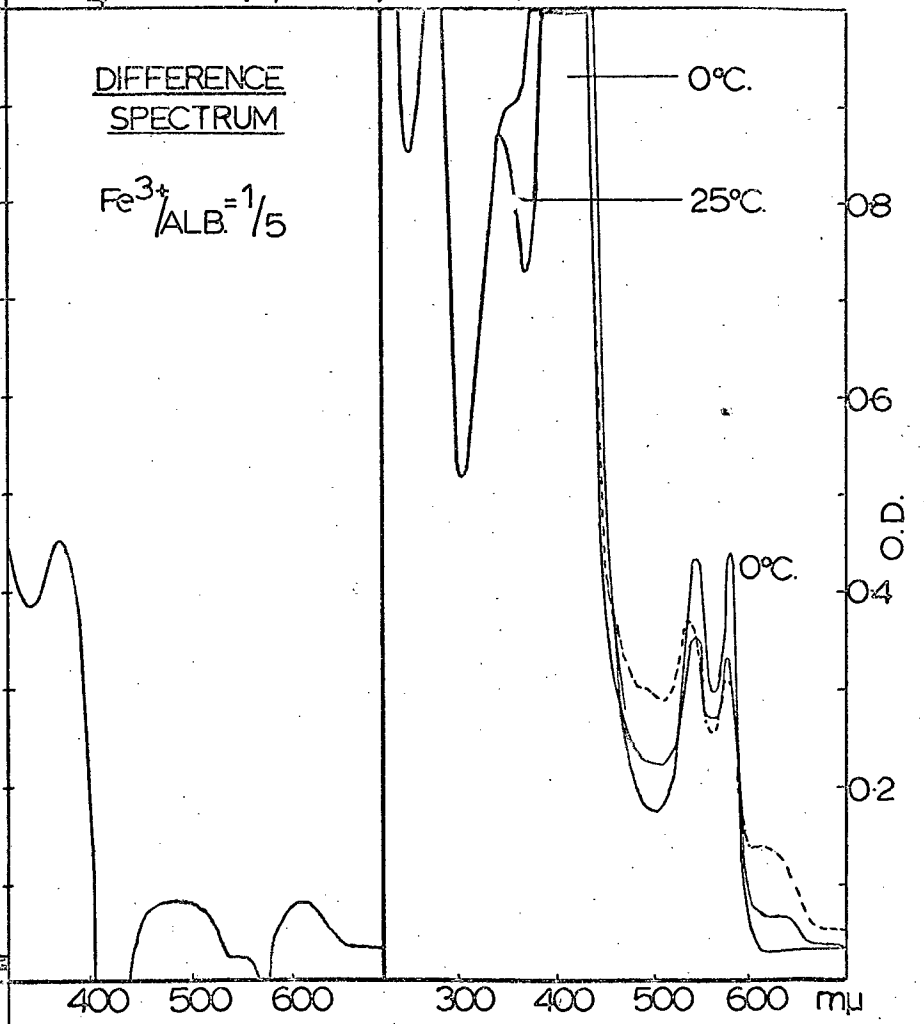


FIG. 29.

CO-HAEMOGLOBIN + ALBUMIN.

in PO_4 BUFFER; pH 7·0; 30 HRS. INCUBATION.



Frequently, spectra of this type were encountered initially and with subsequent incubation, the 370 mμ shoulder disappeared, accompanied by a rise in the Soret maximum of methaemalbumin. In the experiments at room temperature and neutral pH, usually only 20 - 30% of the albumin present, was involved in 1:1 stoichiometric binding of ferrihaem.

(iii) Influence of Temperature.

A neutral solution of haematin was added to albumin in a molar ratio of 2.2 to 1, assuming a molecular weight of 633.5 for haematin and 68,000 for albumin.

Haematin was added after the albumin solutions had been equilibrating in phosphate buffer, pH 7.0 for 30 minutes at 16°C and at 40°C. Haem blanks were incubated at the same time. At selected time intervals, the optical density of each sample at relevant wavelengths was read (Fig. 31), and their absorption spectra scanned. Difference spectra were obtained by comparing the albumin-haem mixtures with the respective haem blanks (Fig. 32). The relative quantities of free haematin and of methaemalbumin in each test sample (Table 13) were calculated from their extinction coefficients at 370 mμ and at 403 mμ (E mM = 46 and 39 for haematin, and E mM = 50 and 83 for methaemalbumin respectively (Rosenfeld and Surgenor[108])), by applying the following general formulae:

$$D_1 = xa_1 + yb_1$$

$$D_2 = xa_2 + yb_2$$

where a and b are the component pigments (e.g. haematin and methaemalbumin), x and y are the respective concentrations of each in the mixture, a_1 and b_1 are their corresponding extinction coefficients at the first wavelength (i.e. 370 mμ) and a_2 and b_2 are the extinction coefficients at 403 mμ. D_1 and D_2 are the observed densities of the mixture at 370 mμ and 403 mμ respectively (Hunter[158]).

TABLE 13.

	16.5°C			40°C		
	Ferrihaem alone	Albumin-Ferrihaem		Ferrihaem alone	Albumin-Ferrihaem	
	H	H	MHA	H	H	MHA
	μM	μM	μM	μM	μM	μM
0	15.1	15.1	(6.5 as albumin)	15.1	15.1	(6.5 as albumin)
3 min.	-	14.2	0.5		14.1	0.6
5 hr.	13.9	13.6	1.0	12.8	11.9	3.0
10-11 hr.	13.8	13.6	1.1	12.9	8.2	6.3
24 hr.	13.4	13.1	1.4	12.6	6.1	7.9

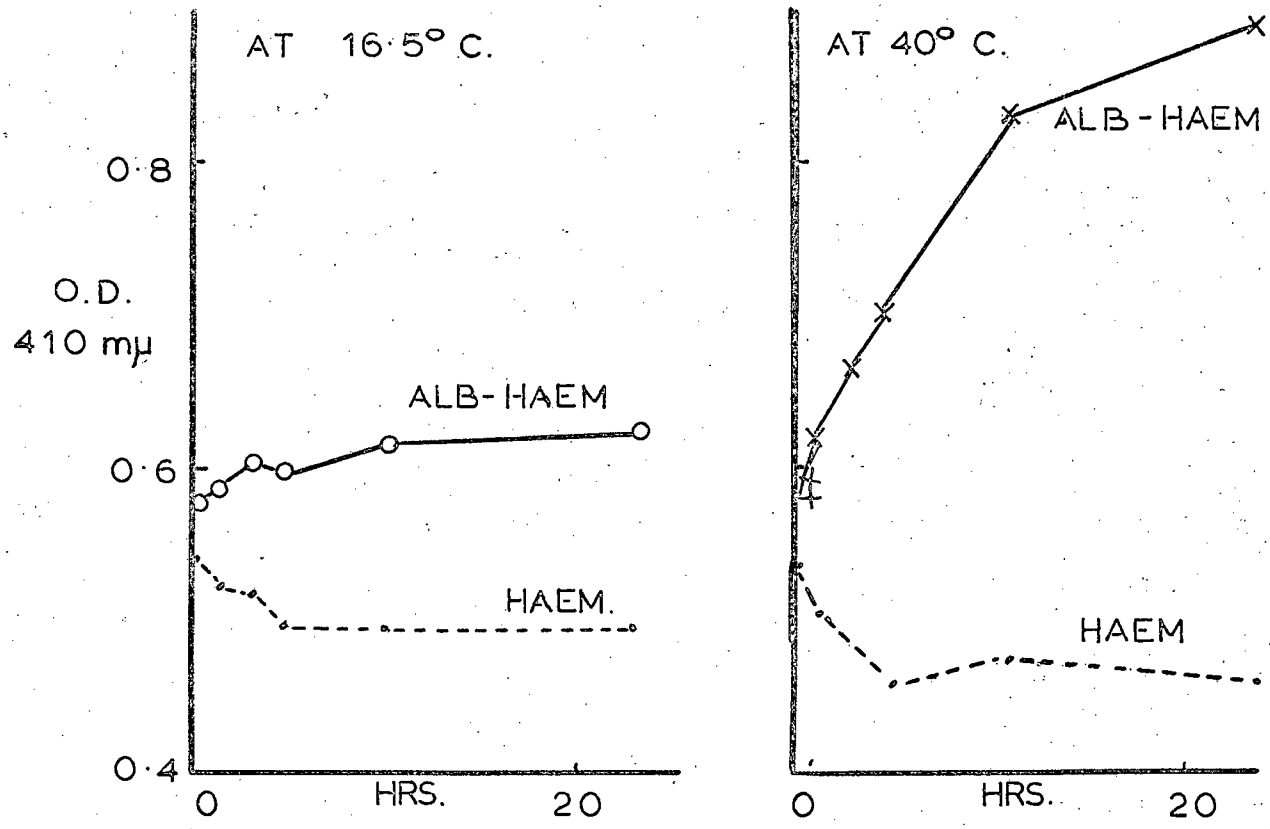
H = ferrihaem.

MHA = methaemalbumin.

The percentages of the original ferrihaem either oxidized or bound in 24 hours reveals the potent effect of increased temperature on the system, i.e.

FIG. 31.

EFFECT OF TEMPERATURE ON METHAEMALBUMIN FORMATION.



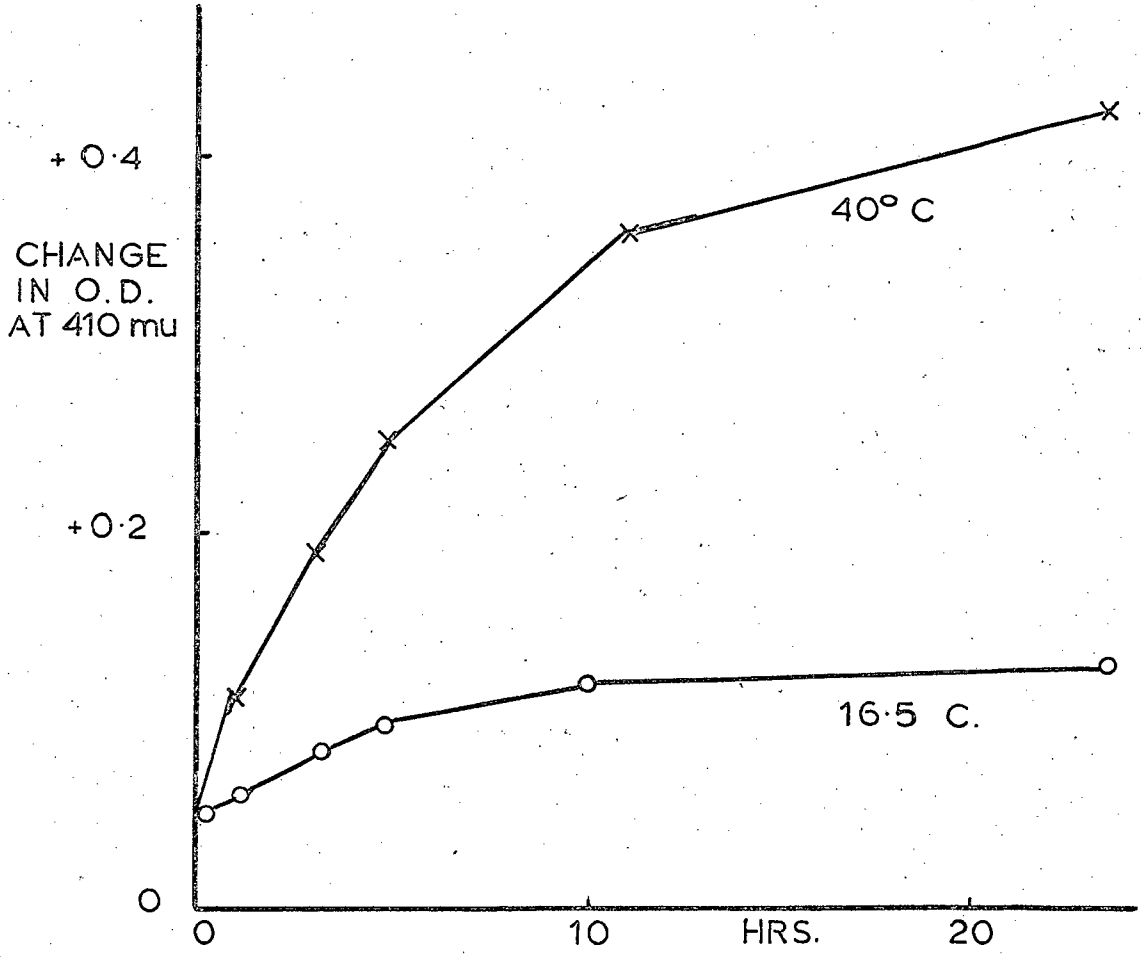
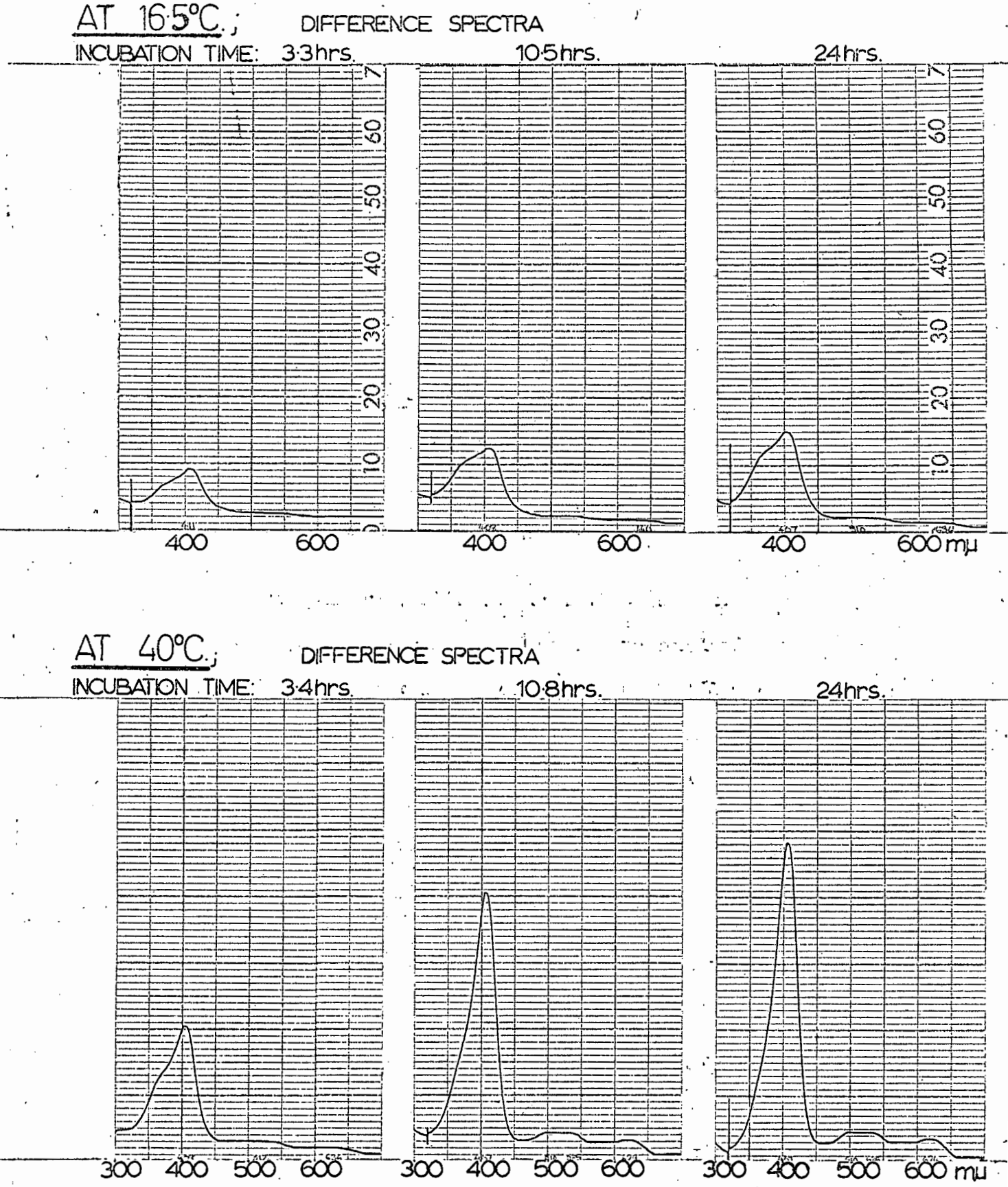


FIG. 32.

INFLUENCE OF TEMPERATURE ON METHAEMALBUMIN FORMATION.



		16.5°C	40°C
		%	%
Ferrihaem oxidized in blanks		11	17
Test:	ferrihaem bound	9	52
	free ferrihaem	87	40
	ferrihaem oxidized	3	8
	albumin used (1:1 haem)	22	120
	albumin used (1:2 haems)	11	60

At room temperature, it can be seen that at most, only 22% of the albumin molecules present in solution were involved in the binding of ferrihaem. Raising the temperature by about 24°C markedly increased the albumin affinity for haematin resulting in some molecules at least, binding more than one molecule of ferrihaem.

From the data, one can also see the extent to which albumin prevented haematin decomposition.

Ferrihaem undergoes autoxidation when exposed to oxygen in the air and this decomposition is enhanced by raising the temperature.

Since the ferrihaem blanks were not true controls under these circumstances, their optical densities were plotted together with the tests in the graphs of Fig. 31. The lower graph shows the relationship between the respective test samples after subtraction of the blank values.

The difference spectra consequently did not represent solely the spectral results of the interaction between ferrihaem and albumin

but also reflected the non-specific protective effect of the protein against haematin oxidation.

(iv) Influence of pH.

(a) Influence of pH on ferrihaem alone.

The solubility of haematin (ferriprotoporphyrin) in aqueous solution is maintained by means of the ionized carboxylic acid groups of the propionate side chains (Shack and Clark[155]). Therefore, in increasingly alkaline solutions, the ionization of the carboxyl groups is enhanced, rendering the protoporphyrin molecule more soluble.

The pigment in our neutral or slightly alkaline haematin preparations had a slight tendency to form aggregates on standing for long periods, and we found that, on acid titration, considerable haematin precipitation occurred at a pH of 6.5; and when the hydrogen ion concentration was increased still further, one could produce complete precipitation of pigment. Cowgill and Clark[159] encountered a similar influence of protonation (adding H^+ ions) on their solutions of ferrimesoporphyrin (a compound in which the 2 vinyl groups of protoporphyrin are replaced by 2 ethyl groups).

In aqueous alkaline and neutral solutions, haematin molecules form associated dimeric units, loosely bound together as colloidal aggregates. To form dimers, one of the 2 free iron ligands on a

single haem molecule co-ordinates in some manner, probably involving hydroxyl ions or water molecules (Shack and Clark[155]), with that of another; while the 2 remaining free ligands of the metal undergo co-ordinate bonding with water molecules; or, in alkali, with hydroxyl ions.

In their potentiometric studies, Shack and Clark found that in moderately alkaline solution (~pH 9), ferriprotoporphyrin occurs as a dimeric unit containing 1 hydroxyl ion per Fe atom[155]. On acid titration the compound behaved as if it were a univalent base, the ionization constant being between 7.4 and 7.6. Cowgill and Clark[159] estimated that pH 7.0 is the half-transformation point of ferrimesoporphyrin with respect to loss and gain of H^+ or OH^- at the co-ordination centre. The transformation points given by Phillips[148], however, are at considerably lower pH's; protohaemin $(Fe^{3+} - 2H_2O)^+$ being 50% transformed to protohaematin $(Fe^{3+} - H_2O - OH^-) + H^+$ at pH 5.9, while the corresponding mesohaemin, having less electrophilic side chains, is converted at pH 6.6.

Since, in our preparations, ferriprotoporphyrin was no longer soluble at a pH of 5.9, the determinations of Cowgill and Clark[159] appear to be more suitable for comparison in this case. In their experiments on iron ligand binding which were performed at neutral and alkaline pH's, their data indicate that the carboxyl groups of the porphyrin are completely ionized in this pH range and play no part in ligand or proton binding involving the co-ordination shell

of the Fe atom.

Rapid acid titration of an alkaline haematin solution (absorption maxima 610 mμ and 388 mμ) to pH 1.7 avoiding precipitation, resulted in a colour change from greenish-brown (alkaline haematin) to reddish-brown; the pigment having a spectrum with absorption maxima at 645 mμ and 375 mμ. It is not known whether this produced an altered form of ferrihaem or a reduction to ferrohaem.

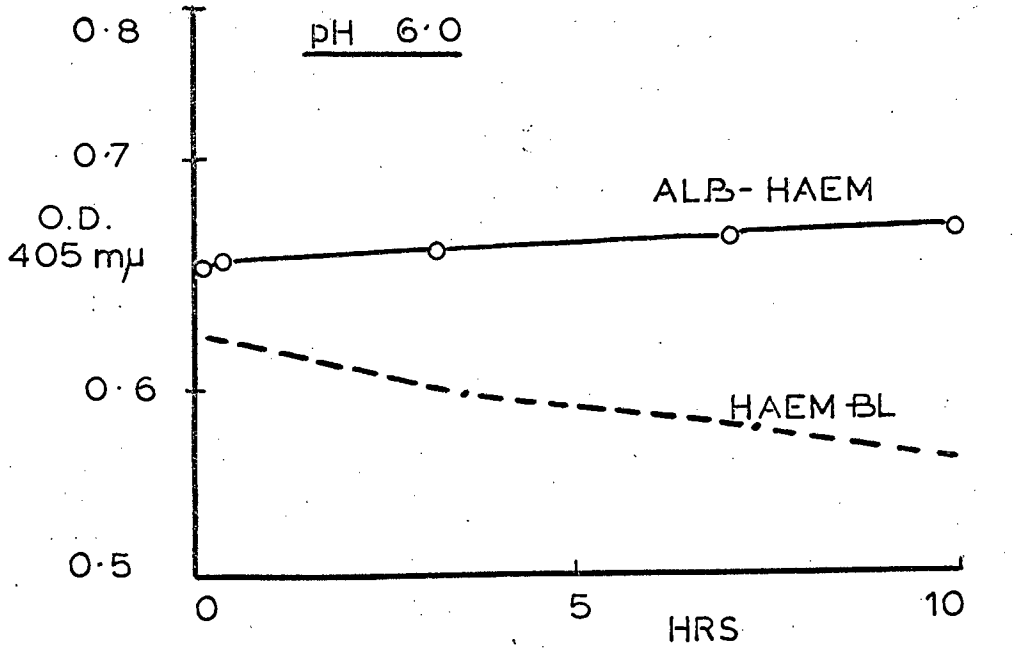
(b) Influence of pH on the ferrihaem-albumin system.

A neutral solution of ferrihaem was added to albumin in Sörensen phosphate (M/15) buffer solutions at pH's 6.0, 6.5, 7.4 and 7.7 in a ratio of 3.4 ferrihaem molecules to 1 of albumin. The test mixtures and ferrihaem blanks were incubated at 37°C for 24 hours. Aliquots were removed for spectrophotometric analysis at chosen times.

The optical densities at 405 mμ (representing methaemalbumin formation) were plotted over the initial 10-hour period and are shown in Figs. 33 and 34. The decrease in absorption of the respective haem blanks are presented in order to illustrate the contribution of this phenomenon to the difference spectra, obtained during the experiment (Fig. 35) and to the corrected curves in Fig. 36.

The graphs in Fig. 37 are compiled from the methaemalbumin formed (as evidenced by increased absorption in the Soret region)

FIG. 33. INFLUENCE OF pH ON METHAEMALBUMIN FORMATION



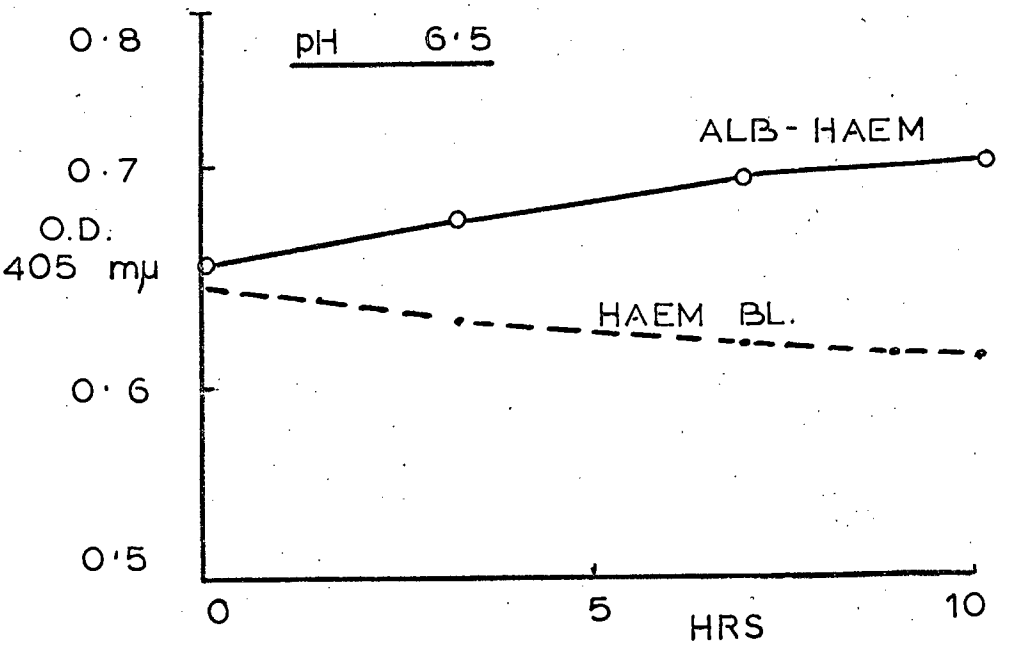
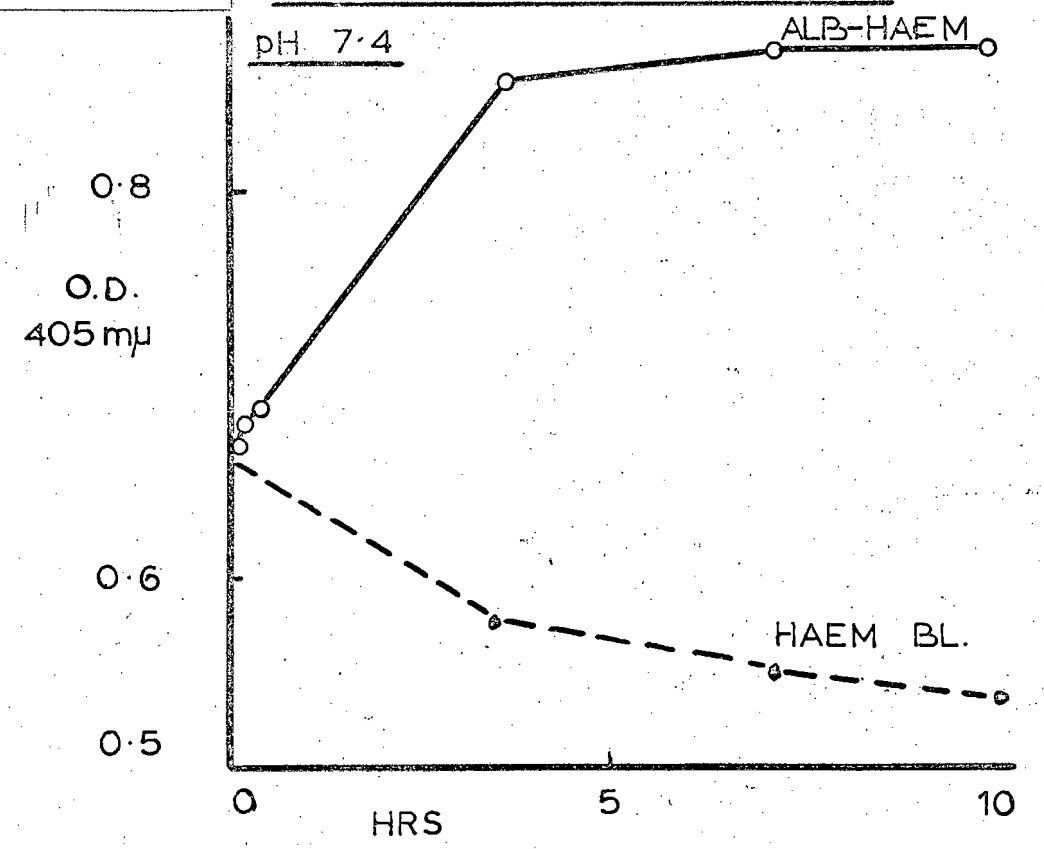


FIG. 34.

INFLUENCE OF pH ON METHAEMALBUMIN FORMATION



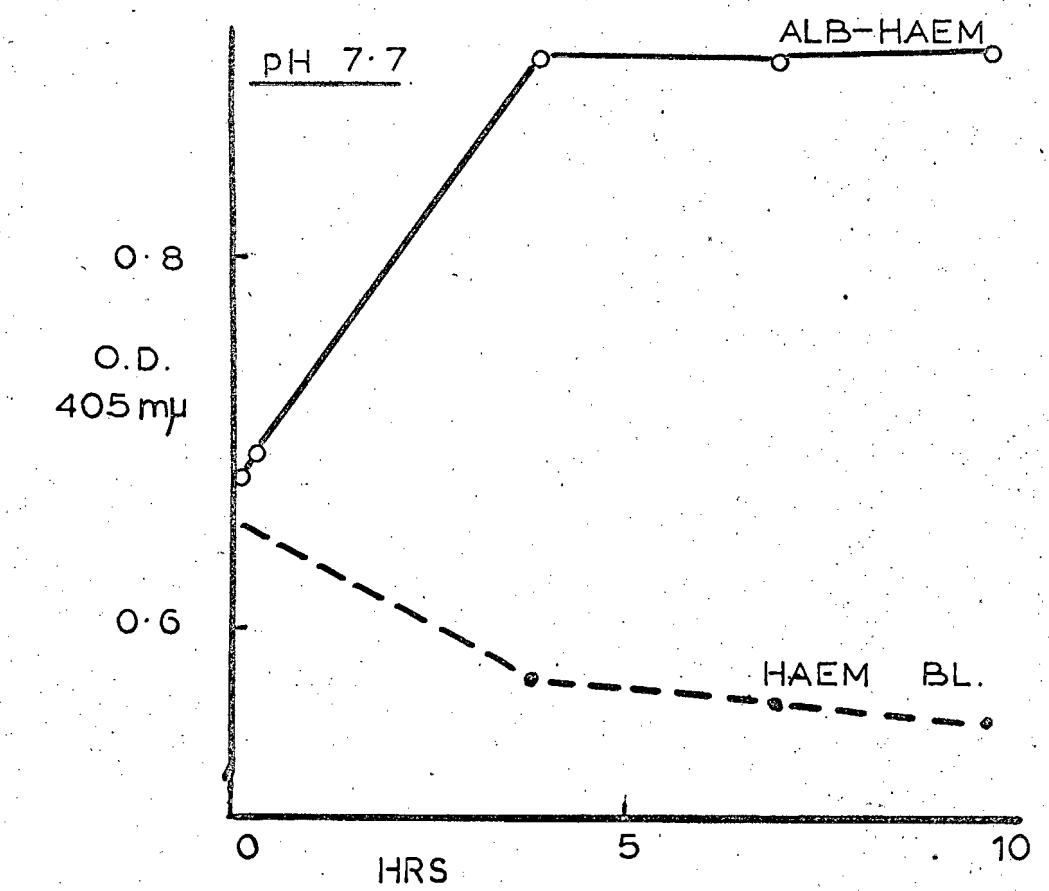
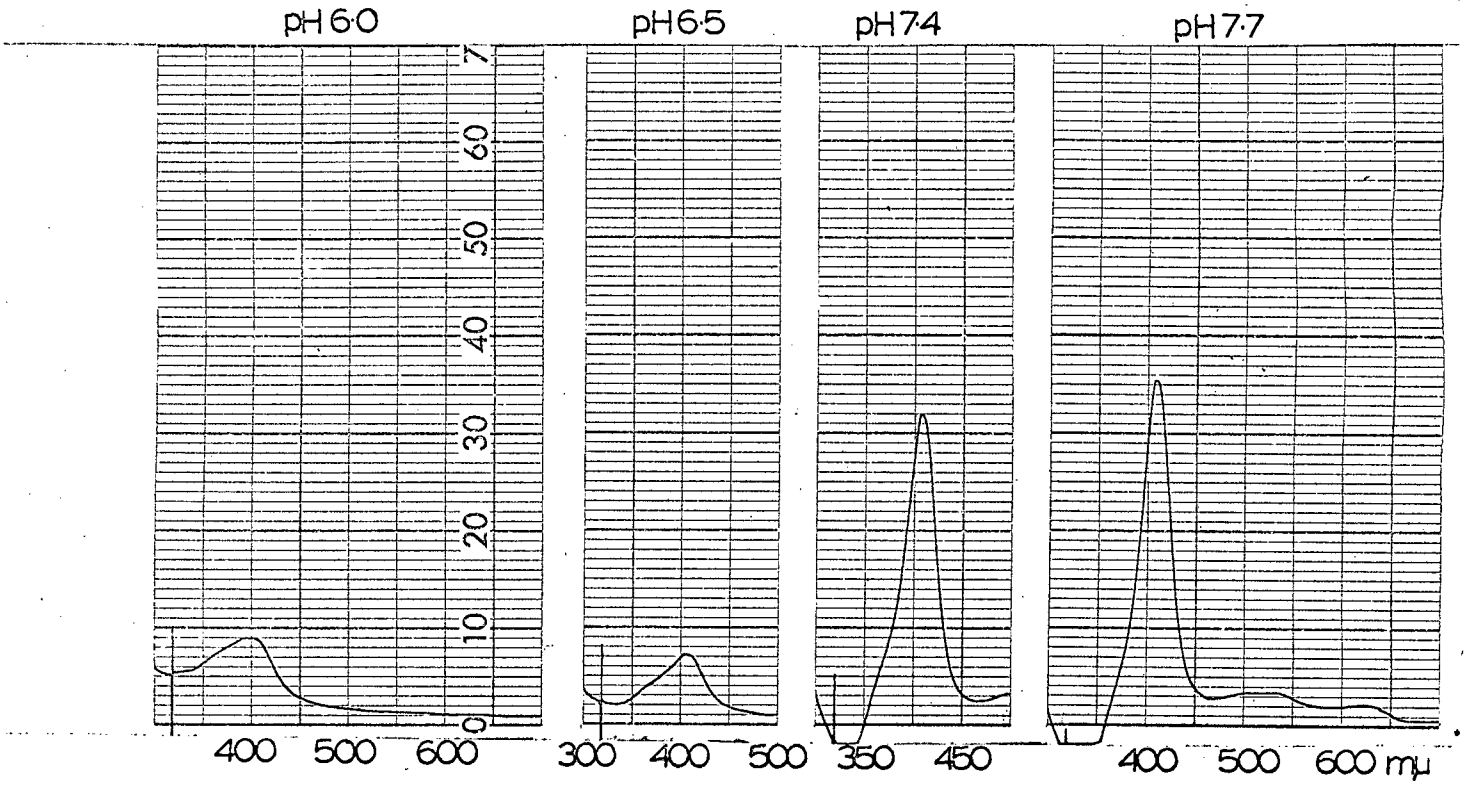


FIG. 35.

INFLUENCE OF pH ON METHAEMALBUMIN FORMATION.

DIFFERENCE SPECTRA AFTER 3-4 HRS. INCUBATION. AT 37°C.



DIFFERENCE SPECTRA AFTER 24 HRS INCUBATION AT 37°C.

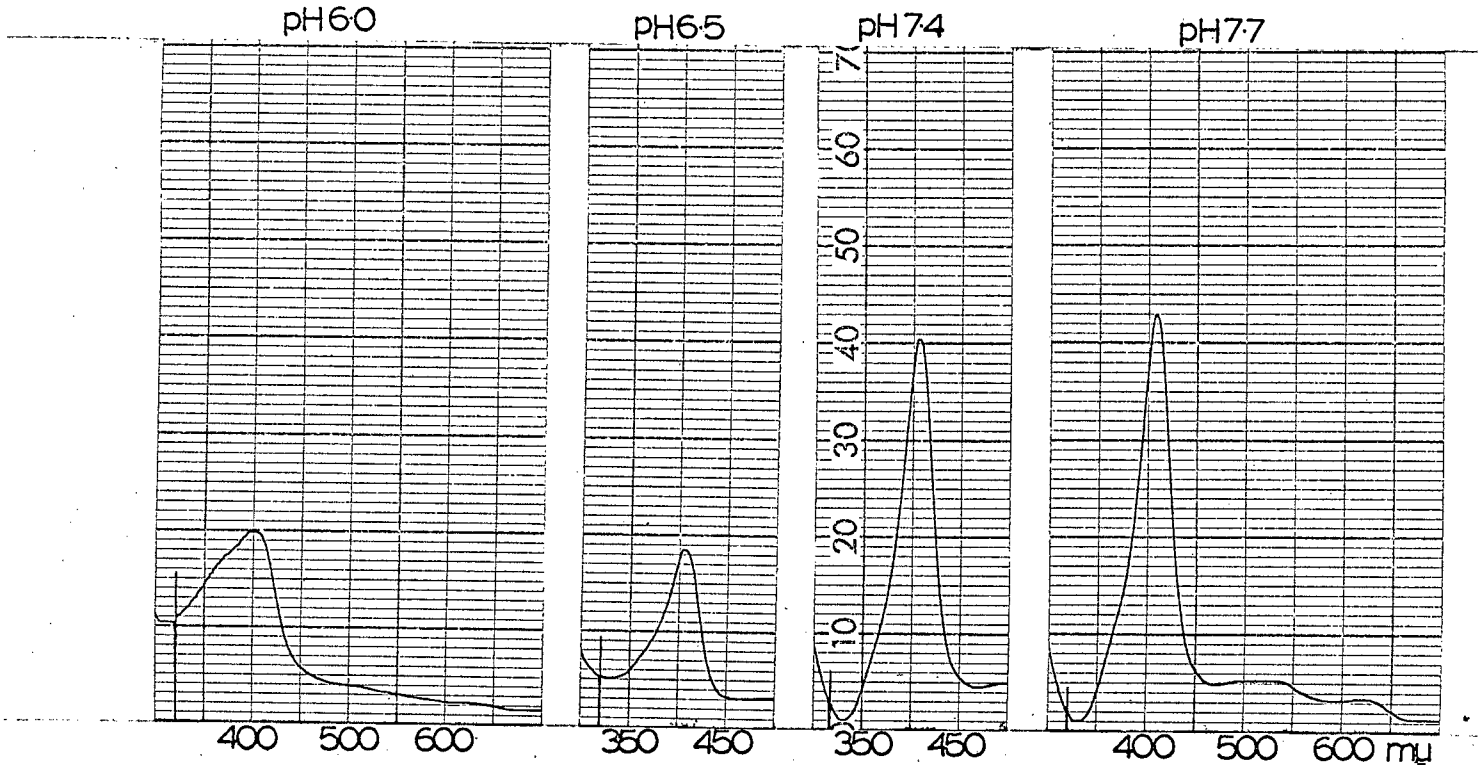


FIG. 36.

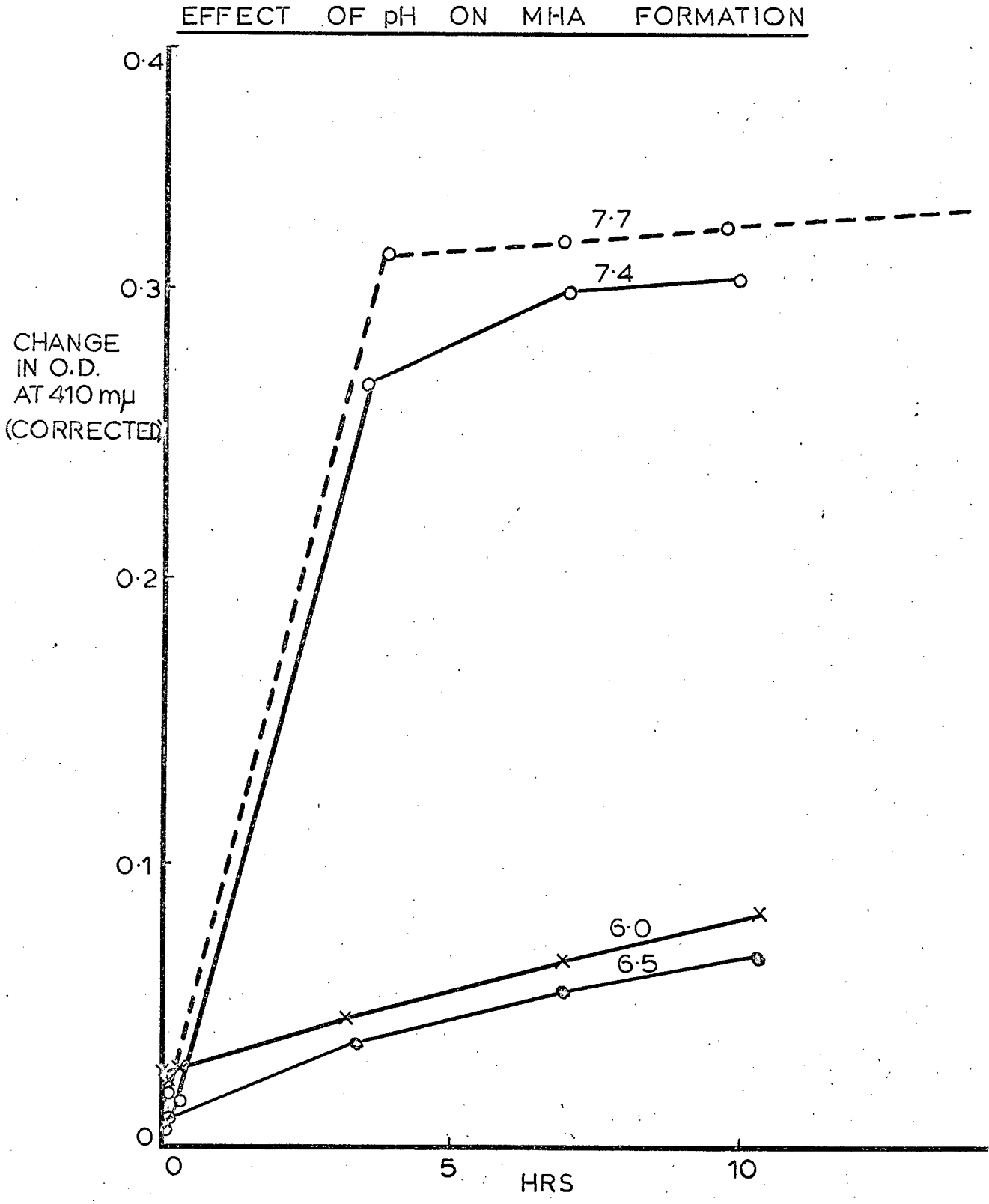
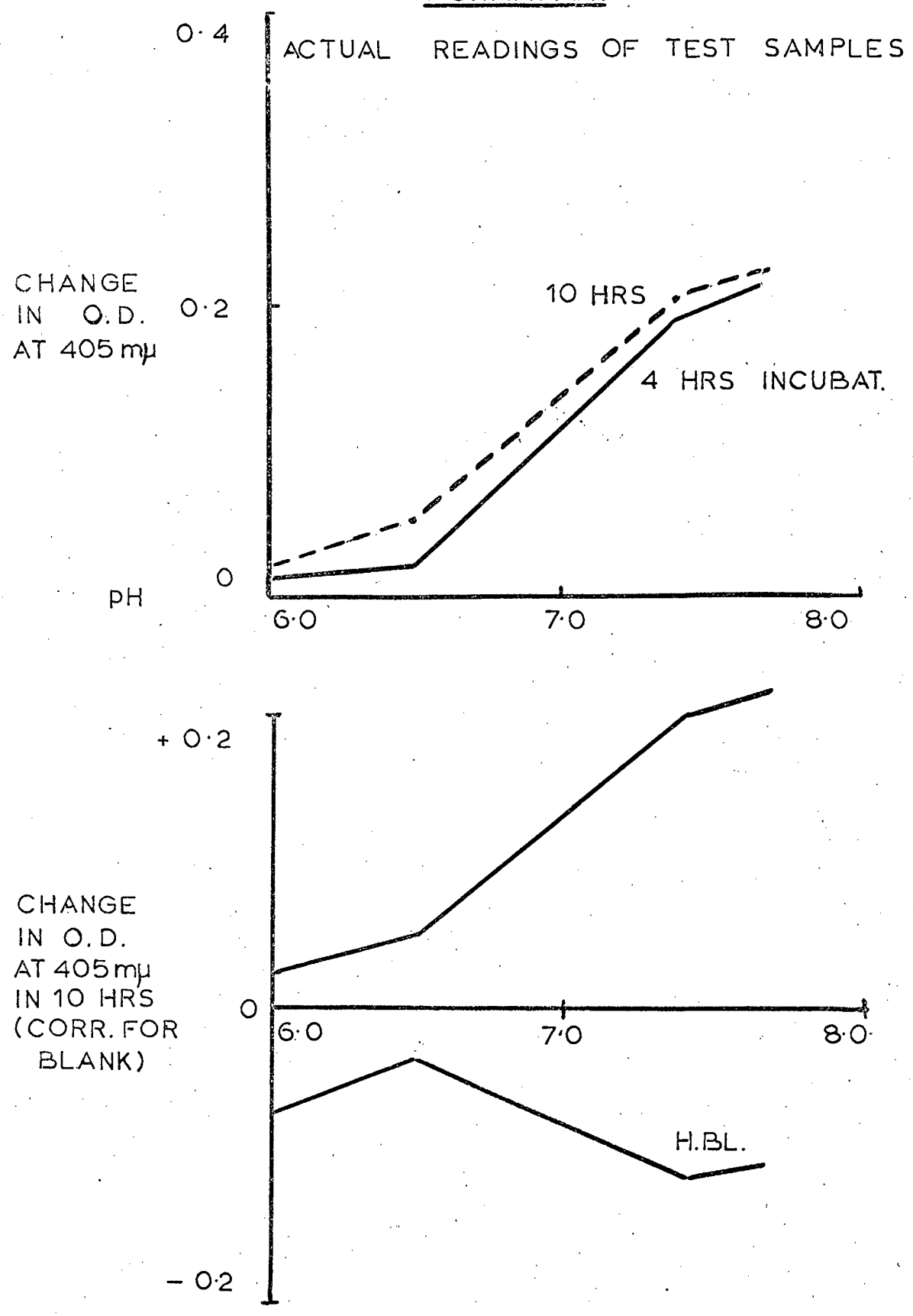

EFFECT OF pH ON MHA FORMATION
0·4
0·3
0·2
0·1
0
CHANGE IN O.D. AT 410 mμ (CORRECTED)
7·7
7·4
6·0
6·5
0
5
10
HRS

FIG. 37.

INFLUENCE OF pH ON MHA FORMATION



at the different pH's at 4 and 10 hours respectively. Fig. 38 shows 2 additional points (at pH 7.0 and 4.4) obtained from other experiments carried out under slightly different conditions and with lower molar concentrations of ferrihaem. Since the ratio to albumin was always more than 2 to 1, however, the ferrihaem concentration was not a limiting factor.

The relative quantities of ferrihaem and methaemalbumin in the mixtures were calculated as before from the optical densities at 370 mμ and 403 mμ. The results are shown in Table 14.

The percentage of ferrihaem which was oxidized or bound in each sample, and the proportion of albumin involved in binding in the 24-hour experimental period was as follows:

TABLE 15.

	pH 6.0	pH 6.5	pH 7.4	pH 7.7
	%	%	%	%
Haematin oxidized in blanks	23	8	13	14
Test: haematin bound	9	16	38	41
haematin oxidized	8	5	11	10
albumin involved in 1:1 binding.	30	53	130	138

For calculating the relative proportions of pigments in the incubated mixtures the extinction coefficient of methaemalbumin, in which ferrihaem is bound in a 1:1 ratio to albumin, was used.

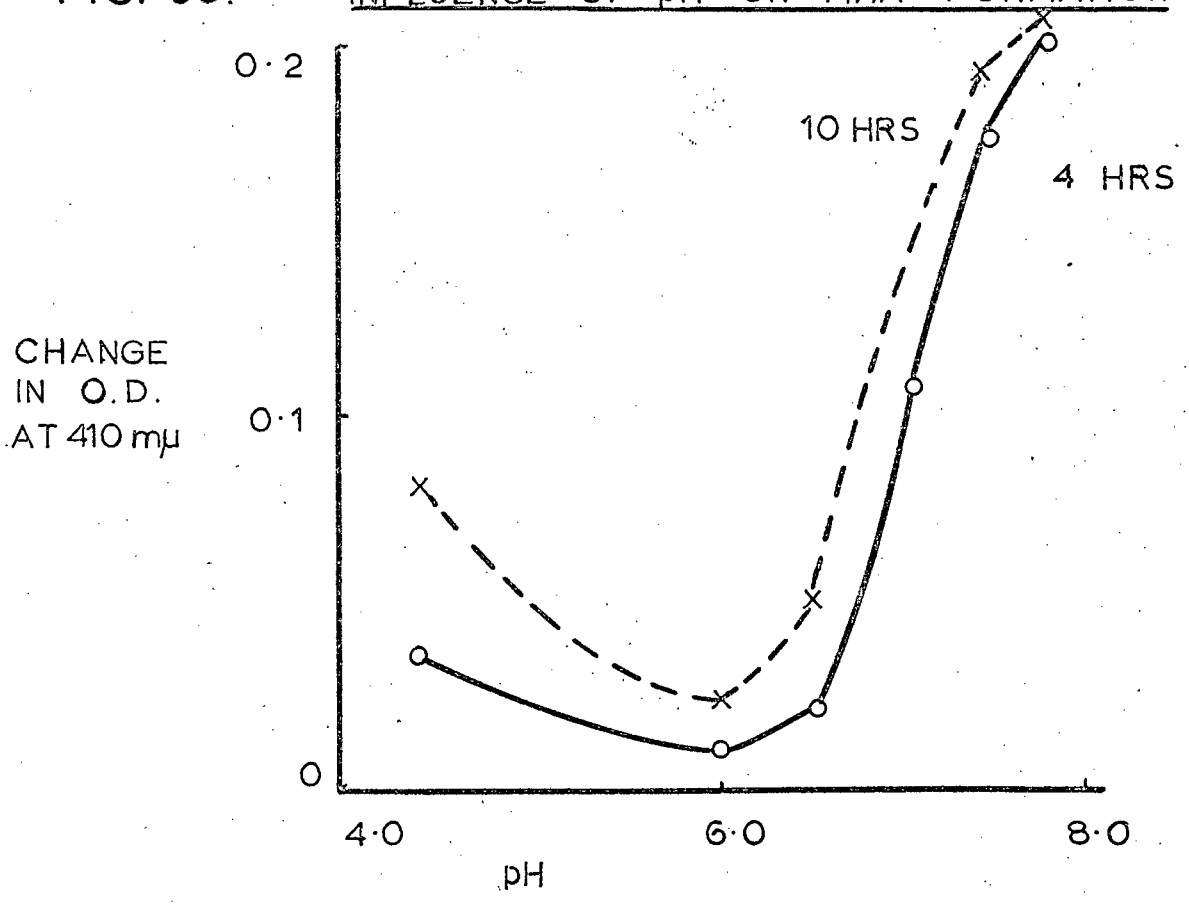
TABLE 14.

pH	6.0			6.5			7.4			7.7		
Sample	Ferrihaem alone	Test		Ferrihaem alone	Test		Ferrihaem alone	Test		Ferrihaem alone	Test	
Component	H	H	MHA	H	H	MHA	H	H	MHA	H	H	MHA
Time	µM	µM	µM	µM	µM	µM	µM	µM	µM	µM	µM	µM
2 mins.	16.4	15.2	0.6	17.0	15.5	0.5	17.0	15.5	0.5	17.0	15.4	0.6
3-4 hrs.	15.5	15.0	0.8	16.5	15.1	0.9	15.9	10.5	5.0	15.9	9.1	6.1
7 hrs.	14.9	15.0	0.8	16.1	14.7	1.3	15.1	9.4	5.7	15.5	8.8	6.2
10 hrs.	14.4	14.7	1.0	15.7	14.4	1.6	14.6	8.9	5.9	15.0	8.7	6.4
24 hrs.	13.1	13.5	1.5	15.7	13.4	2.7	14.7	8.6	6.5	14.7	8.5	6.9

MHA - methaemalbumin

H - ferrihaem

FIG. 38.


INFLUENCE OF pH ON MHA FORMATION
0·2
0·1
0
CHANGE IN O.D. AT 410 mμ
10 HRS
4 HRS
4·0
6·0
8·0
pH

The molar extinction coefficient of the complex containing 2 ferrihaem groups per molecule of albumin is slightly less than twice that value, i.e. $E(1:1)_M = 8.3 \times 10^4$ and $E(2:1)_M = 15 \times 10^4$ (Rosenfeld and Surgenor[108]). At the alkaline pH's, where at least some albumin molecules take up 2 ferrihaem groups, the calculations may produce spurious results, although we found the values generally matched the results shown in the spectra.

The high level of "oxidation" ascribed to the blank at pH 6 probably reflects the diminished solubility at this pH. The relatively greater fall in blank absorption at pH 6.0 is also shown in the difference spectra in Fig. 35. The slightly different shape and higher level of absorption of the pH 6.0 spectrum, as compared with that of pH 6.5, is due to decreases in the blank and not to changes in absorption of the test sample. The large difference in haematin-binding capacity of the albumin between pH's 6.5 and 7.4, led us to conclude that the increased affinity of albumin for ferrihaem at alkaline pH's is almost entirely due to the higher ionization of the propionic acid side chains. If it was due to transformation of ferriprotoporphyrin to ferriporotoporphyrin-hydroxide the curve of Fig. 38 would be shifted towards more alkaline pH's. At no time, was the haematin concentration a limiting factor; over half the initial quantity was still unbound after 24 hours at pH 7.7 (molecular ratio 3.4 haems : 1 albumin). Rosenfeld and Surgenor also reported on the influence of pH on methaemalbumin formation stating that it varied between pH 4.5 and 7.0 and with buffer

composition, and thereafter, i.e. from pH 7.0 to 10.0, maximal binding occurred[108]. Our results do not quite fit this picture but this is probably due to our concentrating on differences in a narrower pH range, nearer the physiological.

Cowgill and Clark claimed, in their studies on ligand binding (imidazoles) by ferrimesoporphyrin, that at neutral and alkaline pH's the porphyrin carboxyl groups were highly ionized and did not contribute to differences in the ligand binding properties - these being altered solely as a function of competition between OH^- ions for the sixth co-ordinating link of the Fe atom[159].

In the light of this evidence, the variations we encountered in the vicinity of pH 7 clearly indicate little influence of iron ligand bonds on methaemalbumin formation, other than their possibly slight effect on the side chains, by altering electron densities within the molecule.

The slight rise in binding affinity for haematin seen at pH 4.4 in Fig. 38 is probably due to difficulties encountered with solubility of the ferrihaem in the blank preparations. Even at this low pH, the albumin appears to stabilize the haematin in solution. Conformational changes in the albumin molecule at this pH may also be responsible.

Although we did not perform an experiment to test whether there is a temperature coefficient of the reaction at different pH's, the results obtained at pH 7, in the previous experiment,

suggest that variations due to pH are enhanced by raising the temperature.

(v) Effect of unsaturated fatty acids.

Albumin in plasma is known to bind long-chain non-esterified fatty acids to the extent of 10 - 40 mg. per 100 ml. of plasma (Harper[160]). The binding of small molecules to albumin is sometimes affected by the presence of fatty acids, e.g. indole binding (McMenamy[130]). We wished to see whether added fatty acids would affect the binding of haematin to albumin. There were other reasons for investigating the tertiary relationship of haematin, albumin and fatty acids and these are concerned with the well-known catalytic effect of haematin and its compounds on peroxidation of unsaturated lipids; and studied mainly in connection with the development of rancidity in food products (Tappel[161]). The concomitant destruction of the haematin catalyst during the peroxidation process was first described by Haurowitz, Schwerin and Yenson in 1941[54]. However, Kench and Varma[39] found that erythrocytic membranes, which are composed of lipoprotein complexes, had no effect on their in vitro peroxidative system in which haemoglobin is degraded to bile pigments by coupled oxidation with ascorbic acid in air. Recently, Nishida and Nishida have shown that a peroxidized β_1 serum lipoprotein forms an association with free haematin, when incubated with methaemoglobin[162]. In view of the haemolytic action of long-chain fatty acids on washed mammalian erythrocytes (Greisman[163]) and their potent inhibitory effect (reversed by albumin) on oxidative phosphorylation in isolated

mitochondria (Björntorp[156]), it seemed profitable to investigate their effect on methaemalbumin formation.

Colloidal suspensions of unsaturated fatty acids (only linoleic and arachidonic acids will be considered here) were prepared by dissolving a weighed quantity of the pure fatty acid (British Drug Houses, Ltd.) in 3 ml. 0.1 N NaOH; diluting with distilled water and titrating with 0.1 N HCl until the pH was neutral or just alkaline. The suspension was then diluted to the desired concentration. Under these conditions the opalescent suspension was stable. Heating, or standing at 0°C for long periods, resulted in a decrease in the absorbance of the fatty acid suspensions, due to autoperoxidation.

The co-oxidative chain reaction, whereby haematin catalyses the breakdown of lipid peroxides will be discussed in detail under "Haem Degradation Systems" (para 2.233 E). Suffice to say here, that, depending on the species and on the concentration of unsaturated fatty acid, and on the initial state of peroxidation of the suspensions tested, the interaction between ferrihaem and an unsaturated long-chain fatty acid results in oxidation of the haematin molecule (as judged by a fall in spectral absorption in the Soret region) to unknown products.

Of the 4 long-chain unsaturated fatty acids tested, i.e. oleic, linoleic, linolenic and arachidonic, our preparations of linoleic acid appeared to be the most active in oxidizing ferrihaem and we

chose to use these suspensions for most of the experiments.

Since we were concerned with interactions of a physiological nature we used far higher molar ratios of haem to fatty acid than previous experimenters who were more concerned with haematin acting as a catalyst in the peroxidation reactions (Haurowitz et al.[54]; Tappel[161]; Kaufmann and Kaufmann[164]). For most of the tertiary experiments with albumin, the molar ratio of fatty acid to ferrihaem was between 10 and 20.

Generally, the results of preincubating linoleate with non-defatted or defatted albumin for a short period prior to addition of ferrihaem, were as follows:

(a) The rate of methaemalbumin formation was increased.

(b) The binding of ferrihaem (as seen in difference spectra) was altered; there being a single sharp peak at 405 - 410 mμ compared with the rather diffuse biphasic maximum obtained when incubating albumin and ferrihaem alone as a control in the same experiments. In some cases this was due to coupled oxidation of the associated free haematin with fatty acid.

(c) Usually the ultimate quantity of methaemalbumin formed was more than that in the samples containing albumin and haem alone, but depended on the quantity of ferrihaem oxidized (presumably by unbound fatty acid) and the conditions of temperature, pH, and initial state of peroxidation of the fatty acid by oxygen of the air, as well as the length of incubation time.

(d) Albumin always protected the haematin to some extent from oxidation by fatty acid.

Certain difficulties were encountered in quantitating the results as none of the blanks acted as adequate controls. Opalescence in the samples containing fatty acid increased the optical density in the Soret region by varying degrees, depending on the quantities of haem or albumin present, or both.

The haematin, by catalysing peroxidation of fatty acid, renders it more soluble; thereby decreasing the opalescence of the fatty acid-haem solutions so that the control solution containing fatty acid alone is too turbid to act as a blank. Similarly, albumin binds added fatty acid, thus decreasing turbidity somewhat, but prevents haematin from catalysing peroxidation to the full extent of the fatty-acid-haematin mixtures.

Calculation of the quantities of haematin and methaemalbumin in the samples (Hunter[158]) solves this problem as it depends on a comparison between the optical densities at 2 wavelengths in the Soret region (370 mμ and 403 mμ) which we assumed to be equally affected by turbidities due to added fatty acid.

The results of 4 experiments performed under varying conditions of pH, fatty acids bound to albumin, and length of incubation time of fatty acid with albumin prior to addition of ferrihaem, are presented on the following pages. The graphs of the first experiment (Fig. 39A and B) illustrate the differences between plotting the optical densities observed and the calculated values.

TABLE 16.

EXPERIMENT I: EFFECT OF LINOLEATE ON METHAEMALBUMIN FORMATION.

	Ferrihaem alone	Linoleate + ferrihaem	Albumin + Ferrihaem		Albumin + Linoleate + Ferrihaem	
	H	H	H	MHA	H	MHA
	mM x 10^{-3}	mM x 10^{-3}	mM x 10^{-3}	mM x 10^{-3}	mM x 10^{-3}	mM x 10^{-3}
2 mins.	15.3	13.0	14.5	0.5	13.9	0.7
15 mins.		11.8	14.3	0.7	12.5	1.5
30 mins.		11.6				
2.5 hrs.		11.0	13.4	1.7	8.6	4.2
3.3 hrs.	14.5					
~5 hrs.		10.8	12.6	2.5	8.0	4.8
6.2 hrs.	13.0					
10 hrs.	12.5	10.6	10.0	4.7	7.3	5.1
At 10 hrs.:						
Haem oxidised	18%	31%	4%		18%	
Haem free			65%		48%	
Haem bound as MHA				31%		34%
Albumin involved in 1:1 binding.				72%		79%

H - Ferrihaem MHA - Methaemalbumin

The experiment was performed at pH 7.3 in 0.1M phosphate buffer at 38°C. The final concentrations of the components were as follows:

Albumin	6.5×10^{-3} mM
Linoleate	2.5×10^{-1} mM
Ferrihaem	15.3×10^{-3} mM

in a molar ratio of 1:37:2.

The fatty acid was preincubated with albumin for 40 minutes before adding ferrihaem.

Results are graphed in Fig. (39 B).

FIG. 39. EFFECT OF LINOLEATE ON MHA FORMATION

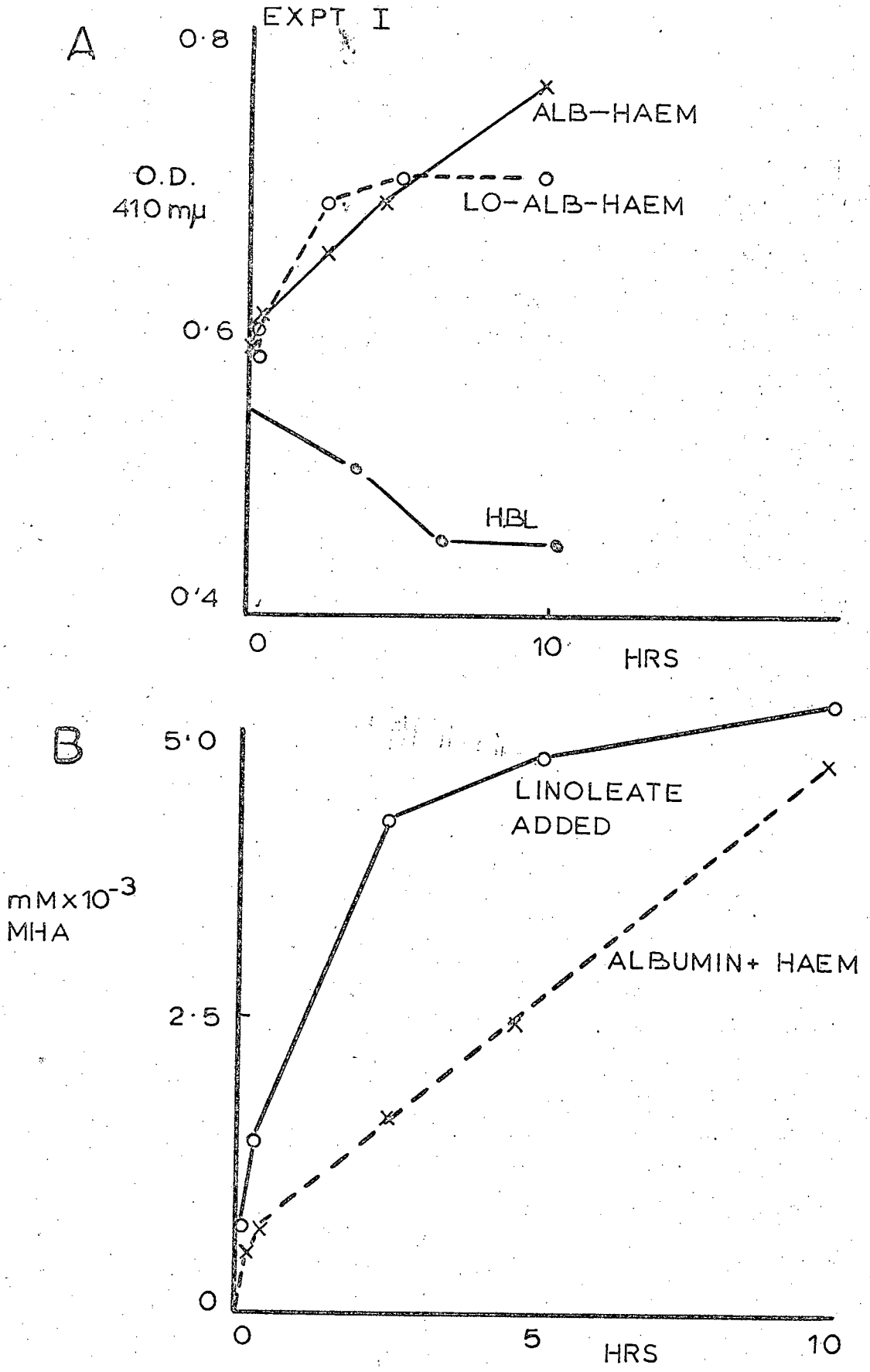
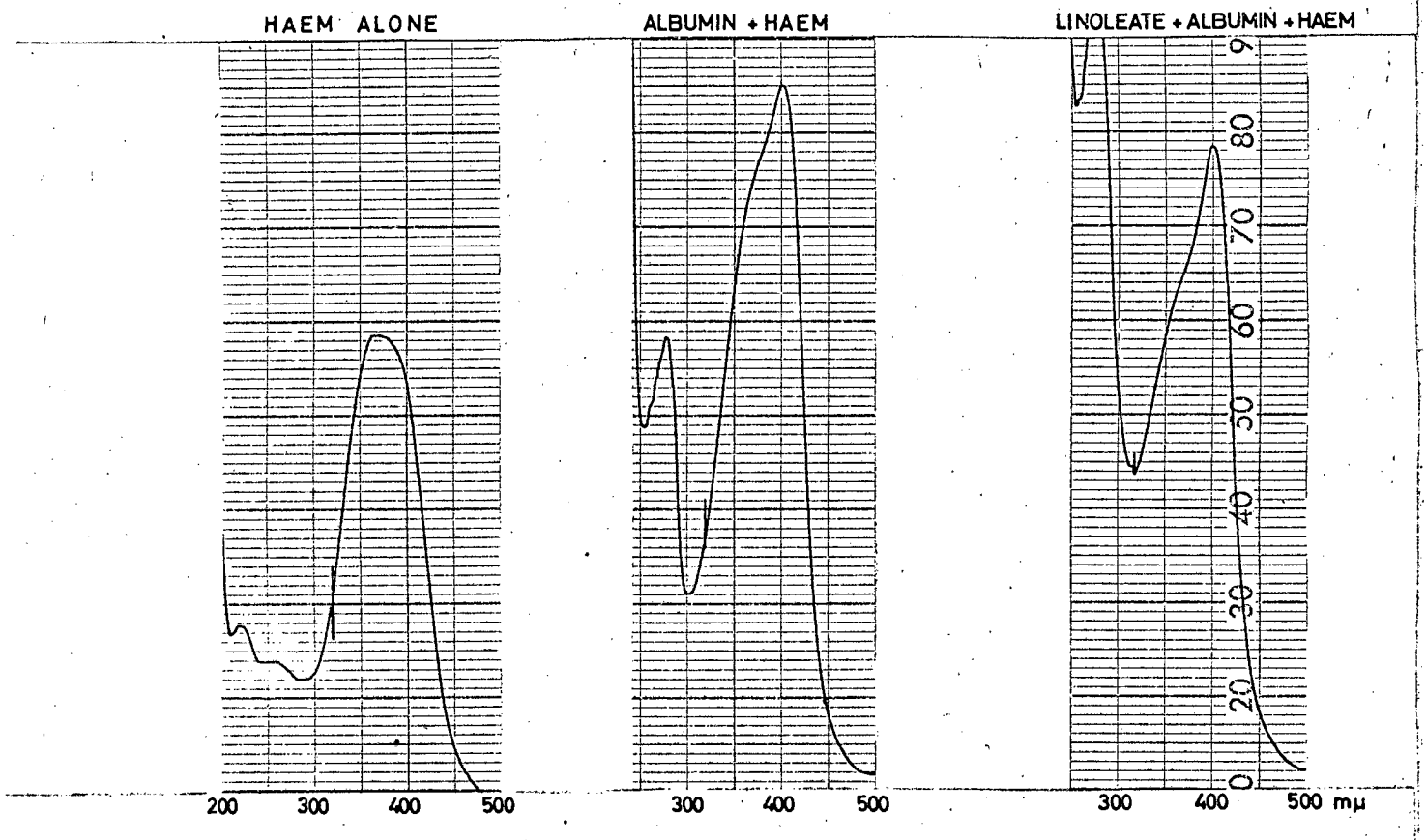


FIG. 40.

EXPT. I EFFECT OF LINOLEATE ON METHAEMALBUMIN FORMATION AT pH 7·3.

Spectra after 10 hrs. incubation at 38°C.



Difference spectra after 11 hrs. incubation at 38°C.

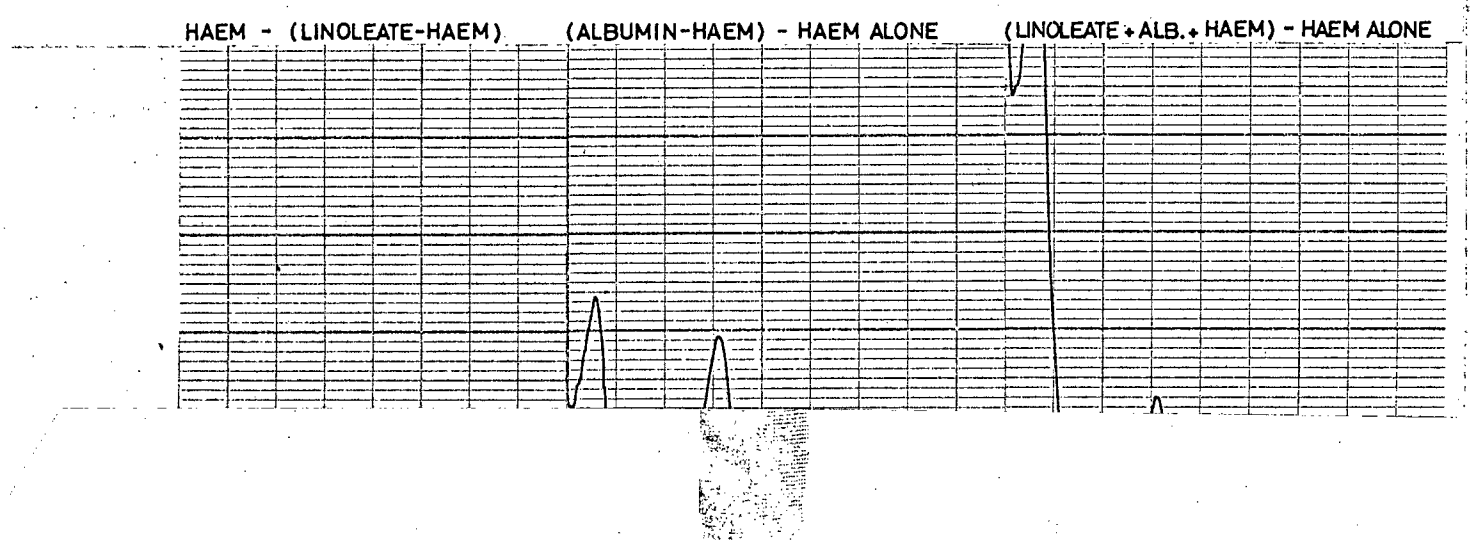


TABLE 17.

EXPERIMENT II: EFFECT OF ADDING LINOLEATE ON METHAEMALBUMIN FORMATION FROM DEFATTED ALBUMIN.

	Ferrihaem alone	Linoleate + Ferrihaem	Albumin + Ferrihaem		Albumin + Linoleate + Ferrihaem	
	H	H	H	MHA	H	MHA
	mM x 10^{-3}	mM x 10^{-3}	mM x 10^{-3}	mM x 10^{-3}	mM x 10^{-3}	mM x 10^{-3}
1 min.		14.0				
2 mins.	15.4				14.3	0.4
3 mins.		13.3				
4 mins.			14.7	0.5		
5 mins.					14.0	0.6
18 mins.		12.4	14.6	0.5	13.2	1.1
2.5 hrs.	14.7	11.5	14.1	1.1	11.0	2.5
4.8 hrs.	14.5	11.3	13.8	1.5	10.0	3.3
8.4 hrs.	12.9	11.0	13.2	2.1	8.8	4.1
At 8.4 hrs.:						
Haem oxidised	16%	29%	0%		16%	
Haem free			86%		57%	
Haem bound as MHA				14%		27%
Albumin involved in 1:1 binding				33%		65%

H - Ferrihaem
MHA - Methaemalbumin

The samples were incubated at 37°C in 0.1M phosphate buffer at pH 7.2. The defatted albumin, prepared by the method of Goodman, was incubated with fatty acid for 90 minutes before ferrihaem was added. The final concentrations in the samples were as follows:

Albumin	6.3×10^{-3} mM
Linoleate	2.5×10^{-1} mM
Ferrihaem	15.4×10^{-3} mM

in a molar ratio of 1:38:2. Fig. 41 illustrates these results.

FIG. 41.

EXPT II EFFECT OF LINOLEATE ON METHAEMALBUMIN FORMATION

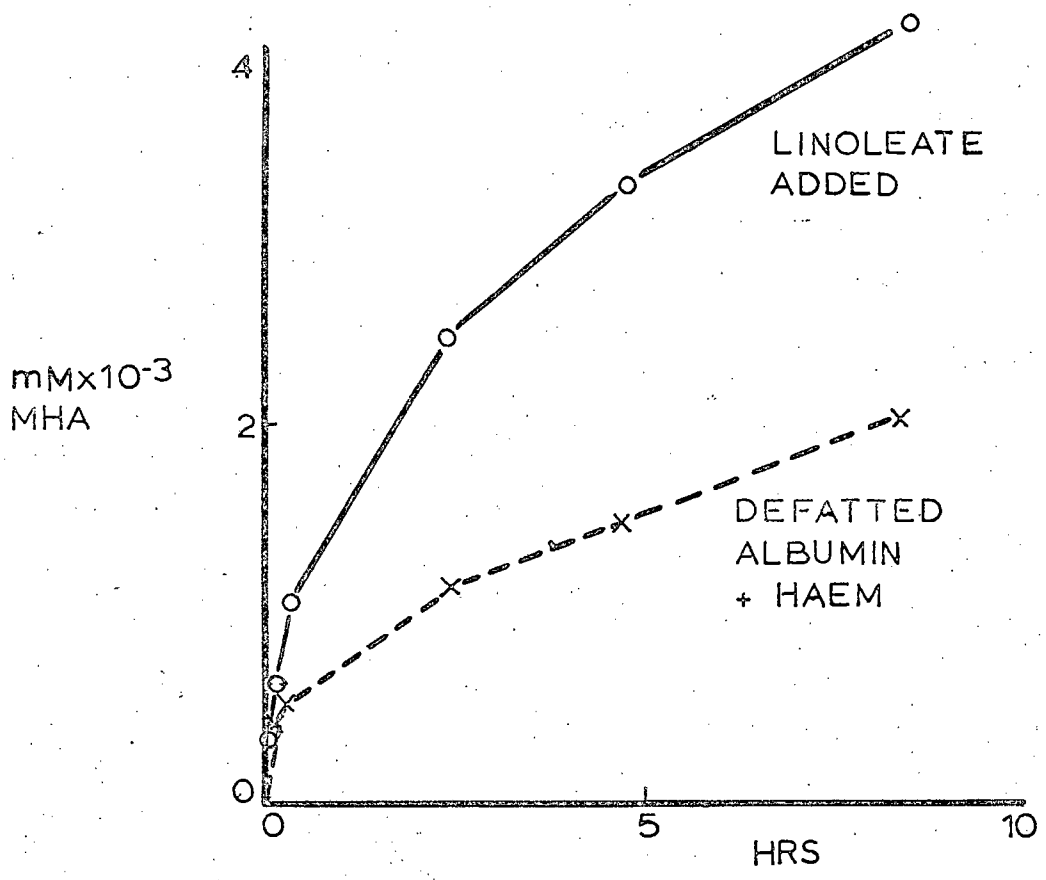
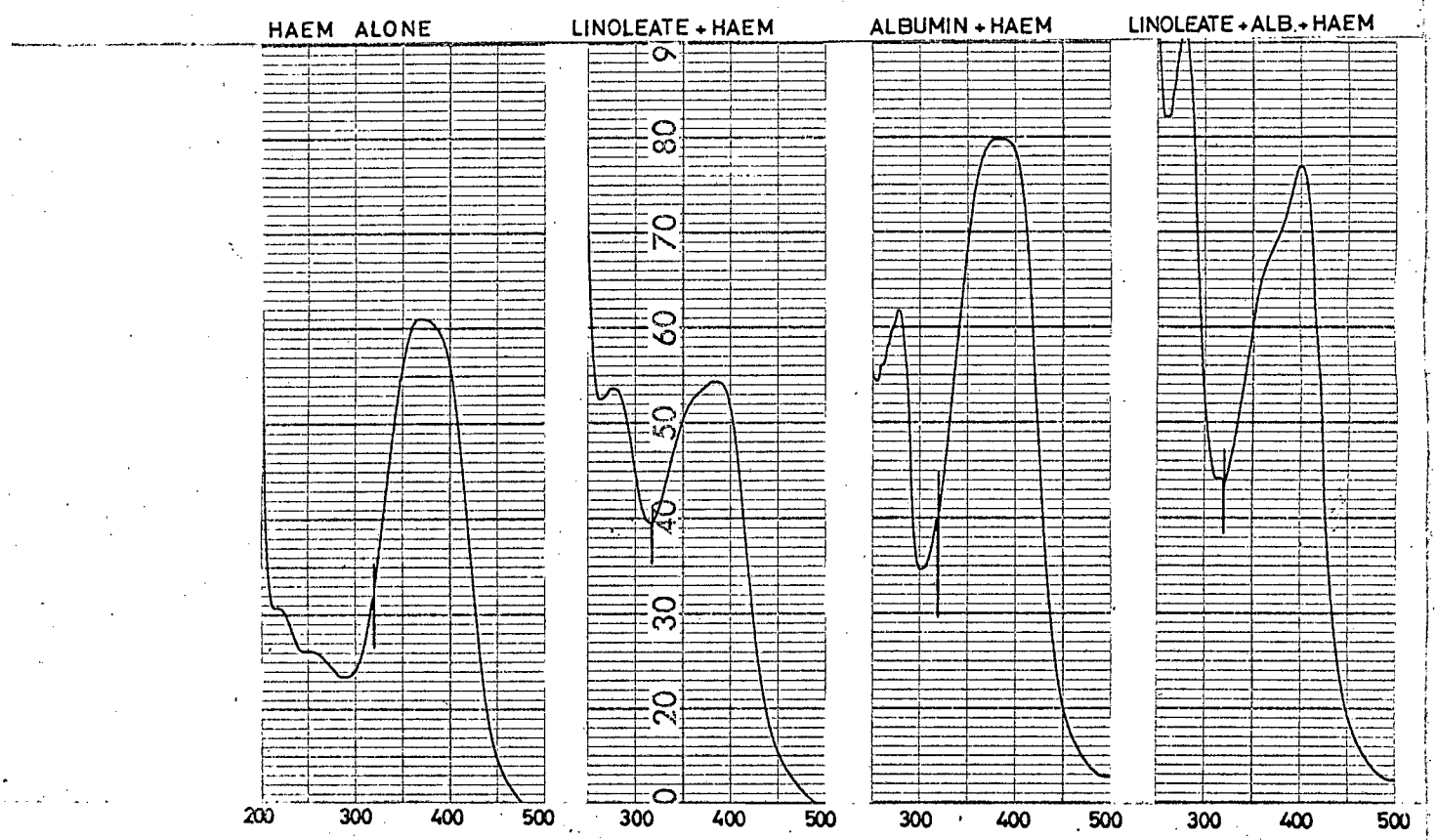


FIG. 42.

EXPT. II EFFECT OF LINOLEATE ON METHAEMALBUMIN FORMATION FROM DEFATTED ALBUMIN.

Spectra after 9hrs. incubation at 37°C.



Difference spectra after 9hrs. incubation at 37°C.

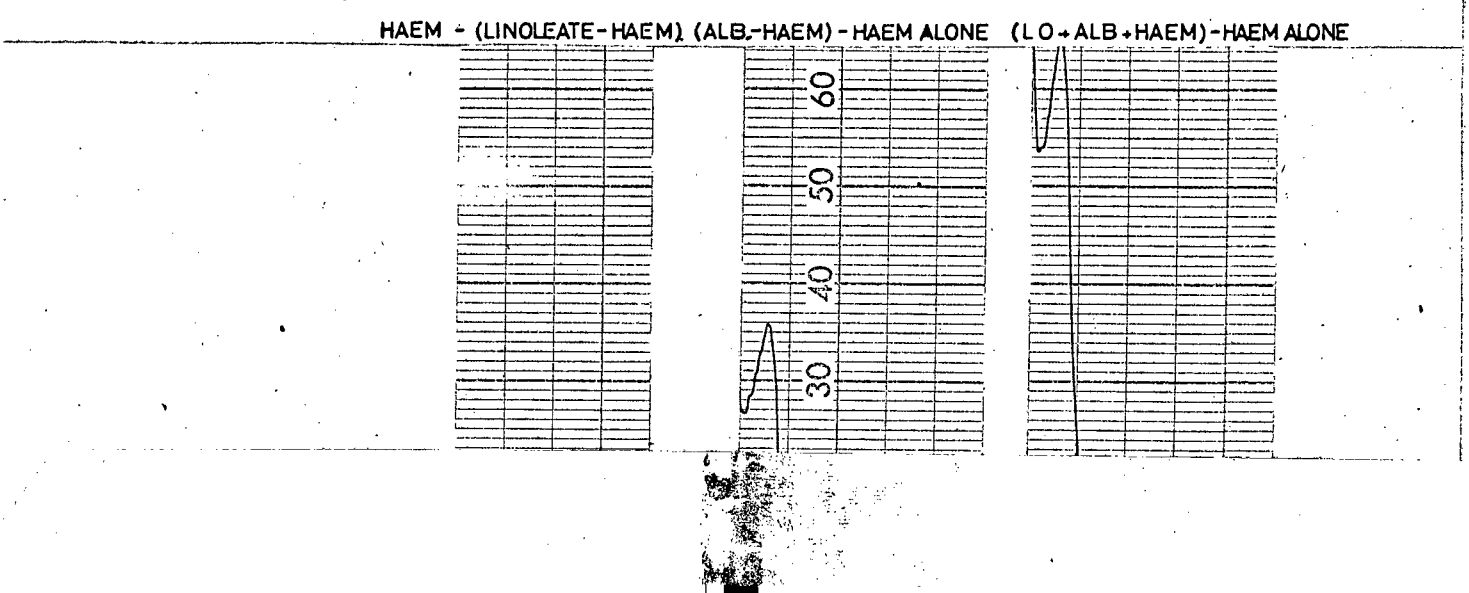


TABLE 18.

EXPERIMENT III: INFLUENCE ON METHAEMALBUMIN FORMATION OF PROLONGED PREINCUBATION OF LINOLEATE WITH ALBUMIN.

	Ferrihaem alone	Linoleate + ferrihaem	Albumin + Ferrihaem		Albumin + Linoleate + Ferrihaem	
	H	H	H	MHA	H	MHA
	mM x 10^{-3}	mM x 10^{-3}	mM x 10^{-3}	mM x 10^{-3}	mM x 10^{-3}	mM x 10^{-3}
2 mins.	14.9	16.6 (opalescent)			14.2	0.9
5 mins.		16.3	14.5	0.6	11.5	3.5
18 mins.			14.5	0.6	8.8	6.2
2 hrs.			14.3	0.8	8.8	6.2
4 hrs.	14.3	15.3				
20 hrs.			14.2	1.4	9.5	6.7
22 hrs.	13.7	14.8				
At 20 hrs.:						
Haem oxidised	8%	11%	0		0	
Haem free			94%		63%	
Haem bound as MHA				9%		44%
Albumin involved in 1:1 binding.				17%		84%

H - Ferrihaem MHA - Methaemalbumin.

The experiment was carried out at pH 7.15 (phosphate buffer) in a waterbath at 25 - 30°C.

The final concentrations of the test substances were as follows:

Albumin	8.0×10^{-3} mM
Linoleate	2.1×10^{-1} mM
Ferrihaem	14.9×10^{-3} mM

i.e., molecular ratios = 1:27:2.

Albumin and fatty acid were incubated for 4 hours prior to addition of ferrihaem. In this experiment, the optical density measurements were not radically affected by turbidity of the fatty acid preparation. This was mainly due to lack of prior formation of lipoperoxides resulting in low peroxidation levels in all samples. The graph (Fig. 43) which follows, is taken from the optical densities at 410 mμ, and not the calculated values.

FIG. 43.

EFFECT OF LINOLEATE ON METHAEMALBUMIN FORMATION AT pH 7

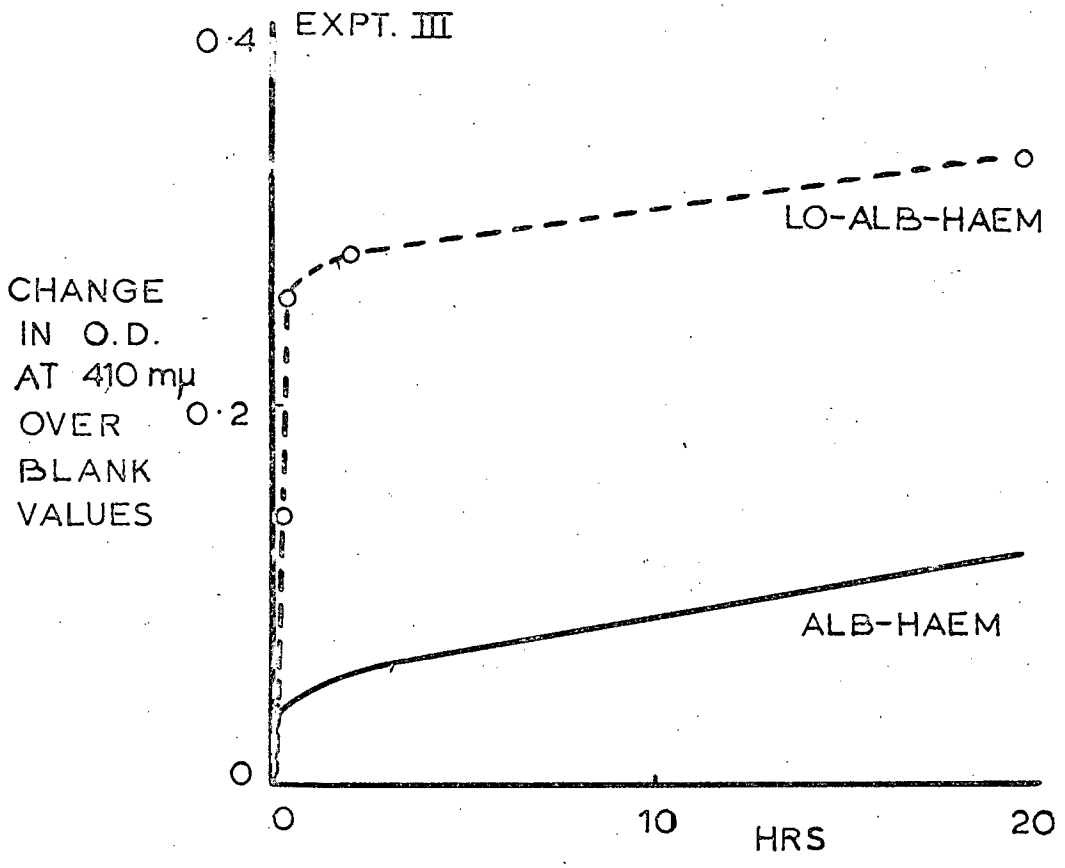


FIG. 44.

EXPT. III: EFFECT ON METHAEMALBUMIN FORMATION OF PROLONGED PRE-INCUBATION OF LINOLEATE WITH ALBUMIN.

Spectra 10 mins after addition of ferrihaem

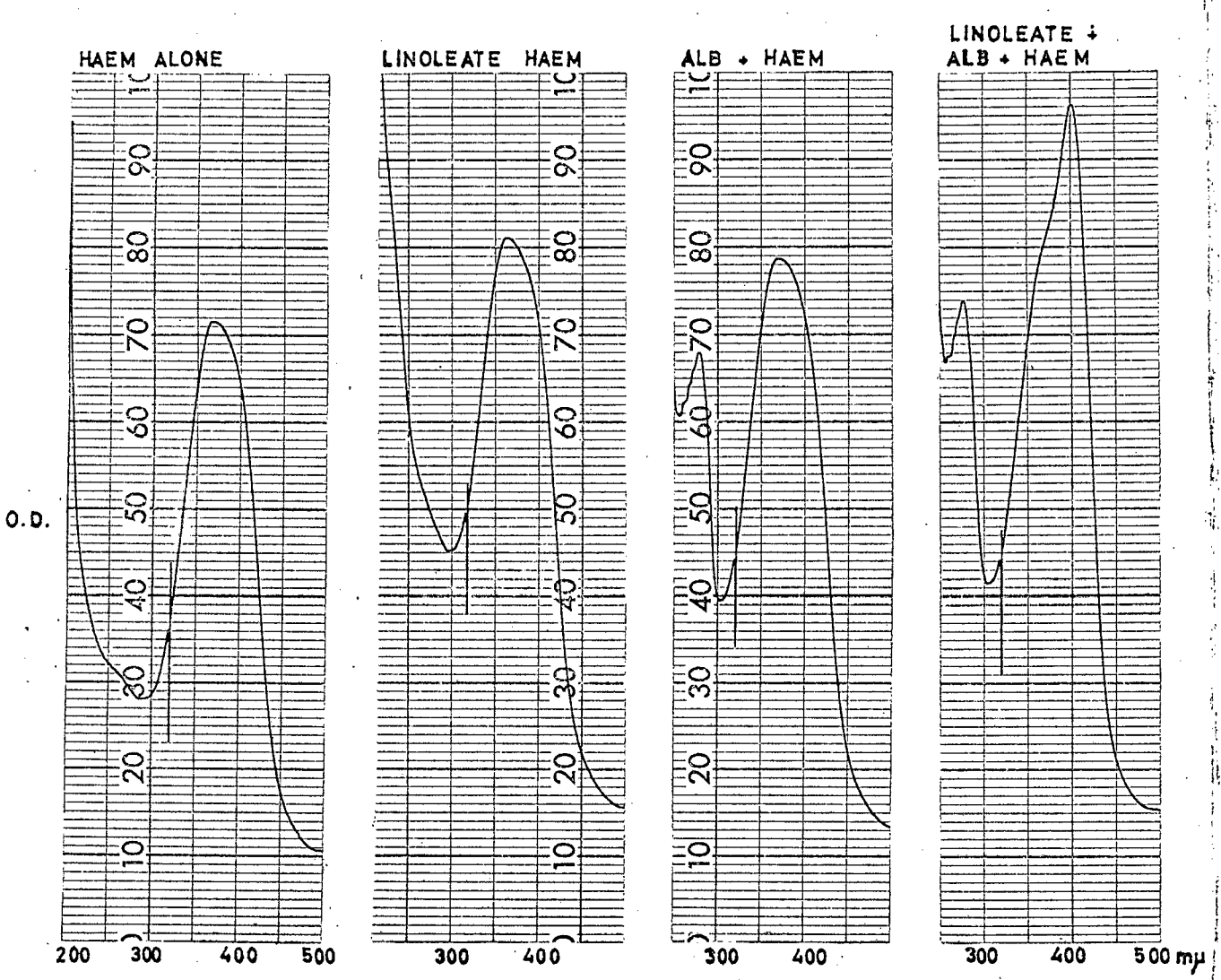


TABLE 19.

EXPERIMENT IV: EFFECT OF LOW pH ON THE TERTIARY SYSTEM.

	Ferrihaem alone	Linoleate + ferrihaem	Albumin + Ferrihaem		Albumin + Linoleate + Ferrihaem	
	H	H	H	MHA	H	MHA
	mM x 10^{-3}	mM x 10^{-3}	mM x 10^{-3}	mM x 10^{-3}	mM x 10^{-3}	mM x 10^{-3}
0	16.0					
2 mins.	13.8 (haem precipitation)	13.0 (haem precipitation)	14.8	0.5	15.6	0.6
4 mins.			15.2	0.3	14.9	0.9
17 mins.					14.4	1.2
2.5 hrs.			14.5	0.9	11.6	3.4
4.1 hrs.			14.2	1.2		
~ 9 hrs.			12.8	2.3	10.7	3.8
In 9 hrs.:						
Haem oxidised	-	-	6%		9%	
Haem free			80%		67%	
Haem bound as MHA				14%		24%
Albumin involved in 1:1 binding				30%		50%

H - Ferrihaem MHA - Methaemalbumin

The reactants were diluted in 0.2 M lactate buffer pH 4.25, each sample having a final pH of 4.5. Albumin was incubated with the fatty acid for 1 hr. 20 min. at 37°C before ferrihaem was finally added to the incubation mixture.

The final concentrations of substances were as follows:

Albumin	7.6 x 10^{-3} mM
Linoleate	2.5 x 10^{-1} mM
Ferrihaem	16.0 x 10^{-3} mM

in an approximate molar ratio of 1:37:2.

FIG. 45.

EXPT. IV EFFECT OF LOW pH ON THE TERTIARY SYSTEM

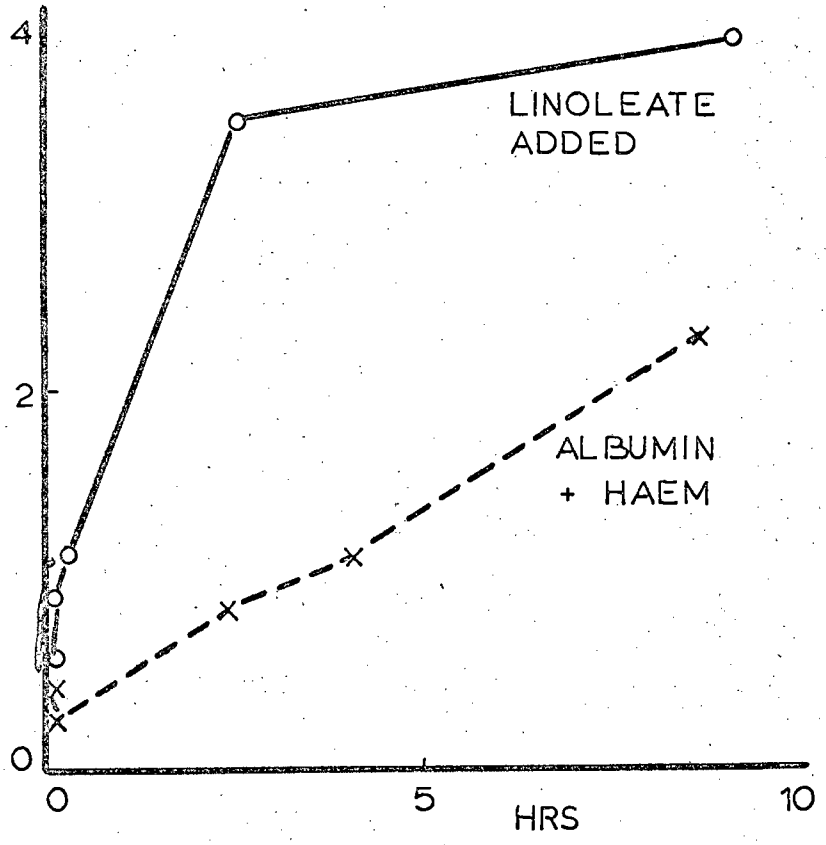


FIG. 46.

EXPT. IV. EFFECT OF LOW pH ON THE INTERACTION BETWEEN ALBUMIN, LINOLEATE AND FERRIHAEM.

Spectra 2·6hrs. after adding ferrihaem.

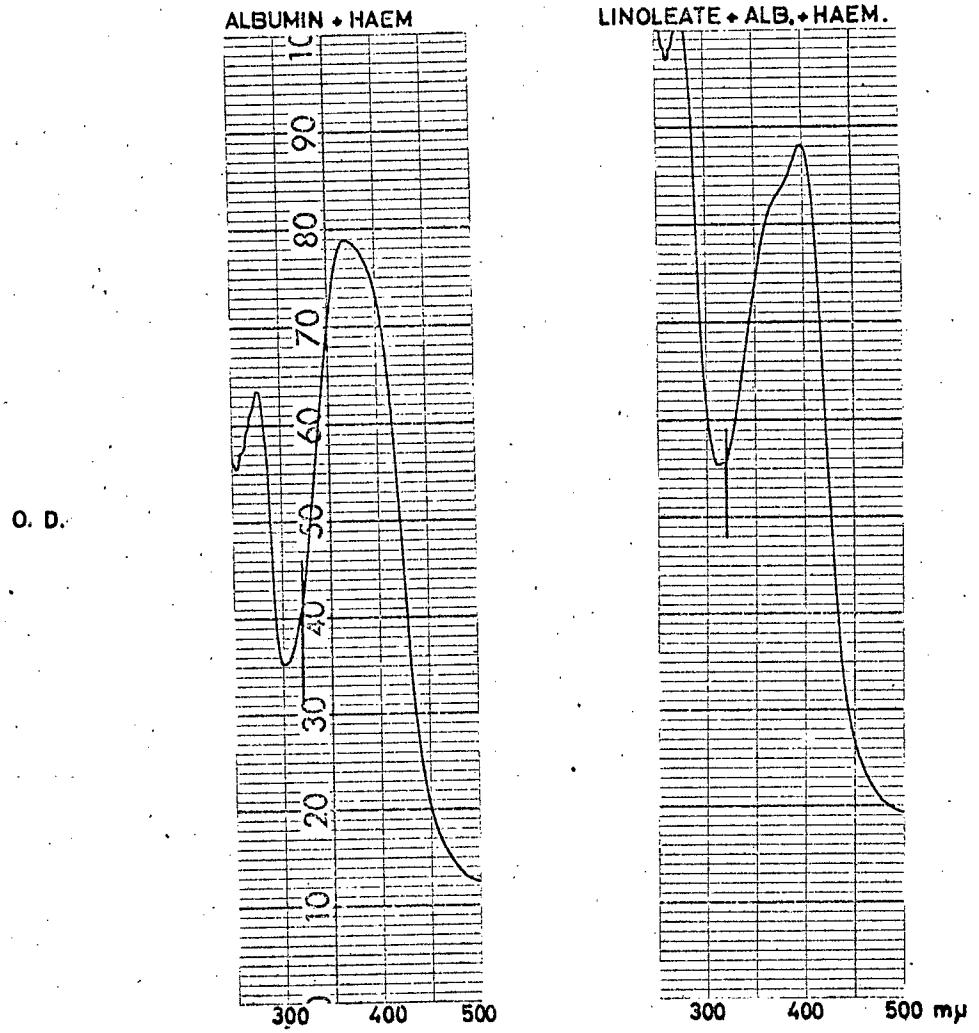


TABLE 20.

EXPT. V.: EFFECT OF ARACHIDONATE ON METHAEMALBUMIN FORMATION.

	Ferrihaem alone	Arachidonate + Ferrihaem	Albumin + Ferrihaem		Albumin + Arachidonic + Ferrihaem	
	H	H	H	MHA	H	MHA
	mM x 10^{-3}	mM x 10^{-3}	mM x 10^{-3}	mM x 10^{-3}	mM x 10^{-3}	mM x 10^{-3}
At 2 mins.	4.7	5.9 (very turbid)	4.6	0	4.8	0
2.5 hrs. at 20°C		4.9	4.5	0.2	3.2	1.5
5.3 hrs. at 20°C	4.3					
After 0°C overnight.	4.0	3.9	4.5	0.3	3.1	1.6
After 3.5 hours incubation at 41°C.	4.0	3.1	4.3	0.4	2.9	1.7
At end of experiment:						
Haem oxidised	15%	46%	1%		3%	
Haem free			91%		61%	
Haem bound as MHA				8%		36%
Albumin involved in 1:1 binding.				8%		38%

H - Ferrihaem
MHA - Methaemalbumin

The samples were at neutral pH but unbuffered. The concentration of each substance in solution was as follows:

Albumin	4.6 x 10^{-3} mM
Arachidonate	7.3 x 10^{-2} mM
Ferrihaem	4.7 x 10^{-3} mM

Molar ratio = 1:16:1.

The incubation time of albumin with arachidonic acid before addition of haematin was not recorded.

In all the experiments, fatty acid stimulated methaemalbumin formation. At the end of the experiments, mixtures supplemented with linoleate, contained more methaemalbumin than those without. This was not always evident from the optical density measurements as mentioned before and shown in Fig. 39A. Nor was this observed in the difference spectra - the test samples without fatty acid invariably having higher Soret values. This was because larger amounts of haematin were present in the albumin-ferrihaem samples, as compared with a ferrihaem blank, than in those in which fatty acid is responsible for oxidation of some of the unbound ferrihaem.

Experiment I (Table 16) is the only one in which the 2 samples attain similar levels of methaemalbumin production in spite of a faster rate in the presence of fatty acid. This is because methaemalbumin formation is enhanced by the slightly alkaline pH (7.3) and the incubation temperature ($38^{\circ}C$). The low pH of Experiment IV inhibits the interaction between albumin and ferrihaem (Fig. 45) and the defatted albumin in Experiment II appears to have been altered slightly during the defatting procedure, thereby decreasing its affinity for haem and probably also fatty acid (the shape of the curve representing methaemalbumin formation in the tertiary system - Fig. 41 - does not flatten as in the other experiments).

In Experiment III (Fig. 43, methaemalbumin formation is inhibited in albumin-haem samples by the comparatively low incubation temperature ($25 - 30^{\circ}C$). The effect of fatty acid on this system is striking. The probable reasons for this are 3-fold:

(a) In contrast to the other experiments performed here, linoleate was made up the day before the experiment was performed. Although the pH of the suspension was more alkaline than in previous preparations, it was much more opalescent and contained few or no lipoperoxides (absorption maximum at 232 mμ). The catalytic action of haematin on unsaturated fatty acids depends on initial auto-peroxidation of the fatty acid (Tappel[161]). At the relatively high levels of haematin used here, the induction period for catalytic peroxidation is greatly increased (Kaufmann and Kaufmann[164]) and, in fact, the co-oxidation of haematin with linoleate in the control is of the same order as the oxidation of haematin with molecular O_2 (in the haem blank) (Table 18). In the other experiments, in which the colloidal suspension of linoleate was optically clear, it had been stored in air for a period of weeks and not only contained lipoperoxides (absorption maximum = 232 mμ) but also breakdown products due to slow autocatalytic action (absorption maximum = 277 mμ) (Tappel[161]). Therefore, in the experiments in which this preparation was used, there was no induction period before co-oxidation of haematin and fatty acid occurred.

Since the presence of albumin prevents haematin oxidation to a greater or lesser extent even in these experiments, this does not fully explain the results of Experiment III.

(b) An explanation requires an analysis of the interaction between all 3 constituents of the system. Shack and Clark have attributed the differences in spectra of ferriporphyrins in aqueous

(diffuse absorption) and detergent (sharp maxima) solutions to conversion from a dimeric species in aqueous media to a monomeric form in detergent solutions[155]. Fatty acids in neutral or alkaline media form colloidal suspensions with detergent activity, i.e. the ionized hydrophilic groups on the molecule being responsible for holding the internally hydrophobic colloidal particle (containing the hydrocarbon chains) in suspension.

Peroxidation of the linoleate produces increased solubility, the solutions become less opalescent, and detergent activity is decreased.

It is not known whether binding to albumin occurs more readily when the haematin is in the monomeric form but clearly since the dimers form aggregates in solution the greater dispersal of the molecules as monomers would enhance the changes of collision. Furthermore, a reassessment of the "associated" haematin in the difference spectra obtained with albumin and haematin alone, suggests that the molecules of ferrihaem bound to albumin, still retained their dimeric form and that this fraction, loosely bound by iron ligands to their dimer partners attached to the albumin molecule, forms the incompletely bound haematin, described before.

(c) The conversion to monomeric haem by fatty acid in suspension may be the main factor responsible for the results obtained but in most instances a large proportion of the unsaturated fatty acid added is probably bound to the albumin molecule itself.

Fatty acid molecules are known to alter the binding properties of albumin and could alter the binding affinity of the molecule in any number of ways depending on the relative positions of the binding sites and so forth. Since the preincubation period in Experiment III was so prolonged, any promotion of haematin binding affinity encountered in the other experiments because of associated fatty acid molecules, is likely to be increased in Experiment III.

The effect of adding arachidonic acid ($C_{20}H_{32}O_2$; 4 unsaturated linkages), although the experimental conditions were slightly different, followed the same pattern (Table 20).

(vi) Effect of globin.

Globin was prepared by the method of Rossi-Fanelli et al.[143] and the lyophilized powder was stored at -15°C. We had difficulty in reconstituting haemoglobin at pH 7.0 as described by these authors. Eventuallywe used a procedure suggested by O'Hagan[110] in which the pH of the globin solution was brought to 7.8, left at room temperature for 1 hour, and at 0°C for 3 days. The denatured protein was then removed by centrifugation. The resultant clear solution had a $\frac{280\ m\mu}{408\ m\mu}$ absorbance ratio of 4 (rather low), but was more stable at room temperature. The technique of investigation in all these experiments was spectroscopic. Since we did not consider it worthwhile, at this stage, to perform the experiments at 0°C, as recommended by Winterhalter and Huehns[153], all the investigations described below, were carried out at room temperature. An alkaline solution of haematin was prepared, 0.05 mM in 0.1N NaOH. All mixtures were diluted appropriately with M/15 phosphate buffer pH 7.8.

Winterhalter and Huehns have shown that their globin, similarly prepared, exists as αβ-globin subunits, which, on addition of alkaline cyan-haematin, formed natural cyanmethaemoglobin containing 4 polypeptide chains with absorption maxima at 540, 418, 357 and 274 mμ[153].

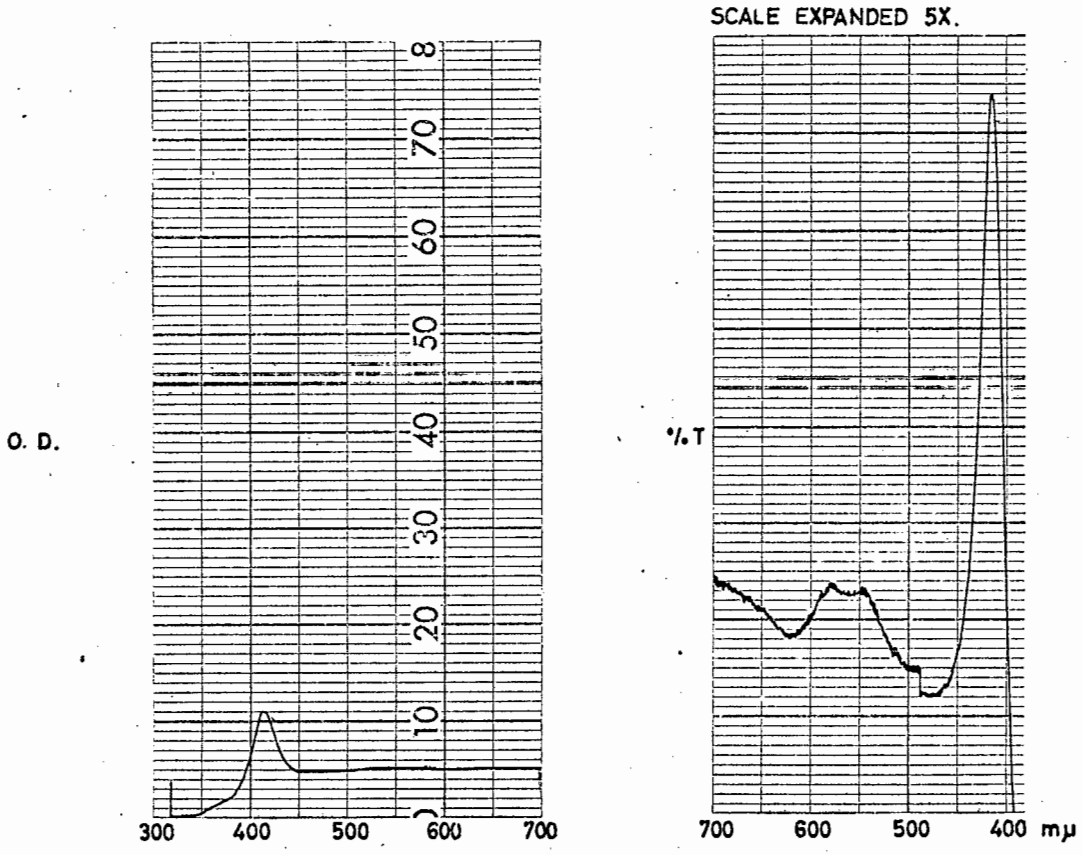
In our experience, spectroscopic observations of the reaction between alkaline ferrihaem and globin (molar ratio = 3 haems to 1 tetrameric globin molecule) showed, initially, very rapid formation (less than 60 secs.) of a ferrohaemoglobin (Absorption maxima at 411, 541 and 579 mμ). This change was followed by dissociation of the haematin, which caused a decrease in the Soret maximum and disappearance of the peak at 579 mμ. At 1 hour, by expanding the recording scale, one could still detect a small maximum at 577 mμ in the difference spectrum (Fig. 47). However, in most of the experiments, longer incubation periods resulted in disappearance of this band with only the 540 - 550 mμ and Soret maxima remaining.

There appears to be a rapid triphasic interaction between ferrihaem and globin involving an almost immediate association of the molecules producing a haemoglobin with α and β bands resembling those of oxyhaemoglobin. This was detectable, after approximately 1 minute after mixing, in one experiment only; that in which ferrihaem was added to globin in equivalent quantities (Table 23). During the other experiments using lower concentrations of ferrihaem, readings at 1 - 2 min. after mixing reflected a situation, in which dissociation, involving some of the ferrihaem molecules was coming to an end. Usually, about 3 minutes later, the absorption at the

FIG. 47. REACTION BETWEEN FERRIHAEM AND NATIVE GLOBIN.

Difference spectrum (Haem-Globin) - (Ferrihaem alone + Globin alone)

1hr. after mixing.



Soret maximum began to rise again and slowly continued to do so for a number of hours usually accompanied by disappearance of the α band of haemoglobin. These spectral changes suggested that methaemoglobin or parahaematin globin-haemochromogen was being formed.

Gibson and Antonini have investigated the kinetics of the reaction between different haems and native globin using a stopped-flow apparatus in which they were able to make very rapid spectrophotometric determinations[165]. They showed that there are 2 stages in the reaction. In the case of protohaematin and native globin, the first involves a rapid reversible combination between haem and globin which takes place in the first 0.15 sec. and is followed by conversion of this intermediate complex to a stable haemoglobin molecule, requiring 15 seconds for completion. The rate of combination with carboxyprotohaem, which is monomeric in solution, is much faster.

These reactions obviously take place at too fast a rate to be observed by our techniques; however, our results seem to indicate a similar type of reaction occurring at a much slower rate possibly due to a higher degree of aggregation of haematin molecules in solution. Also, our results show that the globin preparation contained a large quantity of inactive protein. Only in one experiment was it possible that all the globin protein was completely involved and even then only in the ratio of 1 ferrihaem molecule bound to each globin tetramer. However, since Gibson

and Antonini found very little difference in the reaction kinetics when using denatured globin instead of native globin[165], our results are difficult to interpret unless the extinction coefficients of intermediate-type ferrihaem-globin complexes (i.e. globin tetramers binding less than 4 ferrihaem molecules) are higher than one would expect. Since in only one of the experiments performed (Table 23) was enough ferrihaem added for complete haemoglobin reconstitution, it is obvious that unless the ferrihaem is distributed amongst the globin molecules so as to form only complete haemoglobin, the haemoglobins encountered in the other 3 experiments must be of the intermediate type. The variation in Soret maxima observed in ferrihaem-globin mixtures points to this possibility (Figs. 47, 49, 51 and 53). The difference spectra of Experiments I, II and III, however, indicate a single binding site for haem on each tetrameric unit of globin. In each case, i.e. on adding 1, 2 and 4 haems per tetrameric globin molecule, the increase in absorption in the Soret region of these mixtures, due to haem-globin interaction, showed marked similarity.

Experiment I (1 haem/globin tetramer) O.D. 408 mμ (40 min.) = 0.177

Experiment II (2 haems/globin tetramer) O.D. 416 mμ (26 min.) = 0.177

Experiment III (4 haems/globin tetramer) O.D. 417 mμ (2.7 hrs.)= 0.164

The slower interaction and dissociation we encountered as compared to Gibson and Antonini[165] was possibly due to haematin redistribution, during which the protein, in the presence of a haem deficiency, attains some sort of equilibrium state between

completely and incompletely reconstituted haemoglobin molecules, the spectral evidence suggesting a single haem-binding site on the globin molecule in our preparations. Winterhalter and Huehns found that addition of low concentrations of haemin to globin resulted in the production of a haemoprotein having mobility intermediate between that of globin and haemoglobin on starch-gel electrophoresis[153].

The main source of the problem, however, more than likely lies in the method of calculation in which in solving for the 2 components in the mixture, i.e. haematin and haemoglobin, the extinction values employed were EmM at 387 mμ = 48 for ferrihaem (Rosenfeld and Surgenor[108]) and 213 for oxyhaemoglobin (Vlés[166]); and EmM at 412 mμ = 28 for ferrihaem (Rosenfeld and Surgenor[108]) and 540 for oxyhaemoglobin (Rossi-Fanelli et al.[141]).

We used values for oxyhaemoglobin rather than ferrihaemoglobin on the basis of the spectral chracteristics observed initially, i.e. Fig. 41, but this may be a false assumption.

Interactions of the tertiary system: ferrihaem, albumin and globin.

Three experiments were performed in which ferrihaem was added to mixtures of albumin and globin in different molar ratios. All the experiments were carried out at room temperature at pH 7.8. At this pH, albumin associates readily with ferrihaem. Gibson and Antonini have shown that the reconstitution of haemoglobin is effected with great rapidity above pH 7[165]. Thus, the conditions were

suitable for testing the results of competition between the 2 protein reactants for the ferrihaem moiety.

Experiment I (Table 21; Fig. 48 and 49).

The relative proportions of the reactants in the test preparation were 1 molecule of ferrihaem to 1 of albumin to 1 globin tetramer (i.e., 4 haem equivalents of globin).

The absorption curves, obtained by scanning the control and test solutions, 10 - 11 minutes after addition of ferrihaem (Fig. 49, show that the absorption spectrum of the test preparation containing the three reactants bears a marked similarity both in shape and Soret maximum to the control containing ferrihaem and globin (Soret maximum = 407 mμ). The absorption spectrum of the ferrihaem-albumin mixture has a more diffuse Soret band with an absorption maximum at 403 mμ. The recordings of the difference spectra obtained 20 minutes later show less defined characteristics. The Soret maxima of both controls and of the test mixture were at 408 mμ and the absorption in the tertiary mixture was intermediate between that of the controls, each of which contained 2 components. The spectral results suggested a higher affinity of the globin for the ferrihaem, but that the presence of albumin afforded a certain amount of competition for ferrihaem molecules, thus depressing the absorption to values below that recorded when ferrihaem and globin are allowed to interact alone.

The concentrations calculated by applying a formula to solve

TABLE 21.

Experiment I: Binding of haem to albumin and globulin (1:1:1)

	Haemoglobin formation		Methaemalbumin formation	
	Haem + globin alone	+ albumin	Haem + albumin alone	+ globin
	mM x 10^{-4}	mM x 10^{-4}	mM x 10^{-4}	mM x 10^{-4}
At 2 mins.	14.8	3.8	20.6	28.0
5 mins.	15.4	3.8	21.2	28.2
20 mins.	16.2	5.3	21.6	27.2
45 mins.	16.8	6.1	21.9	25.1
2.6 hrs.			22.2	
4.2 hrs.	16.8	5.7		26.2
Maximal activity during experiment:				
Globin protein (1:1) i.e. binding of 1 haem molecule to 1 haem equivalent of globin.	12%	5%		
Albumin (1:1)			65%	83%
Haematin fraction involved in maximum binding.	60%	22%	79%	101%

The results are presented in the form of haem equivalents (i.e. haemoglobin has a M.W. of 16,700).

The samples were made up in phosphate buffer pH 7.8 and incubated at 18 - 21°C.

The concentrations of the components in haem equivalents were as follows:

Haematin 28 x 10^{-4} mM
Albumin 34 x 10^{-4} mM
Globin 135 x 10^{-4} mM

in an approximate molar ratio of 1:1:4 or if the globin occurred in tetrameric form, 1:1:1.

FIG. 48.

EXPT. I

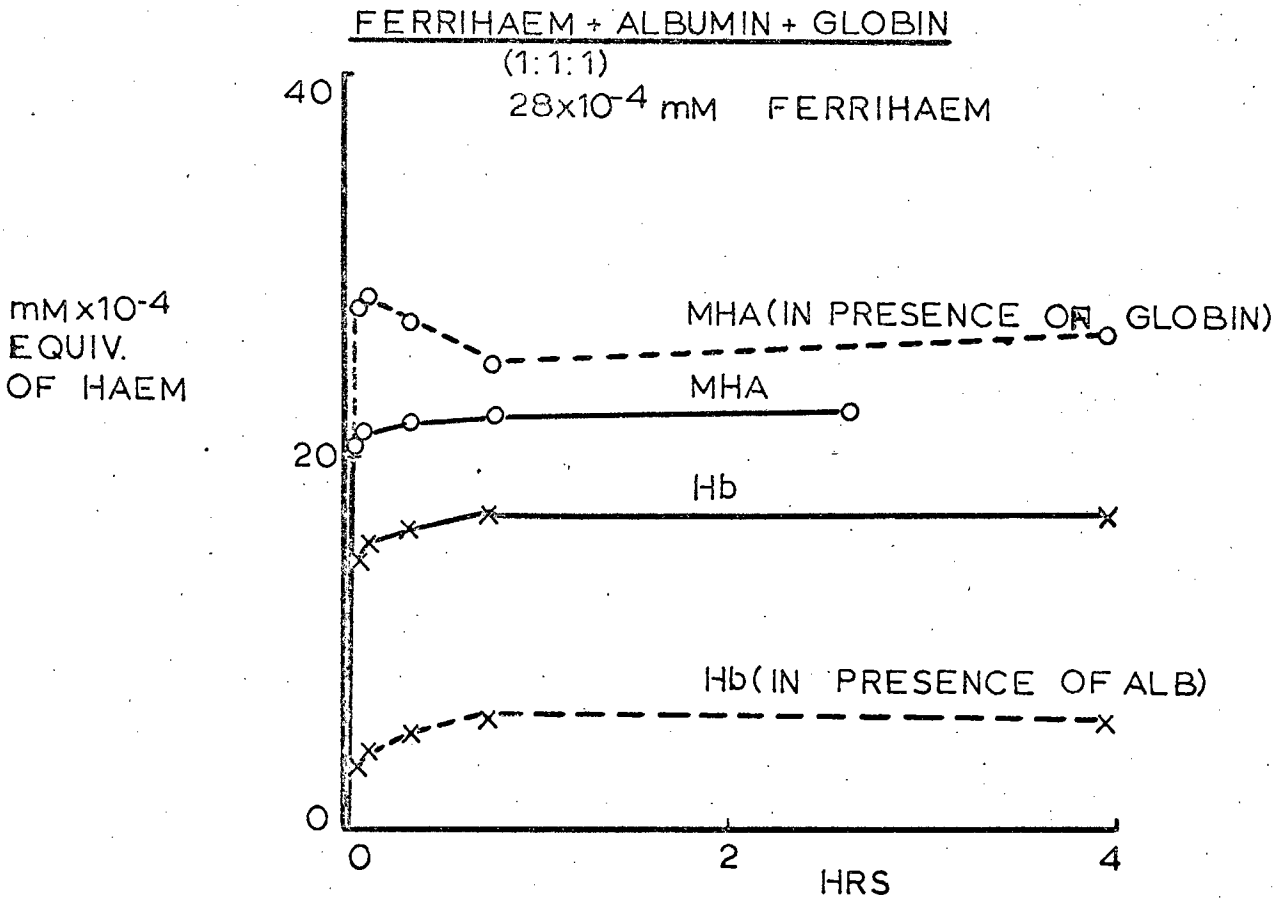
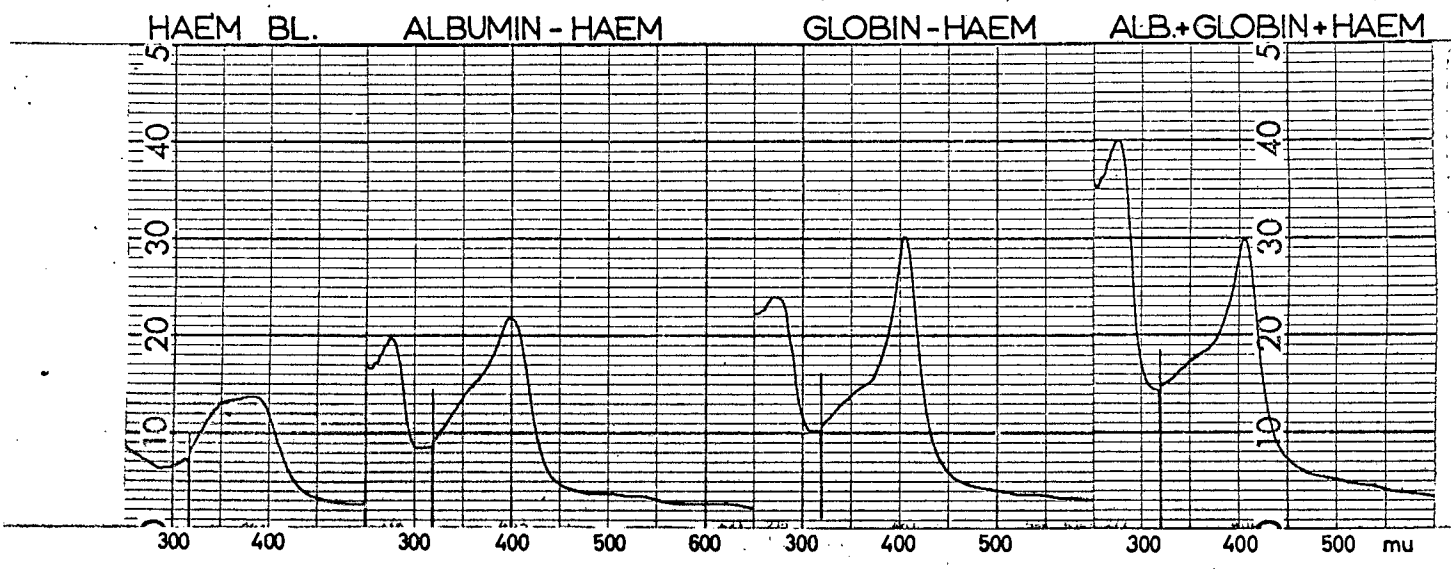

FERRIHAEM + ALBUMIN + GLOBIN
(1:1:1)
28x10⁻⁴ mM FERRIHAEM
mM x10⁻⁴ EQUIV. OF HAEM
40
20
0
MHA (IN PRESENCE OF GLOBIN)
MHA
Hb
Hb (IN PRESENCE OF ALB)
0
2
4
HRS

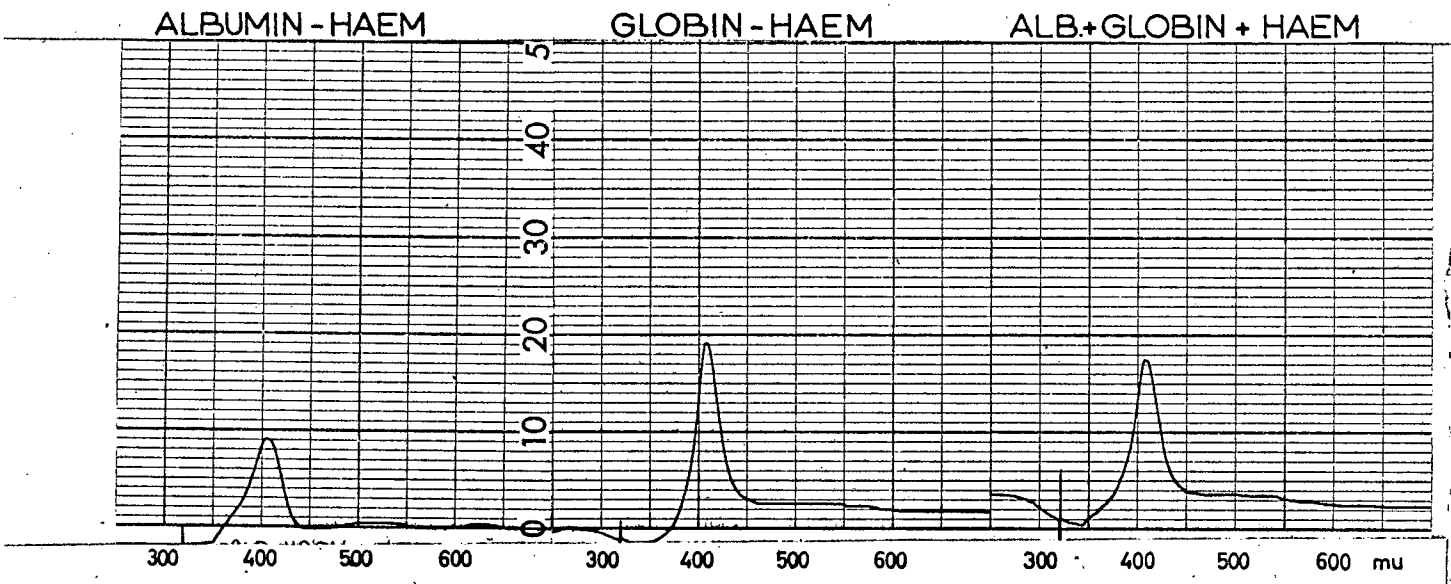
FIG. 49.

EXPT. I : FERRIHAEM + ALBUMIN + GLOBIN (1 : 1 : 1)

Spectra of samples 10 mins. after adding ferrihaem.



Difference spectra after 30 mins. incubation.



for 3 components of a mixture on the basis of their optical densities at 3 different wavelengths (Hunter[158]), i.e. at 385, 403, and 412 mμ, revealed a rather different situation (Fig. 48 and Table 21).

As expected, haemoglobin production, in the presence of albumin, was depressed below the level of the control, representing a 74% decrease in formation. However, globin appeared to stimulate methaemalbumin formation causing a rapid initial rise (approximately 36%) above the control level, followed by dissociation of haem during the first 45 minute period and a very gradual rise thereafter. The level of calculated methaemalbumin in the tertiary system was always higher than in the mixture containing albumin and ferrihaem alone.

The most plausible explanation for this sequence of events was that the ferrihaem formed a complex with the globin as described before. This association, which is extremely rapid (Gibson and Antonini[165]), was not detected. Later, the haem, which is dissociable from the haem-globin complex, was bound by albumin, and in the following 45 minutes ferrihaem was competitively removed from the albumin as the globin protein attained a stable configuration. Ultimately, methaemalbumin increased slowly in a manner similar to that observed previously, when albumin was incubated with free native haemoglobin.

The reason for the higher levels of methaemalbumin in the test

mixture could be explained in 2 ways:

(a) Possibly, globin only combines with monomeric ferrihaem, thereby causing dispersal of the molecules in solution when they dissociate from their initial complex with globin and thus increasing the quantity of monomeric ferrihaem available for albumin binding.

(b) At pH 7.8, the ferrihaem preparation probably contains a small quantity of ferrohaem. There is evidence that albumin will bind ferrohaem only very weakly. Globin has a higher affinity for ferrohaem (Gibson and Antonini[165]) and appears to form a ferro-type complex with the ferrihaem preparation initially, after which the spectrum alters as a stable conformation is attained. It is possible that the globin apoprotein, in the reaction steps following initial haem binding, facilitates conversion of any existing ferrohaem to ferrihaem, thereby increasing the quantity of ferrihaem available to the competing albumin molecules:

Experiment II (Table 22; Figs. 50 and 51).

Equivalent quantities of ferrihaem, albumin and globin were mixed together in a ratio of 2 molecules of ferrihaem to 5 haem equivalents of protein, i.e. 1 albumin molecule and 1 globin tetramer.

In the controls, the addition of an extra haem equivalent raises both haemoglobin and methaemalbumin similarly, i.e. haemoglobin expressed as haem equivalents increases by 32%;

TABLE 22.

Experiment II: Binding of haem to albumin and globin (2:1:1)

	Haemoglobin formation		Methaemalbumin formation	
	Haem + globin alone	+ albumin	Haem + albumin alone	+ globin
	mM x 10^{-4}	mM x 10^{-4}	mM x 10^{-4}	mM x 10^{-4}
At 2 mins.	19.5	10.8	27.5	15.6
5 mins.	20.3	11.2	27.8	15.5
20 mins.	21.0	10.7	28.7	16.6
45 mins.			28.7	
1.2 hrs.		9.1		18.7
22.5 hrs.	20.6	9.0	29.4	19.9
Maximal protein binding during experiment:				
Globin (1:1)	18%	10%		
Albumin (1:1)			88%	59%
Haematin fraction involved in maximum binding.	42%	22%	59%	40%

The results are presented in the form of haem equivalents (i.e. haemoglobin has a M.W. of 16,700).

The reactants were incubated in phosphate buffer pH 7.8 at 18°C. The final concentrations were as follows:

Haematin 50 x 10^{-4} mM
Albumin 33.5 x 10^{-4} mM
Globin 117 x 10^{-4} mM as haem equivalents.

The molar ratio was thus 1.5:1:3; or, if the globin molecule consisted of 4 polypeptide chains, the approximate ratio was 2:1:1.

FIG. 50.

EXPT. II

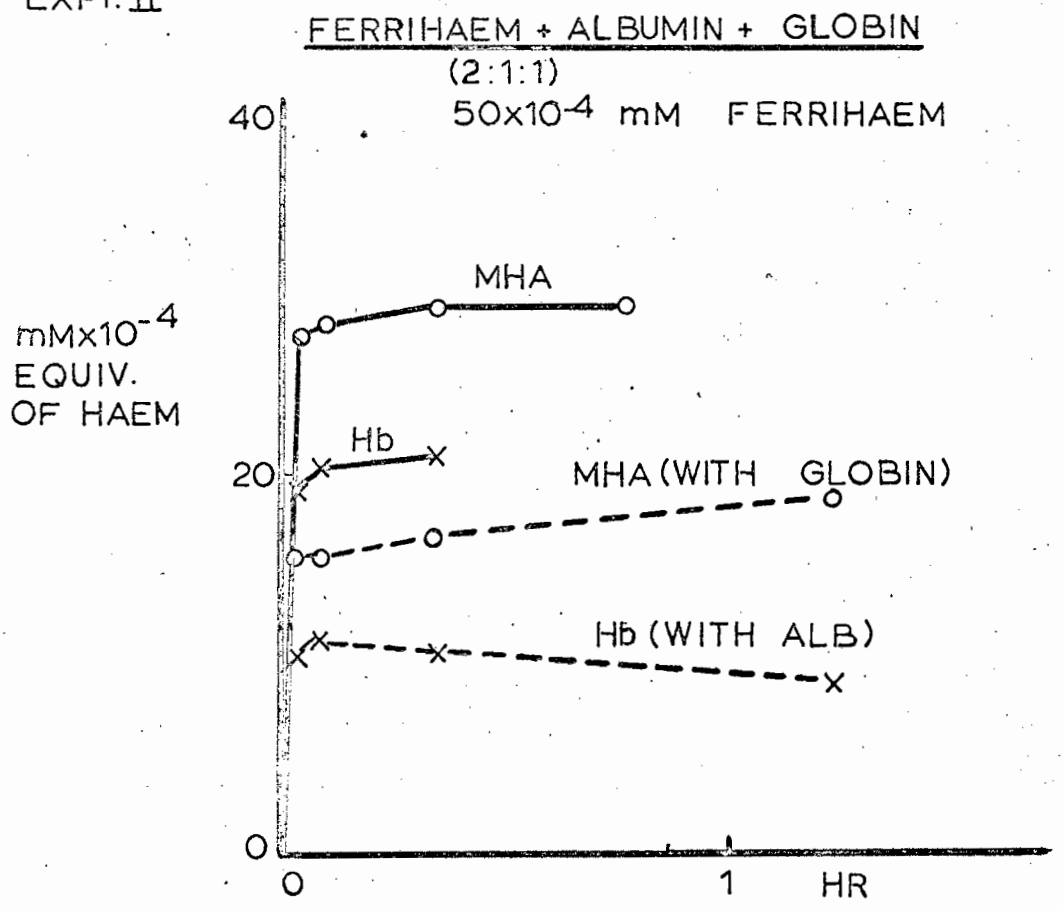
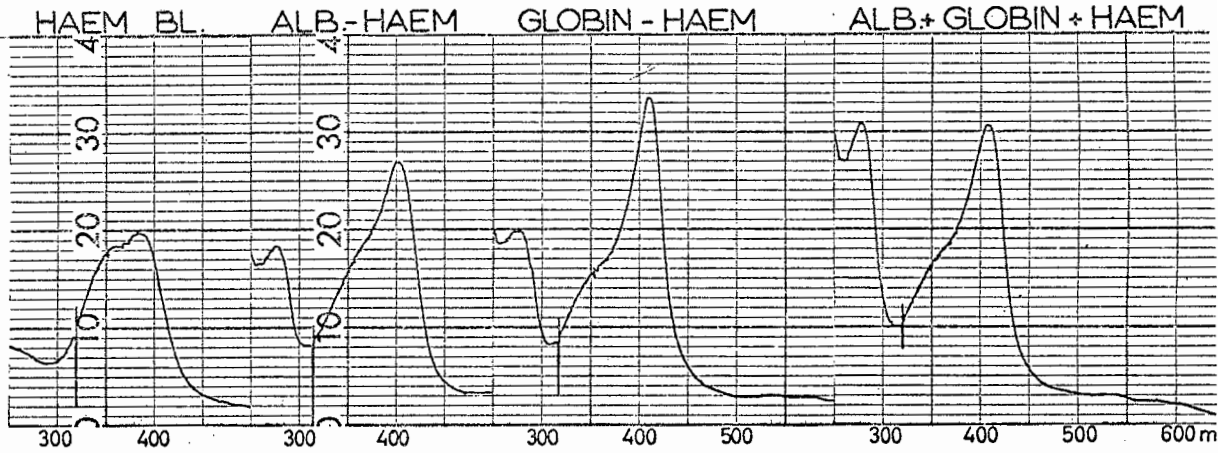


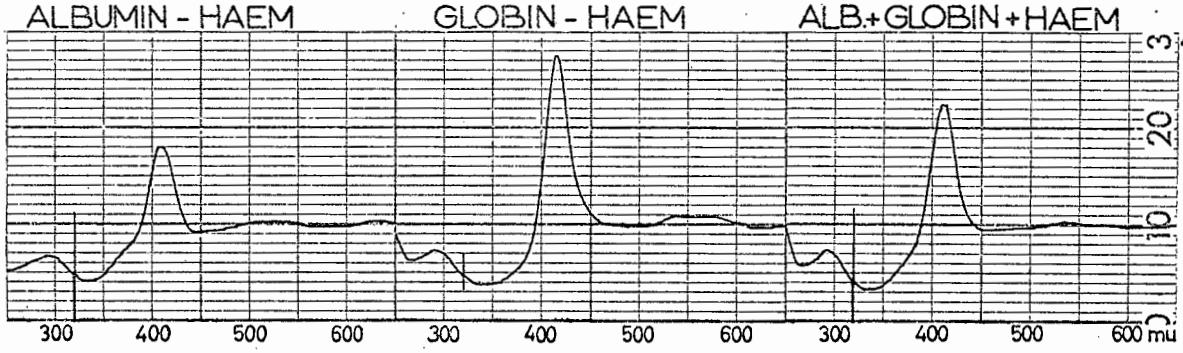
FIG. 51.

EXPT. II : FERRIHAEM + ALBUMIN + GLOBIN (2:1:1)

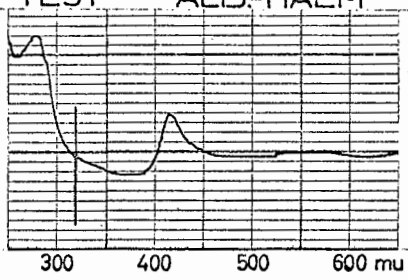
Spectra of samples 11mins. after adding ferrihaem.



Difference spectra after approx. 30mins. incubation.



Difference spectrum (1hr. incubat.) TEST - ALB.-HAEM



the methaemalbumin content is 33% above the level calculated for Experiment I. The overall difference spectra, recorded at roughly 30 minutes after addition of ferrihaem are, however, almost identical in both experiments. Thus, the calculated 30% rise in methaemalbumin and haemoglobin, encountered in the controls, must be due to the extra ferrihaem added. This adds weight to our original contention that calculations on the basis of our findings in this particular system must be regarded with reservation. Possibly, had we selected different wavelength maxima, the results could have been more readily understood, e.g. for the experiments on albumin-ferrihaem interactions, we used the extinction values at 403 mμ and 370 mμ. In these experiments the absorption values at 403 mμ and 387 mμ (alkaline ferrihaem) were used. Their closer proximity may account for the overlap in calculated values.

It seemed advisable, therefore, that we should present our definite qualitative findings in this series of experiments and that we should not attempt a detailed quantitative analysis until further experiments have been performed.

Our investigations showed that

(a) at pH 7.8, there was always competition between albumin and globin for ferrihaem, although, except in one instance (Experiment I), there appeared to be free ferrihaem available. It is not known whether the initial appearance in the haem-globin controls of a haemoprotein having the spectral characteristics of oxyhaemoglobin

is due to preferential binding of small quantities of ferrohaem present in solution or whether globin, after complexing with ferrihaem, caused its conversion to ferrohaem. The subsequent disappearance of the absorption band at 576 mμ, the decrease in Soret absorption and the persistence of the band at 540 mμ are indicative of formation of methaemoglobin or parahaematin globin-haemochromogen. Since the relative proportions of the ferrous and ferric forms in the preparations of alkaline haematin were unknown, these possibilities await further clarification.

(b) In none of the experiments, neither in the controls nor tertiary mixtures, did we ever achieve the binding of 2 ferrihaem molecules to one of albumin or 4 to one of globin. The latter finding was surprising in view of the evidence that denatured globin has roughly the same affinity for carboxyhaem as native globin (Gibson and Antonini[165]). Our evidence suggests a single haem-binding site on the globin protein.

(c) It seems unlikely in view of the evidence, that there is any association between the albumin and globin proteins, which are incubated together, prior to the addition of alkaline haematin. The interaction between haem and globin is probably more rapid than that with albumin (Gibson and Antonini[165]) and it is unlikely that during the initial period in which we have been unable to collect data, there is competition between haemoglobin and albumin molecules. However, Fig. 48, 50 and 52 show definite competition between the proteins thereafter.

(d) There is usually a certain quantity of inactive haem with which both proteins appear reluctant to associate. In Experiment I, the scarcity of haem molecules in the test preparation ensured complete haem utilization (Table 21). However, the graph of Experiment II (Fig. 50), in which only 2 haem molecules were available to every 5 haem equivalents of protein (and which must also be considered to contain a relative paucity of haem molecules), shows albumin competing for globin-bound haem, which decreases as the methaemalbumin content rises, rather than for the calculated 40% of free ferrihaem available. Similar results were obtained in Experiment III (Table 23, Figs. 52 and 53).

In view of the reservations with which these results must be interpreted we do not wish to infer, at this stage, what effects these interactions may have on haemoglobin synthesis in erythropoietic tissues, or its intravascular or intracellular degradation.

2.233 E. Haem Degradation.

(i) By molecular oxygen.

In each of the above experiments on chemical interactions involving ferrihaem, a control, containing only ferrihaem, appropriately diluted in buffer or distilled water, was used for comparison. Ferrihaem in aqueous solution, however, undergoes coupled oxidation

TABLE 23.

Experiment III: Binding of haem to albumin and globin (4:2:1)

	Haemoglobin formation		Methaemalbumin formation	
	Haem + globin alone	+ albumin	Haem + albumin alone	+ globin
	mM x 10^{-4}	mM x 10^{-4}	mM x 10^{-4}	mM x 10^{-4}
At 1 min.	~ 26			
2 mins.	-	15.2	48.5	34.3
4 mins.	18.0			
5 mins.		14.0		32.0
23 mins.		11.6		32.1
30 mins.	13.2			
2 hrs.	13.6	11.6	47.8	33.3
5.6 hrs.	14.0	9.6	48.3	38.5
19 hrs.	14.4	11.2	47.3	35.2
Maximal protein binding during experiment:				
Globin (1:1)	27%	16%		
Albumin (1:1)			113%	90%
Haematin fraction involved in maximum binding.	29%	17%	54%	43%

The results are presented in the form of haem equivalents (i.e., haemoglobin has a M.W. of 16,700).

The mixtures were incubated in phosphate buffer pH 7.8 at 20°C. The final concentrations of reactants were as follows:

Haematin	90 x 10^{-4} mM
Albumin	43 x 10^{-4} mM
Globin	95 x 10^{-4} mM

i.e., approximate molar ratio (M.W. of globin = 16,700) was 2:1:2 respectively (4:2:1 if globin molecule occurred as a tetrameric unit).

FIG. 52.
EXPT. III

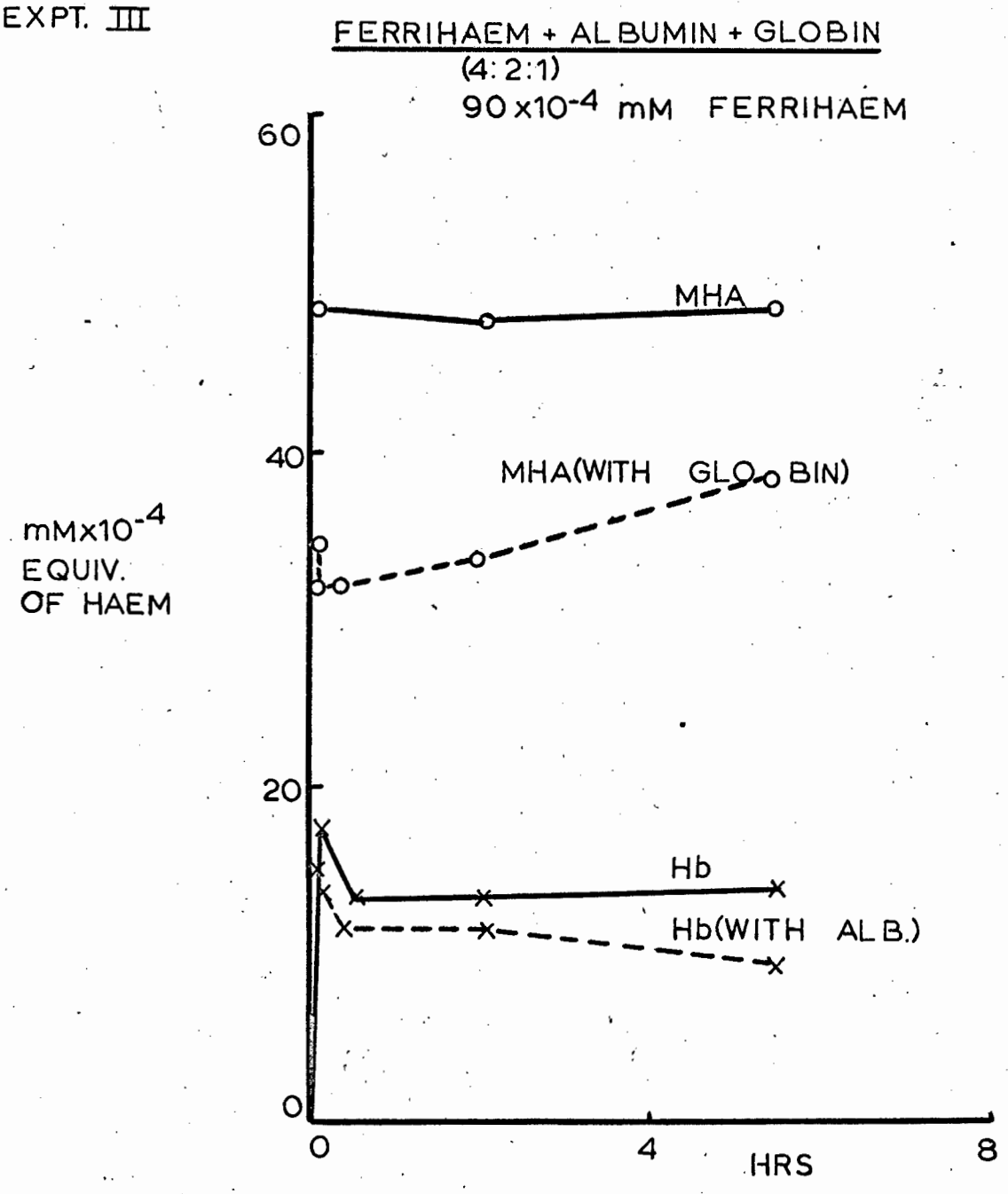
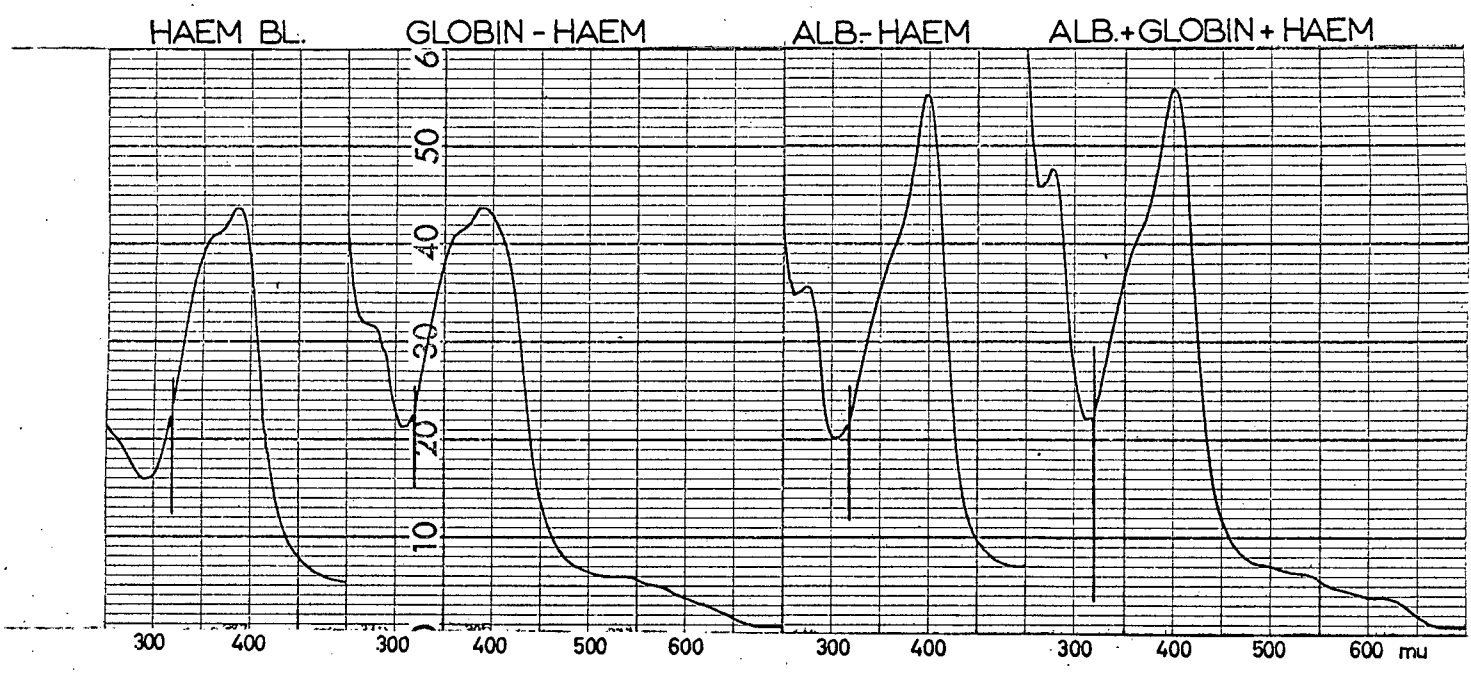


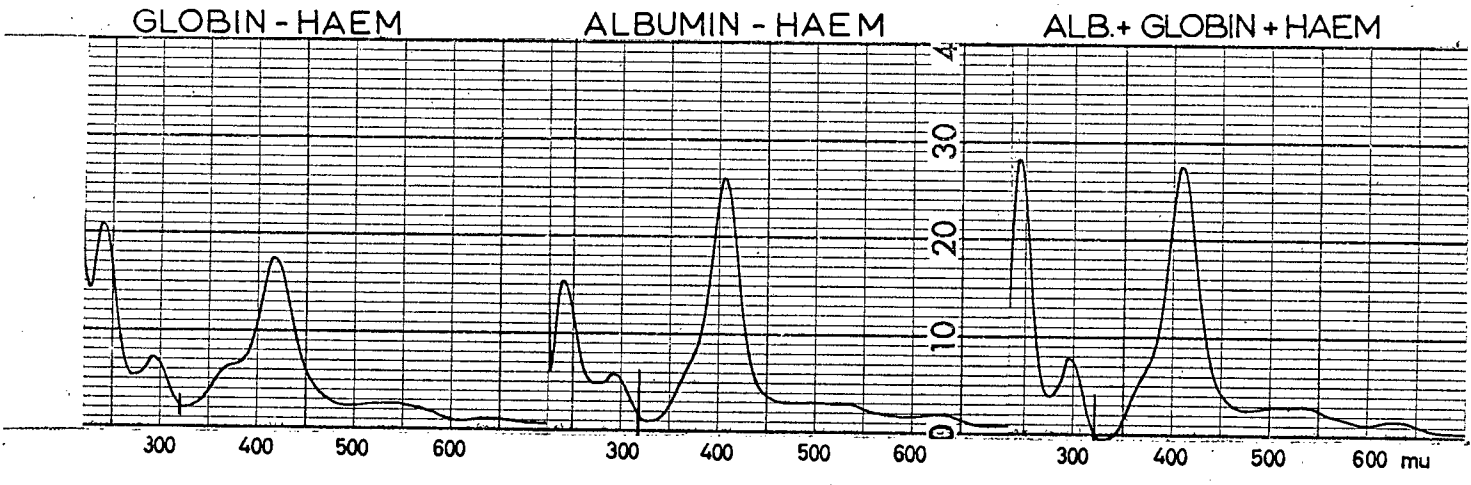
FIG. 53.

EXPT. III : FERRIHAEM + ALBUMIN + GLOBIN (4:2:1)

Spectra of samples 9mins. after adding ferrihaem.



Difference spectra after 2·5hrs incubation.



with oxygen when exposed to air. All the experiments were performed under aerobic conditions, therefore the optical density in the Soret region of these control solutions diminished, the extent varying with the conditions of experimentation, i.e. the percentage fall in the absorption peak in the Soret region rose with time and increasing temperature. Although incubation at different pH's appeared to alter the extent of oxidation (Fig. 37 - lower graph) no particular trends were noted and this aspect requires further experimentation.

The rate of oxidation, as judged by decrease in the Soret absorption maximum, which varied in wavelength from 368 mμ to 389 mμ depending on the pH, appeared to be biphasic. The first 5 hr. period following dilution of the ferrihaem, was characterized by a higher oxidation rate as compared with that of the period (up to 20 hrs.) thereafter (Fig. 31). Since the solutions were not shaken in air, it is possible that the initial period represented coupled oxidation with dissolved oxygen, which continued only at a low rate once most of the O_2 had been utilized autocatalytically by the ferrihaem.

It was found that the presence of albumin in solution protected even unbound ferrihaem from oxidation in air. This property was also noted by Rosenfeld and Surgenor[108]. They ascribed it to a non-specific stabilizing effect of a soluble protein.

The end product of this coupled oxidation has not been characterized. At the concentrations of ferrihaem used, it was

not possible to glean any information from optical densities in the red region of the spectrum (i.e. at wavelengths at which choleglobin and biliverdin (Lemberg and Legge[28]) have absorption maxima). Neutral or slightly alkaline solutions of ferrihaem exhibit an absorption band at 630 - 640 mμ in the red region. The optical density tends to decrease in conjunction with the fall in the Soret region.

(ii) Haem degradation by peroxide.

It has been known for many years that haem pigments are oxidized when treated with peroxide (Fischer and Orth[1]). They act as peroxidases decomposing the peroxide while they themselves undergo coupled oxidation.

In the ascorbic acid-oxygen systems (Lemberg[29]), peroxide is produced when the ascorbic acid is oxidized. This is broken down by ferrihaem and other haemoproteins (Kench[14]) with the production of bile pigment precursors, which on treatment with acetic acid, yield free biliverdin. Addition of leucocytes to this coupled peroxidative system, causes marked inhibition in the formation of bile pigments from haemoglobin (Kench and Varma[39]). They suggest that the myeloperoxidase of the leucocytes has a higher affinity for the peroxide produced, than has haemoglobin.

In mammals there appear to be a number of protective mechanisms preventing interaction of haemoproteins with peroxide produced in

vivo. These include the above-mentioned peroxidase of leucocytes (Kench and Varma[39]), glutathione peroxidase (Mills[167]) and catalase of erythrocytes, and serum levels of α-tocopherol which prevents formation of lipoperoxides (Pokrovskii and Abrarov[61]).

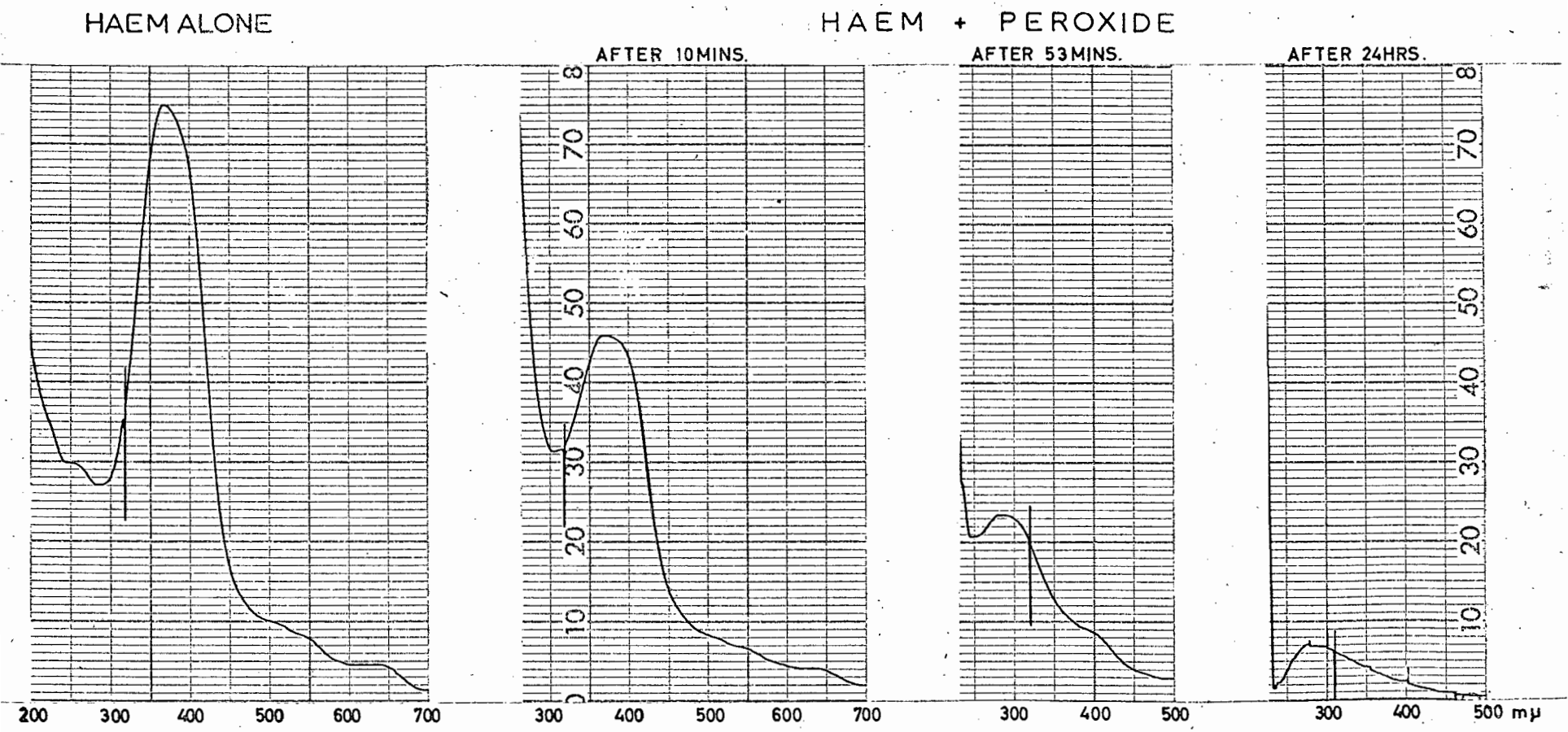
Addition of peroxide to aqueous ferrihaem in a molar ratio of approximately 4,000 to 1 results in complete oxidation of the brownish ferrihaem to a colourless product within 30 minutes (Fig. 54) having a small absorption maximum at 290 mμ.

Experiments were performed to investigate the effect of adding histidine to the ferrihaem-peroxide coupled oxidation system. Cowgill and Clark showed that in the binding of imidazole base ligands to ferrimesoporphyrin, the nitrogens of the imidazole ring compete with hydroxyl ions for the iron co-ordinating link[159]. At neutral and acidic pH's when the ferriporphyrin binds water molecules rather than hydroxyl ions, the affinity for the ligand is decreased. Therefore, ligand binding will only take place with ease at pH's higher than the half-transformation point of ferriporphyrin-ferriporphyrin hydroxide (approximately pH 7.6 for ferrihaem) but not so alkaline that the affinity for hydroxyl ions is greater than that for the nitrogenous base. Cowgill and Clark[159] performed most of their binding experiments at pH's of 8 - 10.

One would thus expect very little effect of histidine at pH 7.1 (Table 24) whereas at pH 8.7 (Table 25), it should be

FIG. 54.

EFFECT OF PEROXIDE ON FERRIHAEM.



bound to ferrihaem, and influence haem degradation. However, the concentration of histidine was probably too low for it to be able to compete favourably with hydroxyl ions.

In the present experiments, only small differences were noted between oxidation of ferrihaem alone and of ferrihaem-histidine mixtures. The main action of histidine appeared to be protective as regards oxidation of ferrihaem by molecular oxygen of the air (Table 25). Although the fall in Soret absorption due to peroxide was similar in both, histidine also promoted a greater increment in the absorption maximum of the peroxidation products at 290 - 300 mμ (Fig. 55).

(iii) Haem degradation by unsaturated fatty acids.

Haurowitz and his colleagues in 1941 proved that haemin and haemoglobin were destroyed by the action of unsaturated fatty acids and molecular oxygen(54). Using a colloidal solution of linoleate at pH 6.8 in a ratio of 130 - 150 molecules fatty acid to 1 of haem, they were able to break down 85% of the haematin and 50% of the haemoglobin in 150 minutes at 38°C. The degree of destruction was estimated by determining the quantity of released inorganic Fe and the benzidine test for peroxidase activity. These workers found that of eleven unsaturated fatty acids tried, only linoleic and linolenic acids were at all active.

Tappel has shown that the rate of linoleate oxidation in the presence of increasing concentrations of a haematin catalyst was

TABLE 24.

Effect of Histidine on Oxidation of Ferrihaem by Peroxide at pH 7.1.

	Ferrihaem alone	Ferrihaem + H_2O_2	Ferrihaem + Histidine	Ferrihaem + Histidine + H_2O_2
	10^{-3} mM	10^{-3} mM	10^{-3} mM	10^{-3} mM
At 2 mins.	14.8	14.0	14.8	13.9
4 mins.		13.9	14.9	13.8
15 mins.		13.7	14.9	
2.5 hrs.		12.2		12.1
3.0 hrs.	13.7		13.7	
23 hrs.	12.5	11.6	12.7	11.9
Change in Soret absorption in 3 hrs.	-7.5%	-17.2%	-7.4%	-18.4%
Change in absorption at 285 mμ in 3 hrs.	-3.3%	-0.8%	-4.4%	-1.6%

Ferrihaem was incubated with histidine in phosphate buffer (pH 7.1) at 40°C, prior to addition of peroxide. The final concentrations of the reactants were:

Ferrihaem	15×10^{-3} mM
Histidine (as histidine-hydrochloride)	50×10^{-3} mM
Peroxide	33×10^{-3} mM

in a molar ratio of 1:3:2.

TABLE 25.

Effect of Histidine on Oxidation of Ferrihaem by Peroxide at pH 8.7.

	Ferrihaem alone	Ferrihaem + H_2O_2	Ferrihaem + Histidine	Ferrihaem + Histidine + H_2O_2
	10^{-3} mM	10^{-3} mM	10^{-3} mM	10^{-3} mM
At 1 min.		11.1		
2 mins.	15.0		14.9	10.9
4 mins.		10.9		
5 mins.			14.9	10.6
18 mins.		10.4	14.8	10.3
1 hr.	14.4			
3 hrs.		8.7		8.7
3.7 hrs.	13.6		14.1	
4.5 hrs.		8.6		8.7
5.3 hrs.	13.1		13.6	
Change in Soret absorption in 4 hrs.	-9%	-43%	-5%	-42%
Change in absorption at 290 mμ in 4 hrs.	-17%	+4%	-14%	+15%

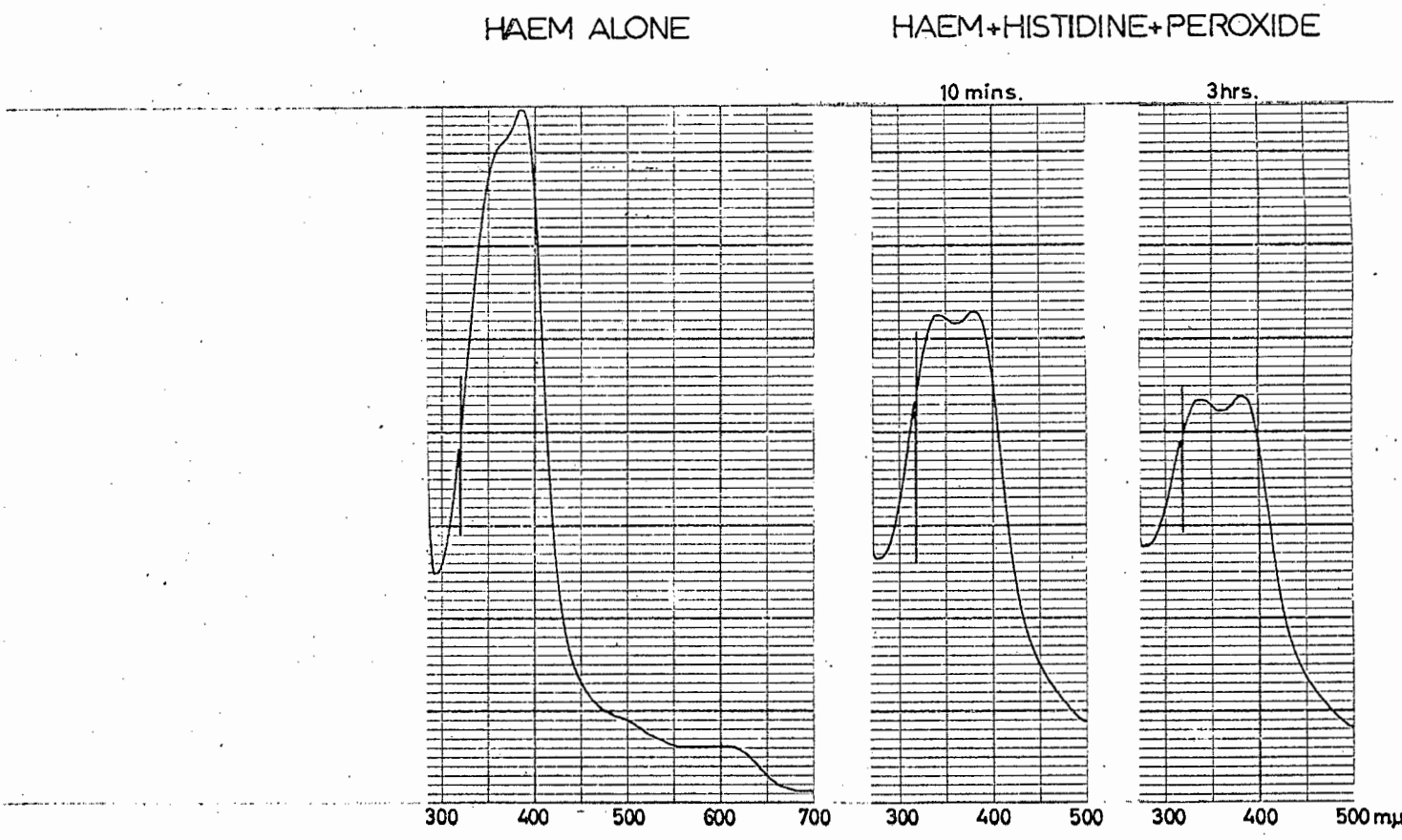
Peroxide was added to ferrihaem or ferrihaem + histidine mixtures in barbitone-acetate buffer pH 8.7 at 39°C. The final concentrations of components were as follows:

Ferrihaem	15×10^{-3} mM
Histidine	67×10^{-3} mM
Peroxide	250×10^{-3} mM

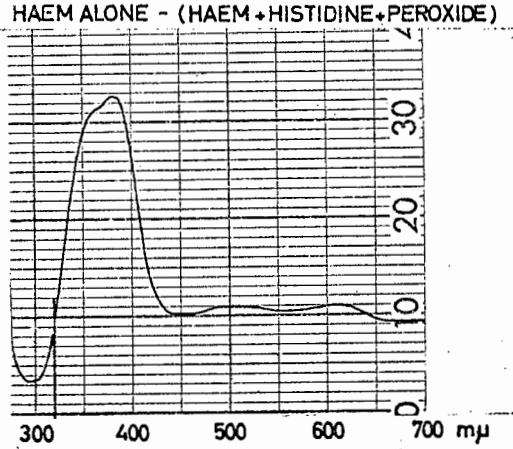
in a molar ratio of 1:4.5:17.

FIG. 55.

ACTION OF PEROXIDE ON A MIXTURE OF FERRIHAEM AND HISTIDINE AT pH 8·7.



DIFFERENCE SPECTRUM AFTER 3hrs.



dependent on temperature, the type of apohaemoprotein used and the state of peroxidation of the linoleate preparation[161].

In our experiments, generally much lower concentrations of fatty acid to haematin were used (4 - 60 moles linoleate/mole ferrihaem).

(a) Effect of different fatty acids on the peroxidation system.

Four unsaturated fatty acids were tested. All were obtained from British Drug Houses, Ltd. These were as follows:

Fatty acid	Length of C-chain	No. of unsaturated linkages.
Oleic	18	1
Linoleic	18	2
Linolenic	18	3
Arachidonic	20	4

Stable colloidal suspensions of the fatty acids were prepared by dissolving a weighed quantity in 2 - 3 ml. 0.1N NaOH, diluting, titrating to neutral or just alkaline pH with 0.1N HCl and bringing the suspension to the desired concentration by further dilution with distilled water.

Destruction of ferrihaem was measured by the fall in absorption of the Soret band and the development of an absorption maximum in the ultraviolet (270 mμ - 285 mμ) reflecting accumulation of peroxidation products of an aldehydic or ketonic nature (Tappel[161]).

Tappel usually found a marked rise in this region with a maximum at 277 mμ during haematin-catalysed linoleate peroxidation[161]. In our experiments, using higher concentrations of ferrihaem, the products of the porphyrin co-oxidation would be expected to affect the absorption spectrum, and it was found that as the Soret band decreased, the wavelength of the absorption maximum in the ultraviolet became longer, i.e. roughly 285 mμ.

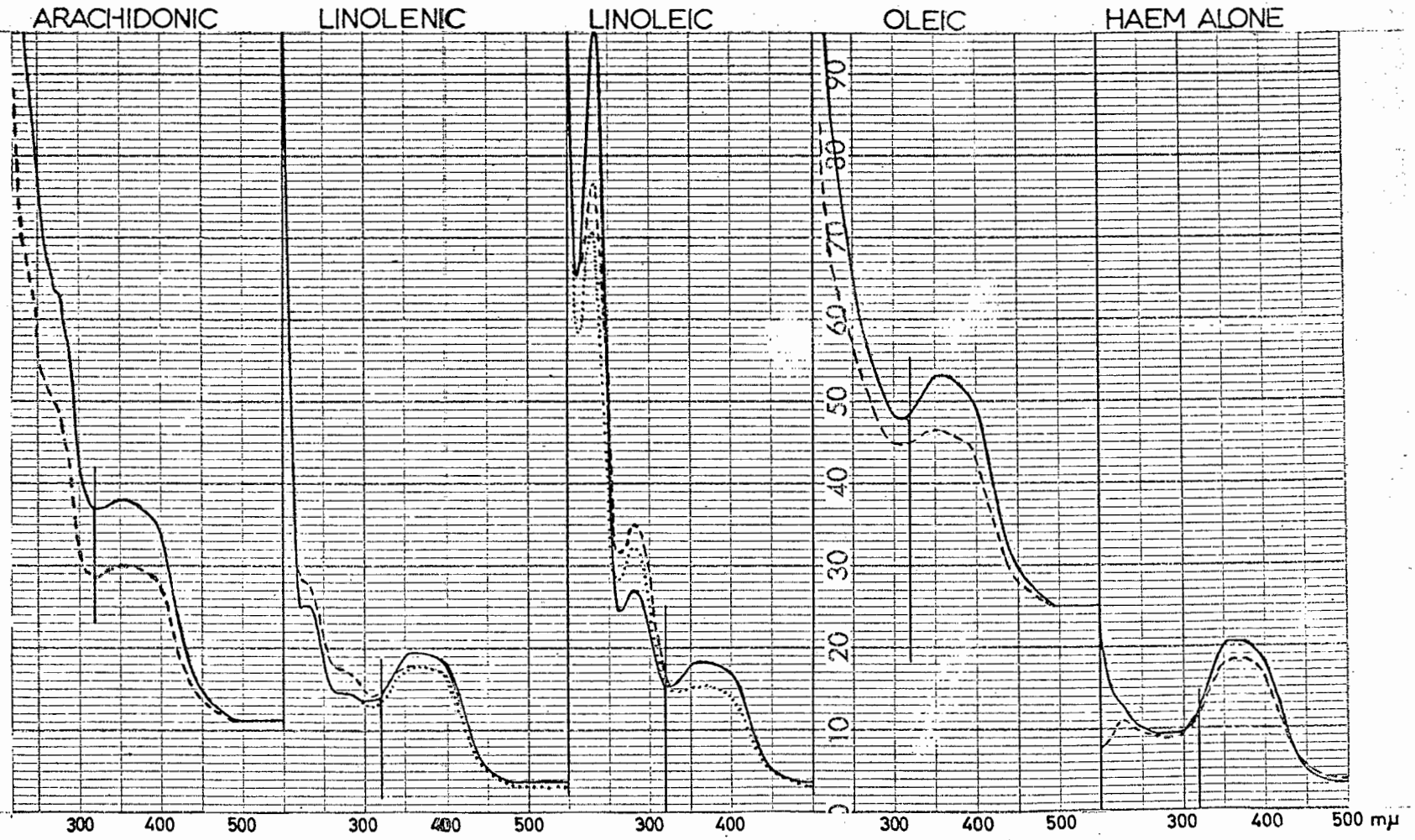
All the above fatty acids in unbuffered neutral solutions produced decrements in the Soret absorption maximum of ferrihaem (Fig. 56). Only linoleic and linolenic, however, were at all effective. This could be correlated with the degree of prior auto-peroxidation in the lipid preparations.

The linoleate preparation was the only one with an appreciable absorption maximum at 233 mμ and on reacting with haem, it produced a rapid decrease in Soret absorption at room temperature. Incubation at 41°C produced slight increments at 230 and 284 mμ but caused little change in Soret absorption.

Linolenic acid, having a much lower peroxide content, caused a lesser fall in Soret absorption and only exhibited a rise at 274 mμ after incubation.

The oleic and arachidonic preparations containing no detectable peroxides were relatively inactive in haematin co-oxidation. The general decrease in optical density on standing was due to a lessening in turbidity of the preparations presumably by slow auto-

FIG. 56. EFFECT OF VARIOUS UNSATURATED FATTY ACIDS ON THE DEGRADATION OF FERRIHAEM.



peroxidation in air. The peroxides formed during the experimental period, were, however, insufficient to induce catalysis by ferrihaem.

Later experiments on this coupled oxidation system of fatty acid peroxides and ferrihaem were performed on linoleate preparations.

(b) Ferrihaem destruction.

In only one experiment performed in unbuffered alkaline solution was ferrihaem almost completely peroxidized. Linoleate was present in a molar ratio of 52 to 1 haem, and the peroxidation products had an absorption maximum at 287 mμ (Fig. 57).

(c) Effect of buffering on the reactants.

The effect of buffering the linoleate-ferrihaem mixtures with 0.1M phosphate buffer at pH 7.2 was stimulatory on the coupled oxidation system as compared to the reactant mixtures diluted with distilled water (final pH = 6.8). (Fig. 58).

(d) Effect of fatty acid concentration on the coupled oxidation system.

Fig. 59 shows the effect of increasing concentrations of linoleate (4 - 44 moles/mole of haem) on the change in 4 hours in the optical density of ferrihaem-linoleate mixtures incubated at 42°C. The absorption at 3 different wavelengths was determined.

FIG. 57.

COUPLED OXIDATION OF FERRIHAEM AND LINOLEIC ACID.

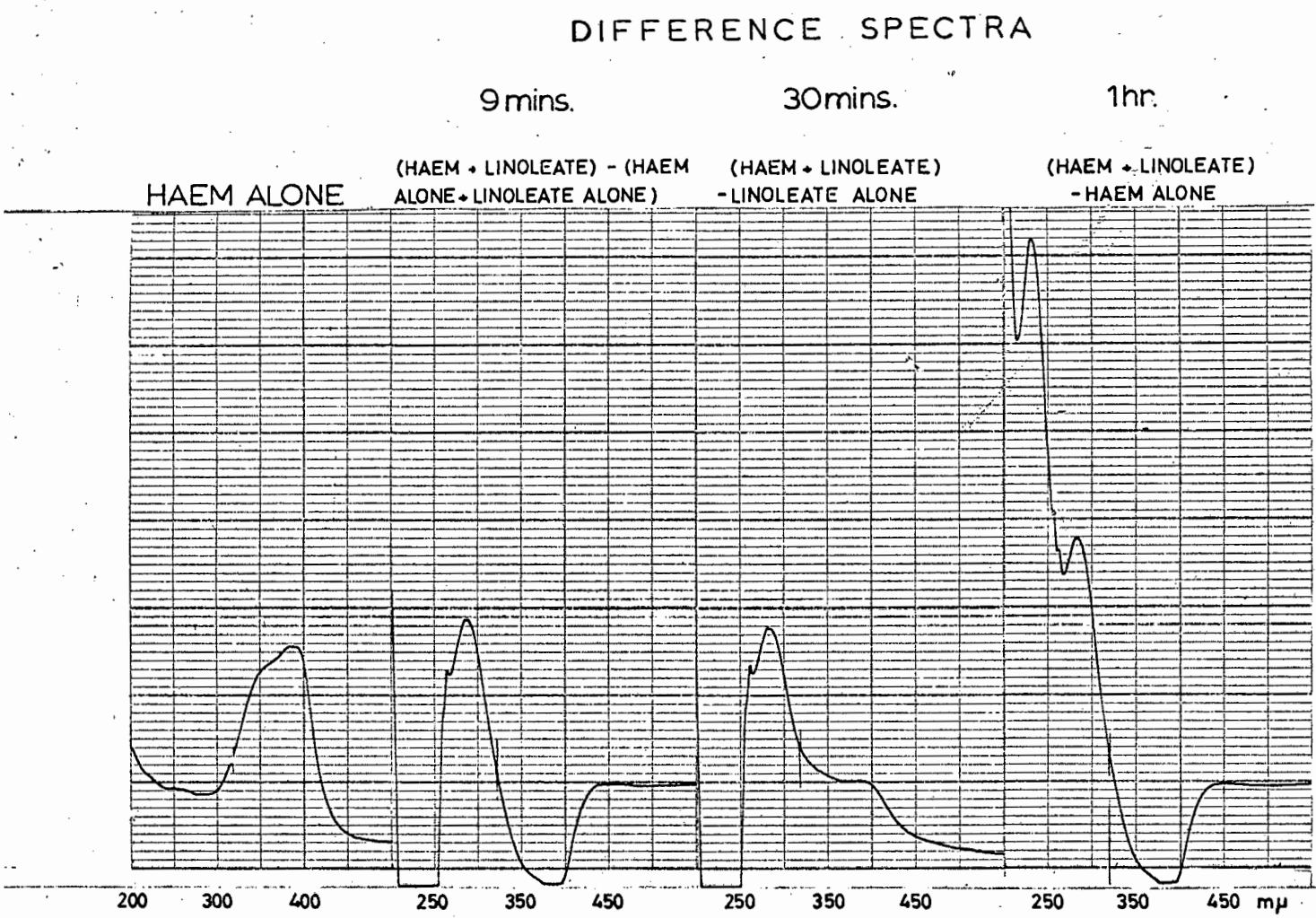


FIG. 58. COUPLED PEROXIDATION OF LINOLEATE AND FERRIHAEM. EFFECT OF BUFFERING THE REACTANTS. (pH 6·8).

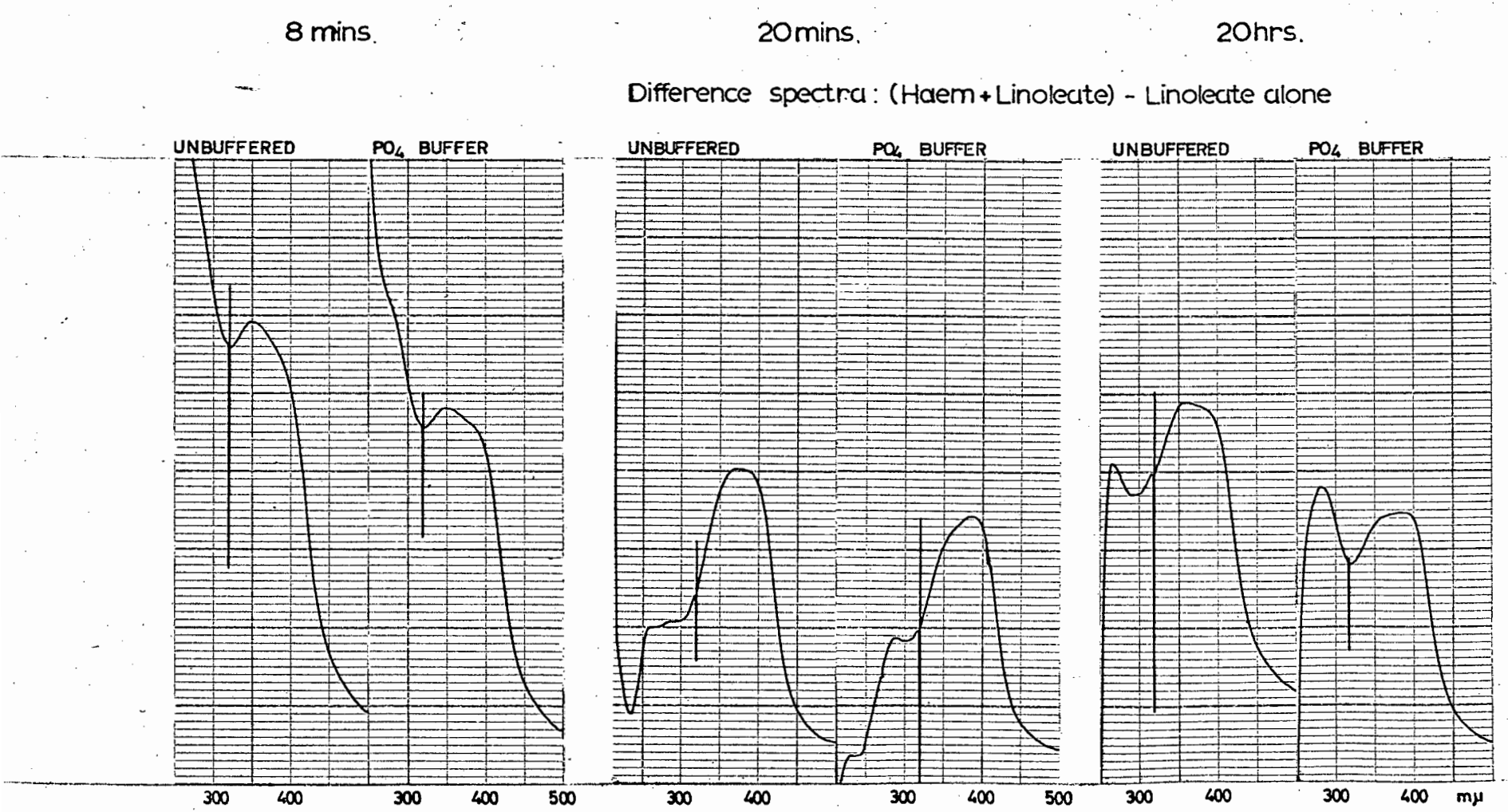
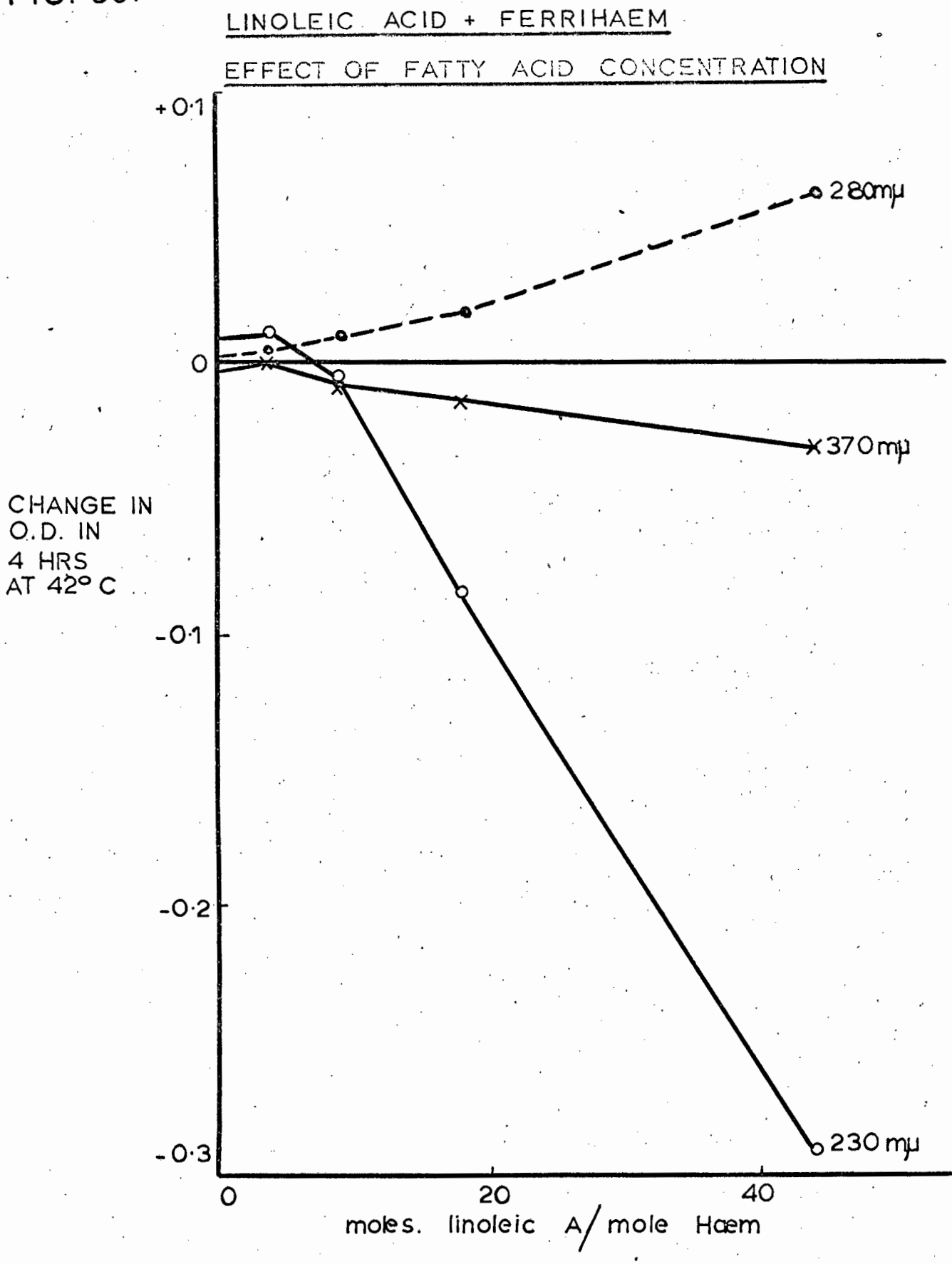


FIG. 59.


LINOLEIC ACID + FERRIHAEM
EFFECT OF FATTY ACID CONCENTRATION
+0·1
0
-0·1
-0·2
-0·3
CHANGE IN O.D. IN 4 HRS AT 42° C
280mμ
370mμ
230mμ
0
20
40
moles. linoleic A/ mole Hæm

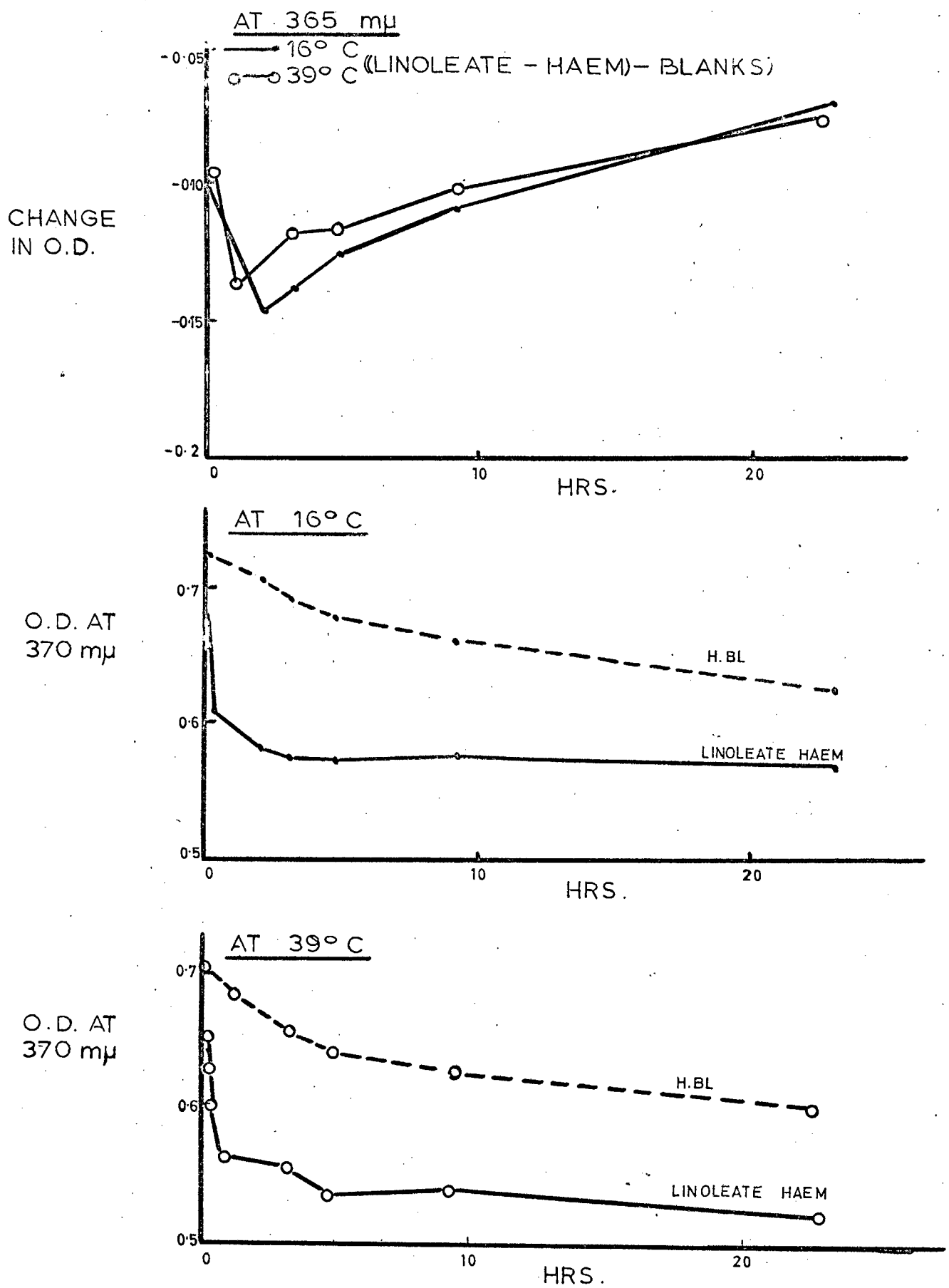
At fatty acid concentrations of less than 7 molecules per haem group, the induction period for peroxidation, which increases with increasing catalyst concentration, (Tappel[161]; Kaufmann and Kaufmann[164]) was longer than four hours. Consequently, the value at the 230 mμ maximum increased slightly with incubation as the linoleate present, underwent autoperoxidation. At higher fatty acid concentrations, scission of lipid peroxides with concomitant haematin destruction occurred (Tappel[161], producing decrements at 230 and 370 mμ. This was followed by the development of an absorption maximum at 280 mμ as peroxidation products accumulated.

(e) Influence of temperature.

We examined the influence of temperature on fatty acid oxidation catalysed by ferrihaem. Linoleate was added to haem in a molecular ratio of 17 to 1. The reactants were buffered to a pH of 7.2.

Fig. 60 shows the almost negligible influence of temperature on the course of haematin destruction estimated by reading the optical density of the mixtures at 370 mμ. The fall in absorption of the samples containing ferrihaem alone, which were incubated concurrently at 16°C and 39°C, was also plotted. One can see that peroxidation due to the presence of fatty acids is far more dramatic than that due to molecular oxygen.

FIG. 60. INFLUENCE OF TEMPERATURE ON FERRIHAEM + LINOLEATE PEROXIDATION



Although haematin destruction appeared unaffected by changes of temperature, optical density readings at 285 mμ and 230 mμ, at the same time, revealed a greater temperature dependence.

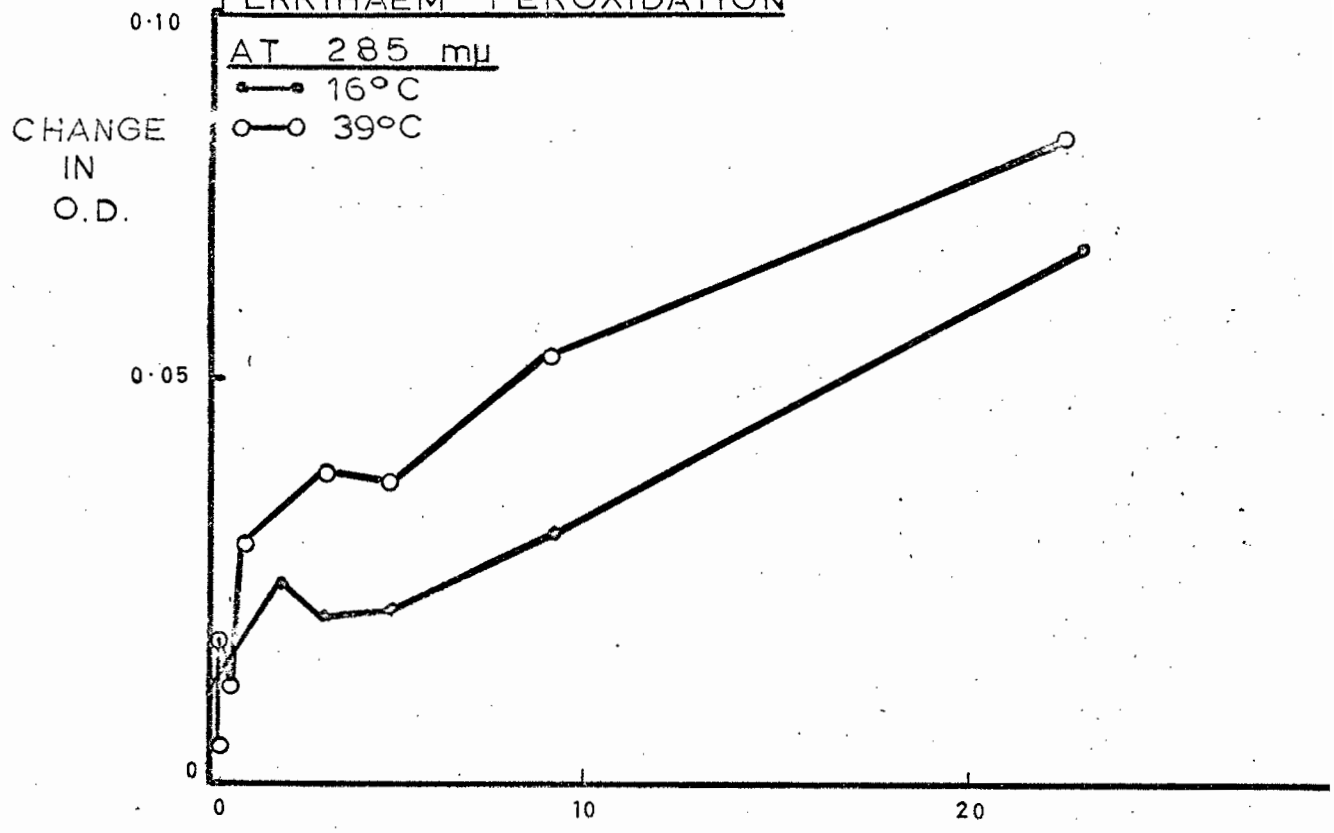
At 285 mμ (Fig. 61): there was a sharp initial rise at both temperatures, coincident with the fall at 370 mμ in the test mixtures. This was followed by a gradual decrease at 285 mμ in the sample at 16°C and a slight rise in that at 39°C. The absorption in the controls, linoleate and ferrihaem incubated separately, fell almost linearly during the incubation period although the decrease was sharper at the lower temperature. Nevertheless, the corrected curves (blanks subtracted) showed a greater rise at 285 mμ on incubating at a higher temperature.

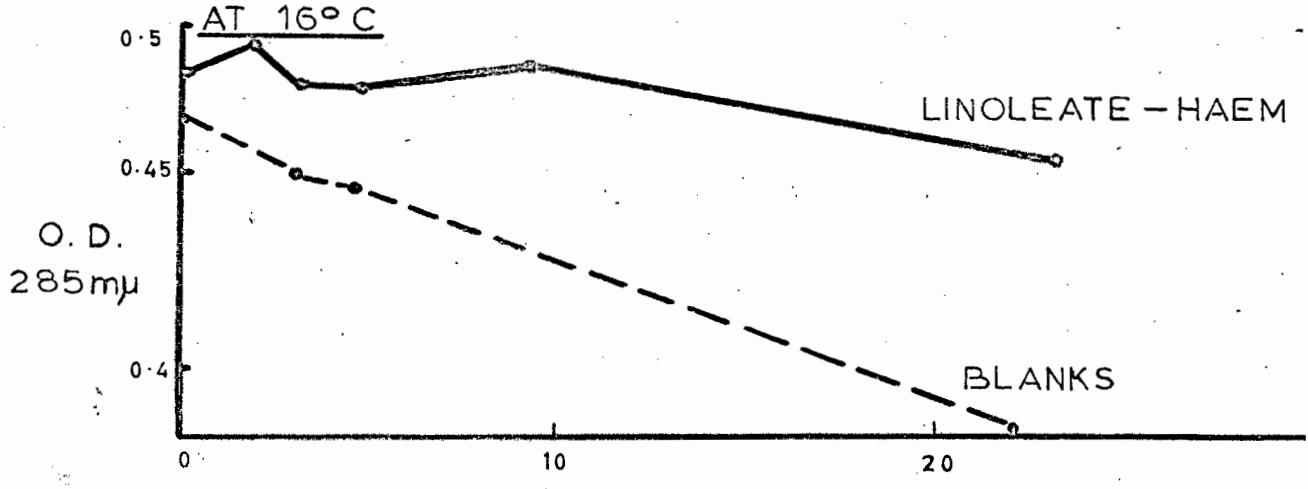
At 230 mμ (Fig. 62): there is a very rapid initial drop at both temperatures as the lipoperoxides present in the linoleate preparations, react with ferrihaem and are broken down. The subsequent incubation produces a greater increment at this wavelength at a higher temperature.

Tappel has shown that increasing the incubation temperature of haem catalyst-linoleate mixtures from 0° to 37°C produces a very definite rise in oxidation rate as measured by O_2 consumption in a Warburg apparatus[161].

Although, at first our results appeared to be contradictory to this finding, a closer examination revealed that there was actually a good correlation between them and the reaction mechanism

FIG. 61. EFFECT OF TEMPERATURE ON LINOLEATE + FERRIHAEM PEROXIDATION





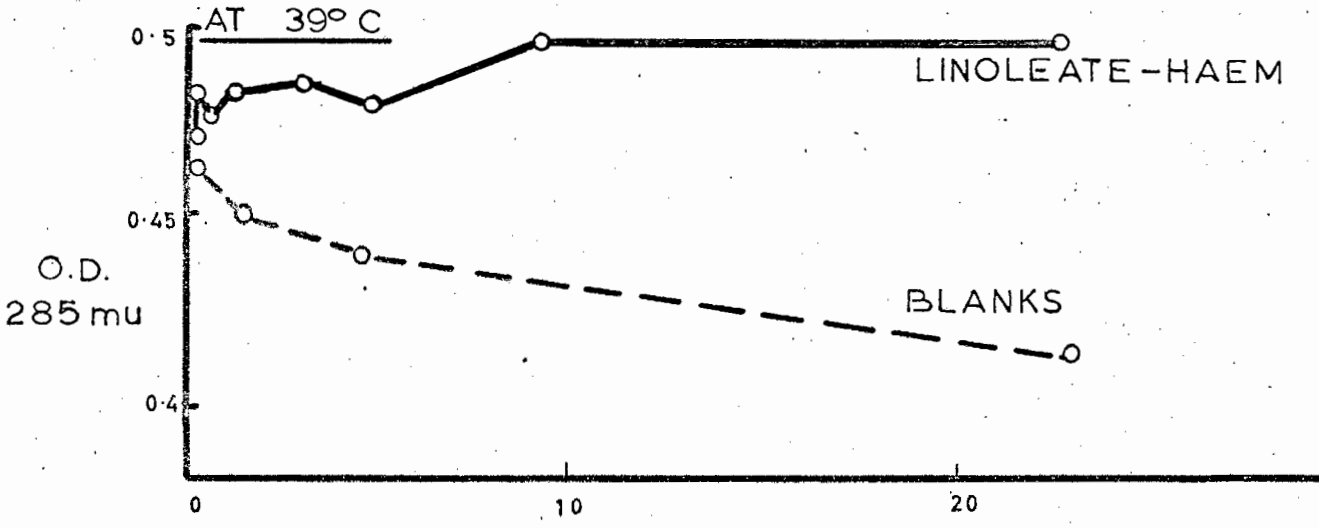
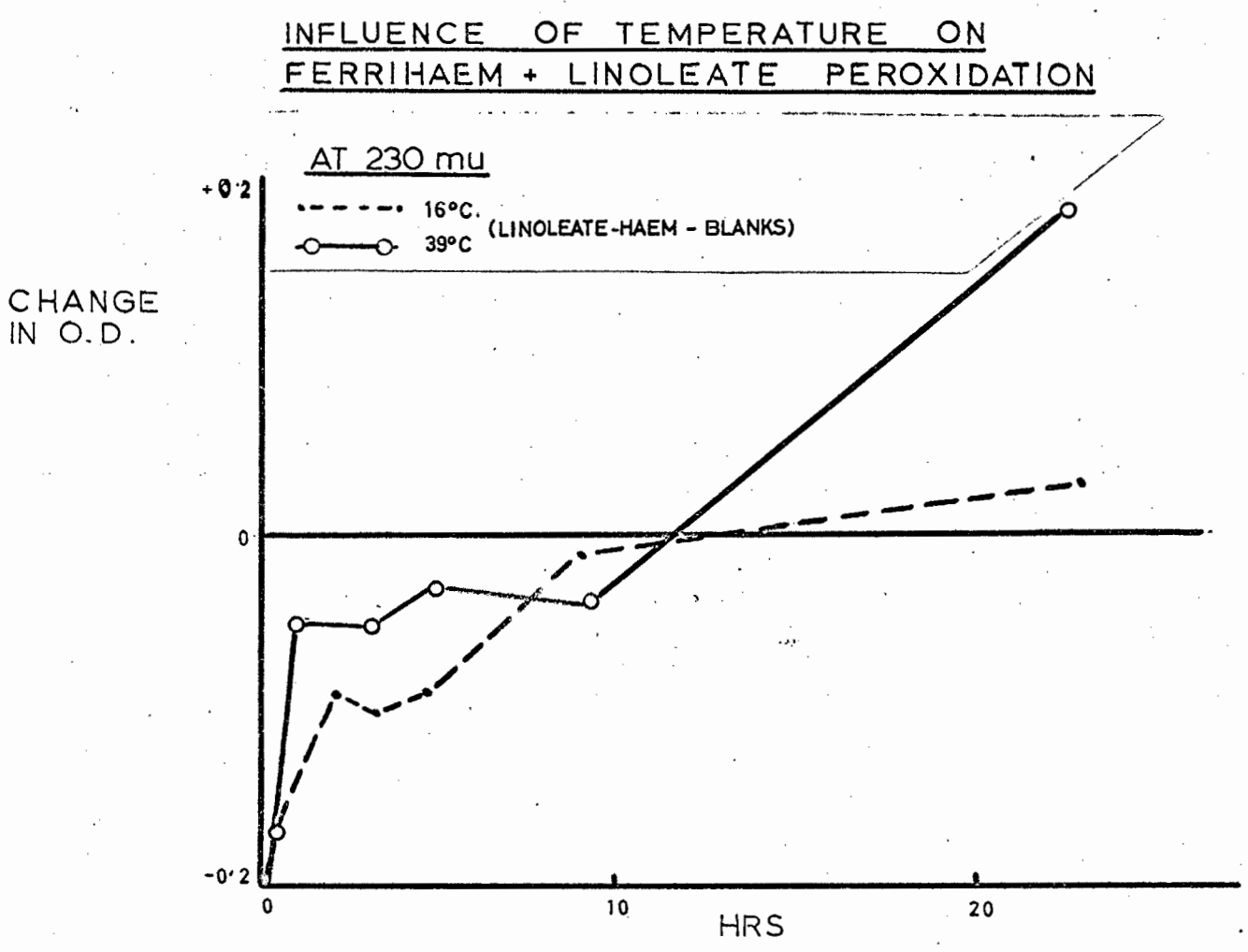


FIG. 62.


INFLUENCE OF TEMPERATURE ON
FERRIHAEM + LINOLEATE PEROXIDATION
AT 230 mu
16°C.
39°C
(LINOLEATE-HAEM - BLANKS)
CHANGE
IN O.D.
+0·2
0
-0·2
0
10
20
HRS

proposed by Tappel[161]. This involves a first step in which a lipoperoxide is formed by a means other than haematin catalysis. We have shown that this step is influenced by temperature. However, our fatty acid preparations always contained a certain quantity of lipoperoxides, consequently there was no induction period over which heating could exert any influence.

Initiation of the peroxidative chain reaction proposed, would require the formation of a ferrihaem-linoleate peroxide as a transitory intermediate and which, on breaking down, would yield 2 radicals, capable of causing propagation of a free-radical chain reaction along the carbon chains of the fatty acid in the presence of oxygen. The haematin is presumably oxidized during this interaction and it is this part of the sequence, shown in Fig. 60 which is unaffected by temperature.

Subsequent interactions between the shorter chain linoleate radicals, peroxides and haematin to terminate the chain reaction produce carbonyl compounds which have ultraviolet absorption. Their final production also seems to be accelerated by a higher incubation temperature (Fig. 61).

(f) Product of ferrihaem oxidation by linoleate.

Ferrihaem oxidation in this system was characterized by a fall in Soret absorption and was not accompanied by an equivalent increase anywhere in the visible region of the spectrum. The intensity of the brown colour in the reaction mixtures decreased

with no apparent change in hue. Although low concentrations of ferrihaem were used, it seemed highly unlikely that any bile pigment was formed.

The only concomitant spectral increase was in the ultraviolet (270 - 285 mμ). This more intense absorption was presumably caused by accumulation of colourless peroxidative products from both linoleate and haematin.

The mixtures did not react with Ehrlich's aldehyde reagent nor did they exhibit any fluorescence. The products of haematin degradation in this system appear to be essentially the same as those produced by the action of peroxide on ferrihaem.

(g) Effect of albumin on linoleate-ferrihaem peroxidation.

The investigations on this 3-component system are described in a previous section (2.233 D (v)).

Linoleate appeared to act in 2 ways:

(1) It formed a coupled oxidation system with a small proportion of the unbound ferrihaem. However, haem breakdown by fatty acid was inhibited by roughly 50% in the presence of albumin.

(2) It increased the affinity of albumin for haem, enhancing both the rate and quantity of methaemalbumin formed.

Albumin appeared to protect both bound and unbound ferrihaem against coupled peroxidation.

It is not known whether the 2 effects outlined above represent the different activities of free linoleate and of albumin-bound fatty acid.

No experiment was performed to test the effect of adding more fatty acid after ferrihaem had reacted with the preincubated albumin-linoleate mixtures.

It has been shown by Tappel that haemoglobin and particularly catalase are less effective as catalysts of fatty acid oxidation than haematin, while cytochrome c is the most active of the 4[161]. It will be interesting to see, therefore, how methaemalbumin functions in this system and whether its activity in this regard, sets it apart from the other haemoproteins.

(h) Effect of α-tocopherol.

The anti-oxidant action of α-tocopherol (vitamin E) is well-known. Its inhibitory effect on haematin-catalysed oxidation of unsaturated fatty acids, vitamin A and carotene has been shown by Tappel[168].

Serum levels of α-tocopherol in the rat have been shown to increase the resistance of erythrocytes to peroxide (Pokrovskii and Abrarov[61]), probably by inhibiting peroxidative breakdown of the lipoprotein-containing cell membranes of erythrocytes.

We thought it possible that if we could selectively inhibit the peroxidative chain reaction in which fatty acid breakdown is catalysed by ferrihaem, it might be possible to produce a situation in which only one methene bridge of each haem molecule is oxidized, i.e. a situation in which bile pigments are formed, instead of the complete oxidative breakdown which seems to occur with fatty acids alone.

We decided to test the effect of α-tocopherol on the linoleate-ferrihaem system in a preliminary qualitative experiment.

Crystalline haemin and a small quantity of α-tocopherol were dissolved in ethanol. The mixture was divided into 2 parts, to one of which one drop of linoleic acid was added. The 2 mixtures were incubated at 37°C for 2 hours, after which the sample containing both linoleate and α-tocopherol was visibly greener than the one containing only α-tocopherol. The spectra on dilution showed absorption bands at 596 and 480 mμ and a sharp Soret peak at 401 mμ - this is the spectrum of ferrihaem-chloride in ethanol. The difference spectrum showed falls in absorption of the greener mixture at 633 and 506 mμ with a slight decrease at 425 mμ. There were slight increments at 685 and 441 mμ.

The components of the mixtures were separated on alumina columns. By development with chloroform and ethanol, the linoleate and α-tocopherol were eluted. The alumina containing the fraction of haem pigment adsorbed onto the top of the column was extracted

with an acetic acid-ether mixture. The ethereal extract of the sample containing α-tocopherol and haemin was brownish, that of the linoleate-supplemented mixture was pale green. Spectral estimations were, however, inconclusive.

Nevertheless, it appeared that some biliverdin might have been formed and since we are vitally interested in the physiological aspects of haem degradation, this line of investigation will be continued in the future.

(iv) Haem degradation by ascorbic acid and oxygen.

During our investigations on the coupled oxidation system of ferrihaem and unsaturated fatty acids, no detectable bile pigments were produced. However, another chemical system, also involving coupled oxidation, namely, the ascorbic acid-oxygen system, is known to produce bile pigments from haematin and haemoproteins in vitro (Kench[14]).

We suspected that the quantities of bile pigment which might have been formed in the above experiments on linoleate-haem peroxidation would have been too small to detect spectroscopically. It was decided, therefore, to investigate by the same method, the interactions between ascorbic acid, ferrihaem and albumin under similar conditions of concentration, pH and temperature as those pertaining in the experiments with unsaturated fatty acids.

Ferrihaem was added to a mixture of ascorbic acid and albumin

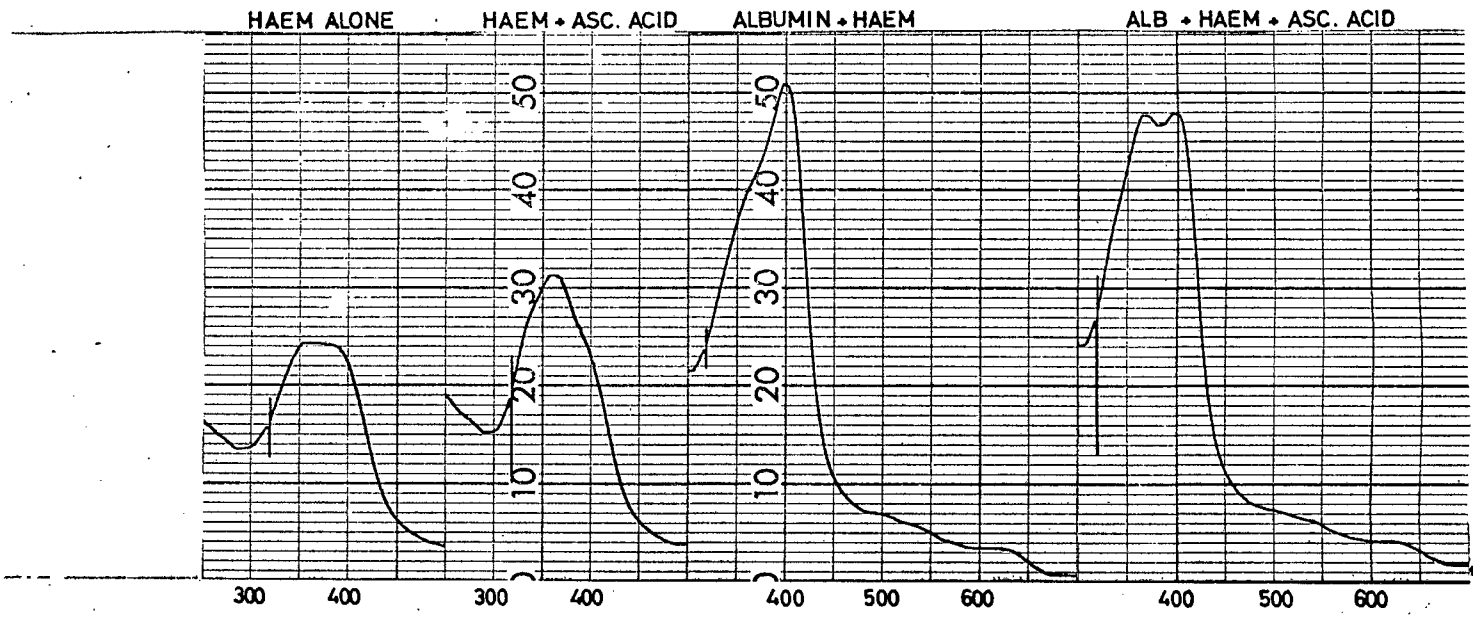
in phosphate buffer pH 7.0 at 37°C. The approximate molar ratio of ascorbate to ferrihaem was 11 to 1 and that of ferrihaem to albumin, 1.5 to 1.

At this concentration ascorbate appeared to have little effect. After 4 hours incubation the control, containing ferrihaem and ascorbic acid showed only a slight fall in absorption at 417 mμ compared with the ferrihaem blank (Soret maximum at 368 mμ). The test mixture containing albumin produced slightly less methaemalbumin than the control with no ascorbic acid. After 5 hrs. incubation, more ascorbic acid solution was added but this made very little difference to the spectra recorded 14 hrs. later (Fig. 63). The bifringent Soret peak exhibited by the mixture containing ascorbic acid, albumin and ferrihaem is due to a selective breakdown of the ferrihaem absorbing maximally at 385 mμ (possibly ferrihaem-hydroxide) whereas the free ferrihaem which has an absorption peak at 368 mμ is not attacked; nor is the methaemalbumin, although slightly less is formed in this mixture than in the control containing no ascorbic acid.

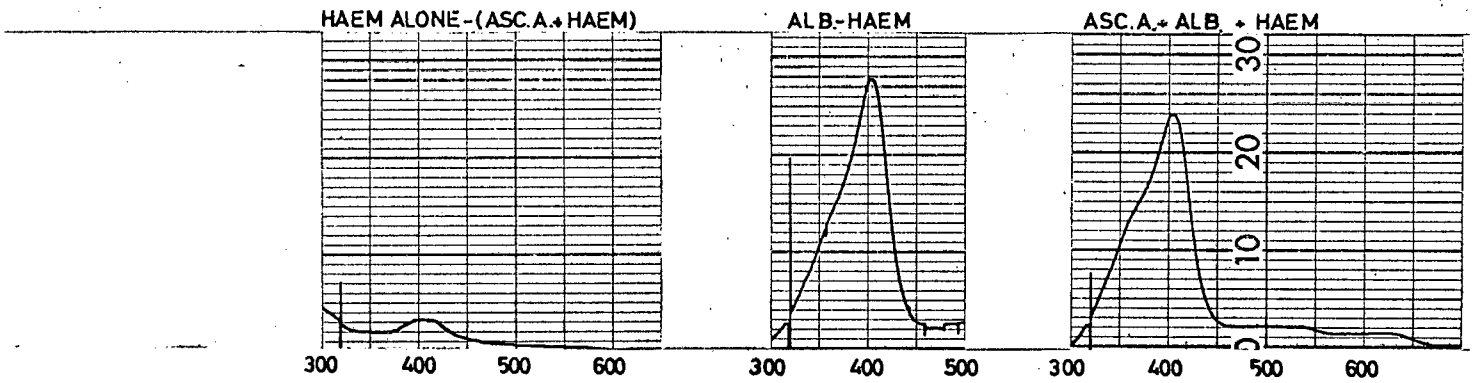
In these interactions, the only very slight changes, caused by the presence of ascorbic acid, can most likely be ascribed to the addition of too small a quantity of ascorbate. Although in his investigations on the oxidation of haematin and methaemalbumin in ascorbic acid oxygen systems, Kench used similar molar ratios, i.e. from 2 to 20 molecules ascorbic acid per molecule of haem (pH of final mixture was acidic), to produce yields of bile pigment

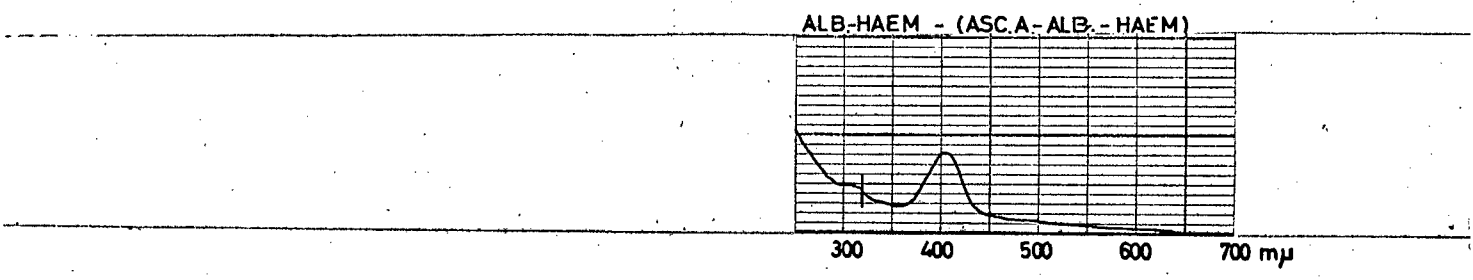
FIG. 63. ACTION OF ASCORBIC ACID ON ALB.-HAEM MIXTURE.

Spectra after 18hrs. incubation.



Difference spectra:





of up to 16% , vigorous oxygenation of the mixtures was continued throughout the experiments, thereby regenerating dehydroascorbic acid[14]. Furthermore, the final concentration of the reactants in our experimental mixtures would make spectroscopic detection of such yields, if attained, well-nigh impossible. In the experiments with linoleate, presumably each molecule can form 2 peroxide radicals, one at each unsaturated linkage. Dehydroascorbic acid on reduction, produces 1 active oxygen atom per molecule. Thus the concentration of ascorbic acid in the above experiment was rather low even compared with our previous degradative experiments. Nevertheless, certain differences appear to exist between these 2 peroxidative systems.

The coupled oxidation of haematin with ascorbic acid-oxygen systems involves the formation of free peroxide radicals in solution. These are decomposed by the peroxidatic action of ferrihaem molecules, the methene bridges of which are cleaved in the process. In the case of unsaturated fatty acids, the peroxide radicals occur as lipoperoxides in solution and it has been postulated (Tappel[161]) that ferrihaem forms a complex with the lipoperoxide which then breaks down to yield fatty acid radicals and carbonyl compounds. Presumably, it is during this interaction that the ferrihaem molecule is oxidized. This type of reaction may result in much more vigorous oxidation of the protoporphyrin ring than is the case with ascorbic acid.

It can be assumed that under the conditions of these

experiments, the concentrations of pigments in the reaction mixtures were always too small to give rise to measurable quantities of bile pigments. Although it seems unlikely that the peroxidation products of linoleate-ferrihaem interactions are bile pigments, this possibility cannot as yet be ruled out.

F. Haemoglobin-albumin interactions - quantitative experiments.

Our basic interest in all the studies described above was directed towards elucidating the physiological mechanisms whereby haemoglobin is degraded in complex biological systems. Some of the investigations on the influence of certain factors on the relatively simple chemical interactions of ferrihaem and albumin were repeated, using haemoglobin instead of ferrihaem.

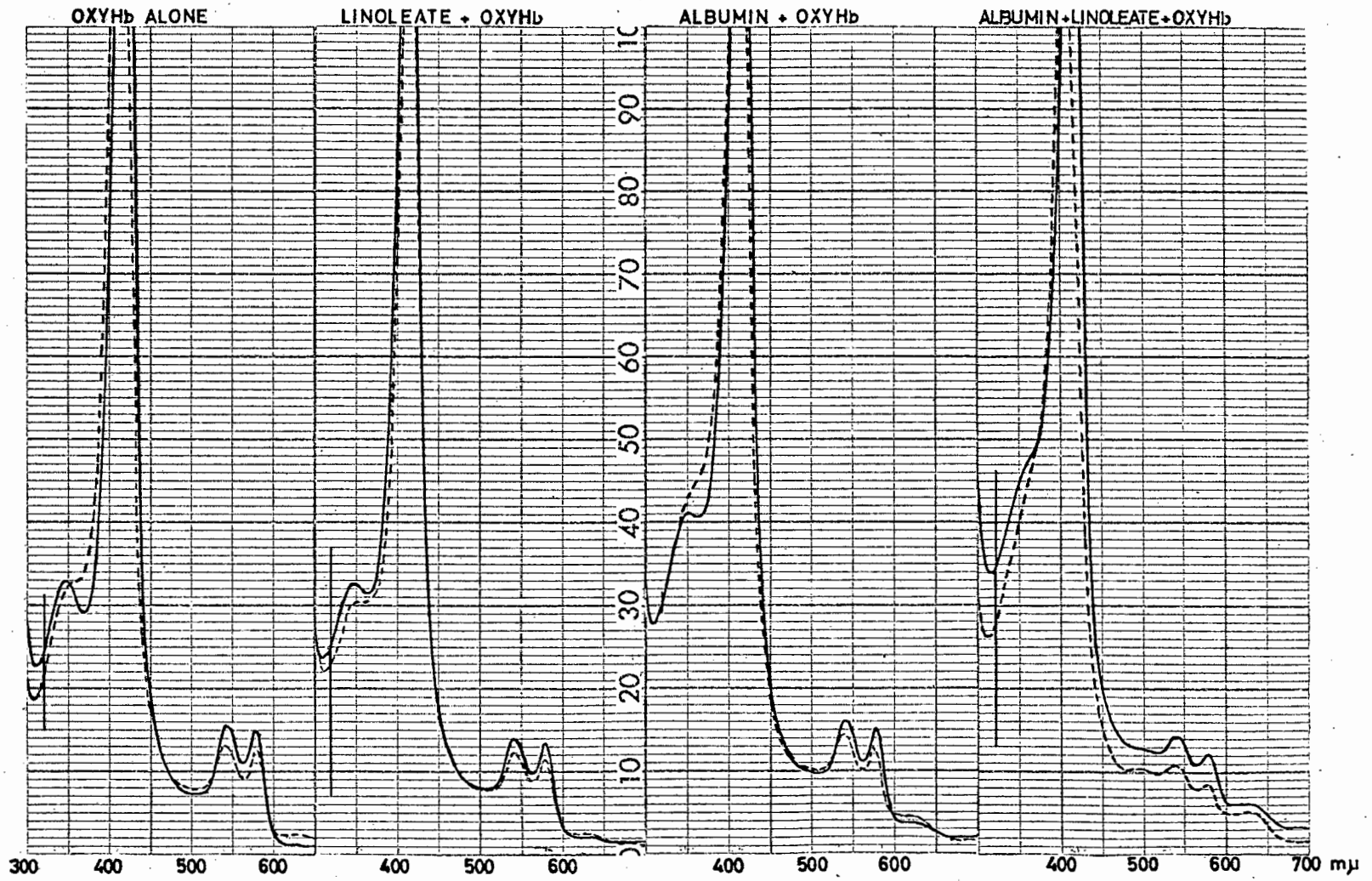
(i) Reaction between oxyhaemoglobin and albumin; effect of added linoleate.

2 haem equivalents of oxyhaemoglobin were added to a mixture containing unbuffered linoleate and albumin (8:1) at a pH of 6. The final concentration of oxyhaemoglobin was 18.4×10^{-3} mM. Controls were set up at the same time. The samples were incubated at 25°C for 7 hrs., stored at 0°C overnight and then incubated at 40°C for 4 hrs.

The spectra of the various controls and the test mixture are shown in Fig. 64. It can be seen that with prolonged incubation the absorption maximum at 347 mμ in all samples tends to disappear

FIG. 64.

EFFECT OF LINOLEATE ON REACTION BETWEEN OXYHAEMOGLOBIN AND ALBUMIN.



——— After 7hrs. at room temperature.

------- After 3·5hrs. at 40°C.

as the minimum at roughly 370 mμ increases. This can be correlated with methaemoglobin formation in the blank which contained only oxyhaemoglobin. This effect is, however, much more pronounced in the samples containing albumin, and occurs in conjunction with the rise at 620 mμ, which indicates formation of methaemalbumin (Fig. 65).

The preparation containing albumin and linoleate is much more effective in the production of methaemalbumin than that containing albumin alone. This is almost exactly the situation encountered with ferrihaem. The difference spectra in Fig. 65 show that there was an almost immediate rise in absorption at 620, 500, 390 and 278 mμ. Similar increments occurred in the control only after heating.

All the reactions, including degradation of the oxyhaemoglobin by linoleate, were much slower than in the similar experiments on ferrihaem. This can be correlated with (a) the findings of Tappel that haemoglobin was a less effective catalyst of linoleate oxidation than haematin[161], (b) the probable necessity for methaemoglobin formation before ferrihaem can dissociate from the globin apoprotein and be bound to albumin and (c) the acidic pH which is less condusive to methaemalbumin formation.

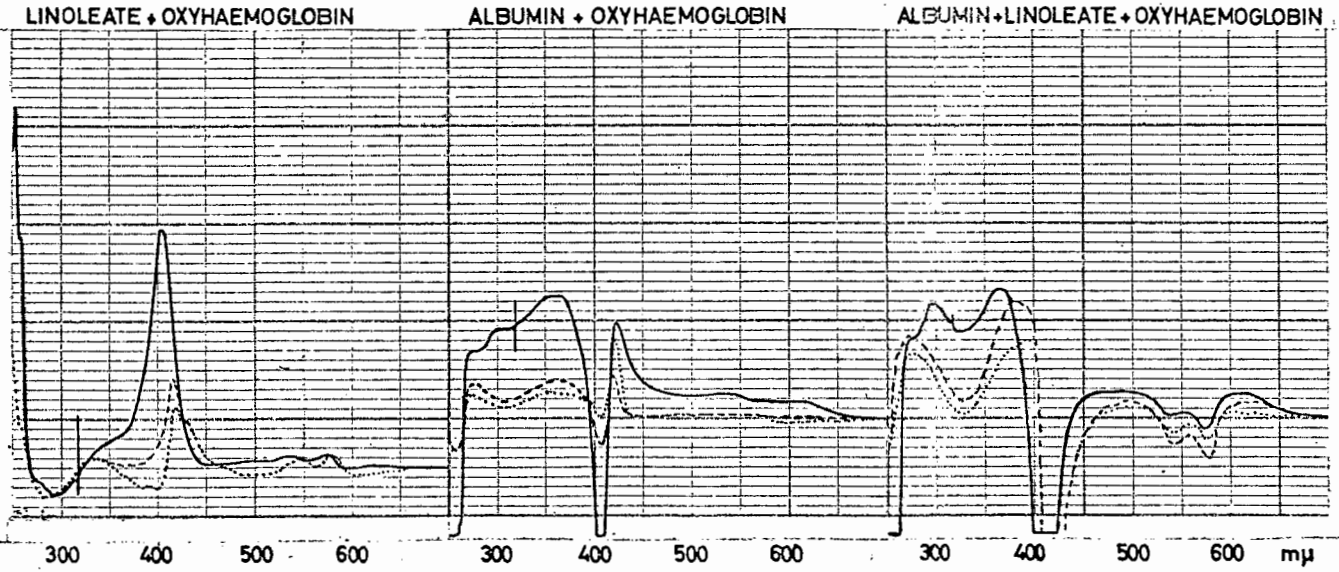
The percentage change in the samples at 3 different wavelengths is shown in Table 26.

The alterations in the difference spectra (Fig. 65) are quite complicated but seem to indicate that,

FIG. 65.

EFFECT OF LINOLEATE ON REACTION BETWEEN OXYHAEMOGLOBIN AND ALBUMIN.

DIFFERENCE SPECTRA :



····· 49 mins. at room temp.	····· 24 mins. at room temp.	····· 12 mins. at room temp.
- - - - - - 180 mins. at room temp.	- - - - - - 130 mins. at room temp.	- - - - - - 119 mins. at room temp.
——— 4 hrs. at 40°C.	——— 4 hrs. at 40°C.	——— 4 hrs. at 40°C.

TABLE 26.

Effect of linoleate on the chemical interaction between oxyhaemoglobin and albumin.

mμ	Percentage Changes in Optical Density			
	Oxy Hb alone	Linoleate + Oxy Hb	Albumin + Oxy Hb	Linoleate + Albumin + Oxy Hb
624	+ 450	+ 120	+ 400	+ 850
418	- 10	- 22	- 17	- 24
350	- 10	- 8	+ 20	+ 29

Oxy Hb = oxyhaemoglobin.

(a) linoleate initially causes the dissociation of haem from oxyhaemoglobin in both the control containing these 2 components and in the test mixture containing albumin as well (as judged by falls in absorption relative to the blanks at 578, 544, 418 and 340 mμ and increments at 390 mμ).

(b) it would be presumptious to infer that haemoglobin exerts its catalytic action on linoleate peroxidation long after dissociation of its haem molecules, i.e. that free haematin is the actual catalyst; especially in view of the rapid production of peroxidative products at 284 mμ in the control mixture which contains linoleate and oxyhaemoglobin. However, the initial increment at roughly 390 mμ representing free haematin decreased rapidly during the following 8 hours whereas the Soret peak of haemoglobin fell markedly only after incubation at 40°C when there was little or no evidence of further peroxidation (absorption maximum at 285 - 295 mμ remains the same).

(c) in the mixture containing albumin, oxyhaemoglobin and linoleate, the appearance of free haematin (rise at 390 mμ) within 12 minutes at 25°C was accompanied by increases at 624 and 500 mμ and by a much greater drop in the Soret maximum of oxyhaemoglobin than in the control in which linoleate was incubated alone with haemoglobin. The wavelength of overall maximal absorption shifted from 418 mμ to 409 mμ within 2 hours. The increment ascribed to free haematin at 390 mμ shifts with time to shorter

wavelengths to give the difference maximum at 370 mμ normally associated with methaemalbumin formation from the haemoglobins.

(d) the formation of methaemalbumin in the albumin-oxy-haemoglobin control was almost negligible with only a very small increment at 624 mμ in 2 hrs. and very little decrease in the Soret maximum of haemoglobin. Incubation at 40°C resulted in more pronounced spectral changes but the magnitude of these never approached the differences exhibited by the test mixture containing linoleate.

The effect of linoleate on methaemalbumin production in this system in which oxyhaemoglobin was used, was even more dramatic than in most of the experiments with ferrihaem. The preincubation period of linoleate with albumin prior to addition of oxyhaemoglobin was roughly 4 hours (cf. Experiment III, 2,233 D (v)) adding weight to the theory that it is the actual binding of fatty acid to the albumin molecules which enhances methaemalbumin formation.

In recent work by Rabinowitz, Chayen, Schen and Goldschmidt[169], it was found that a serum lipoprotein fraction catalyses the oxidation of inorganic ferrous iron.

Furthermore, Nishida and Nishida have shown that incubation of low density lipoproteins containing preformed lipohydroperoxides, with ferrihaemoglobin, at low temperatures under nitrogen, resulted in dissociation of the haematin prosthetic group which became associated with the lipoprotein fraction[162]. Fresh or peroxide-

free lipoproteins did not induce dissociation of haematin from ferrihaemoglobin under nitrogen gas. In air, however, these preparations not only caused oxidative dissociation of haematin but also led to destruction of the pigment to a colourless product.

Kench and Varma found that in experiments on ascorbic acid-oxygen peroxidation of haemoglobin, resulting in bile pigment production, the lipid-rich stroma of erythrocytes had no effect on the bile pigment production in this system[39]. Since yields of bile pigment in the ascorbic acid-oxygen system were only approximately 10% of that theoretically possible, this finding does not preclude the possibility that the rest of the haemoglobin was degraded by some means other than bile pigment formation in the presence of the stromal lipoproteins. Since, on the basis of the available evidence (our experiments and those of Haurowitz et al.[54] and Nishida and Nishida[162]), it seems unlikely that bile pigments are produced as a result of oxidative haematin destruction by lipoperoxides, it is possible that when erythrocytes undergo destruction in the cells of the reticulo-endothelial system and the mechanisms preventing lipid peroxidation in the intact erythrocyte are no longer operative, some of the haemoglobin may be degraded by interaction with the stromal lipoproteins also present in the cells. Such an eventuality might explain the 20 - 45% of administered haem-C^{14} not recovered as bilirubin-C^{14}, by Ostrow et al.[50] when the radioactive dose was injected into

rats in the form of sensitized red cells. However, even when haemoglobin as such, is injected this effect is observed, although the recoveries of bilirubin-C^{14} are greater.

(ii) Comparison of oxy- and methaemoglobin in methaemalbumin formation.

The above experiments and those of other authors (Hollocher and Buckley[150]; Steinhardt, Polet and Moezie[170]) as well as the present theories on the mode of attachment of the haem prosthetic group to the globin molecule (Perutz[171]) suggest that ferrihaem is more easily dissociable from methaemoglobin than is ferrohaem from oxyhaemoglobin. We decided to compare the activities of these haemoglobins in the chemical production of methaemalbumin.

Methaemoglobin was prepared from oxyhaemoglobin by treating it with potassium ferricyanide followed by exhaustive dialysis. The haemoglobin preparations were added to separate aliquots of albumin in molar ratios of 1 to 2 in each case (i.e. haem/albumin ratio = 2/1). The samples and controls were diluted with phosphate buffer, pH 7, and incubated at 37°C.

Only very slight changes even after 24 hrs. incubation were noticeable in the spectra. Analysis of the results was complicated by the gradual oxidation of oxyhaemoglobin to methaemoglobin throughout the experimental period. This caused falls in Soret absorption, which therefore could not be considered as evidence

of methaemalbumin formation.

The difference spectra at roughly 6 hrs. after incubation had commenced, showed that the production of methaemalbumin, albeit very small, was greater when methaemoglobin was used as the haem donor (greater increments at 625 and 362 mμ) (Fig. 66).

Optical density measurements at 625 mμ were the only ones to reveal the differences between oxy- and methaemoglobin in this system and the readings are plotted in Fig. 67. The haemoproteins had very low optical densities at 625 mμ in the concentrations used in this experiment, and, therefore, the changes shown are very small as compared with the concomitant alterations in Soret absorption and must be considered as only tentative results until experiments are performed using higher concentrations of haemoglobin.

The curves in Fig. 67 can be explained as follows:

(a) Methaemoglobin alone has an absorption maximum at 630 mμ. The slight decrease in optical density at 625 mμ in the blank is duplicated at 407 mμ (the Soret absorption maximum) and is due to oxidative decomposition in air.

(b) The rapid rise within 1.3 hrs. at 625 mμ in the mixture containing albumin indicates that the ferrihaem prosthetic group of methaemoglobin is easily available for complex formation with albumin. The fact that there is no increase at 625 mμ on further incubation indicates complete interaction between the haem and albumin.

FIG. 66.

COMPARISON BETWEEN OXY- AND METHb IN THE FORMATION OF METHAEMALBUMIN.

Difference spectra after 6hrs. incubation.

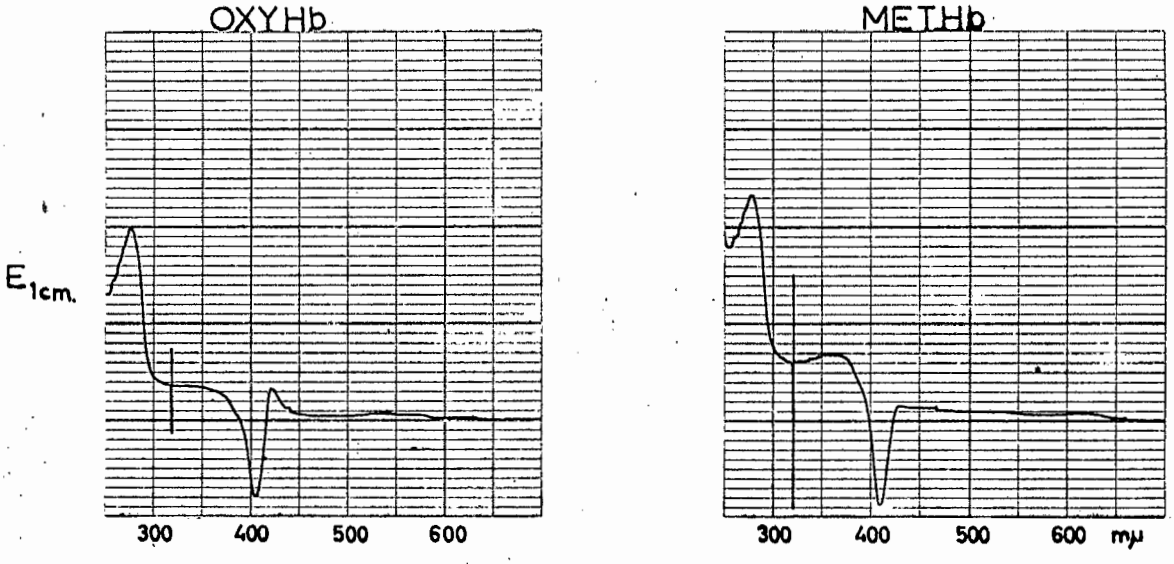
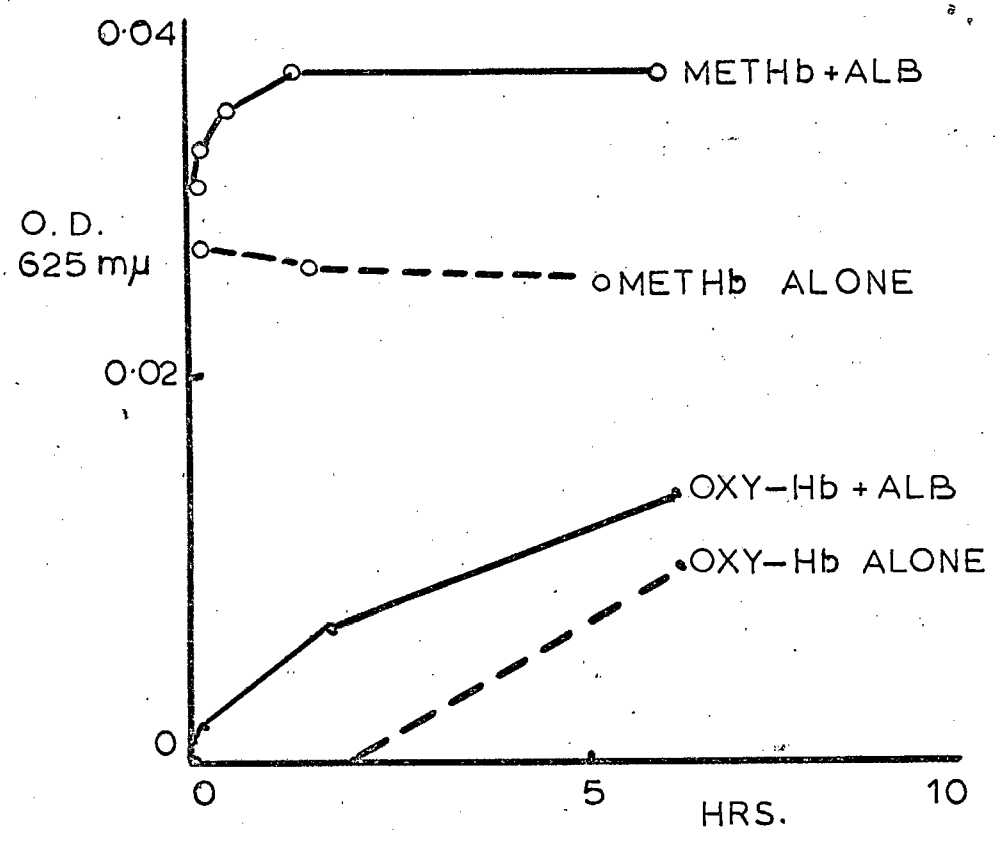


FIG. 67. METHAEMALBUMIN FORMATION FROM OXY- AND METHAEMOGLOBIN



(c) The rise at 625 mμ in the spectrum of oxyhaemoglobin incubated alone for 2 hrs., indicates oxidation to methaemoglobin.

(d) The almost parallel, though greater, increase at 625 mμ of the mixture containing oxyhaemoglobin and albumin, suggests that methaemalbumin is formed solely from methaemoglobin. In the first 30 minutes of incubation, however, the increase at 625 mμ in this mixture is not parallelled by oxidation of the oxyhaemoglobin in the blank, and must be due either to dissociation of ferrohaem from oxyhaemoglobin or increased oxidation of oxyhaemoglobin to methaemoglobin due to the presence of albumin, followed by ferrihaem dissociation and methaemalbumin formation. Evidence from previous experiments suggests that the latter explanation is the most likely to be correct.

(iii) Influence of pH on methaemalbumin formation in methaemoglobin-albumin mixtures.

Since the binding of ferrihaem to albumin is dependent on the pH of the medium, the effect of pH on the formation of methaem-albumin from ferrihaemoglobin was studied to ascertain whether the globin moiety modifies the influence of environmental pH on the ferrihaem molecule.

Methaemoglobin was added to albumin in a molar ratio of 1 to 2 at 4 different pH's. M/15 phosphate buffer was used to dilute the samples at pH 6.5, 7.4 and 7.7 and barbitone-acetate buffer for the mixture at pH 8.7.

The final concentrations of methaemoglobin in these test preparations, were greater than those of the previous experiment. This procedure was employed so as to ensure that differences in optical density at 625 mμ would have greater significance.

Unfortunately, the absorption spectrum of methaemoglobin changes with pH, probably reflecting the change from the high-spin type of iron-porphyrin bond to the low-spin variety as the pH rises and methaemoglobin-hydroxide is formed (Hollocher and Buckley[150]). The 2 absorption bands at pH 6.5 i.e. at 636 mμ and 503 mμ decrease as the pH rises. Small maxima appear at 577 and 540 mμ until at pH 8.7 these are the only maxima, the absorption bands at 636 and 503 mμ having completely disappeared (Fig. 68). Nevertheless, the corrected readings, i.e. initial blank readings subtracted from the optical densities of the respective methaemoglobin-albumin mixtures, show an almost linear relationship between the rise at 625 mμ (i.e. methaemalbumin formation) and pH (Fig. 69). This pH effect is reflected in the difference spectra after 3 - 5 hrs. incubation (Fig. 70). Other features associated with methaemalbumin formation were also noted. As the pH rose and consequently methaemalbumin formation increased, the absorption maxima at both 625 mμ and 370 mμ increased. The wavelength of maximal absorption of these bands also became longer with increasing pH. The fall in Soret absorption increased progressively, indicating breakdown of methaemoglobin, and complete methaemalbumin formation appeared to have been achieved very rapidly at pH 8.7 compared with the slow

FIG. 68. ABSORPTION SPECTRA OF METHb AT DIFFERENT pH S.

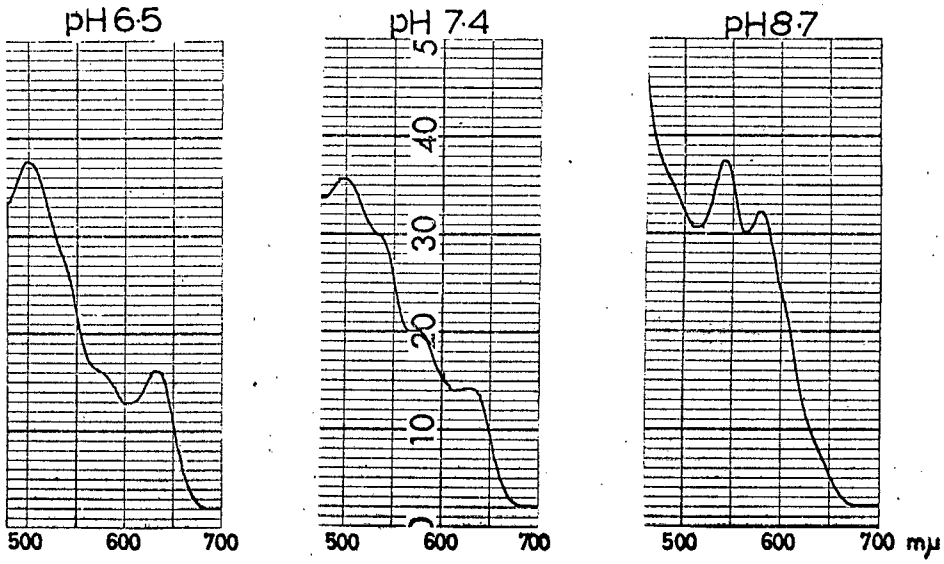


FIG. 69. METHAEMALBUMIN FORMATION FROM METHAEMOGLOBIN

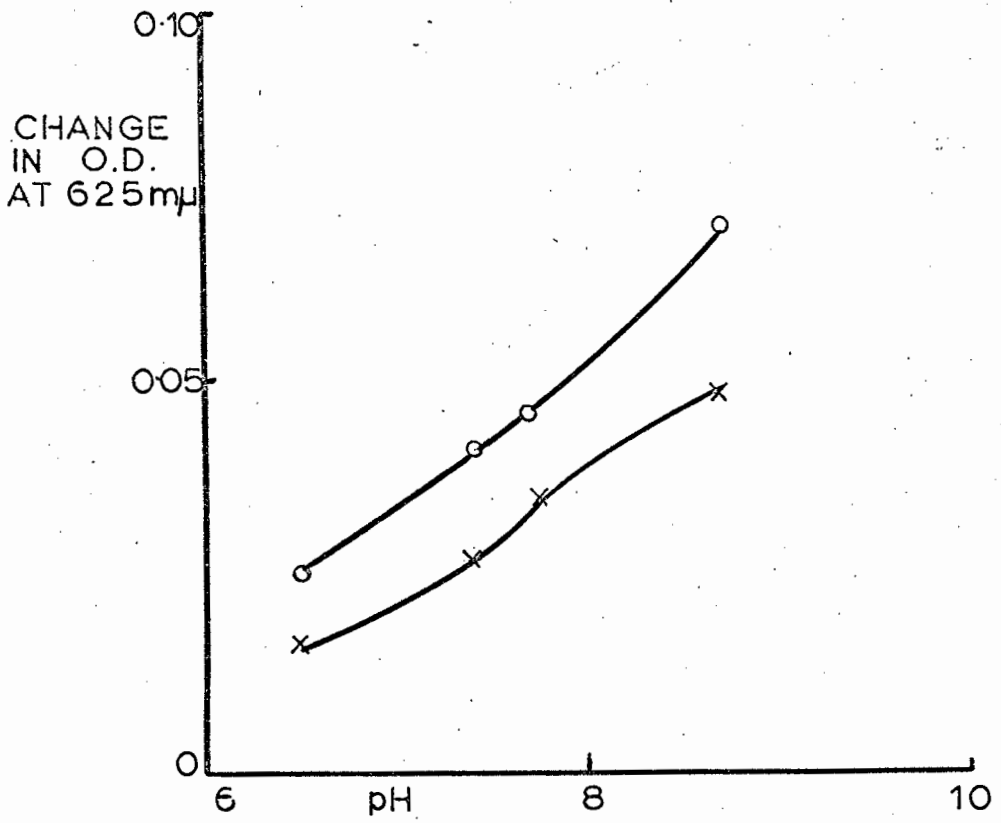
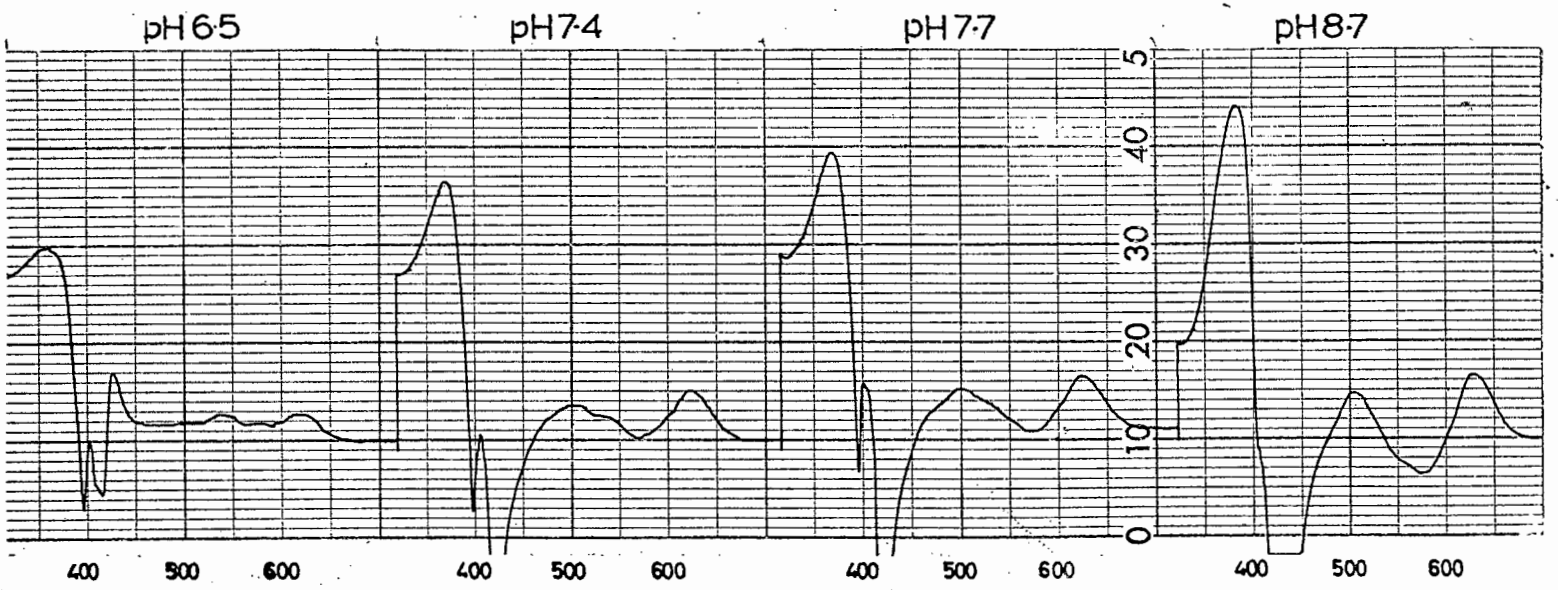


FIG. 70. INFLUENCE OF pH ON INTERACTION BETWEEN METHAEMOGLOBIN AND ALBUMIN.

Difference spectra: 3 - 5 hrs. incubation.



interaction with oxyhaemoglobin in previous experiments.

Another interesting finding was that within an hour, the mixture incubated at pH 8.7 showed marked turbidity. This was followed by the development of similar turbidities within 2 hours, in the test samples at pH 7.7 and 7.4. At pH 6.5, where very little methaemalbumin is formed, only very slight turbidity was noted.

Thus, it seemed that the turbidities must be due to denaturation of the globin apoprotein once the haematin moiety had been removed. Since the greatest denaturation occurred in the sample in which the most methaemalbumin was formed (this being at pH 8.7), the idea suggested previously in the qualitative experiments that this effect might have been due to acid denaturation, falls away.

Korinek and Dvorakova also noted this denaturation when methaemalbumin was formed in serum from added methaemoglobin[152].

PART 3.

DISCUSSION.

PART 3. DISCUSSION.

3.1 IN VIVO EXPERIMENTS ON GEESE.

Our experiments on geese reveal considerable differences in the rate of clearance of injected Fe^{59}-labelled haemoglobin from the plasma and in its subsequent tissue distribution as compared with the findings obtained from similar investigations on mammals.

The rate of clearance of injected haemoglobin was much slower than the rates reported for mammals, i.e. the 50% clearance time of haemoglobin from rabbit plasma was 25 minutes (Murray et al.(67)); in rats, the time required for plasma clearance of half the label was 10 - 20 minutes (Ostrow et al.(50); Keene and Jandl(72)). In the goose we found that half-clearance of injected haemoglobin required a $2\frac{1}{2}$ - 3 hr. period. In both the experiments performed, the haemoglobin load exceeded the binding capacity of the haptoglobins and resulted in haemoglobinuria, the extent depending on the load administered. Nevertheless, in 3 hrs., the Fe^{59} excreted when the plasma was grossly overloaded (approximately 300 mg. haemoglobin/100 ml. whole blood), was only 10% of the total dose. This agrees with Wise's finding that in rats, haemoglobin exceeding the haptoglobin binding capacity is not necessarily excreted by the kidneys and furthermore can act as a source of bile pigment(8).

However, after 3 hours, we were unable to account for roughly 40% of the injected dose which was cleared from the plasma and not excreted. Only 4% was present in the liver. This observation is extremely surprising particularly in view of the fact that the reticulo-endothelial

system in geese is reputed to be almost entirely confined to the liver (McNee[6]). Keene and Jandl have investigated the proportional tissue distribution of Fe^{59}-labelled haemoglobin in normal rats[72]. They found that 30 mins. after injecting the dose, 71% of the haemoglobin taken up by the reticulo-endothelial organs was in the liver, 8% in the spleen and 21% in bone marrow. However, in these investigations, radioactivity measurements were made on portions of the intact tissues and, since after 30 minutes, roughly 40% of the injected dose was still present in the circulation, these findings are probably not comparable with ours. Possibly the method used to remove extracellular haemoglobin from the liver tissue disrupted the cells. It is also possible that gross over-loading in our experiment resulted in increased haemoglobin uptake by the kidneys, which, by the time the animal was killed, had not yet been excreted.

Ostrow et al. found that after 22 hrs. roughly half the Fe^{59} injected into rats as C^{14}-, Fe^{59}-labelled haemoglobin was recoverable in the kidneys and urine although haemoglobinuria accounted for only 4% of the dose[50].

The fact that in our 26-hour experiment, half the radioactive dose was also unaccounted for, probably reflects a situation similar to that found by Ostrow et al.[50] in which Fe^{59} had been split off from the haem and taken up by the kidneys while the C^{14} label was excreted as bilirubin. Additional evidence for this having occurred can be seen in Table 6, which shows the activities of various fractions derived from the first 2 hr. collection of faeces and urine which had been separated on Sephadex G-50. Although most of the activity was associated with haemoglobin, the highest

specific activity was found in a small quantity of non-haem containing protein of large molecular size.

Clearly, the use of Fe^{59}-haemoglobin without concomitant labelling of the haem pyrrole nucleus has limitations as regards the isolation of intermediates, and in investigations of the degradative pathway.

Our results from fractionations of goose liver showed progressive incorporation of Fe^{59} from the cell supernatant into organelles. Gel fractionation of the non-particulate cell supernatant revealed gradual binding of Fe^{59} to non-haem containing proteins of large molecular size ($> 70,000$), presumably ferritin. Thus at 26 hr. after giving the isotope, 30 - 40% of activity was associated with molecules of M.W. 30,000 or less, whereas 48 hrs. after the injection of phenyl hydrazine only 2 - 7% remained attached to such small compounds.

The distribution of Fe^{59} in the particulate fractions 48 hrs. after the onset of intravascular haemolysis is worthy of mention.

The discovery by Omura and Sato[52, 53] of a CO-binding haemoprotein ("P-540") present in liver and adrenal microsomes suggests that under conditions of increased haemoglobin degradation, during which the α-methene bridge of the haem prosthetic group is cleaved with the production of CO, synthesis of the microscomal haemoprotein might be stimulated concomitantly, in order to bind the excess CO produced. Microsomes also contain cytochrome b_5 while all the other cytochromes of the electron transport chain are present in the mitochondria. The incorporation of one third of the Fe^{59} present in the liver homogenate into mitochondria and the high specific activity of

the microsomes (Table 8) are suggestive of Fe^{59} incorporation into these haemoproteins.

3.2 IN VITRO EXPERIMENTS ON LIVER TISSUE.

Nakajima and co-workers in Japan have demonstrated the presence of an enzyme system, present in guinea-pig liver which converts pyridine haemochromogen to a product with absorption at 656 mμ which they claim to be a precursor of biliverdin[40]. They have called this enzyme α-methenyl oxygenase although they have not shown whether the green pigment or the biliverdin produced therefrom, was the IX α-isomer which is the only known form of naturally occurring bile pigment (Petryka et al.[37]). Having performed most of their experiments, using pyridine haemochromogen as the substrate, Nakajima et al. found that although haemoglobin and haematin were not effective as bile pigment producers in this system, complexing of haemoglobin, particularly methaemoglobin, to haptoglobin produced high yields of the biliverdin precursor[40].

Incubation experiments which we performed on both guinea-pig and human foetal livers revealed no difference in the capacity of free haemoglobin or haptoglobin-bound haemoglobin to produce a rise in the red region of the spectrum. In fact, although heat labile components which were active in producing bile pigment precursors (as judged by a rise at 630 mμ), were detected in the liver tissues, yields from these substrates were poor (Kench et al.[98]).

Wise[8], in attempting to repeat the experiments of Nakajima et al.[40], confirmed that guinea-pig liver homogenates produced a slight increase in spectral absorption at 656 mμ when pyridine haemochromogen was used

as the substrate, whereas rat liver homogenates were not at all active in this respect.

A wealth of evidence from in vivo (Wise[8]; Keene and Jandl[72]; Ostrow et al.[50]; Murray et al.[67]) and liver perfusion experiments (Wise[8]; Robinson et al.[11]) indicates that binding of haemoglobin to haptoglobin had little effect on the rate of conversion of haemoglobin to bile pigments or on the rate of reutilization of haemoglobin iron.

Binding did, however retard plasma clearance rates of Fe^{59}-haemoglobin by preventing excretion of the labelled haemoglobin by the kidneys and it therefore also increased the total uptake of haemoglobin by the tissues (Murray et al.[67]). All these experiments were, however, performed on rats or rabbits and the occurrence of species differences in this regard, cannot therefore be excluded.

The only cell-free enzymic system which has been shown to produce the IXα isomer of biliverdin is that of the haemophagous organ of the placenta of the dog, (Wise and Drabkin[97]) which effectively degraded haemin and haemoglobin, both poor substrates in the guinea-pig liver system (Nakajima et al.[40]) and was inactive when pyridine haemochromogen was used as the substrate.

The physiological importance of an enzyme which catalyses the degradation of pyridine haemochromogen is obviously questionable. We have been unable to confirm the subsequent finding of Nakajima and his colleagues[40] that haemoglobin complexed with haptoglobin was an active substrate in this system. Bile pigment yields were no better than those obtained in ascorbic acid-oxygen systems in vitro (Kenoh[14]).

3.3 CHEMICAL INTERACTIONS.

Most of the physico-chemical aspects of the experiments performed were discussed in Part 2. We will confine ourselves here to discussing the more important physiological applications of the results obtained.

Haem transfer from unbound haemoglobin to albumin has been shown to occur in human sera (Neale et al.[104]; Liang[73]; Allison and Rees[121]; Aber and Rowe[112] and Nyman[111]). The fact that in vitro, methaealbumin formation in serum could be increased by prolonging the period of incubation or raising the temperature, was noted by Korinek[74] and Valeri et al.[113] This finding prompted us to investigate whether a "haem transferase" might be present in human serum. Our discovery that this transfer of haem could be effected by a purely chemical interaction between the 2 protein molecules in solution, was extremely surprising. One would expect, in view of the rapid binding affinity of native globin for haem (Gibson and Antonini[165]) that albumin would not be able to compete successfully with globin for haem groups. The only other report of haem transfer occurring between proteins in solution without the aegis of an enzyme, comes from Rossi-Fanelli and Antonini[114] who have shown that haem transfers occur between different apohaemoproteins presumably possessing varying affinities for haem, but albumin cannot be classed as an apohaemoprotein of thistype.

Our evidence suggests that the binding by albumin of haem derived from native haemoglobin, may only occur when the haem has been oxidized to the ferric form. The mere presence of albumin, however, appears to enhance this oxidation. This may be due to the residual quantity of unsaturated fatty acid always associated with purified albumin

(Goodman[132]). It has recently been shown (Rabinowitz et al.[169]) that a lipoprotein in serum catalyses the oxidation of inorganic ferrous iron to the ferric form. Our own experiments in which linoleate was added to a mixture of albumin and oxyhaemoglobin in solution, suggest that the unsaturated fatty acid promoted dissociation of haem in the ferric form from oxyhaemoglobin, thereby enhancing methaemalbumin formation.

Nishida and Nishida have observed a similar interaction when they incubated methaemoglobin with peroxidized serum low-density lipoproteins. Ferrihaem was dissociated from the globin moiety and was found to be associated with the lipid layer when the mixtures were ultracentrifuged[162]. However, solutions of albumin and linoleate exhibit important differences from these lipoproteins. Nishida and Nishida performed all their experiments under nitrogen gas, finding that incubations in air resulted in complete destruction of both the methaemoglobin and free ferrihaem[162].

Our results show that in air, the presence of albumin protein inhibited the peroxidative destruction of ferrihaem by unsaturated fatty acids.

The interaction of albumin with linoleate in aqueous solution, appears, therefore, to ensure maximum methaemalbumin formation in the presence of ferrihaem or oxyhaemoglobin. The nature of this interaction, i.e. whether the fatty acid is bound to albumin or has some surface effect is not known.

The striking influence of pH on the binding of ferrihaem to albumin can be ascribed to the influence of pH on the degree of

ionization of the propionic acid side chains, by means of which ferrihaem is bound to albumin. It is clear from the only slight differences in methaemalbumin formation between pH's 7.4 and 7.7 that at the pH of human plasma, the acidic side chains are almost completely ionized and that conditions are optimal for the binding of ferrihaem to albumin.

It has been assumed that methaemalbumin formation from free haemoglobin occurs only in disease states when, due to excessive intravascular haemolysis, the binding capacity of the serum haptoglobins has been exceeded.

Tentative evidence from experiments on human and goose sera suggests that the sequence of events considered by many workers to occur under conditions of normal intravascular haemolysis may not be as clear-cut as was originally thought. Recent findings that intravascular haemolysis may account for 10 - 30% of daily haemoglobin turnover (Garby and Noyes[68]; Freeman[69]) and that binding of plasma haemoglobin to haptaglobin does not appear to alter significantly the organ distribution (Keene and Jandl[72]) or the rate of bile pigment formation from labelled haemoglobin (Ostrow et al.[50]), add weight to the possibility that albumin may act as a carrier of haem groups under normal circumstances.

Wise[8], in experiments in which he perfused isolated rat liver with different haemoproteins and studied the bilirubin produced therefrom, encountered difficulties when using native haemoglobin and methaemalbumin - the isolated liver was unable to degrade the native proteins. When, however, these were denatured with alkali and added to the perfusion medium, the rate of degradation to bile pigments was comparable with that when the native proteins were infused into intact rats. Wise[8] in

discussing this finding cites the work of Cohen and Gordon[172] in which they found that heat-denatured plasma albumin is catabolized more rapidly than native albumin by the perfused rat liver. It is usually assumed that the passage of a protein across the cell membrane is a rate limiting step in the degradation of that protein. Denaturation of the protein molecules may facilitate their entry into cells. The fact that there is usually a lag period of 30 - 60 minutes in the production of bile pigment from injected haemoglobin in intact rats (Wise[8]; Ostrow et al.[50]) has prompted the conjecture that a mechanism for protein alteration may exist in the intact animal before proteins can enter the liver cell to be degraded.

We have shown that under physiological conditions of pH and temperature, large quantities of methaemalbumin were formed in solutions containing pure albumin and methaemoglobin. We observed that the preparations rapidly became turbid. This effect was also noted in experiments in which carboxyhaemoglobin and oxyhaemoglobin were used although the turbidity was not as marked and required longer periods of incubation to develop. Electrophoresis of these mixtures (Fig. 26) revealed that only methaemalbumin and a denatured haem-containing protein were present. There did not appear to be any free haemoglobin.

The precipitating effect of increasing methaemalbumin formation on homologous globin, has also been observed in sera to which methaemoglobin was added (Korinek and Dvorakova[152]).

Here then, is a means by which plasma haemoglobin might be denatured in vivo. Since some haem appears to remain attached to the denatured globin, in the chemical systems, it is possible that albumin is able to

facilitate the removal of only some of the 4 haem groups, the others being too tightly bound to the globin apoprotein.

Clearly, discussion of the role of albumin in this respect is, at present, highly speculative. There is, however, a considerable lack of knowledge concerning the control of haemoglobin turnover in vivo. The part which might be played by intermediates in this regard has hardly been elucidated. It is obvious that the formation of even small quantities of methaemalbumin under normal conditions might have far-reaching implications as regards both the synthesis and degradation of haemoglobin.

The factors controlling haemoglobin and red cell turnover in vertebrates is little understood. The balance between synthesis and degradation is delicately controlled, and accumulation of intermediates is not observed under normal conditions. Ultimately, however, synthesis and degradation must be regulated in all tissues, at the level of the enzyme-catalysed steps in the biosynthetic and degradative pathways (Lascelles[173]).

At present, in the case of haemoglobin, the known controlling mechanisms appear to exist at different levels i.e. tissue, cellular and biochemical. For example, slightly damaged red cells appear to be sequestered preferentially by the spleen; more markedly injured erythrocytes and plasma haemoglobin produced by intravascular haemolysis are taken up chiefly by the liver (Keene and Jandl[72]). The reticulo-endothelial system of bone marrow, the chief erythroid tissue of adult mammals, has a relatively greater avidity for haemoglobin and immature red cells.

Its sequestering function, which is normally minor, is strikingly stimulated by depletion of the blood-forming elements as occurs during protein deprivation (Keene and Jandl[72, 174]). Variations in the site of red cell destruction under normal conditions probably produces small daily variations of albumin-bound unconjugated bilirubin and transferrin-bound-iron in each individual.

Commensurate with the oxygen-carrying function of haemoglobin is the finding that its synthesis and degradation is regulated by the oxygen tension of the environment.

Hypoxic conditions stimulate the synthesis of erythrocytes in erythroid tissues through the action of the hormone erythropoietin. That hypoxia also stimulates the synthesis of haem by affecting a number of steps in the biosynthetic pathway has been shown in recent years. Its initial effect is probably the diversion of succinyl CoA towards tetrapyrrole synthesis when the rate of turnover of the citric acid cycle is decreased under conditions of low oxygen tension (Onisawa and Labbe[175]; Lascelles[173]). The conversion of porphobilinogen to uroporphyrinogen is oxygen-sensitive (Falk and Porra[176]), while the synthesis of protoporphyrin from the porphyrinogens requires a "reduction potential" in the synthesizing cell to prevent irreversible oxidation to the inactive porphyrin forms (Schmid[26]).

Conversely, haem and haemoglobin synthesis and red cell formation are depressed by inspiration under conditions of high oxygen pressure. It has been shown that inhibition of globin synthesis under conditions of hyperoxia is due to the effect of oxygen on haem synthesis and that addition of haem to a cell-free system of avian erythrocyte nuclei

overcomes the inhibitory effect of high oxygen tensions on protein synthesis (Hammel and Bessman[177, 178]). Evidence is accumulating that the presence of haem is required before ribosomal synthesis of globin can be completed (Waxman and Rabinowitz[179]; Granick[180]).

We have been more concerned in this research, however, with agents which bring about haemoglobin degradation and the factors which control them. Even less is known about these. Clearly there must exist, in vivo, a level of oxygen tension above which, the oxidative destruction, rather than the oxygen-carrying function of haemoglobin is promoted.

The recent advent of surgical techniques involving the use of oxygen under high pressure has stimulated investigations on the effect of hyperoxia on circulating erythrocytes. Having found that a patient developed haemolytic anaemia after brief exposure to oxygen under high pressure (Mengel, Kann, Heyman and Metz[181]), Mengel and Kann[182] investigated the effect of high oxygen tensions on mice. They found that haemolysis was induced only in α-tocopherol-deficient mice. Those which had received diets supplemented with α-tocopherol did not exhibit intravascular haemolysis. The lytic sensitivity of the erythrocytes to peroxide in vitro, parallelled their susceptibility to hyperoxic conditions in vivo. They also established that hyperoxia caused the in vivo formation of lipid peroxides in the tocopherol-deficient mice, prior to the onset of haemolysis. Earlier investigations had already established the antioxidant function of α-tocopherol both in vivo and invitro in relation to erythrocyte resistance to peroxide (Pokrovskii and Abrarov[61]) and Tappel has shown that haematin-catalysed lipid

peroxidation is inhibited by α-tocopherol[168].

Additional evidence for the role of lipid peroxidation in causing red cell lysis comes from recent studies on patients suffering from paroxysmal nocturnal haemoglobinuria, a haemolytic anaemia characterized by accelerated intravascular haemolysis during sleep. In 1949, Dacie and Mollison showed, by cross-transfusion studies, that the defect responsible for the haemolysis was intracorpuscular[183]. However, the basic mechanism responsible for haemolysis remains unknown despite extensive studies during the ensuing years, directed mainly towards finding some stromal defect, e.g. in stromal phospholipids (Harris, Prankerd and Westerman[184]; Munn and Crosby[185]), shown to be no different from normals by Bradlow and Rubenstein[186].

Recently, Meriwether and Mengel found that the lipid extracted from the erythrocytes of patients suffering from paroxysmal nocturnal haemoglobinuria consistently formed twice as much lipid peroxide as the lipid extracted from normal erythrocytes, when these preparations were exposed to ultraviolet light[187].

Mengel, Kann and O'Malley in a recent paper, reported the occurrence of increased haemolysis, after intramuscular administration of iron-dextran, in 4 patients suffering from paroxysmal nocturnal haemoglobinuria[188]. They ascribe this phenomenon to the known catalytic action of ferrous iron on lipid peroxidation, acting in conjunction with the enhanced tendency of erythrocytes from paroxysmal nocturnal haemoglobinurics to form lipid peroxides.

Thus, although there is no evidence to show that haemoglobin is

degraded by co-oxidation with unsaturated fatty acids in vivo, its catalytic action on lipid peroxidation may be of paramount importance in causing the stromal changes associated with "aging" of red cells and in regulating the quantity which undergo intravascular haemolysis under normal conditions. Senescence in the erythrocyte is associated with decreased glycolytic activity, oxidation of sulphydryl groups, e.g. G.S.H., and a general diminution in the reducing capacity of the cell. The defence mechanisms normally employed by the erythrocyte against hydrogen peroxide formed in the body, e.g. action of glutathione peroxidase and methaemoglobin reductase, are no longer active. Possibly the hydrogen peroxide initiates the production of peroxides in the stromal lipids. It has been proposed that only very slight alterations in erythrocyte stroma would be sufficient to promote uptake by the reticulo-endothelial system (Jandl[189]). Nevertheless, the possibility of intravascular haemolysis occurring could be increased with only very slight changes in a number of parameters, e.g. increases in H_2O_2 production, in the plasma concentration of ferrous and cuprous ions, ascorbic acid and haemoglobin and falls in plasma α-tocopherol, and possibly other anti-oxidants, such as manganese, cobalt and selenium.

The eventual inability of erythrocytes to metabolize hydrogen peroxide is not the only mechanism whereby sequestration or intravascular haemolysis may be promoted, but it can be seen that the significance of hydrogen peroxide in initiating the formation of lipid peroxides, the break-up of which can then be catalysed by haemoproteins or ferrous ions, is only just beginning to be realised.

A comparison of the action of hydrogen peroxide on haemoglobin

in vitro with that on erythrocytes in vivo, illustrates an important feature of mammalian biology. Both theories of haemoglobin degradation postulate the intermediate formation of hydrogen peroxide to effect the oxidative cleavage of the α-methene bridge of the haem groups. In vitro, a number of systems in which coupled peroxidation occurs, result in the production of bile pigments, e.g. ascorbic acid-oxygen (Lemberg[29]), and azide (Mills[94]) while others appear to be more powerfully oxidizing, resulting in colourless degradation products, e.g. lipoperoxides of unsaturated fatty acids and hydrogen peroxide itself.

In vivo, the packaging of haemoglobin into erythrocytes renders it unsusceptible to random degradation by coupled peroxidation systems. The biological advantages of having large quantities of haemoglobin contained in discrete units are well known. The presence of erythrocytes in the blood of vertebrates, and particularly in the circulation of warm-blooded animals, ensures that large quantities of oxygen can be carried to peripheral tissues to maintain the high rates of cellular respiration characteristic of homeotherms.

Nevertheless, by virtue of its lipoprotein membrane, the mammalian erythrocyte remains as susceptible to peroxidative agents as the individual haemoglobin molecules it contains. Therefore, the mechanisms which operate to degrade haemoglobin in vivo are similar, although their mode of action is altered. Thus hyperoxia which will cause autoxidation of haemoglobin in vitro, promotes intravascular haemolysis and increased red cell destruction in vivo. The fact that a number of factors seem to operate to prevent bile pigment formation in the circulation, both in the red

cells and the plasma, is probably also advantageous to the organism. Cellular degradation of haemoglobin ensures that feedback and other controlling mechanisms of the degradative pathway can operate to their fullest extent, and that bile pigments are produced at sites where they are able to exert their maximum influence on intermediary metabolism.

We have shown that simple chemical systems, although not directly analogous to the situation pertaining in vivo, illustrate a number of important characteristics and interactions which certain proteins may undergo in biological systems. It is commonly assumed that no reaction occurs in the body unless it is thermodynamically feasible. Conversely, it is unlikely that nature will miss an opportunity to utilize chemical interactions, which take place between compounds normally found in mammalian systems, and which occur with comparative facility. We propose, therefore to continue with this work and to show conclusively its importance in vivo.

PART 4.

SUMMARY AND CONCLUSIONS.

PART 4. SUMMARY AND CONCLUSIONS.

1. In vivo experiments were performed on geese in which the rate of clearance of Fe^{59}-haemoglobin from the plasma was measured. The time required for half the isotope to be cleared was $2\frac{1}{2}$ - 3 hrs. Only a small proportion of the activity was associated with the liver and of this, most was present in the cell supernatant. After 26 hrs. all the labelled haemoglobin had been cleared from the plasma; 40% of the isotope was present in the liver and widely distributed throughout the cell fractions. In both cases, 40 - 50% of the label was unaccounted for. Possible reasons for this finding have been discussed. It is concluded that haemoglobin uptake by the tissues in the goose is slow compared with mammals and that the rate and site of haemoglobin degradation may also differ in geese.

2. The production of haemolysis of Fe^{59}-labelled erythrocytes by administration of acetyl-phenyl hydrazine to a goose resulted in high specific activities being found in association with the microsomal and mitochondrial fractions of liver cells after 48 hrs.

3. Guinea-pig and human foetal liver homogenates were found to be no more active than chemical peroxidation systems in degrading haemoglobin, when either haemoglobin or a haptoglobin-haemoglobin complex was used as the substrate. We can only conclude that either the substrates which we used are not the actual substrates of the enzyme systems present in the liver or that they were supplied in such quantities so as to inactivate the degrading enzymes. It is possible that the enzyme system is extremely labile or that the concentrations of the co-factors provided were too low to activate it.

4. When pure albumin was mixed with native haemoglobin in aqueous solution, haem was transferred from the globin apoprotein and methaemalbumin was formed.

5. The rate of haem transfer and binding was found to be highly dependent on pH and temperature - being maximal at pH's above 7.0 and at temperatures higher than 25°C.

6. The transfer was expedited by oxidation of ferrohaemoglobin to ferrihaemoglobin.

7. When haem was removed from its attachment to haemoglobin, the liberated globin moiety was simultaneously danatured, and precipitated out of solution.

8. The presence of unsaturated fatty acids both accelerated the rate and increased the quantity of methaemalbumin formed.

9. The factors influencing ferrihaem and haemoglobin-catalysed peroxidation of unsaturated fatty acids were examined in the light of the previous finding. It was found that albumin protected ferrihaem and haemoglobin from coupled peroxidation with unsaturated fatty acids.

10. The physiological implications and biological importance of these chemical reactions were discussed.

PART 5.

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PART 5. BIBLIOGRAPHY.

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PART 6.

ADDENDUM.

Reprinted from S.A. Journal of Laboratory and Clinical Medicine, Vol. 9, No. 4, December 1963

SOME OBSERVATIONS ON THE BREAKDOWN OF HAEMOGLOBIN

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Our knowledge of the early steps of haemoglobin breakdown is fragmentary, and lags sadly behind that of the subsequent metabolism of bilirubin in the liver. So far no intermediates have been firmly characterized as the products of enzymic action on haemoglobin. The special interest attached to the mode of degradation of this important protein is matched by the technical difficulties of the study.

The principal sites of haemoglobin catabolism are the bone marrow, spleen and liver,[1, 2] complex organs of which reticuloendothelial cells form only a part. Other non-phagocytic cells in the tissues may be important, but it is difficult to assess their contribution to the activity of tissue extracts and crude enzymic preparations. Haemoglobin does not appear to undergo degradation to bile pigments in normal circulating erythrocytes.[3]

Karrer *et al.*[4] in 1933 succeeded in degrading the prosthetic protohaem to bile pigments by coupled oxidation with ascorbic acid, and later Lemberg[5] was able to prepare crystalline bile pigments in a similar manner. Fischer and Libowitzky[6] elucidated many of the chemical changes involved in the conversion of coprohaem to coprobiliverdin. During the past 25 years Lemberg and his colleagues in Australia have made a systematic study of the chemical structure of bile pigments and of their formation *in vitro* from haemoglobin.[7, 8] Haemoglobin incubated with ascorbic acid in air at pH 7·4 and 37°C. was shown to give rise to biliverdin. They obtained spectroscopic evidence of the presence of an intermediate, choleglobin, absorbing maximally at 629 mμ, whose structure differed from haemoglobin only in the attachment of two atoms of oxygen to the αmethene bridge of the four haem prosthetic groups. Later the αmethene carbon was replaced by oxygen and the protein thus formed was named verdoglobin. From this protein, the central iron atom was now removed and, lastly, biliverdin detached from globin.

Neither haematin nor porphyrin was considered to be an intermediate in the catabolic process. These views were supported by Yamaoka Imai,[9] and Sjoestrand[10] was able to detect the carbon monoxide that arises from destruction of the αmethene bridge.

Lemberg's views were challenged by Kench,[11] who found that, in a similar chemical system, various haem compounds, including free haematin, were converted to bile pigment, and only cytochrome C and protoporphyrin were not degraded in the presence of ascorbic acid and oxygen.

Bile pigment was formed from isotopically-labelled haematin and protoporphyrin given intravenously to dogs,[12, 13] but it is possible that methaemalbumin or another haemoprotein may have arisen which was a true intermediate of haemoglobin catabolism. A very recent and important finding

with regard to the role of haematin as a bile-pigment precursor is the prompt labelling of stercobilin following administration of ^{15}N glycine to a patient suffering from erythropoietic protoporphyria.[14]

It has, undoubtedly, been generally appreciated by the workers engaged in this field that the chemical systems employed could only provide a guide to the possible pathways which could obtain *in vivo*. Randomization might occur and be misleading. That such, in fact, is the case has been shown recently in a most important and elegant paper.[15] The bilirubin formed by coupled oxidation of pyridine haemochromogen and ascorbic acid in air is a mixture formed by random breakdown of α, β, γ and δ bridges. *In vivo*, only biliverdin IXα is formed, and the obvious implication is that a specific directive enzyme is there responsible.

In a series of publications Mills[16] has demonstrated the degradation of haemoglobin by hydrogen peroxide generated in rat liver during the catabolism of various purine compounds.

Other recent reports describe experiments directed towards the detection and isolation of enzymes active in the expedition and control of the degradation of haemoglobin to biliverdin. Kench and Varma[17] found that the splenic pulp from a patient suffering from haemolytic anaemia markedly enhanced the production of bile-pigment precursors, and a part of the increased yield arose from the action of a heat-labile component of the tissue. Normal human splenic tissue was ineffective. Neonatal tissues containing reticulo-endothelial cells —bone marrow, liver and spleen—were also enzymically active, about one-third of the gain being abolished by heating. Diffusible and non-diffusible activators in the neonatal liver contributed about equally to the reaction, but glutathione did not augment production.

Important advances have been claimed by Nakajima and his associates in a series of publications.[18-25] From their work the following conclusions were drawn:

1. Haemoglobin was catabolized to bile pigments in the liver and kidney only, not in the spleen and bone marrow.

2. Haemoglobin was broken down only after it had formed a complex with haptoglobins in the serum.

3. The enzyme concerned was localized to the supernatant, non-particulate fractions of liver and kidney cells; was a sulphydryl enzyme, requiring ferrous ions and reduced nicotinamide adenine dinucleotide phosphate ($NADPH_2$) as cofactors; and was inactivated by oxygen.

We should now like to present very briefly some of our own recent observations on the breakdown of haemoglobin, with especial reference to the importance of enzymes and coenzymes in tissue preparations.

EXPERIMENTS AND RESULTS

1. *Fractionation, on Molecular-weight Basis, of Products of Coupled Oxidation of Haemoglobin and Ascorbic Acid*

Human foetal haemoglobin was complexed with human haptoglobin obtained from pooled sera, and incubated with foetal liver homogenate. To 6 ml. of a solution of Hb-Hp complex containing 0·18 μmoles of haemoglobin was added 1·8 ml. of human foetal liver homogenate (1:1 w/v with phosphate buffer 0·1 M pH 7·3). The products of the reaction were passed through a column of dextran gel (Sephadex G. 100) and the emergent fractions examined for optical density at certain wavelengths in a Beckman U.V. spectrophotometer. Similar experiments have previously shown[17] that in this time of incubation no significant liberation of amino acids occurred. Some fractionation appeared to take place in higher molecular components, as scanned at 260 mμ. (Fig. 1), but what appears to be of particular interest is the presence of haem derivatives in the fraction 96 - 108 ml. (Fig. 2), since these must be relatively small molecular fragments.

2. Comparison of Free Human Haemoglobin with Haemoglobin-Haptoglobin Complex as a Source of Biliverdin

In our hands, the preparation of haemoglobin-haptoglobin complexes by the procedure described by Nyman[26] was somewhat unpredictable. We have found that the complex (MW 153,000) can be readily separated from excess of free haemoglobin (MW 68,000) by a single passage through a column of dextran gel (Sephadex G. 100) —Fig. 3—and this technique has been used to prepare a suitable haemoglobin-haptoglobin complex for study. A crude haptoglobin fraction was prepared from human serum, as the protein fraction soluble in 33% saturated ammonium sulphate but insoluble in 50% saturated ammonium sulphate. To an appropriate dialysed solution of haptoglobin an excess of haemoglobin was added and the formed complex separated on dextran gel. The optical density of the haemoglobin-haptoglobin complex at 542 mμ was about 10% greater than an equivalent concentration of free haemoglobin, but absorptions in the Soret region appeared to be indistinguishable from one another (cf. ref. 26). This procedure was thereafter employed to guide us in the preparation of haemoglobin complexes for incubation experiments.

We were able to detect the presence of heat-labile components in foetal human liver—prepared according to the procedures of Nakajima and his colleagues—which gave rise to compounds with increased spectral absorption at 630 mμ. The formation of such products was greatly enhanced by adding ascorbic acid and reduced nicotinamide adenine dinucleotide phosphate ($NADPH_2$). In general, haemoglobin complexed with haptoglobin was a slightly better source of biliverdin than haemoglobin alone,

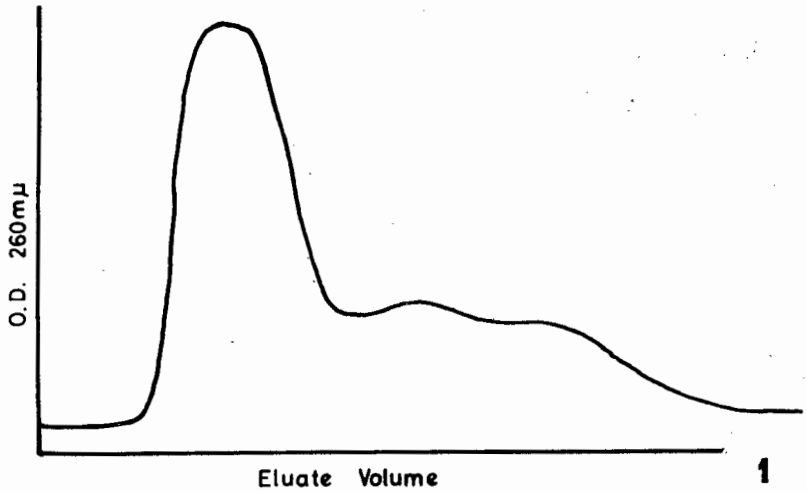

Fig. 1. Fractionation of products of coupled oxidation of haemoglobin and ascorbic acid on a column of dextran gel.

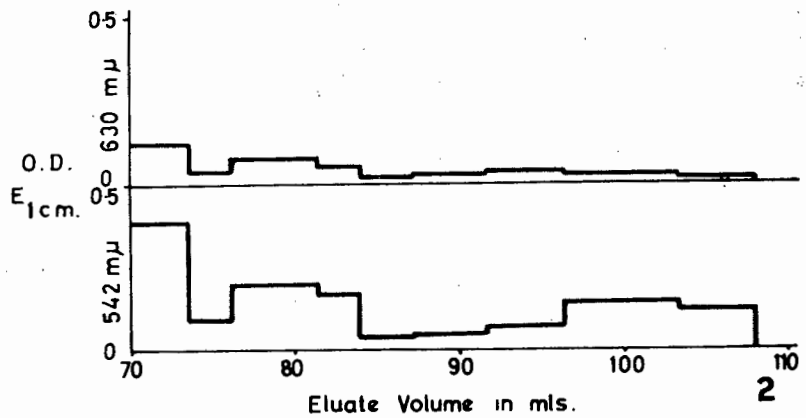

Fig. 2. Optical densities of the products of haemoglobin breakdown, separated on dextran gel.

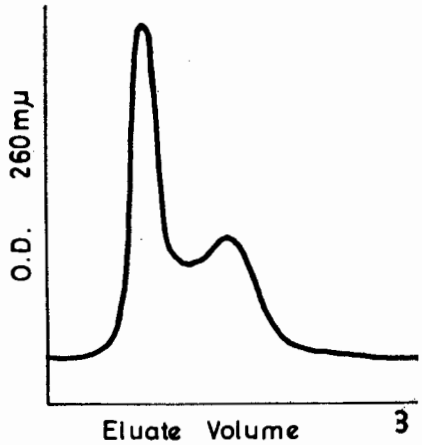

Fig. 3. Separation of free haemoglobin from haemoglobin-haptoglobin complex on dextran gel. The complex, of higher MW, emerged first from the column and is contained in the first peak.

but in no experiment have we succeeded in raising the yield of biliverdin above that obtained with ascorbic acid alone in earlier experiments.[11] In a series of experiments, using a variety of combinations of substrate, enzyme preparations, and cofactors, we have in no instance been able to reproduce the result of Nakajima and his colleagues, namely a 49% breakdown of haemoglobin to bile pigments. We have used foetal and adult human, and guinea-pig liver preparations; foetal and adult human haemoglobin; and bovine haemoglobin either free or complexed with adult human or bovine haptoglobins. Besides $NADPH_2$ a number of other cofactors have been added in attempts to boost the system, but without avail; viz. folic acid (to trap the single carbon fragment), pyridoxal phosphate (to test whether it facilitated decarboxylation), vitamin K (since transhydrogenation might be an important feature of the degradative process). If these substances were necessary, they were presumably present already in adequate quantities in the liver preparations.

Erythrocytic stroma is profoundly inhibitive, but removal of the liver stroma was without beneficial effect on the course of bile-pigment formation.

DISCUSSION AND CONCLUSIONS

Although we have elicited from foetal human liver an enzymic action on the early stages of haemoglobin breakdown, the activity was much less than might be expected of a tissue actively engaged in that process *in vivo*. We are at a loss to account for the discrepancy between our findings and those of the Japanese workers. Whatever the explanation, one additional test implicit in the findings of Gray and his colleagues should be applied: Is the catabolic pathway being channelled to produce only biliverdin IXα? If this criterion cannot be fulfilled, the value of the preparation as an enzyme must be discounted. Inasmuch as the Japanese workers have assayed their materials on pyridine haemochromogen, it is important to substantiate the specificity of their enzyme in the conversion of haemoglobin to precursors of biliverdin IXα. If this can be done, then a tremendous advance will have been achieved, and the enzyme will provide a powerful tool for further study of the chemical nature of the intermediates, and their impact on general metabolism.[17]

We propose, at this stage, to re-examine more closely the degradation of human metmyoglobin, since in earlier experiments[11] this haemoprotein gave the highest yield of biliverdin (36% theoretical) of any haematin derivative then examined. Myoglobin offers several advantages over haemoglobin: it contains one polypeptide chain and only one haem attached, as compared with four of each in haemoglobin. Separation of products on dextran gels should not be such a formidable technical problem, and myoglobin, being much less easily denatured, remains in solution throughout.[11] It is hoped that myoglobin will provide a more suitable substrate for enzyme assay.

Our grateful thanks are especially due to Professors F. J. Ford and J. T. Louw, and their staffs, to Drs. L. S. Smith and M. J. Greeff, of the Government Laboratory, Cape Town, and to the nursing staff of the maternity wards in Groote

Schuur Hospital and the New Somerset Hospital, for their unstinted help. We wish further to acknowledge the kind permission of Professors C. H. Gray and C. Rimington and their coworkers to refer to their paper before its publication.

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DISCUSSION

Schmid: We, too, have tried to repeat the experiments by Nakajima with similar inconclusive results. Whether or not haem is an intermediate in the breakdown of haemoglobin to bile pigment remains undecided, but we have data which reveal an almost quantitative conversion of haem to bilirubin in the rat. Over 80% of haem-C^{14}, injected into such animals can be recovered as bilirubin-C^{14} from the cannulated bile duct. Of course, these observations do not necessarily permit us to consider haem as a metabolic intermediate; they simply indicate that haem is converted to and excreted as bilirubin.