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THE EFFECT OF HEAT TREATMENT ON BLOOD.

A thesis presented in partial fulfilment of the requirements for the degree of Master of Food Technology in Product Development and Marketing at Massey University, Palmerston North, New Zealand.

Helen Margaret Tervit

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I. INTRODUCTION

A. Processing of Blood in New Zealand.

B. Consideration of the Utilisation of Blood as an Animal Feedstuff.

C. Production of Dried Blood for Use in Animal Feedstuffs.

D. Effect of Heat on Blood Proteins.

I. INTRODUCTION

New Zealand is a meat producing and exporting country and therefore has a large meat processing industry. In any industry, the utilisation of waste materials from the production of the main product as "byproducts" aids in the reduction of the manufacturing costs of the primary product, because of increased utilisation of overheads, and results in increased profit. During the processing of animal carcases to produce meat, a large amount of waste materials, including offal, skins, hooves, bones and blood, is produced. One of these waste materials, blood, is drained from the animals immediately after their slaughter and is available in large quantities as a raw material for the production of byproducts. Besides the reduction of meat processing costs, the recovery of blood solids considerably reduces the effluent load of the processing plant, blood solids being mainly organic matter. Unfortunately this latter consideration, i.e. the reduction of the Biological Oxygen Demand of the effluent from meat processing plants, is often the prime consideration in blood processing in New Zealand, as the cost of recovery may exceed the value of the final product; the quality and therefore value of blood products being of little importance.

The major products produced from whole blood are animal feedstuffs (blood meal) and fertiliser (dried blood, and blood and bone), although blood may be processed into black puddings, or utilised as a protein binder in sausage manufacture. Blood is also processed by separating the red corpuscles from the plasma, and manufacturing edible and pharmaceutical products from these separated fractions. This improves the value of the final products obtained from blood, but the advantage of this refinement in processing would depend on the cost of the separation process and the individual processing of the two fractions; this would depend to a large extent upon the throughput of the plant.

A. Processing of Blood in New Zealand

Practically all the blood utilised in New Zealand is used as nitrogenous fertiliser (mainly insoluble) in the form of "dried blood" or "blood and bone". Smaller plants include blood in the general waste materials which are treated to form meatmeals; larger plants often increase the protein content of their meatmeals by the addition of blood solids. In New Zealand blood does not appear to have been considered as a suitable animal feedstuff, only a very small amount of blood being utilised in a few specialised feedstuffs. However, although the statistics do not give a clear distinction between the quantities of dried blood exported as animal feedstuff and that exported for fertiliser purposes, it can be estimated that 60 percent of the dried blood exported from New Zealand is utilised as animal feedstuff.

The values for the various blood products (Table I.1) indicate that the utilisation of dried blood in meatmeal results in a product of lower value ($2.7-3.2 per cwt.) than fertiliser ($3.6-4.6 per cwt.). The blood product which is of highest value is the blood meal which is exported mainly as an animal feedstuff ($4.3-5.4 per cwt.).

B. Consideration of the Utilisation of Blood as an Animal Feedstuff

Blood is approximately 20 percent solids and 80 percent water. For economy in storage and transport facilities the removal of the water, e.g. by drying, is the major processing operation. The blood solids have a very high protein content (80 percent). This protein is of good quality and hence it may be utilised as a source of nutritional protein. This is generally used as an animal feedstuff, although it has great potential as a source of protein for humans.

TABLE I.1 VALUES OF SOME MEAT BYPRODUCTS IN NEW ZEALAND

($ per cwt.)

PRODUCTION

(Calculated from figures given in the "Statistics of Industrial Production", 1965-1966, for the Meat Freezing and Preserving Industry and related industries).

	1963-64	1964-65	1965-66	1967-68
Fertilisers				
Blood manure	3.6	3.8	4.6	
Blood and bone	1.7	2.0	2.1	
Stockfood				
Meatmeal	2.7	2.9	3.2	
EXPORTS (Calculated from Export Statistics)				
Dried Blood	4.3	-	-	5.4

Figures available for the biological value of some of the blood proteins, namely fibrin (83.1,lamb) (Ellis et al.(1956)) and serum proteins (95.4$\pm$ 0.2, rat) (Periatianu (1957)) were higher than those given for casein in the same experiments (72.7 and 91.4$\pm$ 0.2, respectively). However, Grau and Almquist (1944) have shown that the serum proteins and fibrin are of much better quality than the blood cell proteins which constitute 70 percent of the total protein in blood and in which isoleucine is the limiting amino acid. Because of this amino acid imbalance, indiscriminate use of blood solids as a feedstuff may produce detrimental results. Blood, however, is an animal protein and therefore a source of Vitamin B_{12} ("growth promoting factor"), as well as being a good source of other vitamins. Its protein is a rich source of lysine, methionine and tryptophane, and as lysine is usually the limiting amino acid in cereals which often make up the major portion of animal feedstuffs, the supplementation of these feedstuffs with blood (as a cheap source of animal protein and lysine) would improve the nutritional value of the protein in these products. The low mineral content of blood is not significant when blood is used for supplementation of feeds.

C. Production of Dried Blood for Use in Animal Feedstuffs

During the collection and processing of blood which is to be used for edible purposes, contamination of the blood must be minimised. A major problem in this respect is the introduction of disease-producing organisms, particularly spore formers such as *Bacillus anthracis*, through the use of blood from infected animals. This problem can be reduced by avoiding the use of blood from these animals. This would necessitate the collection of blood from individual animals (or small groups of animals) in small containers, the contents of which could be pooled after the carcases have been inspected and found to be free from infection.

The blood from infected animals could be utilised in manure production. As there is a low incidence of condemned carcases in most New Zealand meat processing plants, the size of these batches could be quite large and the risk of contamination of any batch would be very low. Adequate sterilisation of the product must be ensured.

There are two main techniques for the production of dried blood :

1. Direct drying of raw blood without previous coagulation and dewatering.

This may be a continuous or a discontinuous process (generally the latter). This technique requires a large amount of heat energy to remove the large quantity of water in blood (80 percent). Examples of this type of process are spray drying and roller drying, the former being preferable for blood drying because of the tendency of the albuminous proteins to adhere to hot drying surfaces (Hirchberg (1957)) to which they are exposed in the latter process. Concentration of the solution to be dried to 40-50 percent solids, a process which normally precedes spray drying, is avoided in the case of blood because of its viscous nature and the deposition of considerable quantities of sludge during concentration (Hirchberg (1957)).

2. Coagulation, dewatering and drying of blood.

Coagulation (or precipitation) of the blood proteins enables their separation from a large proportion of the water by mechanical means (pressing or centrifuging) before drying, thus reducing the steam requirements of the drying process considerably. This technique is the one which is universally used in New Zealand, and also may be a discontinuous or continuous process.

a. Discontinuous Coagulation.

Batch coagulation of blood in large vessels by the use of indirect (steam-jacketted) or direct (steam injection) heat treatment has been the most common

method of blood coagulation in New Zealand. However batch processing methods are time consuming, have very high labour costs, and often result in low recovery of the blood solids.

b. Continuous Coagulation.

Continuous coagulation procedures have been developed during the last ten years to enable quick recovery of the protein from the ever increasing volumes of blood becoming available for the manufacture of byproducts. In these processes, the blood is generally coagulated by direct steam injection in a specially designed pipe coagulator and the coagulated solids separated by means of a screw press or high speed centrifuge.

c. Dewatering and Drying.

After their separation from the liquid the blood solids may be dried by a wide variety of processes. The most common methods used to dry these solids being by contact driers (such as Iwels or thermoscrews) or by pneumatic driers (such as ring driers).

D. Effect of Heat on Blood Proteins

According to Abrams (1961) the effect of heat on protein di gestibility is as follows :

1. Moist heat treatment of a protein is less destructive than dry heat, other factors being equal (water has a protective effect against high temperatures).
2. The duration of heat treatment influences the digestibility of the protein. Often there is an initial increase in protein digestibility, but prolonged heating diminishes protein digestibility.

3. If high temperatures are attained during heat treatment the protein digestibility tends to decrease (Mitchell (1945)).

Obviously, therefore, the heat treatment which the blood proteins undergo during processing has a profound effect on the quality of the final product (as reflected by the digestibility of the protein). Because of the high temperatures often attained in the preparation of dried blood its protein is often of low digestibility (Hirchberg (1957)). It has been noted that the utilisation of lower temperatures enables the production of a more soluble, digestible product of higher nutritive value (Morrisson (1938)).

By stricter control of the amount of heat used during the processing of blood a higher grade product could be produced.

The main aim of these experiments was to gain a greater understanding of the effect of heat on solutions of blood proteins, in the hopes of ascertaining the conditions of heat treatment which would completely coagulate the blood proteins with minimum deleterious effects (irreversible insolubilisation of the protein and loss of protein digestibility). The later stages of blood processing (separation and drying of coagulated solids) were not considered.

Despite the advocation of increasing use of techniques such as solvent precipitation (Vickery (1968)) and spray drying in blood processing, these methods are generally used economically only in large scale operations, and so it was felt that this study on present processing was justified.

II. LITERATURE REVIEW

A. Protein Precipitation

1. Neutralisation

2. Dehydration

a. Evaporation of solvent water.
b. Sedimentation.
c. Low neutral salt concentrations.
d. High neutral salt concentrations.
e. Organic solvents.

3. Specific Interactions

4. Denaturation

B. Blood Protein Precipitation

1. The Composition of Blood

2. Properties of Blood Proteins

Haemoglobin

Plasma Proteins

Fibrinogen

Serum Proteins

3. Precipitation of Blood Proteins

a. Neutralisation
b. Dehydration

(i) Evaporation of Solvent Water.
(ii) Sedimentation.
(iii) High concentrations of neutral salts.
(iv) Organic solvents.

c. Combinations of the above techniques.
d. Clotting.
e. Denaturation.

C. Effect of Heat on Blood Proteins

1. Influence of Heat on Blood Proteins

a. Plasma Proteins.

(i) Fibrinogen.
(ii) Serum Proteins.

b. Erythrocyte Protein (Haemoglobin)

2. Influence of pH on Heat-induced Changes in Serum

a. Effect of pH on the Protein Fractions of Serum.
b. Effect of pH plus Heat on the Protein Fractions of Serum.

3. Influence of Various Substances on the Heat Coagulation of Blood Protein

4. Influence of Aging on Heat-induced Changes in Serum

a. Effect of Aging on the Protein Fractions of Serum.
b. Effect of Aging plus Heat on the Protein Fractions of Serum.

5. Influence of Preheating on Heat Coagulation of Blood Protein

D. Theoretical Aspects of Heat Coagulation of Proteins

II. LITERATURE REVIEW

A. Protein Precipitation

Proteins, complex nitrogenous compounds which exist in vegetable and animal tissues, are made up of one or more polypeptide chains which consist of a large number of amino acid residues linked by peptide bonds (-CO-NH-). The sequence of these amino acid residues is known as the "primary" structure of the protein. The polypeptide chains can exist in several basic forms, defined as the "secondary" structure of the protein. The most common configurations are the regularly folded helix and the fully extended form. The stability of possible configurations of the chains is influenced by the number and nature of the side chain groups on the amino acid residues, which thereby determine the conformation assumed (i.e. the most stable conformation). This basic protein structure folds into a configuration stabilised by a variety of bonds between the side chains, e.g. hydrogen bonds, Van der Waals' forces between hydrophobic residues, and electrostatic links (ionic bonds) between charged amino acid residues, this folding defining the tertiary structure of the protein.

There are two classes of proteins: fibrous and globular. The fibrous proteins (scleroproteins), e.g. keratin, myosin and fibrinogen, have very regular characteristic foldings, highly dependent upon the secondary structure, which enables molecules of these proteins to assume a very high degree of orientation. The conformation of globular (corpuscular) proteins such as blood serum proteins and egg albumin, however, is highly dependent upon the tertiary structure.

Because of the existence of free amino and carboxy groups at the ends of the polypeptide chains and also on side chains of diamino and dicarboxy amino acid

residues, protein molecules are amphoteric and exist in aqueous solutions in the form of "switterions". They are large, heavily hydrated molecules; typical "hydrophilic colloids" whose behaviour in solution depends to a great extent upon the pH. The pH at which a protein is electrically neutral is characteristic for each protein and is the pH at which that protein is least stable, i.e. most readily removed from solution. This pH is known as the isoelectric point of the protein.

The tendency of a protein molecule to remain in solution is due to the interaction of this molecule with the solvent molecule (Eisenberg and Schwert (1951)). The solubility of the protein molecules is influenced by the nature and amount of protein in the system, pH, salt concentration and temperature.

Methods of removing protein from solution can be classified into four categories :

1. neutralisation
2. dehydration
3. specific interactions, and
4. denaturation.

1. Neutralisation

Adjustment of the pH of many protein solutions to the isoelectric point of the protein, e.g. by the addition of acid or alkali, results in the precipitation of the protein.

2. Dehydration

Concentration of a protein solution by the removal of the solvent water results in the precipitation of the protein because of the association between surface side chains of adjacent molecules (Mirsky and Pauling (1936)); this association being prevented in aqueous solutions by the presence of the hydration layer of water molecules which surround each protein molecule. The water can be

removed by physical means (e.g. evaporation or centrifuging) or by chemical means (e.g. low or high neutral salt concentrations or organic solvents). However, proteins are susceptible to denaturation, a characteristic reaction which is generally regarded as any change in the secondary, tertiary or quaternary structure of the protein. (N.B. Changes in covalent bonding which occur in chemical reactions are excluded by this definition). Denaturation, which often results in the irreversible insolubilisation and aggregation of protein molecules, is influenced by time, temperature and pH, and is caused , under certain conditions, by many of the chemical compounds which could be used to remove water from protein solutions. Naturally conditions under which denaturation occur must be avoided during the dehydration of protein solutions if the native state of the protein is to be retained.

a. Evaporation of solvent water.

The removal of water from protein solutions requires the use of energy. The major problem in drying protein solutions is in the application of this energy with the minimal denaturation of the protein. The process of denaturation is accelerated by increased temperatures, but is inhibited by the absence of water.

High temperatures can be used to dry protein solutions without causing denaturation, provided that the drying process is completed before the temperature of the solid has had time to reach a dangerously high level (i.e. a level which would cause denaturation). As evaporation occurs at the surface of solutions, the rate of evaporation and therefore the drying rate may be increased by exposing the liquid to conditions which induce evaporation in a very thin film. Two "high temperature-short time" drying methods are commonly used, namely roller and spray drying. The latter is the more preferable for protein solutions as it causes less denaturation. These methods are widely used for the drying of milk and eggs.

The use of lower temperatures results in much longer drying times and in the latter stages of these processes the protein molecules are exposed to high salt concentrations (i.e. the possibility of denaturation caused by factors other than high temperatures is increased). However, fairly rapid drying may be achieved by the use of large surfaces (by means of fluidised bed drying techniques or of semi-permeable membranes as in pervaporation) and also by distillation at reduced pressures (Greaves (1946)).

At temperatures below freezing evaporation proceeds as a sublimation of the water molecules from the solid state (Greaves (1946)). No denaturation takes place during this process because the solid state maintained throughout the dessication process has prevented the concentration of salts and protein molecules. Obviously this last method, known as "freeze-drying", is the ideal method for the dessication of protein materials. However this process is the most complex and expensive of all drying methods.

b. Sedimentation.

Physical separation of large particles from suspension rather than true solution can be achieved by subjecting the liquid to gravitational and centrifugal forces. Proteins only sediment under very high centrifugal forces (such as those achieved by ultra centrifugation), whereas protein aggregates may sediment out under normal centrifugal forces.

c. Low neutral salt concentrations.

The precipitation of some proteins by low concentrations of neutral salts is possibly due to a reduction in the number of charged groups surrounding the protein molecules in solution and a consequent decrease in the hydration layer of these molecules.

d. <u>High neutral salt concentrations.</u>

It has been suggested that high concentrations of neutral salts precipitate some proteins because the "hydration required by the inorganic salt ties up enough water molecules to decrease effectively the water available for the solution of the large protein molecule". (A.M.Potts (1965)).

e. <u>Organic solvents.</u>

Organic solvents such as acetone and ethanol precipitate proteins by decreasing their hydration layer. The denaturing action of high concentrations of these chemicals is avoided by the use of very low temperatures.

3. Specific Interactions

Specific interactions of proteins, e.g. with salts of heavy metals such as zinc, mercury or lead, or with trichloracetic acid, often result in precipitation of the protein.

4. Denaturation

As conformation changes occur during denaturation of proteins, the affinity of the surface of the molecules for the surrounding medium (and also for other protein molecules) changes, resulting in changes in the state of dispersion of the protein molecules in solution; for instance, when association, aggregation, coagulation or gelation occurs. In many instances decrease in solubility is used as a criterion of denaturation (Joly (1965)). Association and polymerisation (the formation of completely reversible aggregates of high molecular weight) frequently occurs under very mild conditions. However aggregation (the formation of submicroscopic particles which remain in solution) takes place only when the protein is exposed to somewhat drastic treatments such as extremes of pH, heating, and the addition of concentrated denaturing agents, e.g. urea and guanidine.

Coagulation (the formation of macroscopic aggregates which precipitate) is the ultimate stage of the aggregation process which only occurs if the protein concentration is high enough. Gelation (the transformation of a more or less viscous solution into a highly deformable solid without the separation of a liquid, the whole bulk of the solution becoming more or less rigid but not ceasing to be a single continuous macroscopic phase) can only occur when the pH of the solution is far enough from the isoelectric point of the protein to prevent a total aggregation leading to coagulation or precipitation (Joly (1965)). Hence denaturation under conditions which lead to precipitation may be regarded as a means of removing protein material from solution, although it should be realised that the precipitated protein is no longer in its native state and, in most cases, can not revert to this state. Common denaturing agents are heat, acid, alkali, aliphatic alcohols and urea.

B. Blood Protein Precipitation

1. The Composition of Blood.

Blood is a complex biological fluid which circulates in mammals and other higher-classed animals. It is a medium by which nutritive materials and oxygen are transported to the tissues, carbon dioxide and other waste materials metabolites conveyed between the tissues, carbon dioxide and other waste materials carried to the site of their excretion (lung, kidney, liver, skin,etc.), and it also has many important regulatory and protective functions (carrying antibodies against invading organisms, maintenance of tissue water content,etc.).

The overall composition of blood is approximately 80 percent water and 20 percent solids, the latter being composed chiefly of protein (95 percent). It can be noted (Table II.1) that there is very little difference in bloods from cattle, sheep and swine.

TABLE II.1 COMPOSITION OF BLOOD OF CATTLE, SHEEP and SWINE

(Most figures taken from P.L. Altman)

Species	Cattle	Sheep	Swine
	Percentage of Solids		
Whole Blood	19	18	21
Erythrocytes	38	39	37
Serum	9	8	8
	Amount of Protein (grams per 100 mls. of blood)		
Haemoglobin	11.0	10.8	13.0
Serum proteins	7.3	6.8	7.0
Fibrinogen	0.7	0.35	0.4
	Composition of Serum Proteins (percentage total serum proteins)		
Albumin	45-55	45-55	40
Alpha globulins	10-15	15-20	15-25
Beta globulins	7-14	10-18	15-25
Gamma globulins	20-30	10-20	15-25

Blood plasma which makes up 50-70 percent of the volume of whole blood, contains about 9 percent solids, almost 80 percent of this being protein.

The other major component of blood, the erythrocytes, which make up 30-50 percent of the whole blood volume, contains almost 40 percent solids, 98 percent of these being protein - mainly haemoglobin (95 percent) but also stroma protein. The erythrocytes are 4-8 microns in diameter.

The leucocytes are composed of various cells of different size (6-20 microns in diameter) and diverse function. They make up 5-10 percent of the whole blood volume and contain about 18 percent solids, the composition of which has not been clearly defined.

The thrombocytes are very small (1-5 microns in diameter) and make up a very small proportion of the whole blood volume. Sixty-four percent of the dry weight of thrombocytes is protein material, 15 percent lipid (mainly phospholipid), and 9 percent carbohydrate.

2. Properties of Blood Proteins.

Protein is the major constituent of blood. Haemoglobin accounts for 75 percent of the protein content of blood and plasma proteins the other 25 percent; the stroma proteins contributing less than 1 percent of the total protein in blood.

Haemoglobin

Haemoglobin is a conjugated protein consisting of an iron-containing porphyrin (haeme) linked to a protein moiety (globin). Globin, which forms about 95 percent of the haemoglobin molecule (Thorpe (1952)),is a strongly basic protein which is very easily denatured. Haemoglobin is the oxygen carrying principle of blood. It has a molecular weight of about 38,000.

Plasma proteins

The plasma proteins are generally classified into three main groups: fibrinogen, albumins and globulins.

Fibrinogen

Fibrinogen, a globulin present in plasma, is extremely easily precipitated (25 percent saturation with ammonium sulphate or 50 percent saturated sodium chloride). Its enzymic conversion to fibrin results in the clotting of blood. It has a molecular weight of 340,000 (Caspary and Kekwick (1954)).

Serum Proteins

Serum is expressed from blood clots on standing, in a process known as "syneresis" or "clot retraction". The serum contains all of the plasma proteins except fibrinogen. The serum proteins can be divided into two major groups : albumin and the globulins.

Albumin, which precipitates out at 68 percent saturation with ammonium sulphate, is soluble in distilled water and is coagulable by heat in neutral or feebly acid solutions. It has a molecular weight of 68,000.

The globulins are precipitated out by saturated magnesium sulphate or by 50 percent saturation with ammonium sulphate. They are insoluble in distilled water and hence are often precipitated during dialysis. Their molecular weights are in the 90,000 - 300,000 range. This group can be differentiated into the euglobulins (precipitated by 28-33 percent saturation with ammonium sulphate) an d the pseudoglobulins (precipitated by 34-50 percent saturation with ammonium sulphate) whose properties more closely resemble those of albumin.

The globulins can be further fractionated into alpha-1 and alpha-2, beta and gamma globulins by salt fractionation; however these groupings are far from precise and much overlapping occurs between fractions precipitated at particular salt concentrations. More efficient separation of the different fractions is achieved by electrophoretic methods, some of which distinguish up to 20 different globulin species.

TABLE II.2 PHYSICAL PROPERTIES OF BLOOD PROTEINS

Protein Fraction		Electrophoretic Mobility (cm/sec/voltx10^{-5})	Deduced Isoelectric Point (pH)	Sedimentation Constant	Molecular Weight
Albumins		5.1	4.8	4.6[a]	68,000[b]
Globulins-	alpha-1	4.5	4.9	5[a]	200,000[c]
	alpha-2	4.15	5.1	9[a]	-
	alpha-3	3.7	5.25	-	-
	beta-1	3.1	5.45	7[a]	90,000[c]
	beta-2	2.35	5.7	7[a]	-
	gamma-1	1.8	5.85	7-10[a]	156,000[c]
	gamma-2	1.3	6.05		
	gamma-3	0.9	6.15		
Fibrinogen		-	5.4[a]	9[a]	340,000[d]
Haemoglobin		-	slightly 7	-	68,000[c]

Unless otherwise specified figures are from Ardry (1951a).

a. Cohn *et al.*(1950).

b. Thorpe (1952).

c. Conn (1945).

d. Wintrobe (1961).

3. Precipitation of Blood Proteins.

a. Neutralisation

A very common method of precipitating proteins is by adjustment of the pH of the medium to the isoelectric point of the protein. As blood contains a mixture of proteins with different isoelectric points,(refer to Table II.2) complete recovery of the blood proteins would entail several isolations at different pHs.

b. Dehydration

(i) Evaporation of Solvent Water. Direct drying of raw blood (i.e. without previous precipitation) can be done with minimum denaturation of the proteins by using methods such as spray drying.

(ii) Sedimentation. The separation of blood into two layers (one of erythrocytes and a plasma layer) by centrifuging or by sedimentation on standing, with a consequent increase in the protein content of the erythrocyte layer may be regarded as a means of "precipitating" haemoglobin.

(iii) High concentrations of neutral salts. High concentrations of neutral salts (e.g. sodium, magnesium or ammonium sulphates, or sodium chloride or sulphite) can be used to obtain complete precipitation of blood proteins.

(iv) Organic solvents. Organic solvents (ethanol, ether,etc.) which may be readily removed from the protein by distillation at low temperatures have often been used for the precipitation of blood proteins, particularly in laboratory fractionation procedures (Cohn et al. (1940) and Kekwick (1955)).

c. Combinations of the above techniques.

Various combinations of the techniques outlined above (e.g. salt precipitation using organic solvents to lower the protein solubility and hence

the salt concentration required for precipitation) have been used in a number of fractionation procedures for plasma proteins.

d. Clotting.

During the natural enzymic process of blood clotting which takes place within the few minutes immediately after the blood has been withdrawn from an animal, a clot network which engulfs all blood constituents is formed. On standing, this clot gradually undergoes syneresis and expresses serum. The retracted clot consists of a fibrin network which entangles the blood corpuscles within its structure. The serum contains all the plasma proteins except fibrinogen, this protein being converted into fibrin and forming the basic clot network. Thus the clotting process could be regarded as a method of precipitating haemoglobin and the stroma proteins (about 65 percent of the whole blood proteins). However, because of the release of serum proteins in the fluid, the clotting process can not be regarded as a very efficient method for recovery of total blood proteins.

e. Denaturation.

Denaturation may be brought about by various agents (acid, alkali, heat, alcohol,etc.) resulting in a loss of solubility and precipitation of protein.

Strong denaturing agents, e.g. trichloracetic acid, are used to precipitate blood for analytical work. However, the precipitates formed under these conditions are highly insoluble.

Mild denaturation often improves the digestibility of protein material but severe denaturation often reduces digestibility (to nil), and it is considered justifiable to consider mild denaturation conditions as possible methods of blood protein precipitation. Heat is one of the most common methods of denaturing proteins. In aqueous solutions the denaturing effects of pH and heat are very closely connected so that denaturing processes can only rarely be considered as purely thermal.

Vickery (1968) advocates the use of acidified acetone (0.14 percent HCl), presumably at room temperatures, for the recovery of blood proteins for edible purposes, and under these conditions mild denaturation of the proteins probably occurs.

C. Effect of Heat on Blood Proteins

1. Influence of Heat on the Protein Fractions of Blood.

Reports on the effect of heat on the various blood proteins are often conflicting, because the effect of heat is highly dependent upon the temperature and time of heating, as well as the nature and concentration of different proteins, salts and other substances (Jensen et al.(1950); Hospelhorn and Jensen (1954) and Joly (1965)).

Howell (1942) stated that the "temperature of heat coagulation", i.e. the exact temperature at which coagulation takes place, is a characteristic of each protein. He quoted the temperature of heat coagulation of serum albumin as 70-75°C, noting that it varied with the conditions (e.g. nature and concentration of salts) and also mentioning that it had been asserted that careful heating under proper conditions gave separate coagula at three different temperatures (73,77 and 83°C), indicating the possibility that serum albumin may be a mixture of three different proteins. The serum albumins apparently coagulate upon heating to 75°C in neutral or feebly acid solutions. According to Hammarstein (1913), 1 percent serum albumin has a coagulation temperature of 50°C, but this rises with the salt concentration and is usually given as 70-85°C.

Fibrinogen coagulates on heating at 52-55°C in 5-10 percent sodium chloride, whereas in approximately neutral salt solutions it coagulates at 56°C (Hammarstein

(1913)). Howell (1942) stated that the latter temperature is the temperature of heat coagulation of fibrinogen.

In 5-10 percent salt solution, the other serum globulins coagulate at 69-76°C (Hammarstein (1913)). Hawke and Bergeim (1938) stated that serum globulins coagulate at 65-69°C, but did not indicate the salt content of their solution.

The temperature of heat coagulation of haemoglobin could not be found.

These temperatures of heat coagulation may be related to the temperature of denaturation which is defined (Joly (1965)) as the temperature at which half the protein becomes insoluble in distilled water after a definite heating period.

More specific and hence more useful information on the effect of heat on the various blood proteins is given below.

a. Plasma Proteins

(i) Fibrinogen. Fibrinogen appears to be the most labile of the plasma proteins. Black et al.(1948) associated increased coagulation values (i.e. the increase in turbidity on heating a 1 in 5 dilution of plasma in distilled water for 10 seconds at 100°C) with increased fibrinogen content , but did not find any stoichiometric relationship between these factors, although similar heat treatment of serum produced small or even negative coagulation values. Devron and Plan (1950), however, suspected that different biological and physico-chemical factors (such as physiological inhibitors of coagulation, pH, salt concentration and dilution of plasma) generally masked the relationship between fibrinogen and coagulation value; and, by the addition of varying amounts of a fibrinogen preparation to one plasma, were able to keep these other factors constant and show that the coagulation values obtained by heating a 1:4 dilution at 100°C for 3 minutes were directly proportional to the fibrinogen content. Foster et al.(1959) correlated the amount of precipitate formed by heating plasma at 56°C with the

fibrinogen content of the plasma, and proposed the use of heat precipitation of fibrinogen as a clinical method for fibrinogen determination. They did note that on rare occasions heat-coagulable globulins ("pyroglobulins" - Martin and Mathieson (1953)) were precipitated at 56°C. Maximum precipitate formation in plasma heated in glass capillary tubes (75 mm.long x 1 mm. diameter) immersed in a water bath at 56°C was obtained after 15 minutes.

This precipitation of fibrinogen would account for the prevention of the natural clotting of blood when blood is held for a few minutes at 60°C soon after collection from an animal. (Earle (1965)). Macfarlane (1948) stated that fibrinogen is precipitated at 54°C, this precipitation being the cause of poor clotting of blood at temperatures above 47°C.

(ii) Serum Proteins. There are varied reports on the effect of heat on serum proteins. Apparently complexes between various protein components are formed, the nature and amount of complex formation depending on the heating conditions (temperature and time).

Van der Scheer *et al.*(1941) noted that a new component with a mobility similar to that of beta globulin was formed when horse serum was heated at 65-70°C for 15 minutes. They designated this component "component C". Bozzetti (1956) reported the formation of a similar component after heating human serum at 65°C for 15 minutes. Hoch and Chanutin reported the formation of the "C component" in the alpha globulin area after heating human serum for 4 hours at 55°C, and Okulov (1957) noted an increase in the alpha-2 globulins and a decrease in albumins after 2 hours at 50-65°C. This is in contrast to the many reports of increases occurring in both the alpha and beta regions (Van der Scheer *et al.* (1941); Cagli (1955); Bozzetti and Prina (1955) and Ardry (1951a)), but may be explained by the fact that the location of the new component ("component C") on the electrophoretic pattern depends on the heating temperature (Schulz (1959)) as shown in Table II.3.

Using a heating period of 30 minutes, Schulz showed that heating at 48°C made no apparent change to the electrophoretic pattern (as compared to unheated serum); heating at 49-57°C resulted in a gradual disappearance of the alpha-1 globulin peak and a simultaneous increase of the alpha-2 globulin area; while at 57-58°C the alpha-2 and beta peaks fused forming the so-called "C component" band in the alpha-2 area. Bozzetti and Prina (1955) and Tekman and Ozner (1965) also noted the fusion of these two peaks. Schulz reported that above 55°C the gamma globulin area declined, indicating that it also contributed to the new component. When the temperature was increased from 58 to 64°C, this new "C component" band increased in amount and shifted into the beta area. Between 65 to 70°C this band became less distinct and finally blended into the gamma globulin band. Tekman and Ozner (1965) also found that the globulins migrated as a single fraction after heating at 65°C for 15 minutes. Schulz found that at temperatures above 66°C the presence of precipitated protein distorted the electrophoretic pattern and noted that most of the total serum protein was precipitated above 70°C.

Van der Scheer _et al_.(1941) found that the "C component" was not formed from serum albumin under the same conditions in which it was formed in serum. They suggested that in serum it arose at the expense of the globulins, and then, when produced in large amounts, adsorbed considerable quantities of albumin. The results of Kovdics and Kerekes (1961), which showed a decrease in the gamma globulin fraction of human serum heated for 10 minutes at 60°C and a decrease in both albumin and gamma globulin after 10 minutes at 64°C, and those of Schulz (1959) (Table II.3) which did not reveal any decrease in the albumin fraction after heating at 48-66°C for 30 minutes but a slight decrease after heating at 67 or 68°C for 30 minutes, appear to verify this suggestion.

Although Hardt _et al_. (1946) found that the "C component" was formed in the absence of gamma and beta globulins in human serum, several workers using serum

TABLE II.3 THE EFFECT OF HEAT ON THE COMPOSITION OF HUMAN SERUM PROTEINS

Heat Treatment		Protein Fraction (Values expressed as percentage of total serum protein)								Reference *
Temperature (°C)	Time (minutes)	Albumin	Globulins							
			Alpha			Beta		Gamma		
			1	2	3	1	2	1	2	
						total				
48	30	25.5	9.0	17.7		19.9		27.9		a
49	30	24.3	8.3	17.7		20.5		29.2		
			"Alpha area"							
50	30	25.6	8.0	19.0		19.0		28.4		
52	30	25.2	7.3	19.6		19.5		28.4		
55	30	26.1	6.5	20.6		21.1		25.7		
56	30	27.7	4.8	21.5		20.6		25.4		
57	30	28.7		24.1		21.9		25.3		
						"Beta area"				
58	30	28.3					46.0	25.7		
59	30	27.9					46.6	25.5		
60	30	27.1					49.5	23.3		
63	30	27.6					51.6	20.8		
64	30	27.0					53.4	19.6		
								"Globulin"		
65	30	26.4						73.6		
66	30	25.0						75.0		
67	30	23.6						76.4		
68	30	22.6						77.4		
20	-	60.9	3.1	5.3	2.6	11.2	2.4	3.7	10.8	b
57	10	61.3	4.1	5.3	7.8	7.3	2.2	2.2	9.8	
59	10	59.1	2.7	5.7	10.2	6.6	3.1	2.8	9.7	

*

a D.M.Schulz (1959) - Heated diluted serum (1:1 with buffer); paper electrophoresis using barbiturate buffer, pH 8.6.

b R.Ardry (1951) - Heated whole serum; moving boundary electrophoresis, pH 7.8.

from different species have assumed that complex formation occurred between albumin and the gamma globulins and resulted in the formation of a component of intermediate mobility (Mathews and Buthala (1958); Mathews (1960); and Shan and Rao (1960). Mathews and Buthala (1958) noted a marked decline in both the albumin and gamma globulin fractions of swine serum heated for 30 minutes at 58-65°C, with a concomitant increase of a component in the alpha and beta region. Shan and Rao (1960) reported a similar trend in horse serum. These results are shown in Table II.4. While investigating the inactivation of serum antibodies by heat, Kleczkowski (1941) studied the formation of precipitates in heated mixtures of rabbit albumin and globulin, and concluded that such precipitates represented complexes formed between the two proteins under the influence of heat. Jennings and Smith (1942) elaborated upon this concept by confirming the observation that antibodies in the pure state are more difficult to inactivate by heat than when mixed with serum globulin, presumably because of complex formation under the latter conditions.

Although a species difference was suspected as a possible cause of the different effects noted at similar temperatures, there were conflicting reports on horse serum by Shan and Rao (1960) and Miyamoto et al.(1957); the former reported decreases in albumin and gamma globulins (Table II.4) and the latter noted a decrease of up to 60 percent in albumin but very little change in the globulin fractions after heating at 54-60°C.

Differing results may be caused by variations in the electrophoretic techniques used. In fact, it seems that these variations may be the largest source of the differences between reported results. After heating guinea-pig serum at 56°C for 30 minutes, Weimer et al.(1965) noted that an increase in the alpha-2 globulin fraction and a decrease in the beta-globulin fraction was detected by moving boundary electrophoresis, but that an increase in both of these fractions was shown by filter paper electrophoresis. Eleven of the twenty-one bands separated by starch gel electrophoresis from normal serum were altered in the

TABLE 11.4 EFFECT OF HEAT ON THE COMPOSITION OF SERUM PROTEINS

<table>
<tr><th colspan="3">Heat Treatment</th><th colspan="4">Protein Fraction</th><th>Reference*</th></tr>
<tr><th>Species</th><th>Temperature (°C)</th><th>Time (minutes)</th><th>Albumin</th><th>Alpha Globulin</th><th>Beta Globulin</th><th>Gamma Globulin</th><th></th></tr>
<tr><th></th><th></th><th></th><th colspan="4">(percentage of total serum protein)</th><th></th></tr>
<tr><td>Swine</td><td>Control</td><td>-</td><td>47.5</td><td>17.5</td><td>16.7</td><td>18.3</td><td>a</td></tr>
<tr><td></td><td>58-59</td><td>30</td><td>42.9</td><td colspan="2">39.8</td><td>17.3</td><td></td></tr>
<tr><td></td><td>60-61</td><td>30</td><td>39.1</td><td colspan="2">46.7</td><td>14.2</td><td></td></tr>
<tr><td></td><td>62-63</td><td>30</td><td>27.3</td><td colspan="2">58.5</td><td>14.2</td><td></td></tr>
<tr><td></td><td>64-65</td><td>30</td><td>20.7</td><td colspan="2">70.5</td><td>8.8</td><td></td></tr>
<tr><td>Horse</td><td>Control</td><td>-</td><td>44.1</td><td colspan="2">37.0</td><td>18.9</td><td>b</td></tr>
<tr><td></td><td>55</td><td>30</td><td>16.8</td><td colspan="2">63.9</td><td>19.3</td><td></td></tr>
<tr><td></td><td>65</td><td>30</td><td>-</td><td colspan="2">90.9</td><td>9.1</td><td></td></tr>
</table>

* a J.Mathews and D.A.Buthala (1958) - moving boundary electrophoresis.

b S.L.Shan and S.S.Rao (1960) - paper electrophoresis, pH 8.6.

heat-treated serums (six disappeared completely), but these results were not correlated with the fractions obtained in either moving boundary or paper electrophoresis. Larin (1956) noted that heating serum at 56°C for 30 minutes resulted in increased electromigration in paper electrophoresis, whereas heating serum at 62 or 65°C for 30 minutes considerably reduced migration and the protein was strongly adsorbed on the paper. When investigating the changes induced by heat on the serum proteins, Ardry (1951b) obtained different results between moving boundary electrophoresis and chemical precipitation. He suggested the possibility that these differences were due to internal modifications in the serum protein molecules which could affect chemical but not electrophoretic separation.

It can be concluded that heat denaturation of serum proteins results in modification of their electrophoretic patterns. Santamaria and Ieso (1957) confirmed that these modifications chiefly involved the formation of an aggregate component from molecules of different types, possibly as a consequence of the unfolding of peptide chains and the attraction of some polar groups. This aggregate formation was found to be highly dependent upon the denaturation intensity (i.e. the time and temperature of heating). In serum, some proteins are certainly more heat-labile than others and the presence of the more heat-labile proteins probably causes "coprecipitation" of the less heat-labile proteins. This is verified by the fact that Ardry (1951a) found that isolated albumin and gamma-globulin fractions, heated at 57-62°C for ten minutes, retained their individual state, but aggregation of these proteins was caused in serum after this heat treatment.

Ballou et al.(1944) reported that the addition of alpha globulin did not result in a decrease in the thermal stability of albumin. Other workers (Hink et al.(1957) and Hink (1960)) found that the removal of fibrinogen and gamma globulin from plasma produced a solution which contained 85-90 percent albumin, 6-9 percent alpha globulin and 3-6 percent beta globulin. This was stable at 60°C

for more than ten hours. Nitschman <u>et al</u>.(1956) have also prepared a similar solution. These reports indicate that fibrinogen and the gamma globulins are the more heat-labile plasma proteins.

However, a few conflicting reports (Macfarlane (1961); Krawczynski and Drewnowska (1955); and Santamaria and Ieso (1957)) suggest that the albumin fraction of serum is more easily coagulated than the globulin fractions. Krawczynski and Drewnowska (1955) state that a serum fraction which contained 33 percent beta and 67 percent gamma globulin is more resistant to coagulation than the albumin fraction.

In conclusion it may be noted that many of these experiments have been conducted in the interest of determining changes in serum during the inactivation of the complement activity of serum by heating. The complementary activity of fresh unheated serum depends on the interaction of at least four distinct components. It may be noted here that the activity of different components is related to different protein fractions (Topley and Wilson (1946)) and so it is not surprising that these components vary in thermal stability.

b. <u>Erythrocyte Protein (Haemoglobin)</u>

(i) <u>Effect of Heat on Haemoglobin</u>. Haemoglobin remained a homogeneous component when heated alone for 30 minutes at 56°C (Dimopoullos and Fellowes (1958)). Early reports (Thorpe (1952)) that haemoglobin (in solutions of approximately pH7) at temperatures above 70°C was split into the pigment haematin and globin are apparently incorrect, more recent work indicates that the four haemoglobin subunits dissociate on heating (Joly (1965)). The globin portion of these subunits is very easily denatured but remains attached to the porphyrin haeme group, as the haeme-binding capacity of globin is increased at least six-fold by denaturation (Joly (1965)).

(ii) Effect of Adding Haemoglobin to Serum. Dimopoullos and Fellowes (1958) using paper electrophoresis (barbiturate buffer, pH8.6 and ionic strength 0.05) found that when haemoglobin (an electrophoretically homogeneous component with the mobility of alpha globulin) was added to guinea-pig serum, there was no change in the gamma globulins but there was an increase in the amount of albumins and an increase in the alpha and beta globulins which was greater than that expected from the haemoglobin alone. Increases in some of the globulin fractions had been reported by several workers, and as noted with heated serum proteins, the actual fraction in which the increase was noted was apparently highly dependent upon the buffer used for paper electrophoresis: alpha-2 globulins (phosphate-chloride buffer, pH7.7); alpha and beta globulins (barbital buffer, pH8.6); and gamma globulins (phosphate buffer, pH7.4). Dimopoullos and Fellowes, therefore, concluded that complex formation between haemoglobin and serum proteins (mainly the alpha globulins) occurred.

(iii) Effect of Heat on a Mixture of Haemoglobin and Serum. Heating a mixture of haemoglobin and serum proteins resulted in a significant increase in the beta globulins, indicating increased complex formation.

2. Influence of pH on Heat-induced Changes in Serum.

a. Effect of pH on the Protein Fractions of Serum.

Ardry (1951b) found that the electrophoretic pattern of serum held at room temperature for 12 hours at modified pHs (between 3.8 and 9.8) was only slightly altered. However, at pH 2.8 there was a marked decline in the albumin content, which was balanced by an increase in the alpha globulins. Chemical fractionation using sodium sulphite precipitation showed a decline in the albumin content and an increase in beta and gamma globulins, the amount of alpha globulin apparently remaining unchanged.

b. Effect of pH plus Heat on the Protein Fractions of Serum.

Schulz, working with diluted serum, found that changes in the pH range (3.0-9.0) has very little effect on the heat-induced changes in the electrophoretic pattern of human serum if the serum was heated at temperatures below 60°C for thirty minutes. However, Ardry (1951b) found that whole serum at 57°C formed a yellow transparent gel at pH 2.8 and a white opaque gel at pH 3.8. Therefore only serums brought to pH 5, 6.2, 8.6 and 9.8 could be analysed. In these serums a large amount of a constituent of mobility 3.7 (i.e. an alpha-3 globulin) was formed, this being particularly rapid at pH 5.0. The progressive disappearance of the gamma and beta globulins on heating (to the profit of the alpha-3 globulins) was most marked at raised temperatures. Schulz also found in diluted serum that at higher temperatures (60-70°C) the amount of migrating, stainable globulin was smaller at lower pH values, and suggested that this was probably because more of the globulin was bound up in the precipitated protein.

Measurement of the turbidity, surface tension and viscosity of the pH adjusted serums before heating indicated that pH changes from 5 to 10 did not have a marked effect on these properties (Ardry (1951c)). At pH 3.8, the surface tension was not markedly affected but there was a small increase in viscosity and a large increase in the turbidity. At pH 2.8, a marked increase in the viscosity of the serum and marked decreases in turbidity and surface tension were noted. After heating at 57°C for 10 minutes, the values of all these properties decreased slightly, but after 10 minutes at 62°C or 65°C they increased. The pH did not have a marked influence on the heat-induced changes in surface tension, and pHs between 5 and 8.6 did not have any influence on the heat-induced changes in viscosity. At pH 9.8, the viscosity rose sharply on heating. As the pH was lowered from 10 to 5, the increase in turbidity on heating was more marked.

Drevon and Landrivon (1949) concluded that heat coagulation of plasma was dependent not only on the pH but also the type of buffer used.

3. Influence of Various Substances on the Heat Coagulation of Blood Proteins.

Heat denaturation often becomes more marked in the presence of substances which alone do not have any denaturing effect. The presence of inorganic salts affects heat coagulation of proteins because of their influence on the electrostatic repulsive forces between protein molecules in solution. The importance of these forces in heat coagulation is discussed in Section II.D. Besides this general effect of ionic strength there are also specific effects depending on the nature of the anions and cations (Joly (1965)).

Many substances (e.g. inorganic anions, fatty acid anions, sugars and formaldehyde) have an inhibiting effect on the thermal denaturation of plasma proteins. The inhibitory effect of inorganic anions (e.g. sulphate) and fatty acids (e.g. caprylate) is believed to be due to electrostatic interactions and binding in non-polar regions which prevent the protein molecules from unfolding completely (Markus et al.(1964); Boyer et al.(1946); and Duggan and Luck (1948)). There is no evidence to show that sugars would act in a similar manner (Hardt et al.(1946)). The inhibitory action of formaldehyde is due to its biphasic reaction with free amino groups of proteins (Dawson et al. (1964)).

Acetylation of the free amino groups of albumin imparts a marked resistance to thermal coagulation, which Jensen et al. (1950) suggest is due to the removal of free cationic centres, thereby increasing the net negative charge of the albumin molecules.

4. Influence of Aging on Heat-induced Changes in Serum.

a. Effect of Aging on the Protein Fractions of Serum.

Storage of human blood at 37°C for three months resulted in the modification of the electrophoretic mobility of many serum proteins and of haemoglobin, and

over a longer period some of the protein fractions could no longer be detected (Kaminski and Gajos (1962)). Krejci *et al.*(1956) found that fibrinogen and gamma globulin in blood (preserved with glucose and merthiolate) could not be detected after three years' storage and that the relative amount of beta globulin had decreased, while the component with the mobility of alpha globulin had increased.

They also found similar changes in blood plasma. Storage of human plasma at room temperature for six years resulted in the complete disappearance of fibrinogen, and decreases (as shown by Tiselius electrophoresis) in the beta and gamma globulin and total protein contents (44,20 and 13 percent decrease respectively). Similar changes were found in plasma stored at higher temperatures. Ishimura (1961) found that the incubation of human plasma at 30°C for four days markedly increased the relative amount of alpha globulin fraction (while slightly lowering its electrophoretic mobility) and decreased that of the beta globulin fraction. On incubation at 50°C for 24 hours, these changes were more marked, and the relative amounts of albumin, gamma globulin and fibrinogen fractions decreased, as did the mobility of all fractions. Lowering of the storage temperature below room temperature also affects the aging of plasma. The decrease in the total protein and fibrinogen of plasma after 10 days' storage was greater at 4-8°C than at 20-25°C; this decrease in total protein was mainly due to the decrease in fibrinogen (Mantseva and Lyuboshits (1958)).

On aging human serum for one month at 4°C, decreases in the alpha-2, beta-1 and gamma globulin fractions and small increases in the albumin and beta-2 globulin fractions were noted (Ardry (1951a) - using moving boundary electrophoresis). However, Weimer *et al.* (1965) found small decreases in both albumin and beta globulin fractions (using moving boundary and filter paper electrophoresis) after holding guinea-pig serum at 4°C for two weeks (changes in other fractions differing according to the electrophoretic method). This difference in results was noted in an earlier section (C.1.a.(ii)) and is probably due to

differences inherent in the analytical techniques as used by different workers, although the possibility of species differences has not been completely discounted.

According to Skvaril (1960) human gamma globulin undergoes slow proteolysis into two fractions during the storage of plasma, but no other reports of this were found.

b. Effect of Aging plus Heat on the Protein Fractions of Serum.

The changes which occurred on heating aged human serum (Table II.5) were found to be very similar to those observed in heating fresh human serum (Table II.3) (Ardry (1951a)). He likens the changes in aged serum heated at 59 and 62°C for 10 minutes to those obtained at 57 and 59°C with fresh serum (Table II.3) and, therefore, assumes that aging causes a slight resistance to the changes produced by heating fresh serum.

5. Influence of Preheating on Heat Coagulation of Blood Proteins.

Spektor (1966) found the incubation of serum proteins at 37°C for 60 minutes stabilised the proteins against thermal denaturation, whereas incubation at lower temperatures (4,18 and 30°C) led to the destabilisation of these proteins. This could be due to the formation of "lipid prealbumin" (apparently a simple process of binding free fatty acids to albumin) reported by Losticky (1963); or to changes in protein-bound hexose and seromucoid tyrosine reported by Stepan et al. (1963). The effect of various combinations of two denaturation temperatures (65°C and 80°C) on the heat denaturation of human serum albumin was studied by Sedlacek (1960). He concluded that preheating at 65°C inhibited aggregation at 80°C, whereas preheating at 80°C stimulated aggregation at 65°C. In agreement with these results it has been noted (Kratochvil et al. (1962) that heating at temperatures below the temperature used for heat coagulation retarded the coagulation process, this being interpreted as the result of denaturation at the lower temperature producing molecules which are less prone to aggregation.

TABLE II.5 EFFECT OF AGING ON THE ELECTROPHORETIC PATTERN OF SERUM PROTEINS

Species and Treatment	Protein Fraction								Reference*
	Albumin	Globulins							
		Alpha			Beta		Gamma		
		1	2	3	1	2	1	2	
Human Serum	(expressed as percent of total serum protein)								
Fresh Serum	60.9	3.1	5.3	2.6	11.2	2.4	3.7	10.8	a
Aged Serum	62.8	3.5	3.7	2.2	10.2	4.4	4.5	8.7	
Aged Serum heated at 57°C for 10 min.	63.7	2.2	2.3	7.7	8.9	3.8	2.5	8.9	
Aged Serum heated at 59°C for 10 min.	61.6	3.8	3.7	8.7	11.5	1.6	1.6	7.5	
Aged Serum heated at 62°C for 10 min.	52.7	3.9	8.4	13.1	10.1	3.5	2.5	6.0	
Aged Serum heated at 65°C for 10 min.	41.4	9.0		40.9		2.5		6.2	
Guinea-pig Serum	(expressed in grams. Total serum protein=6.5 grams in all cases)								
Control	2.5	0.5	1.3		1.4		0.8		b
Aged	2.6	0.4	1.4		1.3		0.8		
Control	3.3	0.5	1.5		0.7		0.5		c
Aged	3.4	0.5	1.4		0.6		0.6		

* a R.Ardry (1951a) - Moving Boundary Electrophoresis, Buffer pH 7.8. Serum aged for 1 month at 4°C.

b H.E.Weimer et al.(1965) - Paper Electrophoresis, Veronal buffer, pH 8.6, ionic strength 0.075. Serum aged for 2 weeks at 4°C.

c H.E.Weimer et al.(1965) - Moving Boundary Electrophoresis, Veronal buffer, pH 8.6, ionic strength 0.1. Serum aged for 2 weeks at 4°C.

D. Theoretical Aspects of Heat Coagulation of Proteins

Heat coagulation of proteins appears to consist of two main stages (Ballou et al. (1944); Chick and Martin (1911); Huggins and Jensen (1949); Pederson (1931) and Joly (1965)) :

a) denaturation stage - the proteins are denatured, their molecules becoming unfolded and hydrated; and

b) aggregation stage - in which the unfolded molecules aggregate under suitable conditions of pH, protein concentration and ionic strength, forming light scattering particles which ultimately form a coagulum.

A third stage, one of degradation, may occur at high temperatures or after prolonged heating.

The heat coagulation of proteins is influenced by pH, ionic strength, protein concentration and the presence of specific substances, as well as the time and temperature of heating. Jensen et al. (1950) found that pH, protein concentration, and small amounts of sulphydryl reagents, markedly affected the course of coagulation of serums heated at 100°C. They found that two different types of coagula could be produced from bovine plasma albumin and human serum albumin, depending on the pH of the medium. These types of coagula corresponded to the coarse and fine clots produced by the action of thrombin on fibrinogen under different conditions, which have been described by Ferry and Morris on (1947). Clots of an intermediate nature were also formed (i.e. there is no definite division between the conditions which produce fine clots and those that produce coarse clots).

The major influence which pH has on the nature of the coagulum has been interpreted in terms of the molecular repulsive forces due to the net charge on the molecules. Heat coagulation occurs at most pH levels, the only exceptions

being at very high or low pH regions. In these regions gelation occurs rapidly at room temperatures. (Gelation can only occur when the pH of the solution is far enough removed from the isoelectric points of the proteins in solution to avoid total aggregation and precipitation). Nearer their isoelectric points the proteins remain in solution until heat treatment renders them coagulable.

The mechanism of heat coagulation of bovine and human serum albumin has been studied by many workers; for example, Jensen et al. (1950); Stein rauf and Dandliker (1958); Hospelhorn and Jensen (1954a) and (1954 b); Frensdorf et al. (1953); Levy and Warner (1954); Kerekes (1963); Lumry and Eyring (1958); Munk et al. (1960); and Stokrova and Sponar (1962)). The properties of the coagula formed on heat coagulation of albumin under different conditions indicate the presence of three types of intermolecular linkage (Jensen et al. (1950)) :

i) a three dimensional network in the fine (clear) clots formed by a relatively slow reaction of unknown nature.

ii) additional lateral association in the opaque synerising clots involving the protein sulphydryl groups.

iii) hydrogen bond formation which also leads to turbidity in the coagula.

These are discussed below.

Within the isoelectric region the intermolecular repulsive forces are minimal, and side-by-side aggregation rapidly occurs with the release of free side chain groups on heat denaturation. As the pH is raised or lowered from the isoelectric region, the net charge on the denatured protein molecules increases, and consequently the intermolecular repulsions become stronger, i.e. both network formation and lateral association are inhibited.

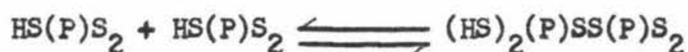
Within the isoelectric region the heat aggregation of bovine serum albumin is not dependent upon the presence of sulphydryl reagents (e.g. cysteine and thioglycollate) (Bro et al. (1958)). This is apparently due to the formation

of intermolecular hydrogen bonds between carboxy and amino groups of neighbouring polypeptide chains (see Figure II.1) as proposed by Mirsky and Pauling (1936).

FIGURE II.1 INTERMOLECULAR HYDROGEN BOND FORMATION

In the presence of free sulphydryl groups aggregation also occurs by the disulphide-sulphydryl interchange reaction (Figure II.2), although sulphydryl reagents have little effect on the opaque coagulum formed rapidly on thermal coagulation at pH 5.5 (cysteine or thioglycollate) (Bro et al. (1958)).

FIGURE II.2 DISULPHIDE-SULPHYDRYL INTERCHANGE REACTION



Near the isoelectric region, bovine serum albumin, aggregation is chiefly of the side-by-side kind as shown by the low anisotropy of the aggregates. Van der Waal's forces are also involved in the aggregation process.

Outside the isoelectric region hydrogen bond formation does not occur, and therefore aggregation can not take place if the sulphydryl groups are blocked by quinone. The sulphydryl-disulphide oxidation reduction reaction (Figure II.2) takes place in the presence of free sulphydryl groups, this reaction being independent on pH.

Stein rauf and Dandliker (1958) reported that the polymerisation of bovine serum albumin was mainly due to the disulphide-sulphydryl interchange reaction. The formation of insoluble material was most rapid at pH 5.6. They attributed the pH dependence of the polymerisation to (1) the availability of sulphydryl

groups and (2) electrostatic repulsion.

In the isoelectric region, the intermolecular repulsive forces are minimal and, therefore, the molecules would be able to approach more closely and this would lead to an increase in the rate of the disulphide-sulphydryl interchange reaction. On heating 1 ml.samples of 0.6 percent bovine serum albumin at 100°C, Steinkrauf and Dandliker noted a 30 second lag period before very rapid internal disulphide-sulphydryl exchange reactions occurred. During this lag period, dimers were formed. This could be accounted for by the presence of one disulphide bond which was more susceptible to reduction than the others. The presence of one such susceptible disulphide bond in human serum albumin had been reported by Markus and Karush (1957).

A low value of electrostatic repulsion is apparently required for heat coagulation. Thus if bovine serum albumin is heated at pH 5.5 in the absence of buffer, only partial coagulation occurs, the pH rising from 5.5 to about 6.9. In contrast, if the albumin is heated at pH 5.4 in the presence of 0.0025M citrate buffer (which prevents pH change due to the release of free acidic or basic groups on denaturation, thus inhibiting the course of denaturation) the pH does not rise above pH 5.9 and all the protein is coagulated (Zubay and Doty (1957)). If the native albumin, without salt, is heated at pH 6.5 for 10 minutes in a boiling water bath no coagulation occurs. This is due to the increase in the negative charge on the albumin molecule (isoelectric point of bovine serum albumin is 4.5) which is probably accounted for by the neutralisation of the imidazole groups of histidine which would be expected to titrate in this region (pH 5.5-6.5). (N.B.There are 18 histidine residues per molecule of bovine serum albumin (Tristam (1953)). The repulsion between albumin molecules becomes great enough above pH 6 to prevent effective contact. This repulsion caused by the charge effect is overcome in a medium of high ionic strength; albumin at pH 6.5 in 0.04M sodium chloride undergoes coagulation on heating. Similarly at room temperature and low pH (about 1.9) bovine serum albumin in solution does not

aggregate in 0.1M potassium chloride, whereas it forms aggregates in 0.5M potassium chloride (Bro et al.(1955)) and 0.1M sodium chloride or acetate (Saroff et al.(1955)).

The addition of salt during heat treatment greatly enhances aggregation : the mean length of aggregates in a 2.4 percent solution of human serum albumin after 10 minutes at 80°C in 0.02M acetate buffer at pH 4.2 is 2700 Å compared with 850 Å in the absence of salts (Bartl and Sedlacek (1958)). The addition of salt after heat treatment increases the aggregation and favours flocculation of the albumin (Joly (1965)).

In conclusion it is noted that disulphide-sulphydryl interchange reactions, electrostatic interactions between side chains of the protein molecules, and hydrogen bond formation may all occur during heat coagulation of albumin. Therefore it is obvious that these reactions would occur during the heating of mixtures of blood proteins, ultimately resulting in their coagulation.

III. EXPERIMENTAL WORK

A. Materials and Methods

1. Collection of Blood.

2. Preparation of Blood Fractions.

a. Preparation of Plasma and Red Cells.
b. Preparation of Serum.

3. Techniques Used in Laboratory Experiments on Heat Coagulation of Blood, Red Cells and Plasma.

a. Heat Treatment.
b. Dilution of Blood, Red Cells and Plasma.

   (i) Preparation for Sampling.
   (ii) Sampling and Dilution.

c. Turbidity Measurements.

   (i) Light Reflectance using the Eel nephelometer head.
   (ii) Light Absorbance using the Hitachi Spectrophotometer.

d. Separation of the Precipitate from the Supernatant.
e. Determination of the Protein Content of the Supernatant.
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g. Protein Analysis - Polyacrylamide Disc Electrophoretic Method.
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   (i) Hitachi Model 101 UV-Vis Spectrophotometer.
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i. Separation of Blood Protein Fractions by Column Chromatography using Hydroxyl Apatite Columns.

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   (ii) Direct Heat Transfer - Steam Injection into a Pipe.

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Laboratory Experiments

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(i) Turbidity.
(ii) Changes in Absorption Spectra.

b. Protein.
c. Formation of Precipitate.
d. Electrophoresis.
e. General Discussion.

2. Influence of Species on the Heat Coagulation of Blood.

3. Effect of Age of Animal on the Effect of Heat on Blood.

4. Effect of Preheating on the Heat Coagulation of Blood.

5. The Effect of Aging on Heat Coagulation of Blood.

6. Effect of Aging Plus Preheating on Heat Coagulation of Blood.

7. Effect of Other Variables on the Heat Coagulation of Blood.

8. The Effect of Heat on the Blood Fractions.

a. Effect of Heat on Plasma.
b. Effect of Heat on the Red Cells.
c. The Influence of the Blood Fractions - plasma and red cells - on the heat coagulation of bood.

Pilot Plant Experiments

1. Effect of Heat on Blood.

a. Indirect Heat Transfer.

(i) Optical Properties.
(ii) Protein in Supernatant.
(iii) Precipitate Formation.

b. Steam Injection.
c. Discussion.

2. Effect of Aging on Heat Coagulation of Blood.

3. Effect of Dilution on Heat Coagulation of Blood.

4. Effect of Sodium Chloride Addition on Heat Coagulation of Blood.

C. Discussion

III. EXPERIMENTAL WORK

A. Materials and Methods

1. Collection of Blood.

Blood was collected from sheep and cattle killed under normal New Zealand meatworks' conditions. The cattle were stunned using a captive bolt pistol, then hoisted by a hind leg on to a rail, and their throats cut. The throats of the sheep were cut while the animals were on a "tipping box". Immediately after the throats of the animals were cut, the blood was drained from the cut into containers (1 litre glass preserving jars or 10 litre plastic buckets). If serum was required, no anticoagulant was added to the blood. In all other cases, the anticoagulant was measured into the jar or bucket before collection of the blood.

The anticoagulant used was disodium ethylene diaminetetraacetic acid (EDTA) at a concentration of one gram per litre of blood. The use of EDTA for these experiments avoided the dilution of the blood which normally occurs when sodium citrate is used. Solid sodium citrate does not readily dissolve in blood and a 3.13 percent solution (1 ml. to 9 ml. of blood) is generally used for anti-coagulant purposes. Oxalate salts (ammonium, potassium or sodium) were not used since they were not readily available in the quantities required to prevent coagulation in these experiments (1.2, 0.8 and 3.0 grams per litre of blood respectively).

Rapid chilling of the blood and maintenance of its temperature below temperatures at which clotting could occur (i.e. below $1^{\circ}C$) was inconvenient in the present experiments. Chilling of blood in 1 litre jars surrounded by ice in the freezer storage rooms was not rapid enough to prevent clotting.

Rapid chilling before clotting took place could be achieved in the subzero temperatures of the works' blast-freezing tunnel, but this was not always available for experimental work.

The collected blood was transported to Massey University and held at $4^{\circ}C$ until required. Except in the aging study, the blood was used within 5 hours of collection.

2. Preparation of Blood Fractions.

a. Preparation of Plasma and Red Cells.

Blood used for the preparation of plasma was centrifuged at 2,700 r.p.m. for 60 minutes and the plasma very carefully decanted from the sedimented red cells. The layer of plasma immediately above the red cell layer was discarded as it was often contaminated by red cells. The top portion of the red cell layer was also discarded because it may have been mixed with plasma during the separation procedure.

b. Preparation of Serum.

Blood to which no anticoagulant had been added was held after collection at room temperature for 4 hours to allow the clotting process to take place relatively rapidly and the process of syneresis to begin. The blood was then held at $4^{\circ}C$ for 20 hours to allow completion of syneresis. The serum was then very carefully pipetted from the clot as usually only small quantities were required. Extreme care was necessary during this separation process as the disruption of the fragile red cells would have resulted in the release of haemoglobin into the serum.

3. Techniques used in Laboratory Experiments on Heat Coagulation of Blood, Red Cells and Plasma.

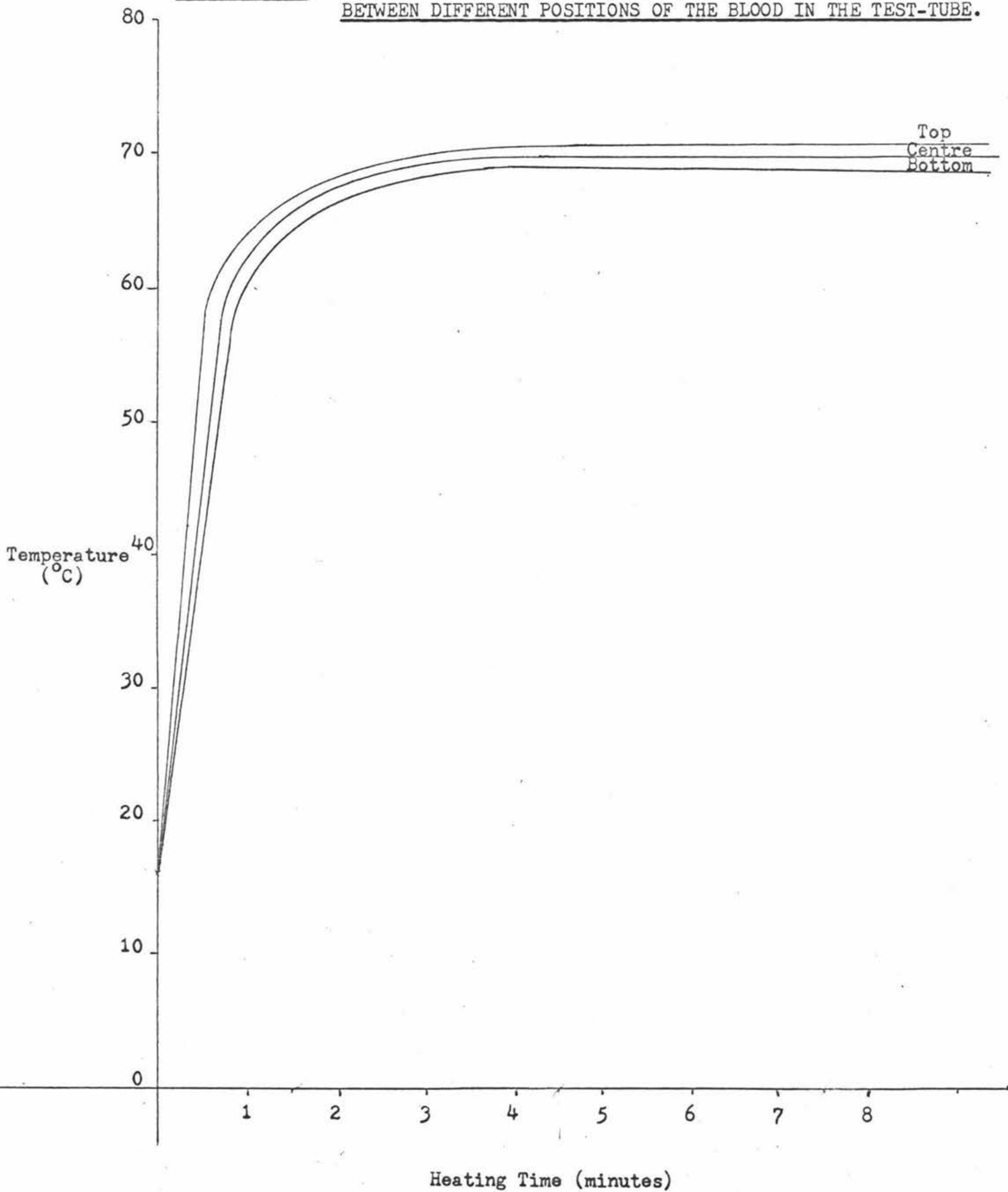
a. Heat Treatment.

Ten ml. aliquots of blood, red cells, or plasma, were pipetted into the required number of 15 cm.(length) x 2 cm.(diameter) pyrex test-tubes. (The variation in external diameter found within batches of these tubes was reduced by the use of only those tubes which had an external diameter of less than or equal to 1.9 cm.). This volume of fluid filled the lower 2.5 cm.(height) of the tubes. The filled test-tubes were then placed in metal racks and were immersed to a depth of approximately 10 cm. in a thermostatically controlled waterbath which had been equilibrated at the specified temperature. In order to avoid a large drop in the temperature of the waterbath on immersion of the tubes, the bath volume was relatively large (approximately 60 litres) compared to the total volume of the tubes immersed at one time (less than 1 litre). The tubes were not shaken or agitated during this heat treatment. At regular intervals, in most cases 15 second intervals, from the time of immersion one tube from the series was taken from the waterbath and immediately immersed in cold water at about 18°C (running cold water was used where possible) for at least 10 minutes.

It was noted that coagulation often began from the air-water interface. This was believed to be mainly due to the difference in temperature between the top and bottom portions of the fluid. Temperature differences between the top, central and bottom portions of the tubes in the waterbath at 70°C are shown in Figure III.1.

Typical heating curves obtained when heating blood samples by this method are given in Figure III.2. Unfortunately no temperature recordings were taken to determine the difference between heating rates of blood, red cells and plasma.

FIGURE III.1. HEATING CURVES WHICH INDICATE THE TEMPERATURE VARIATION BETWEEN DIFFERENT POSITIONS OF THE BLOOD IN THE TEST-TUBE.



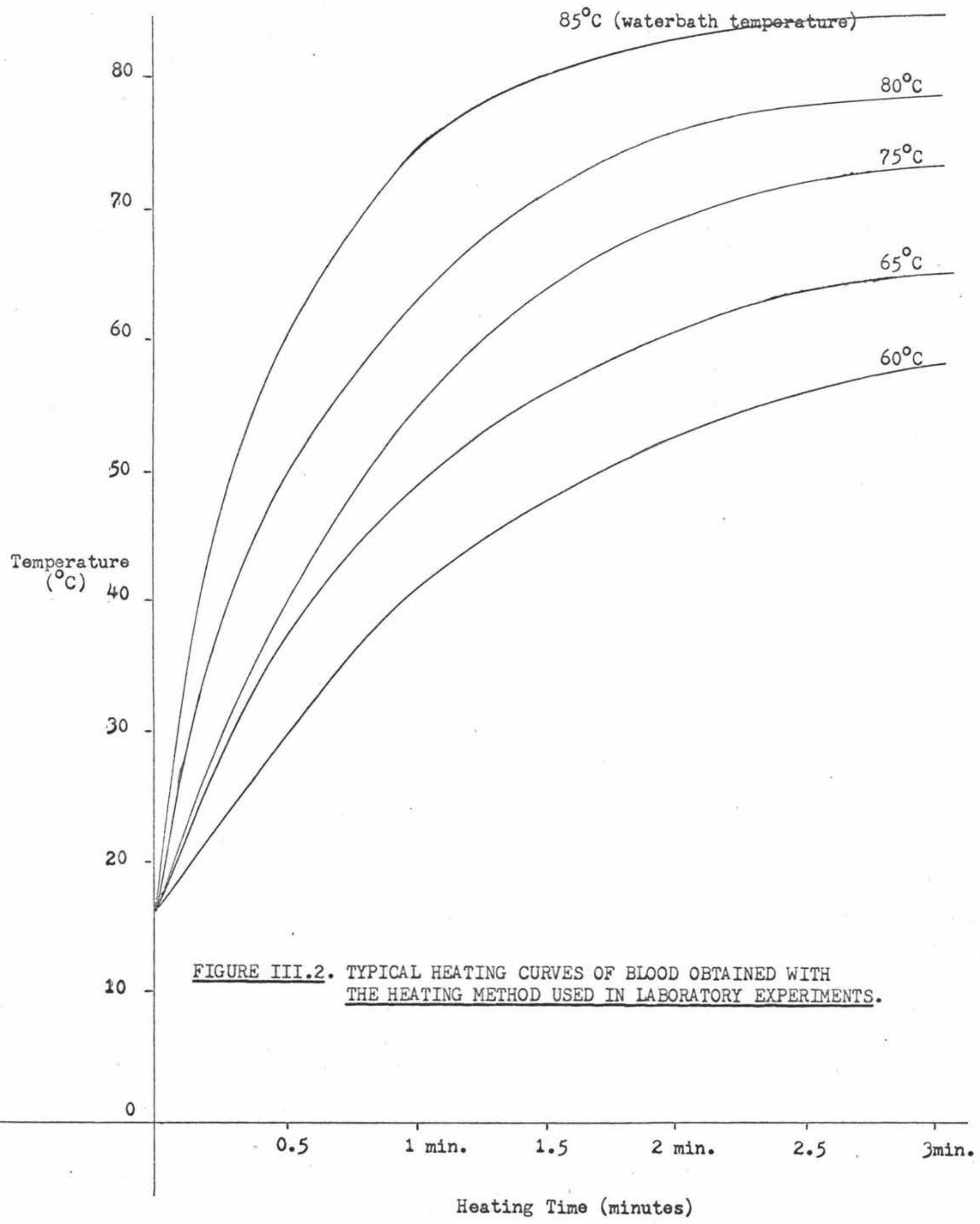


FIGURE III.2. TYPICAL HEATING CURVES OF BLOOD OBTAINED WITH THE HEATING METHOD USED IN LABORATORY EXPERIMENTS.

b. Dilution of Blood, Red Cells and Plasma.

Samples of unheated controls and heated blood, red cells and plasma were diluted in preparation for turbidity measurements (Section 3 c.). In most cases dilution was done within 2 hours of heating but in some experiments, where insufficient time was available, the samples had to be stored overnight at 4°C before dilution. The controls were diluted at the same time as the heated samples. The effects of variations in the times of dilution and of reading on the turbidity measurements are discussed in Appendix 1.

(i) Preparation for Sampling. In order to ensure that representative samples were obtained from each tube, all tubes were thoroughly mixed before the samples for dilution were taken. The more fluid samples of blood and plasma were mixed by inverting the stoppered tubes four times, while tubes containing the more viscous red cells were generally inverted about ten times before the contents were thoroughly mixed. The more solid samples of blood, red cells and plasma were stirred with a clean, dry glass rod until a fluid of uniform consistency was obtained. Completely solid samples, from which a fluid could not be prepared by stirring alone, were mixed with an equal quantity of water (10 ml.- assuming that no evaporation occurred during heat treatment) and then mixed to a uniform consistency by stirring with a glass rod. Two ml. of the solution prepared in this manner was regarded as equivalent to 1 ml. of the heated, undiluted solid.

These experiments were limited by the difficulty of obtaining a representative sample once extensive coagulation had occurred. The method of dilution plus mixing before sampling lessened, but did not avoid the difficulties of pipetting an accurately measured amount of partly solid material. All the samples could have been weighed accurately, but this would have been very time-consuming and not suitable for large numbers of samples.

Weighed samples were only taken in the experiments in which the blood was coagulated by steam injection.

(ii) Sampling and Dilution. Samples were taken from the well-mixed solutions using a wide bore graduated 1 ml. pipette and diluted with distilled water. One or two ml. pipettes, graduated in 0.05 ml. divisions, were used to measure the sample volumes. An adapted syringe enabled relatively quick measurement of a constant volume of distilled water into a large number of test-tubes. The blood samples were diluted to a final dilution of 1 in 15 (i.e. 1 ml. of blood was diluted to 15 ml.); the plasma samples were diluted to a final dilution of 1 in 32 (0.5 ml. plasma was diluted to 16 ml.); and red cells were diluted to a final dilution of 1 in 85 (0.2 ml. red cells was diluted to 17 ml.). The determination of these dilutions is described in Appendix 2.

c. Turbidity Measurements.

The turbidity of the diluted samples was determined by two methods : light reflectance using the Eel nephelometer head, and light absorbance using the Hitachi spectrophotometer.

(i) Light Reflectance using the Eel nephelometer head. Turbidity was determined using an Eel nephelometer head operated in conjunction with an Eel Unigalvo-Type 20. In the nephelometer head, white light from a lamp was directed vertically upwards through the orifice of an annular photo-cell onto the hemispherical base of a standard 6" (length) x 5/8"(diameter) test-tube. A cap, which fitted over the test-tube when the instrument was in use, excluded extraneous light from the sample. When this cap was removed a microswitch operated to disconnect the photo-cell from the galvanometer.

If the solution contained in the test-tube was turbid the light was scattered by reflection from the particles which caused this cloudiness. Such scattered light was collected by a reflector mounted above the photo-cell

and then directed onto the photo-cell itself. The current so generated was fed to the galvanometer on which the readings were indicated on a 1-100 linear division scale (percent reflectance).

The turbidity readings were standardised against a perspex standard (which was taken as 100 percent reflectance) and a distilled water blank (0 percent reflectance).

A filter wheel, interposed between the lamp and the photo-cell, enabled turbidity determinations on coloured solutions to be made. Both the colour and the turbidity of blood, red cells and plasma varied with the heat treatment. If these two factors had varied independently, gross inaccuracies would have been introduced into the turbidity values if they had been determined using an unfiltered white light source.

However, when the turbidity (percent reflectance) of different dilutions of blood was determined (Appendix 2), it was noted that the readings using white light with and without a red filter (OR2) showed similar trends. This indicated that the haemoglobin (red pigment) concentration did not affect the turbidity reading.

Nephelometric turbidity measurements were also made with and without the red filter on a number of heated blood solutions (Figure III.3). As can be seen in Figure III.4, there was a direct relationship between the two sets of readings.

Although the introduction of the red filter into the light beam decreased the sensitivity of the instrument, its use with the blood samples enabled a much wider range of values to be obtained than was obtained using white light (i.e. there was greater distinction between the values of individual samples). Figure III.3 indicates this clearly: with white light (B) the readings are much lower than those obtained with the red filter (OR2). For this reason, the changes in the nephelometric turbidity values on heating blood and red cells were determined using the red filter.

FIGURE III.3. EFFECT OF HEATING ON THE PERCENT REFLECTANCE READINGS OF BLOOD - A COMPARISON OF READINGS TAKEN WITH AND WITHOUT THE RED FILTER.

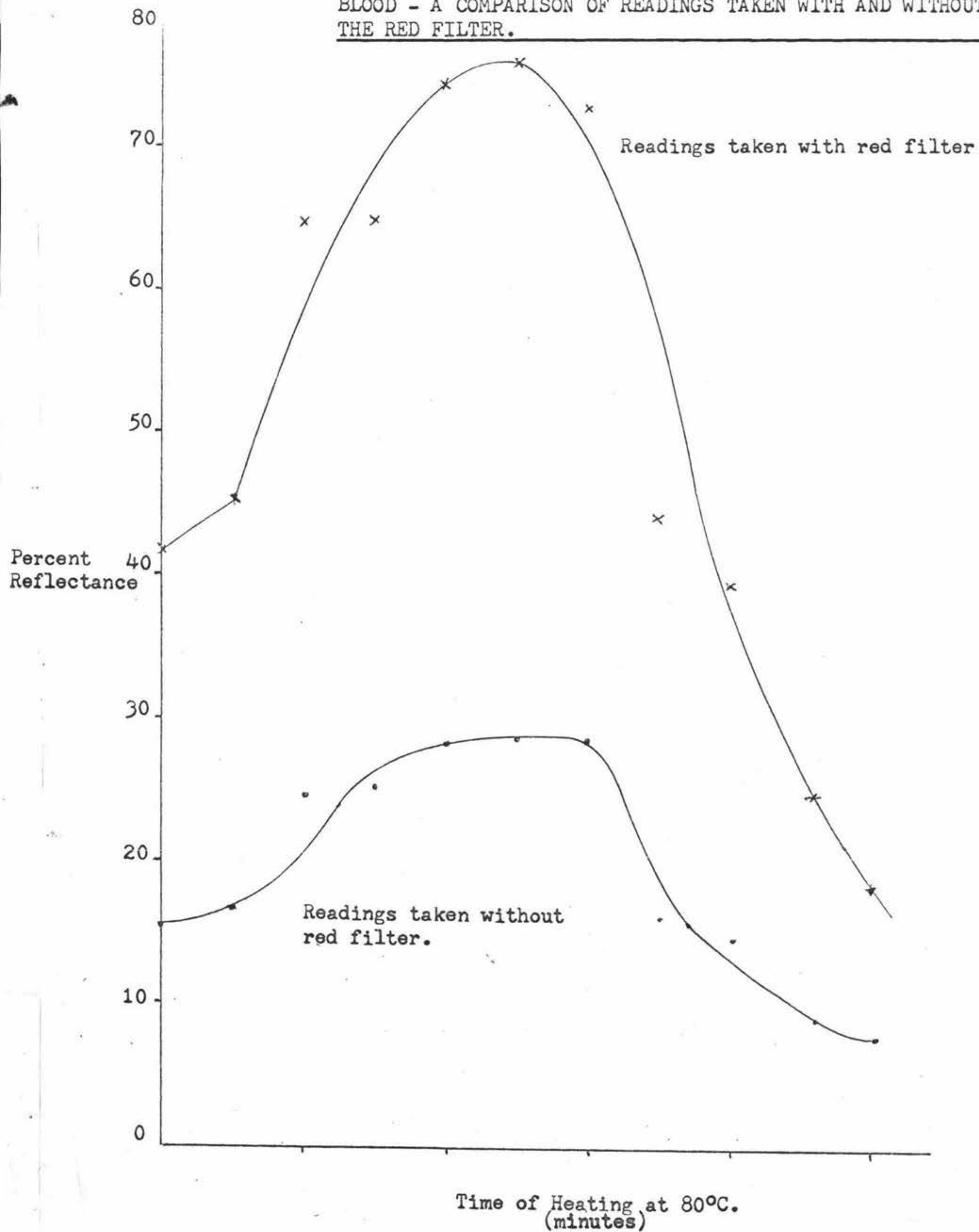
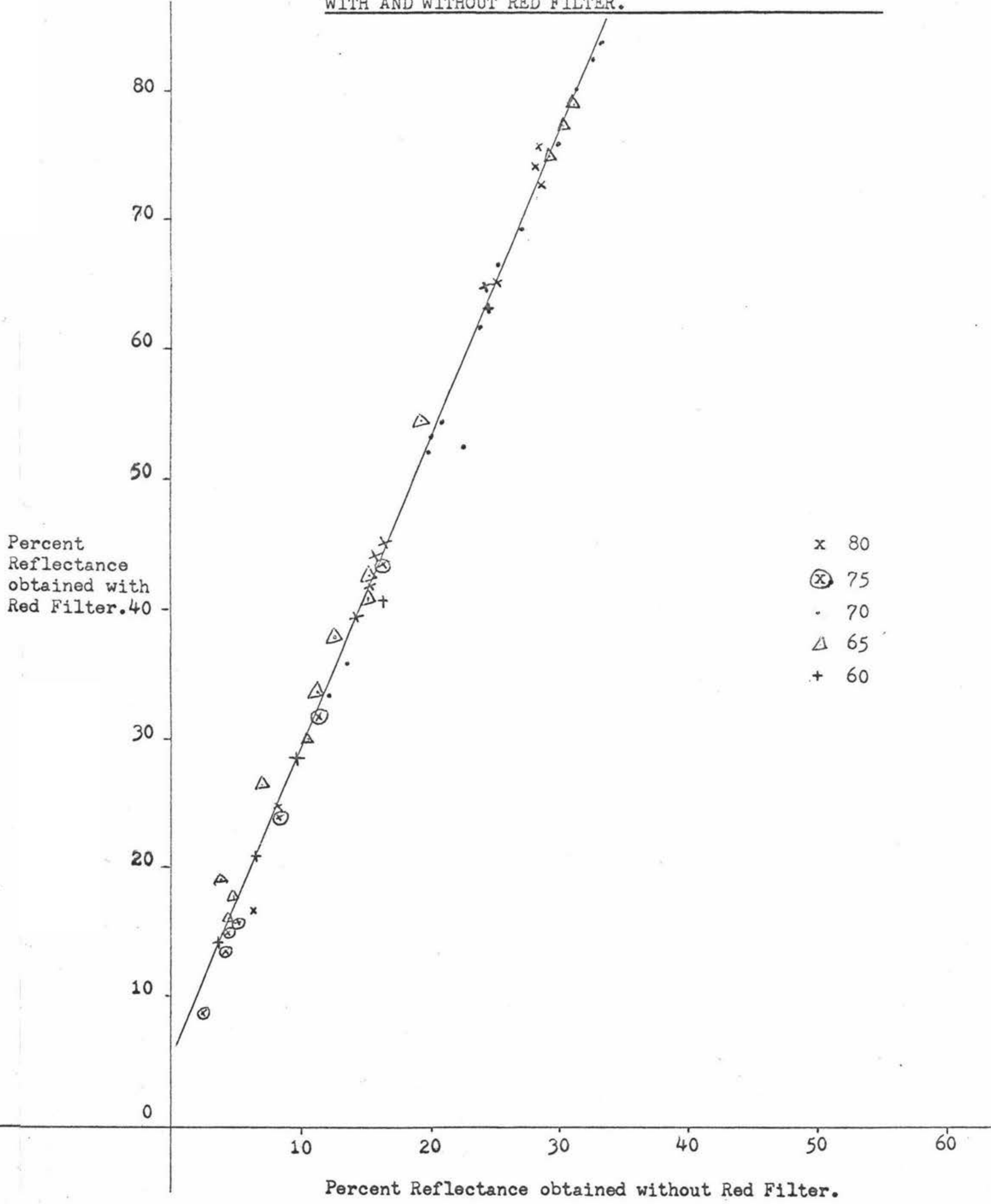


FIGURE III.4. CORRELATION OF PERCENT REFLECTANCE READINGS OBTAINED WITH AND WITHOUT RED FILTER.



The effect of the colour change (from yellow to white) on heating plasma on turbidity changes could not be determined as there was no yellow filter provided with the nephelometer head. White unfiltered light was used for the determination of the changes in the turbidity values of plasma on heating and it was assumed that the colour change was directly related to the changes in turbidity.

(ii) Light Absorbance using the Hitachi Spectrophotometer. The turbidity was also determined by measuring the optical density at a wavelength of 805 mu using the Hitachi Model 101 UV-Vis Spectrophotometer, with a Tungsten lamp light source. Perfectly matched glass cells (1 cm.) were used for these determinations, and the instrument was standardised against a distilled water blank (optical density = zero). The wavelength, 805 mu, was chosen for these determinations as it was outside the range of the visible light region in which haeme pigments absorb light (350-600 mu) and would, therefore, interfere with turbidity determinations. In the 600-900 mu wavelength range, the lowest optical densities of 10-50 percent blood solutions were in the 800-830 mu region. Although there were only very small differences in the optical densities obtained in this region on the blood samples, a possible slight minimum was noted at 805 mu and this wavelength was used for the turbidity determinations.

d. Separation of Precipitate from Supernatant.

After the turbidity of the samples had been measured, the samples were separated into two fractions - the supernatant and the precipitate. Separation by direct filtration through Whatman No.1 filter paper was found to be very slow, and so the samples were either centrifuged at top speed in a Wifug angle centrifuge for 10 minutes, or allowed to stand for 12 to 16 hours at 4°C, in order to sediment the precipitate. Measurement of the volume of precipitate sedimented by centrifugation would have been a rapid method of estimating the amount of precipitate in the samples. However this method could not be used as

suitable centrifuge tubes were not available.

Several millilitres of the supernatant were then pipetted into a clean, dry test-tube in preparation for protein determination (Section 3 e.).

A numbered filter paper (Whatman No.1) was dried at 100°C for 8 hours, cooled in a desiccator and weighed. The remainder of the supernatant was poured through this filter paper; the last two millilitres of the supernatant being thoroughly mixed with the precipitate by agitation of the tube to help transfer of the often sticky precipitate to the filter paper. The filtrate was discarded. The test-tube which had contained the sample was rinsed three times with four millilitre aliquots of distilled water; the washings being used to wash the precipitate on the filter paper. This standardised washing procedure was necessary to eliminate variation between the experiments caused by redissolution of differing amounts of precipitate.

It was noted that if the supernatant solution was held at room temperature for several hours, a white precipitate was formed. This precipitate dissolved when the tubes were shaken, and so it was assumed that it would not affect the amount of precipitate measured as it would have been washed through the filter paper during the rinsing and washing procedure.

e. Determination of Protein Content of Supernatant.

The supernatant samples were diluted and their protein concentration determined by measuring the optical density of the solution at 280 mu. The following dilutions were used :

Blood 0.3 ml. of the supernatant liquid from each blood sample was added to 7.7 ml. of distilled water;

Plasma 0.98 ml. of the supernatant liquid from each plasma sample was diluted with 11.0 ml. of distilled water; and

Red Cells 1.7 ml. of the supernatant from each red cell sample was diluted with 14.3 ml. of distilled water.

The optical density of these diluted supernatants was determined using the Hitachi Model 101 UV-Vis Spectrophotometer, with a hydrogen lamp light source and perfectly matched silica cells. The values obtained by this method correlated with the protein concentration as shown in Figure III.5.

f. Determination of the Amount of Precipitate.

The filter paper containing the washed precipitate was allowed to drain before being completely dried at 100°C for 12 hours in an air oven (or 70°C in a vacuum oven at 25" vacuum for 4 hours). After drying, the filter papers plus precipitates were cooled in a desiccator for at least 1 hour and then weighed. The increase in weight of the filter paper after addition of the precipitate was, therefore, the amount of precipitate in the original sample (Section 3 b.), i.e. in 1 ml. of blood, 0.5 ml. of plasma or 0.2 ml. of red cells.

g. Protein Analysis - Polyacrylamide Disc Electrophoretic Method.

Apparatus

The apparatus used for disc electrophoresis was the "ACRYLOPHOR" apparatus manufactured by Pleuger (Wijnejem, Belgium). It was constructed according to the principles described by Bloemendal (1963 a and b) and Bloemendal et al. (1962). It consisted of a cover plate connected with a circular platinum electrode, an upper electrode vessel supplied with O-rings, and a lower circular platinum electrode attached to the lower electrode vessel. There were eight tubes connecting the two electrode vessels. This apparatus enabled 8 samples to be done simultaneously. Diagram of apparatus is shown in Figure III.6.

Chemicals

Buffer solution : Tris-glycine Buffer, pH 8.5.

Stock solution composition : 3g tris-hydroxymethylaminomethane

and 14g glycine per 100 ml.

This stock solution was diluted 10 times (i.e. 100 ml. to 1 litre) before use.

FIGURE III.5. EFFECT OF PROTEIN CONCENTRATION ON OPTICAL DENSITY AT 280mu.

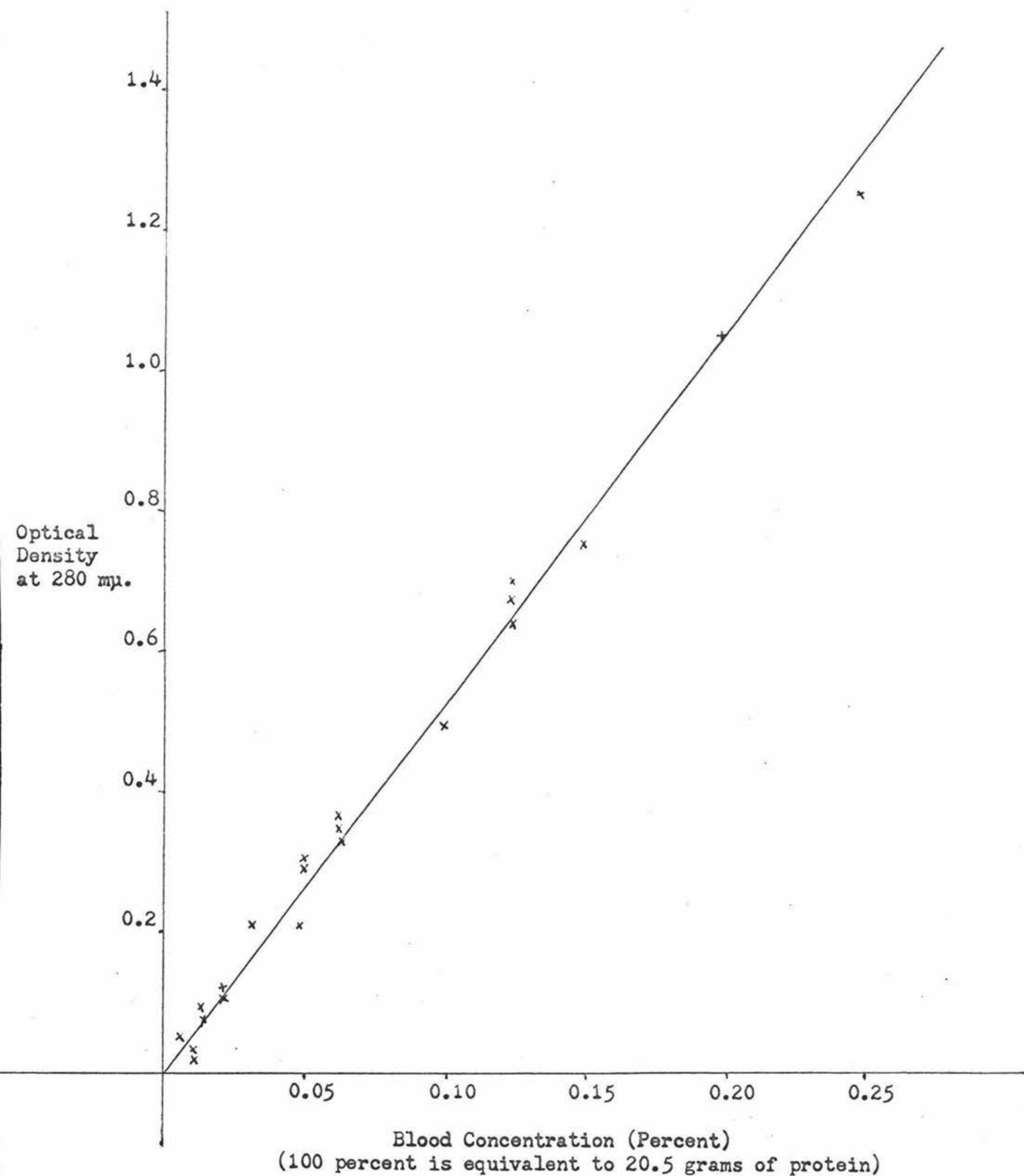
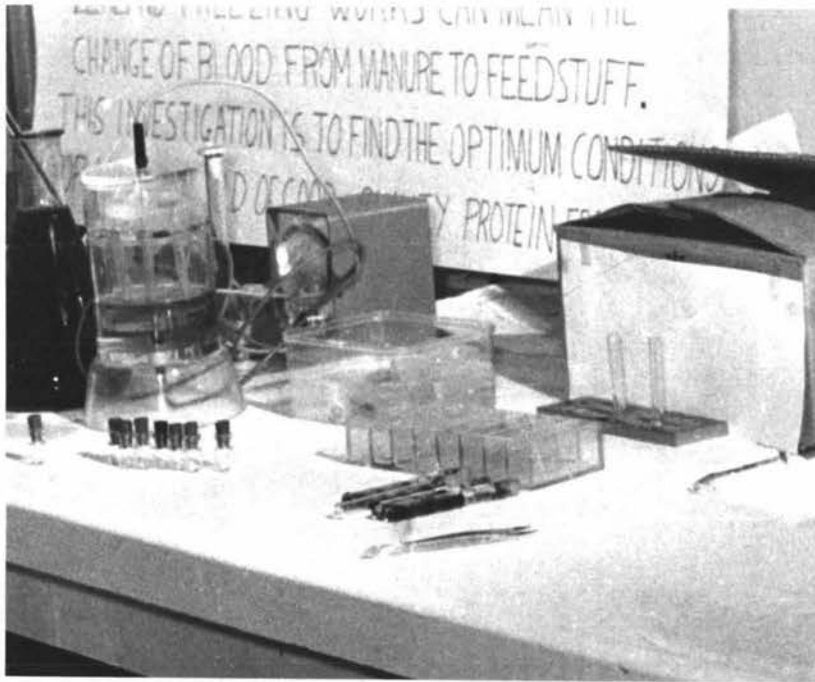


FIGURE III.6

POLYACRYLAMIDE DISC ELECTROPHORESIS APPARATUS.



Polyacrylamide gel.

Stock solutions : (1) 30g Acrylamide and 0.8g N.N'Methylenebisacrylamide, made up to 100 ml. in distilled water and stored in a brown bottle.

(2) 1.6 percent dimethylaminopropinonitril in Tris buffer.

(3) 0.3 percent potassium ferricyanide in distilled water.

(4) 0.48 percent ammonium persulphate in distilled water.

These stock solutions were stored in a refrigerator and allowed to equilibrate at room temperature before the gel was prepared.

Staining Solution.

Saturated Amido Schwarz 10B (about 6 grams per litre) in 7 percent Acetic acid. Any undissolved dye was filtered from this solution before it was used.

Method

Preparation of the gel.

The required number of electrophoresis tubes (maximum = 8) were firmly pressed in a strictly vertical position onto a base plate of plasticine, thus sealing the bottoms of the tubes. Equal volumes of the four gel stock solutions (about 4 ml. of each for 8 tubes) were carefully mixed in the sequence listed above, a swirling action being used to avoid the formation of air bubbles. The mixture was then carefully poured into the tubes to about 4 mm. from the top. With the aid of a bevelled-edged pipette, distilled water was carefully layered on top of this mixture in each of the tubes. At room temperature the gel formed in about 75 minutes. After removing each tube containing formed gel from the base plate, the top of the gel was rinsed with Tris-glycine buffer and the tube fitted into the appropriate place on the upper electrode vessel.

Preparation and Insertion of the Sample.

The sample (blood, haemolysed by 1:1 dilution with distilled water, serum, plasma), heated or unheated, was centrifuged for 5 minutes at top speed in Wifug angle centrifuge to remove insoluble particles before electrophoresis. After centrifuging, a volume of the supernatant which contained 4-20 micro grams of protein, was mixed with 0.1 ml. of a 5 percent sucrose solution containing 0.001 percent bromophenol blue.

The power cables were connected to the apparatus and the lower vessel filled with about 300 ml. of tris-glycine buffer. The upper electrode vessel was placed in position. Care was taken to avoid the presence of air bubbles between the bottom of the gel and the buffer in the lower electrode - this could be achieved by placing the lower ends of the tubes into the buffer at a slant, allowing surface tension forces to fill the lower end of the tube with buffer. Once the upper electrode was in position, the prepared sample could be carefully layered on the buffer-gel interface. If less than 8 tubes were being used the remaining holes were closed with rubber stoppers. The upper vessel was then filled with about 120 ml. of buffer (care was needed to prevent the disturbance of the samples).

Electrophoretic Run

The power supply (regulated at a constant 80 volts) was turned on (current approximately 7 mA/gel). After 45 minutes the voltage was increased to 160V for 15-30 minutes and then the power turned off. In order to get maximum separation the power was not turned off until the albumin band, which was visible during electrophoresis, was within 2 cm. of the end of the gel. This band was visible during electrophoresis because of the formation of a bromophenol blue-albumin complex which did not affect the electrophoretic mobility of the albumin molecule, and formed a blue ring which was preceded by a thin band of free indicator. Following decantation of the buffer in the upper electrode vessel, each tube was

detached from this vessel. The gel was then very carefully removed from the tube by simultaneously rotating the tube and injecting water between the tube wall and the gel surface (from a blunt-needled-hypodermic syringe). If the gel did not slip out easily its removal was aided by squeezing a small rubber bulb fitted onto the end of the tube.

Staining.

Each gel was transferred to its appropriate position in the staining bath (or labelled test-tube) and covered with the staining solution. The gels remained in the stain overnight (about 12 hours). Staining times of at least 30 minutes were recommended in the instruction handbook, but it was found that staining times of up to 2 hours resulted in "tailing" of the pattern during electrophoretic destaining. This "tailing" was caused by unfixed protein in the gel migrating under the influence of the electric current applied during destaining. Shorter staining times were adequate if the destaining was not done by the influence of the electric current.

Destaining.

After staining, the gels were placed in a solution of 7 percent acetic acid for a few minutes, rinsed with the same solution, and transferred to tubes with constricted lower ends (which prevented the gel from falling through). These tubes were attached to the upper electrode vessel (in the same manner as those for electrophoresis). The buffer vessels and tubes containing the stained gels were filled with 7 percent acetic acid, the power supply connected, and the power turned on at a constant voltage of 80V (the current should not exceed 7mA per tube). As the current decreased during destaining, the voltage could later be increased to 160V. Complete decolourisation was usually achieved after about 2 hours.

Destaining could also be carried out by the conventional procedure, i.e. washing with several washes of 7 percent acetic acid. This method, however, took at least 2 days for complete decolourisation.

h. Absorption Spectrophotometry.

(i) Hitachi Model 101 UV-Vis Spectrophotometer. Absorption spectra of heated and unheated samples of blood, serum, plasma and red blood cells were prepared by manual scanning of the wavelength region, 220-900 mu, using the Hitachi Spectrophotometer. Readings were taken at 2.5 mu intervals in regions near maxima or minima of the spectral curves, and at 5 mu intervals in other regions. The hydrogen lamp light source was used in the ultra-violet range (220-340 mu) an d the tungsten lamp light source was used, in conjunction with one of three filters (the filter depending on the wavelength) in the visible light range (340-900 mu). The samples were usually in aqueous dilutions (1 in 400 or 1 in 800) which enabled absorbance readings below 2 to be obtained over the entire wavelength range.

(ii) Unicam SP.800 Spectrophotometer. The Unicam SP.800 recording spectrophotometer was used for one experiment in which the absorption spectra of both the supernatants and the precipitates of blood samples, which had been heated for 0,1,2 and 3 minutes at 90°C, were required. These heated blood samples were thoroughly mixed with twice the sample weight of distilled water and then centrifuged at 2,700 r.p.m. for 30 minutes. The supernatant was then very carefully decanted from the precipitate. Apart from the unheated sample, the supernatants were fairly easily separated from their corresponding precipitates and washing of the precipitate to remove contaminant supernatant was considered to be an unnecessary step. As only a very small amount of precipitate (mainly cell membrane material) was obtained from the unheated sample, this precipitate was not washed with distilled water in order to avoid any loss of this material.

Aqueous 1 in 200 dilutions of the supernatant were prepared. Each precipitate was mixed with 1 ml. of distilled water and then 0.5 ml. 5M urea

($CO(NH_3)_2$,M.W.=60.06) was added to each in order to aid its dissolution. The precipitates were thoroughly shaken, and after standing for 30 minutes all of the precipitates had dissolved apart from a small portion of the sample that had been heated for 3 minutes. The precipitates were then diluted to their final concentrations. (N.B. The use of a mixture of urea and thioglycollic acid to disrupt disulphide bonds formed in this precipitate was only effective in acid solution. Buffering the precipitates at pH 7.4 hindered complete dissolution, but prevented a large decrease in pH which resulted in acid denaturation and, as expected, a completely different spectral pattern).

These diluted supernatants and precipitates were then scanned over the wavelength range 190-700 mu using a Unicam SP.800 spectrophotometer. Distilled water was used as a blank and the reading at 700 mu was adjusted to zero.

i. Separation of Blood Protein Fractions by Column Chromatography using Hydroxyl Apatite Columns.

Hydroxyl apatite, a modified calcium phosphate, was prepared in the manner described by Tiselius et al.(1956), then packed into columns in the absence of pressure. The flow rate of the columns (20 cm.in height x 2 cm.diameter) was adjusted to 15-20 ml.per hour by altering the height of the buffer reservoir above the column.

The column was equilibrated with the starting buffer and then the serum pipetted onto the top of the column. After the serum had penetrated the column the starting buffer was carefully poured onto the column. The serum proteins were eluted using sodium and potassium phosphate buffers at pH 6.8, at concentrations from 0.02 molar to 0.65 molar in a stepwise procedure as described by Hjerten (1959). The effluent was collected in an automatic volume-operated fraction collector, and the concentration of the proteins in the different fractions determined by measuring the absorption at 280 mu with a spectrophotometer (as in Section 3 e.). The protein solutions, which were to be

analysed by polyacrylamide disc electrophoresis and used in heating experiments on the individual fractions and combinations of these, were concentrated by one of two methods.

A rotary film evaporator was used to concentrate fractions eluted from the columns. However, during the initial stages frothing was a problem, which often caused the loss of the sample. This could be controlled by permitting a slight air leak into the system which prevented the reduction of the pressure below an apparently critical point. Once the sample had been partly concentrated the pressure could be further reduced without any recurrence of frothing. This procedure had several disadvantages : The prevention of frothing often was a lengthy procedure; some fractions could not be concentrated unless the temperature was raised to 25-30°C; and also the salt concentration of the samples increased markedly during the concentration procedure. The fact that these last two factors probably resulted in some denaturation was indicated by the low solubility of the concentrated fractions.

The preferred method, using polyethylene glycol to absorb water from the protein solution through dialysis tubing was used once the chemical was available. This method is described by Kohn (1959) and is simple, fairly rapid, does not result in salt concentration and proved very satisfactory at 4°C.

Unfortunately during the later stages of this work the columns packed more closely and very low flow rates were obtained, and as reasonably large quantities of each fraction were required for heating experiments it was decided to abandon these experiments.

4. Equipment and Techniques used in Pilot Plant Experiments on Heat Coagulation of Blood.

a. Description of Heat Coagulation Apparatus.

(i) Indirect Heat Transfer - Pipe Heat Exchanger.

Hot water (70-97°C). A nine-pass stainless steel tubular heat exchanger was used to coagulate blood by indirect transfer of heat from hot water,

circulated from a thermostatically controlled water bath through the jacket of the heat exchanger as shown in Figure III.7. The blood was fed through this heat exchanger by a single-action piston pump. After heat treatment, the blood was cooled by passing it through a stainless steel cooling coil immersed in a bath of running cold water. The blood from this cooling coil was collected in jars from which samples could be taken.

Steam (100°C). The nine-pass tubular heat exchanger was converted to a three-pass heat exchanger by altering the position of one of the end plates and steam was allowed to flow through the jacket of the heat exchanger. A shorter coagulation path was used as the time required for coagulation at the higher temperatures was less than with hot water. As the cooling coil caused frequent blockages at the higher temperature it was removed, and the coagulated samples were cooled by placing their containers in a bath of cold, running water.

(ii) Direct Heat Transfer - Steam Injection into a Pipe (128±2°C).

For convenience, rather than design purposes, one-pass of the heat exchanger was used as the pipe into which steam was injected. The successful steam injection modification to the inlet pipe of the heat exchanger is depicted in Figure III.8. The coagulated blood was collected directly from the end of the pipe and batch-cooled by placing the sample (500 ml.) in a bath of cold, running water.

b. Process Variables and Their Control.

(i) Temperature. The thermostatically-controlled waterbath could be controlled to within 0.25°C, at temperatures up to 97°C. However, because of heat losses during circulation of the water, this temperature was only sufficient to raise the blood temperature at the outlet of the heat exchanger to 94°C. The bath temperature could be held at 100°C by allowing steam to flow

FIGURE III.7A. PILOT PLANT - TUBULAR HEAT EXCHANGER.

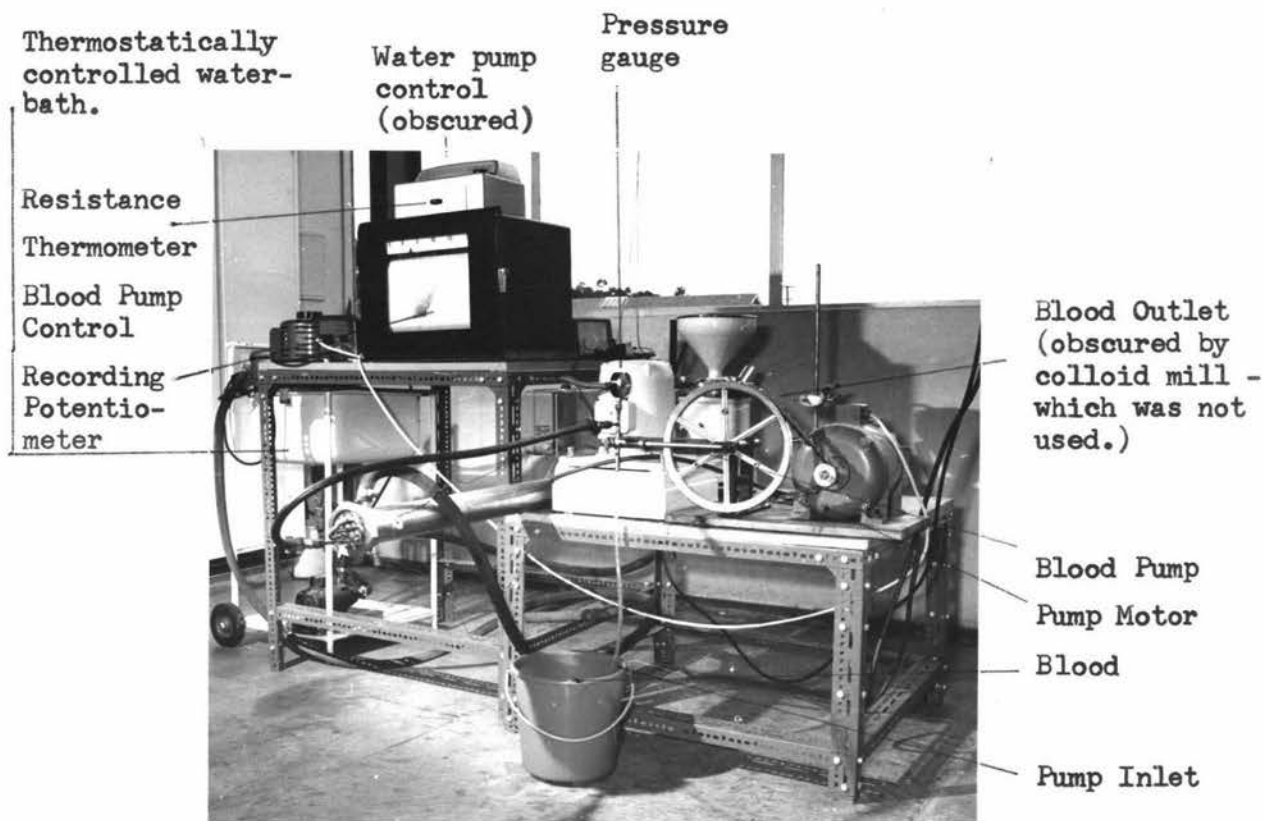
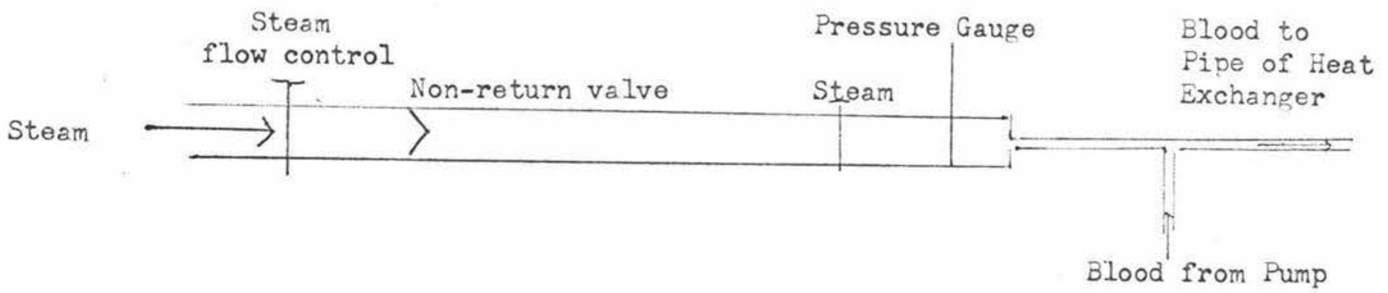


FIGURE III.8 DIAGRAM OF STEAM INJECTION MODIFICATION TO INLET PIPE OF HEAT EXCHANGER.



into the waterbath, but the 2-3°C difference between the bath temperature and the blood outlet temperature still existed and the latter could only be raised to 96°C.

Passing steam directly through the jacket of the heat exchanger enabled outlet blood temperatures of 98-102°C to be obtained.

Injection of steam at 128°($\pm$2°C) into the blood enabled a temperature of 113($\pm$5°C) to be reached.

The temperature of the waterbath, or inflowing steam, was measured with a resistance probe connected to a spot galvanometer calibrated in °F. This reading was checked with an accurate mercury in glass thermometer.

The blood temperatures were measured by thermocouples (36 gauge) inserted into the heat exchanger tubes through glands in the end-plates, and recorded on a multi-point recording potentiometer. The blood outlet temperature was measured with a resistance probe. Unfortunately the thermocouples in the heat exchanger appeared to initiate blockage of the small bore pipe when steam injection was used and, therefore, the temperature history in the tube during steam injection was determined using water instead of blood. Comparison of the heating patterns in the heat exchanger with steam injection, steam in the jacket and water in the jacket is shown in Figure III.9.

(ii) Flow Rate of Blood. The flow rate of the blood through the heat coagulation apparatus was controlled by a transistorised input control on the 3/4 horsepower D.C.motor driving the piston pump. This gave fine control and positive displacement at all speeds and pressures. Generally this pump could be operated at pressures up to 500 psi; pressures above this value indicated excessive build up of blood on the walls of the pipe which resulted in blockage.

The motor speed (revolutions per minute) could be determined during the experiments using a tachometer, and could be correlated with the flow rate of the blood and the residence time of the blood in the apparatus (Figure III.10).

FIGURE III.9 COMPARISON OF TEMPERATURE CHANGES IN BLOOD AT VARIOUS POSITIONS IN HEAT EXCHANGER DURING PILOT PLANT EXPERIMENTS.

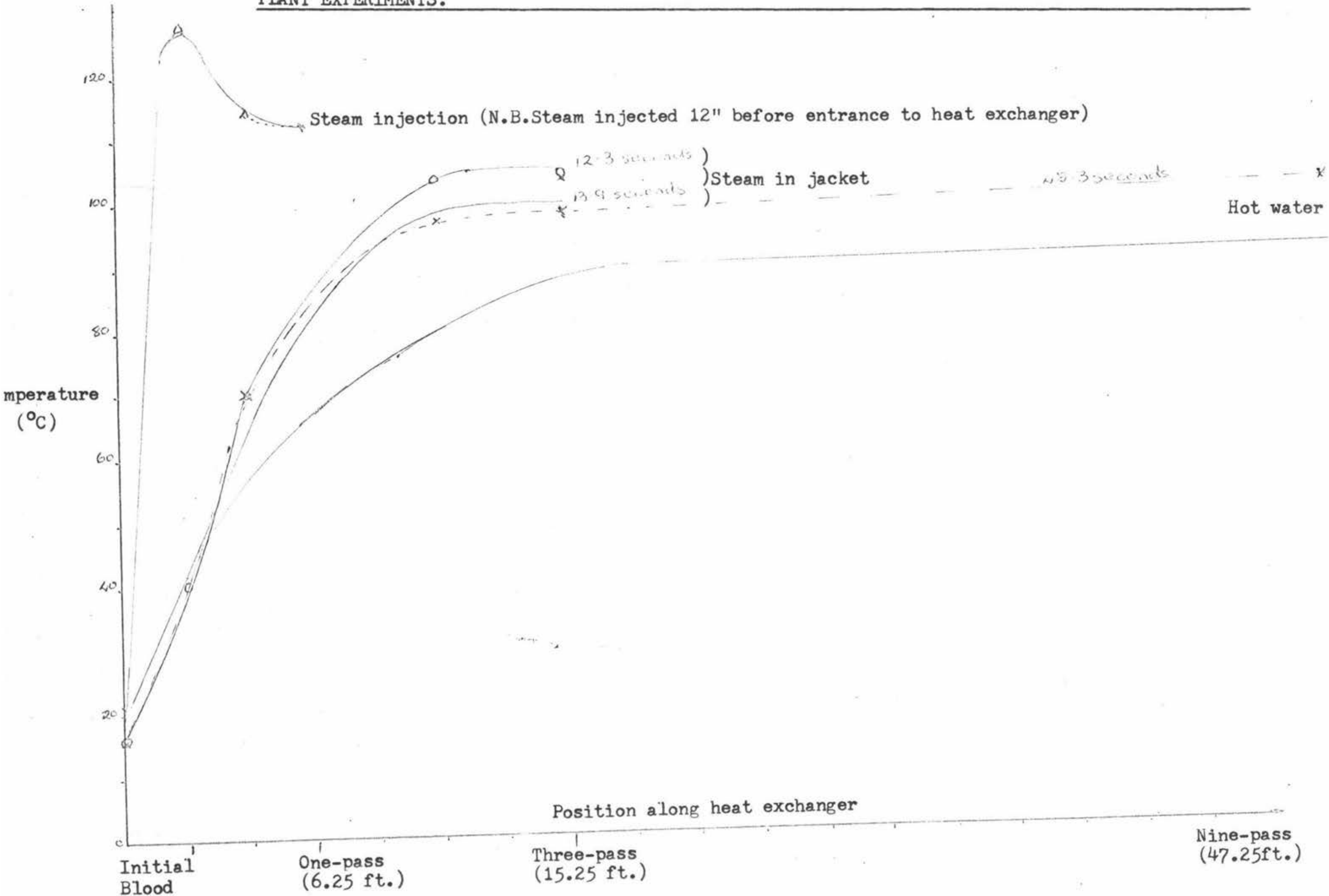
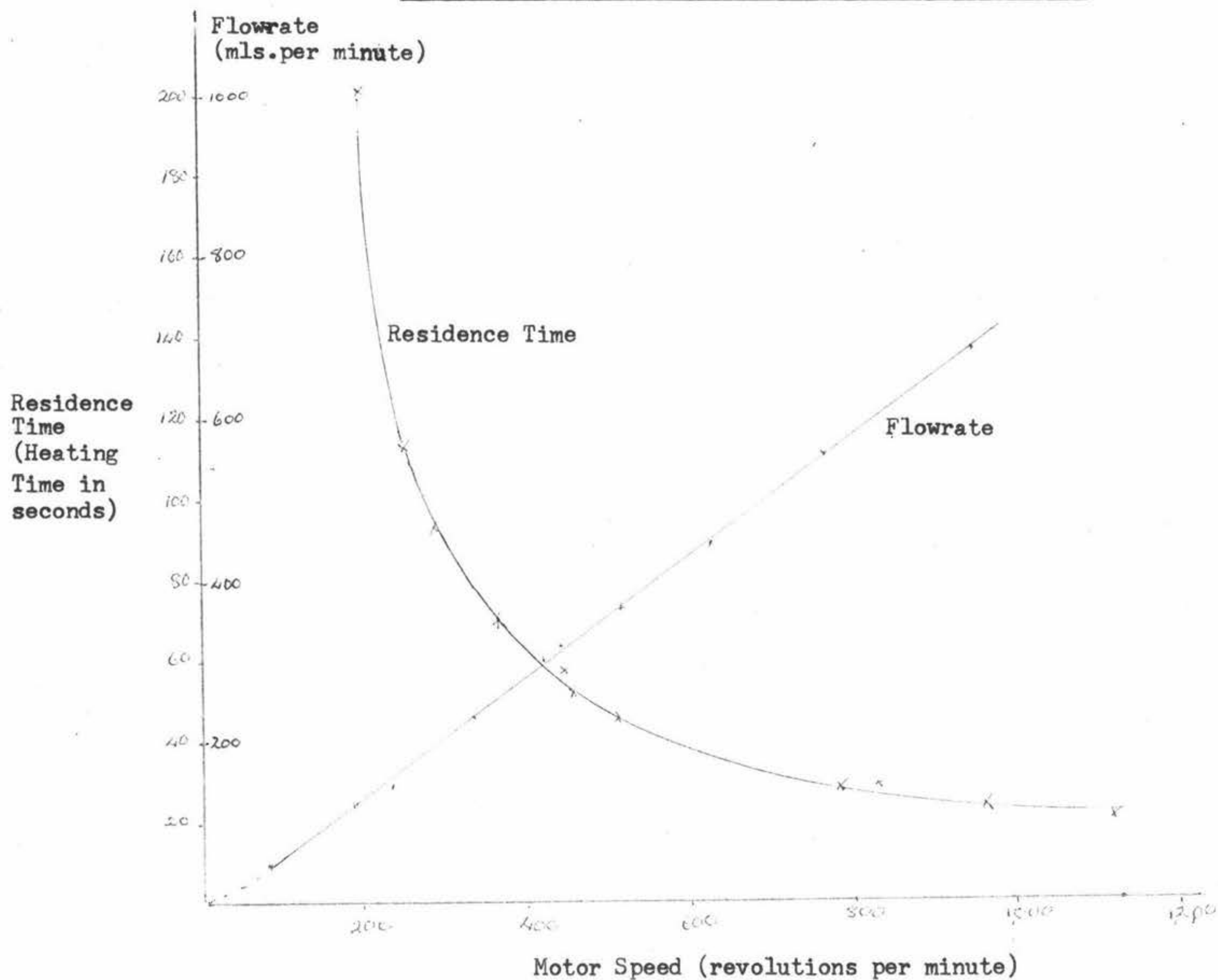


FIGURE III.10 CORRELATION OF FLOWRATE AND RESIDENCE TIME WITH THE MOTOR SPEED.



B. Results

Laboratory Experiments

1. Effect of Heat on Blood.

a. Optical Properties.

(i) Turbidity. The changes in the blood on heating were determined by two methods : light reflectance and light absorbance. The results obtained by these two methods are discussed separately below.

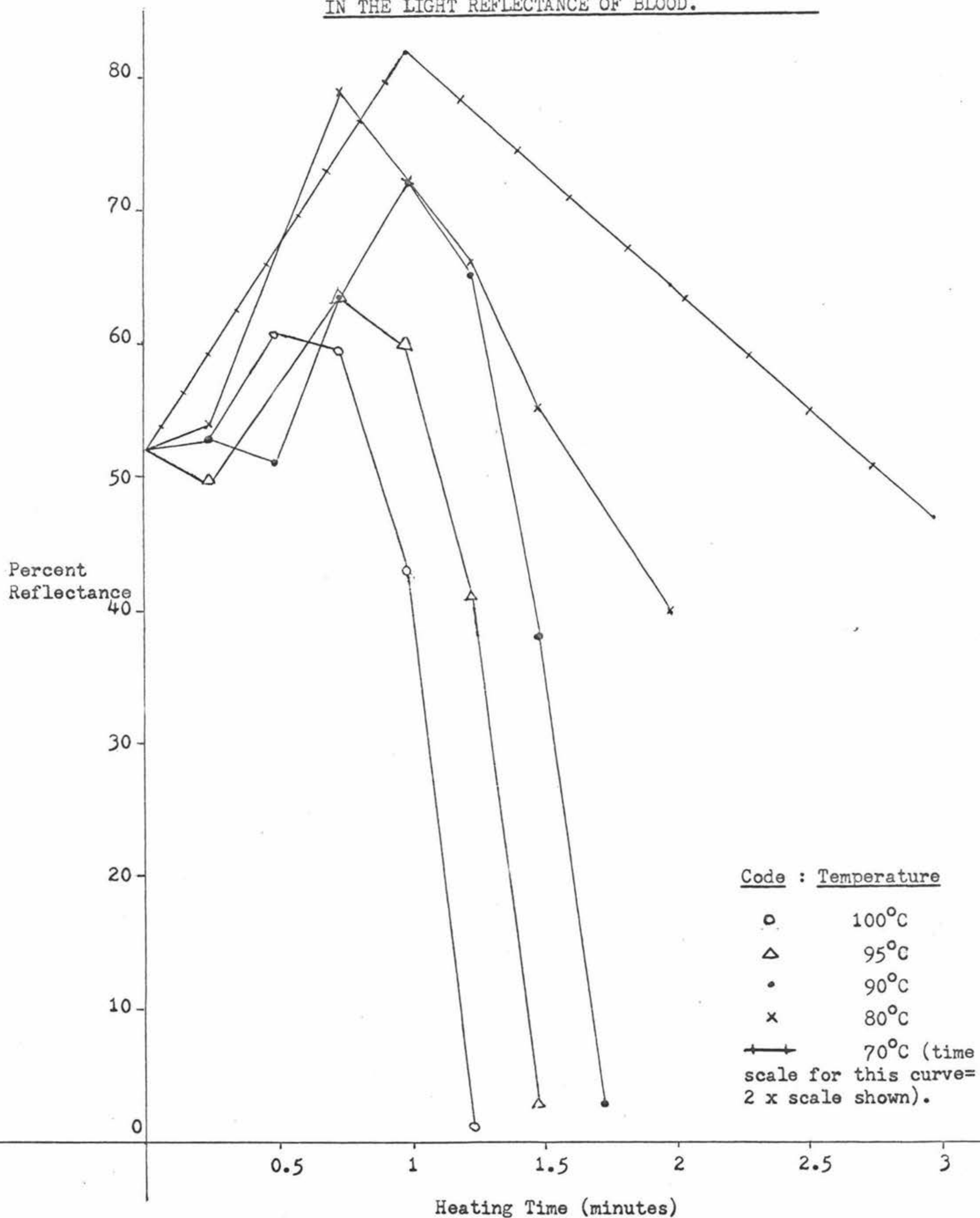
Reflectance: The light reflectance values obtained were found to increase during the initial stages of heating and then decline rapidly. This trend can be noted in the curves plotted in Figure III.11. The maximum reflectance values were attained within much shorter heating periods at higher temperatures. At temperatures above 80°C, the maximum was reached within the first minute of heating, although at 70°C it was not reached until after 2 minutes of heating.

There was a slight delay before the reflectance values began to increase and a slight decrease in these values was often noticed within the first minute of heating. This delay was more noticeable at the higher temperatures (i.e. above 80°C), where there was a more rapid increase in the reflectance value.

The height of the peak (i.e. maximum value of the reflectance) appeared to be influenced by the temperature, the highest peaks being produced by heating at 75°C and decreasing at both higher and lower temperatures.

The rate of decrease of the reflectance values after the maximum reflectance had been reached was greater at higher temperatures. Heating temperatures of 90 to 100°C resulted in extremely low reflectance values (below 5 percent) within one and three quarter minutes. At 60, 70 and 80°C, however, this decline was much slower, reflectance values below five percent being attained after 80, 8 and 2½ minutes respectively.

FIGURE III.11 EFFECT OF TEMPERATURE ON HEAT-INDUCED CHANGES IN THE LIGHT REFLECTANCE OF BLOOD.



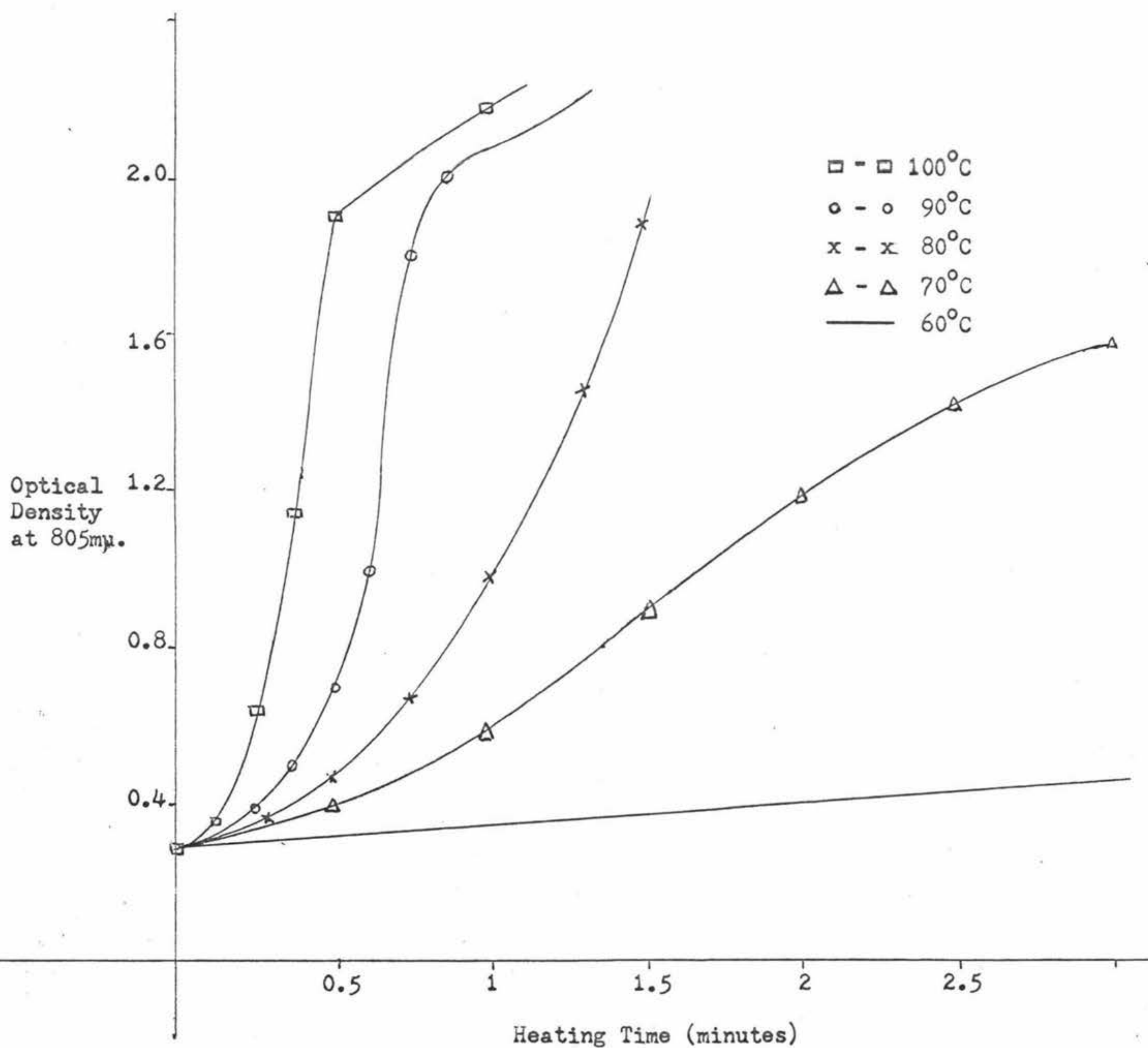
Absorbance at 805mu : The light absorbance of blood solutions at 805mu was increased by heating. There appeared to be two stages in the optical density versus time curves - an initial stage in which there was no marked change in the light absorbance of the solutions, and a succeeding stage in which the light absorbance increased extremely rapidly. As can be seen in Figure III.12, at higher heating temperatures the initial stage was shortened and the rate of increase of the light absorbance values in the second stage was much greater.

These turbidity changes may be related to the changes in the sizes and shapes of the protein molecules as they become denatured. On heating, the hydration of the protein molecules increases and the weaker bonding between different portions of the molecule are broken allowing the molecules to unfold. The unfolded molecules then aggregate.

This aggregation would result in the formation of light-absorbing particles which would increase in size and ultimately precipitate out of solution. The formation of an increasing number and/or size of light absorbing particles could be assumed to be directly related to the light absorbance values of the solution.

The reflectance values of the heated solutions were not directly related to the formation of particles as the values decreased after an initial increase as noted above. There was, however, some relationship between the reflectance and absorbance values, as is indicated by the graph in which reflectance values were plotted against the light absorbance for the same heated samples (Figure III.13). From this graph, it can be noted that there is a direct linear relationship between the reflectance and absorbance (805mu) values when the light absorbance is below 0.5. Above this value however, the correlation is in the opposite direction; that is, the reflectance decreased with increasing absorbance and the correlation curve varied with the heating temperature.

FIGURE III.12 EFFECT OF TEMPERATURE ON HEAT-INDUCED CHANGES IN OPTICAL DENSITY AT 805 mu.



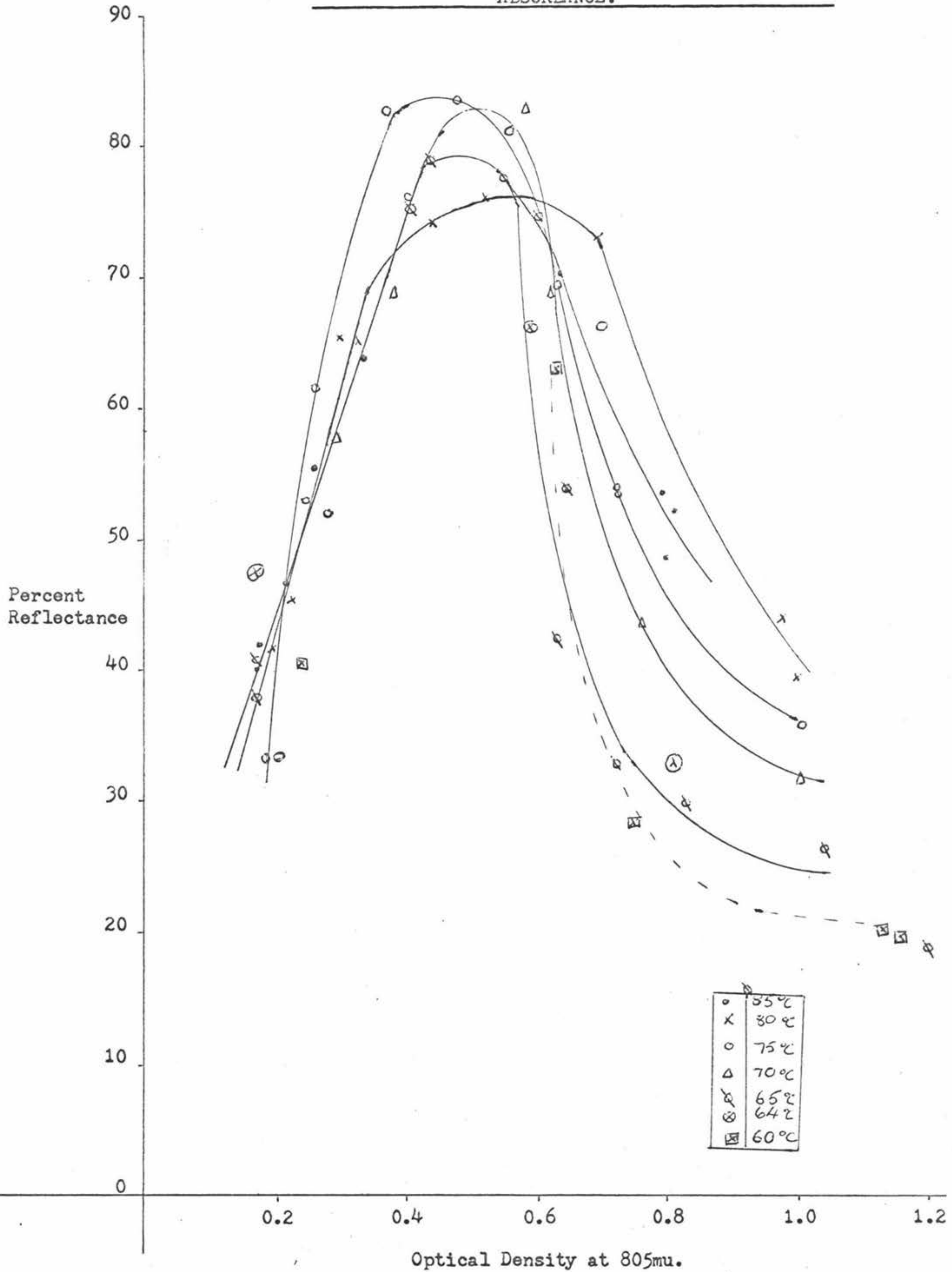


FIGURE III.13 RELATIONSHIP BETWEEN LIGHT REFLECTANCE AND LIGHT ABSORBANCE.

Therefore the initial stage in the reflectance curve is probably due to the increased formation of particles which both reflect and absorb light. The decline of the reflectance values in the second stage is probably due to the increase in the number and size of particles above the point at which maximum surface area is exposed and maximum reflectance values are attained, resulting in further increases in light absorbance but decreasing the amount of light reflected.

(ii) Changes in the Absorption Spectra. No changes in the positions of the peaks in the absorption spectra were observed on heating. However, the peak at 275mu, which was shown to be directly proportional to the protein concentration (Section A e.), gradually diminished in the spectra of the supernatants from heated blood (Figure III.14). This indicated that the protein was being precipitated from the solution.

The ratio of the absorbance at a maximum of one of the haeme peaks to the absorbance at 275mu would indicate the ratio of haemoglobin to total protein in the sample. There was no change in this ratio in the supernatants or precipitates up to 2 minutes' heating at 90°C, indicating that haemoglobin was being precipitated with other proteins. However, after 3 minutes, the haeme to total protein ratio in the supernatant increased indicating that haemoglobin was remaining in solution, while other proteins were precipitating out. The spectra of the dissolved precipitates did not show any marked change with time of heating on the haeme : total protein ratio. However, this ratio was lower than that found in the supernatant, indicating that relatively less haemoglobin than plasma proteins was precipitated.

b. Protein.

Heating blood resulted in the precipitation of the protein from solution. When the protein remaining in the supernatant was determined spectrophotometrically

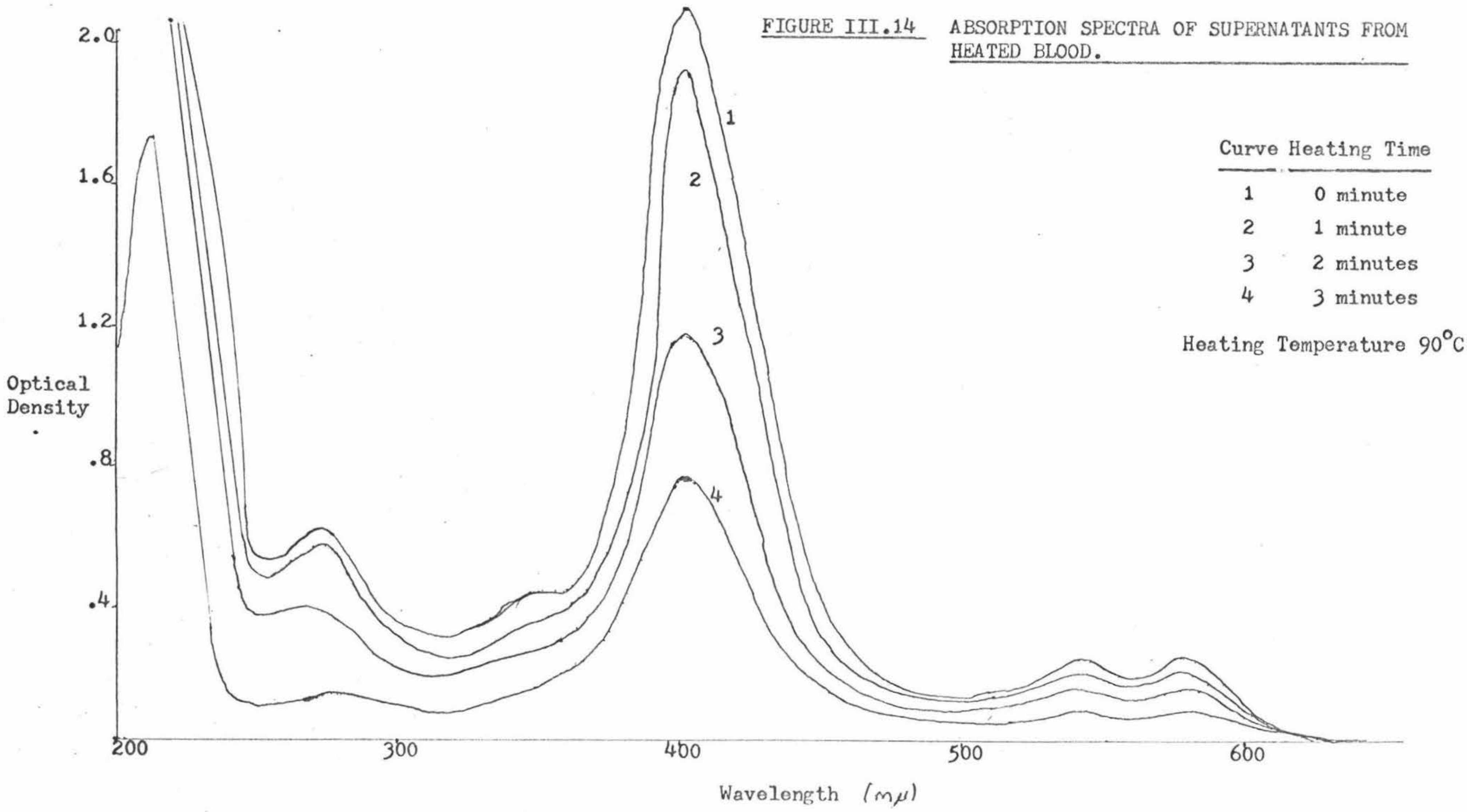


FIGURE III.14 ABSORPTION SPECTRA OF SUPERNATANTS FROM HEATED BLOOD.

at 280mu, there appeared to be three stages in the disappearance of protein from the supernatant on heating :

1st Stage : very little change observed in the protein content.

2nd Stage : relatively rapid decline in protein.

3rd Stage : decrease in rate of disappearance of protein.

The effect of temperature on the protein content of the supernatant is shown in Figure III.15.

The first stage was shorter at higher temperatures, the second stage starting after :

3/4 minutes at 100°C,

1 minute at 95°C,

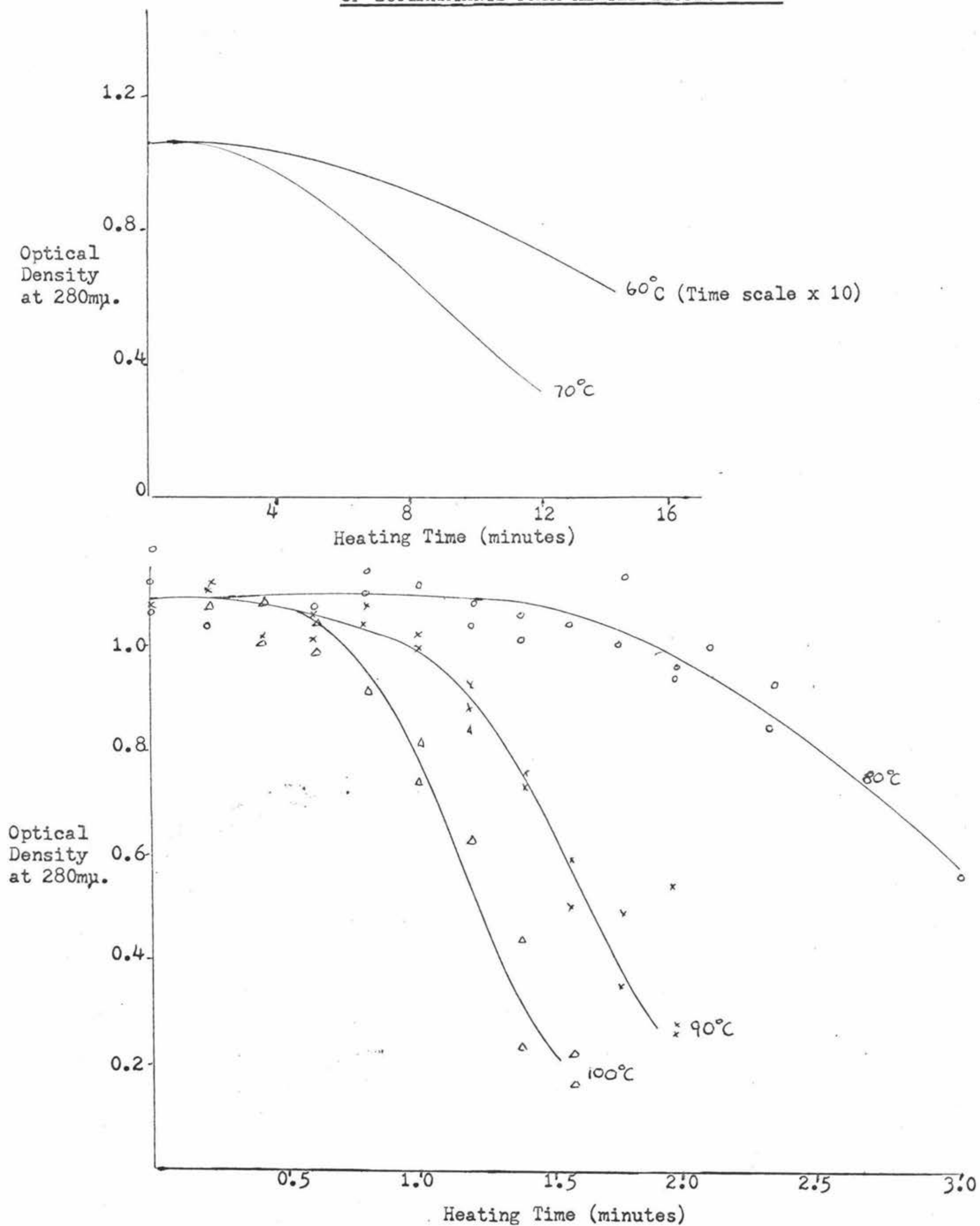
$1\frac{1}{4}$ minutes at 90°C,

$2\frac{1}{4}$ minutes at 80°C.

In most cases, the third stage in the 280mu versus time curve was not detected, as the samples had solidified to such an extent that dilution was difficult before this stage was reached. In the few samples in which the supernatant protein versus heating time curve did level off, it was noticed that the value to which the curve declined depended on the heating temperature. At the higher temperatures, the amount of protein which remained in the supernatant after the rapid loss of supernatant protein was less than at 60 to 80°C.

On heating at 100°C, there was a rapid loss of protein in the supernatant up to 2 minutes, when a minimum value of approximately 0.15 (optical density at 280mu) was reached. This corresponded to 1.8 percent protein in the undiluted supernatant. At 90°C, the rapid decrease in supernatant protein was completed after 3 minutes' heating time and the minimum value obtained corresponded to 2.4 percent protein in the supernatant. The rapid decline in protein content was

FIGURE III.15 EFFECT OF TEMPERATURE ON PROTEIN CONTENT OF SUPERNATANTS FROM HEATED BLOOD.



observed after $3\frac{1}{2}$ minutes at 80°C, but the value at which the protein-heating time curve levelled off was not reached. At 60 and 70°C, the decrease in protein content was much slower and more gradual than at the higher temperatures.

c. <u>Formation of Precipitate</u>.

When blood was heated a precipitate was formed. The amount of precipitate formed was influenced by the heating temperature and time. Typical results which indicate this are shown in Figure III.16.

A small amount of precipitate was found in the unheated samples. A portion of this precipitate, 0.005 to 0.01 grams per ml., could be attributed to the cell stroma, which would precipitate after dilution of the blood had caused haemolysis.

The remainder of the precipitate, 0.010 - 0.015 grams per ml., was assumed to have been precipitated by the aging of the samples which occurred during the analysis. This was inherent in the procedure, the precipitates not being separated from their supernatants until the blood was 2 days' old. (During this period the blood samples were held at 4°C for at least 20 hours and the remaining period at room temperature (16°C)). The increased amount of precipitate found in the heated samples was much greater than this initial precipitate.

The precipitate formation during heating occurred in three stages which corresponded to those observed in the protein content of the supernatant.

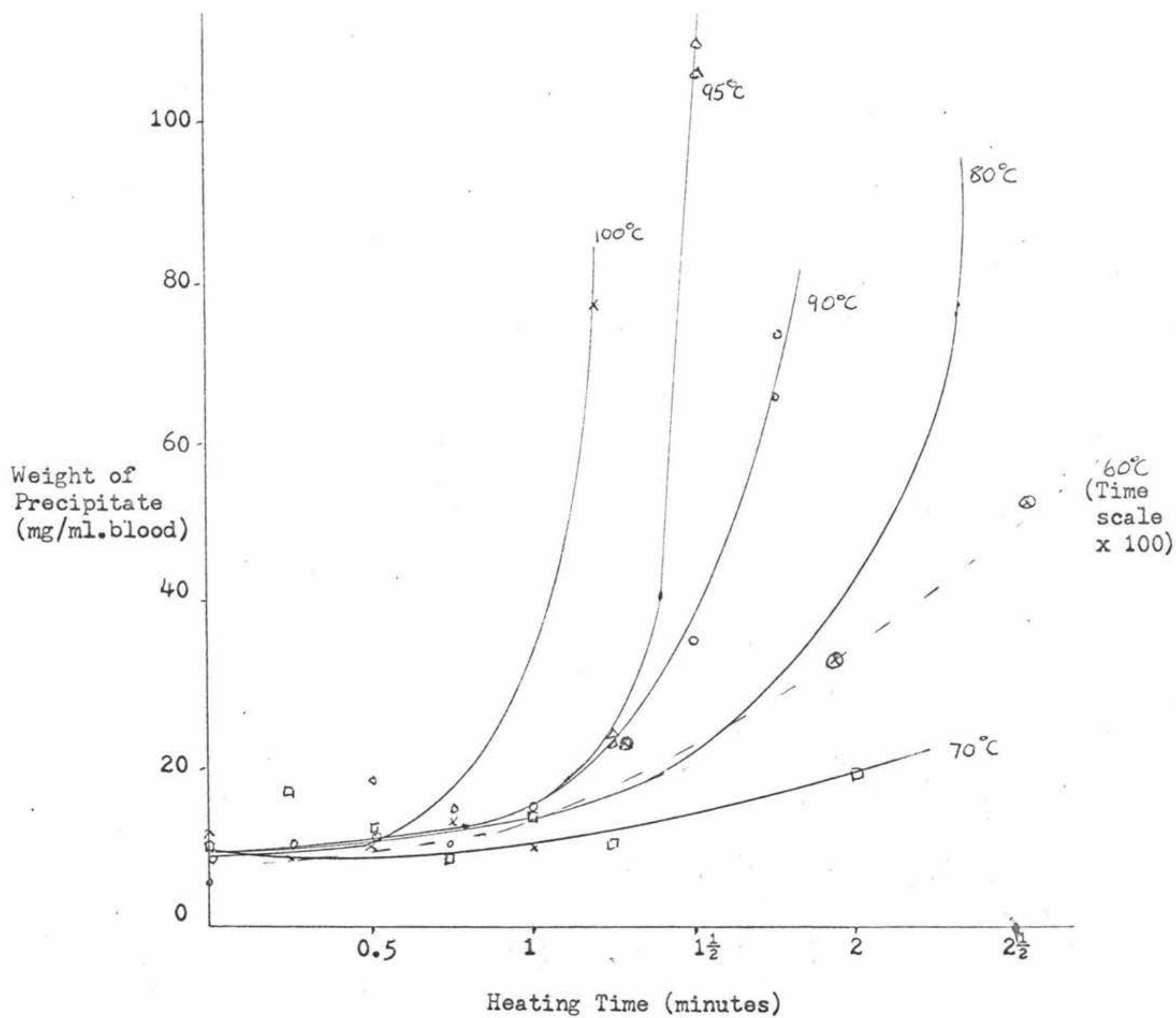
1st Stage : no change in amount of precipitate.

2nd Stage : increase in the amount of precipitate - this increase occurred very rapidly at heating temperatures above 90°C.

3rd Stage : decline in rate of precipitate formation.

As noted in the results of the supernatant protein, the third stage was not observed in many samples as the samples at this stage were too difficult to dilute. After 2 minutes at 100°C, the precipitate weight versus heating time

FIGURE III.16 EFFECT OF TEMPERATURE ON AMOUNT OF PRECIPITATE FORMED DURING HEATING.



curve was beginning to level off as is indicated in Figure III.17. The amount of precipitate found in blood samples which had been heated at 100°C for just over 2 minutes was found to be approximately 0.2 grams per ml. of blood, which is the approximate total solids of blood and, therefore, it could be assumed that these samples had almost undergone complete coagulation.

Variations of one or two seconds in the heating process would have caused quite a large difference in the amount of precipitate obtained during the rapid stage of precipitation. This was probably the reason why results obtained in different experiments often did not duplicate exactly. However, it was noted that about 0.08 g./ml. of blood were precipitated by heating for 1 minute at 100°C, 1½ minutes at 90°C, 2 minutes at 80°C, 6 minutes at 70°C and 3½ hours at 60°C. Approximately 0.18 g./ml. of blood were precipitated after 2 minutes at 100°C, 2½ minutes at 90°C, 2½-3 minutes at 80°C and 8 minutes at 70°C.

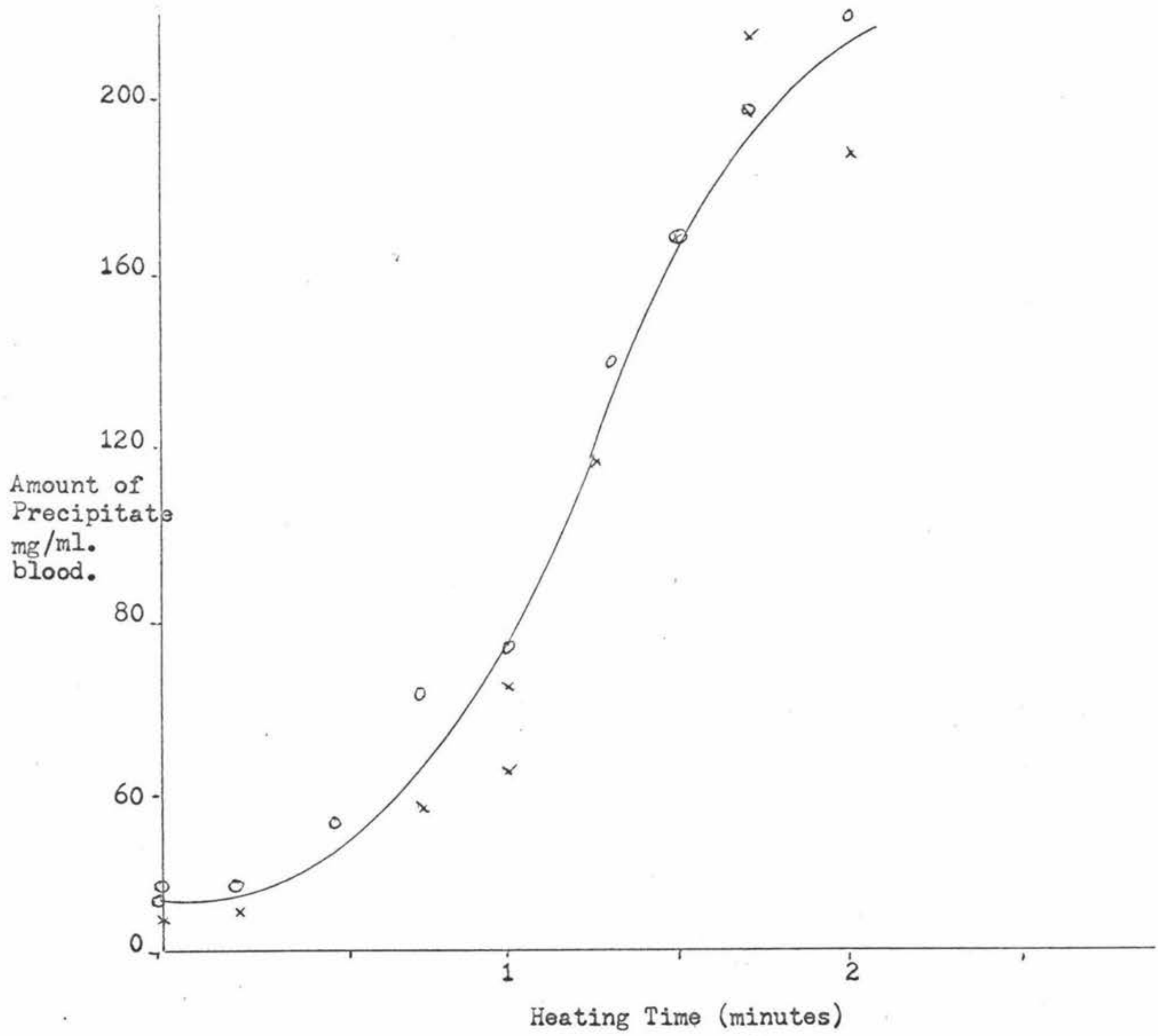
d. Electrophoresis.

Electrophoretic patterns of blood did not show all of the detail of the plasma proteins because of the small amount of sample used (2.5 microlitres). If larger sample quantities (5 microlitres) were used, a wide diffuse haemoglobin band covered most of the gel.

No change in the electrophoretic pattern of the soluble protein was observed after heating at 80°C for 2 minutes. For electrophoresis, the precipitated protein had been removed by centrifuging the heated blood and the electrophoretic sample taken from the supernatant.

In an experiment in which blood was heated at 90°C for up to 3 minutes, the supernatant and the precipitate from the samples were separated and each analysed electrophoretically. After one minute at 90°C, the soluble blood protein pattern had changed; the detail in the alpha-2 globulin area becoming fainter and the haemoglobin and albumin bands becoming narrower, which indicated that proteins in all of these regions had been altered, and probably precipitated

FIGURE III.17 PRECIPITATE FORMATION WHEN BLOOD IS HEATED AT 100°C.



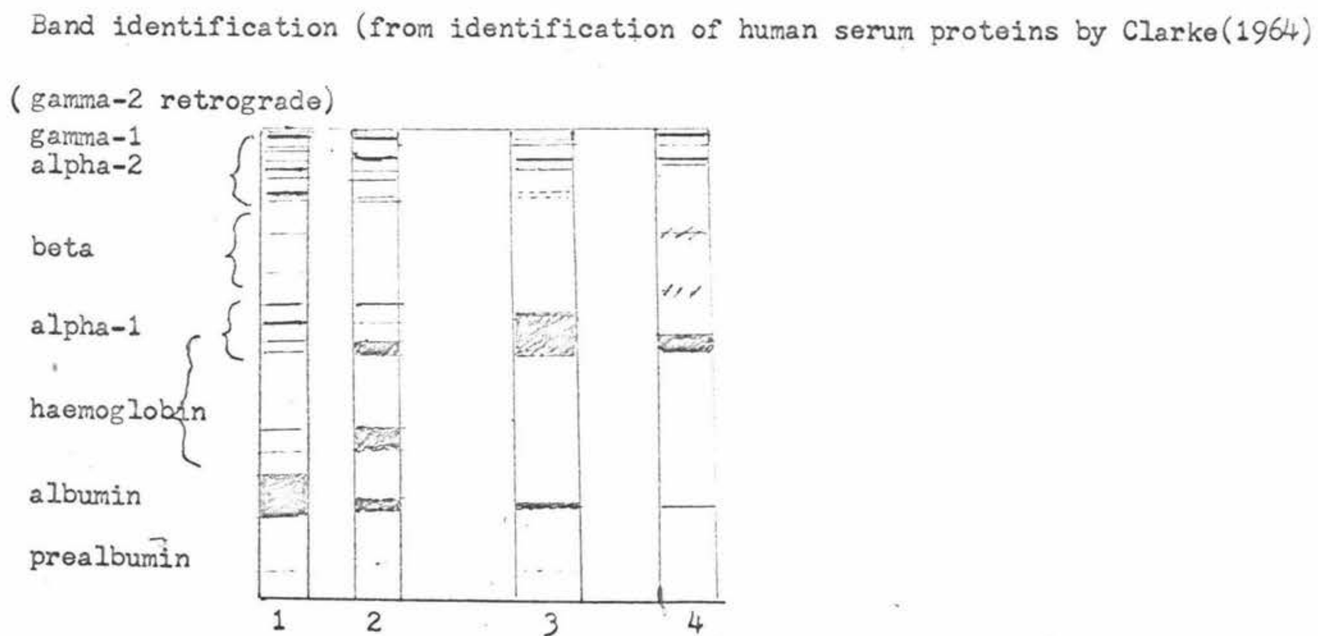
by heat treatment. The faint prealbumin band did not appear to alter even after 3 minutes at 90°C. After 2 minutes at 90°C, the albumin band was only just recognisable and only one of the bands in the alpha-2 globulin area appeared on the gel; the haemoglobin band decreased but remained the only distinct band on the gel. After 3 minutes at 90°C, traces of albumin were still discernible, and the one remaining band in the alpha-2 globulin area was less distinct. The width of the haemoglobin band appeared to remain fairly constant during the third minute at 90°C. After the third minute at 90°C, the albumin peak and the prealbumin appeared to be closer together on the gel. Also after the third minute at 90°C, two very faint bands appeared in the alpha-2 area.

The dissolved precipitates from the samples contained prealbumin, albumin, haemoglobin and four alpha-2 globulins and gamma-1 globulins. After 3 minutes' heating, there was no apparent change in the nature or relative concentrations of these proteins in the precipitate. Line drawings of blood, plasma, and supernatant and precipitates from heated blood are shown in Figure III.18. Only one haemoglobin band was observed in the sheep blood sample used in this experiment, although two bands had previously been observed in a cattle blood as shown in Figure III.18. This difference may have been due to the difference in species.

e. General Discussion.

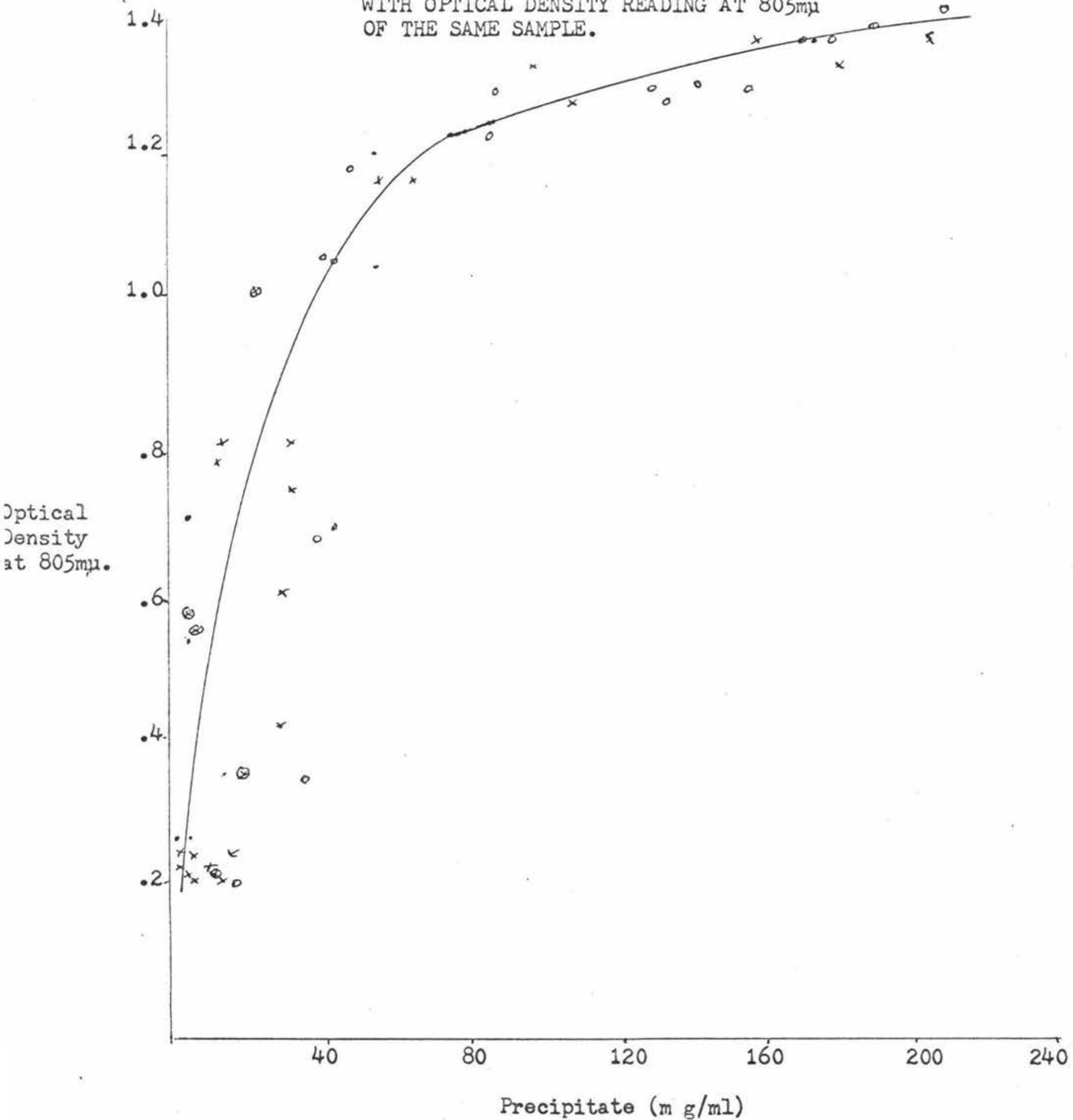
As previously noted, the changes in the turbidity of the blood on heating are related to the formation of particles which increase and ultimately precipitate from the solution. The changes in the turbidity values obtained in these experiments were due to the initial formation and aggregation of particles before much precipitation had occurred. This was deduced from the observation that only a small amount of precipitation had taken place in samples which had absorbance values below 0.8 (Figure III.19).

FIGURE III.18 LINE DRAWINGS OF GEL PATTERNS OF BLOOD, PLASMA, AND SUPERNATANT AND PRECIPITATES FROM HEATED BLOOD OBTAINED FROM DISC ELECTROPHORESIS GELS.



1. Bovine plasma.
2. Bovine blood.
3. Supernatant from bovine blood heated at 90°C for 2 minutes.
4. Redissolved precipitate from bovine blood heated at 90°C. for 2 minutes.

FIGURE III.19 CORRELATION OF AMOUNT OF PRECIPITATE IN HEATED BLOOD WITH OPTICAL DENSITY READING AT 805mμ OF THE SAME SAMPLE.



The stage at which the precipitation of most of the blood protein occurred had a marked effect on the protein content of the supernatant and the precipitate weights. The precipitate weights increased rapidly and the supernatant protein decreased rapidly. As would be expected, the protein in the supernatant decreased as the precipitate increased (Figure III.20).

In order to confirm that there was formation and aggregation of particles, blood heated at 100°C was examined microscopically. In the initial stages of heating, an increase in the number of particles in the solution could be easily observed. When these particles were inspected under magnification, they appeared to be very irregularly shaped and there was quite a large variation in particle size within each sample. In order to ascertain whether the size of these particles was dependent upon the heating time, the average particle size was determined on precipitates of blood samples that had been heated at 100°C for varying lengths of time. The shape of the particles was very irregular - varying from elongated, rod-like particles, to rough-edged spheres. Two dimensions of each particle were measured : the longest dimension and the dimension perpendicular to it at the centre of the length. The product of the two dimensions was calculated and considered to be the "area" of the particle and, therefore, an indication of the particle size. The average particle areas obtained by averaging the areas calculated from the dimensions of twelve particles in each sample are presented in Table III.1.(The particles measured were selected by choosing one particle for measurement, then moving the stage in one direction until six particles had been located and measured, then moving the stage in a direction perpendicular to that used previously until a total of twelve particles had been measured).

FIGURE III.20 CORRELATION OF AMOUNT OF PRECIPITATE IN HEATED BLOOD WITH PROTEIN IN THE SUPERNATANT OF THE SAME SAMPLE.

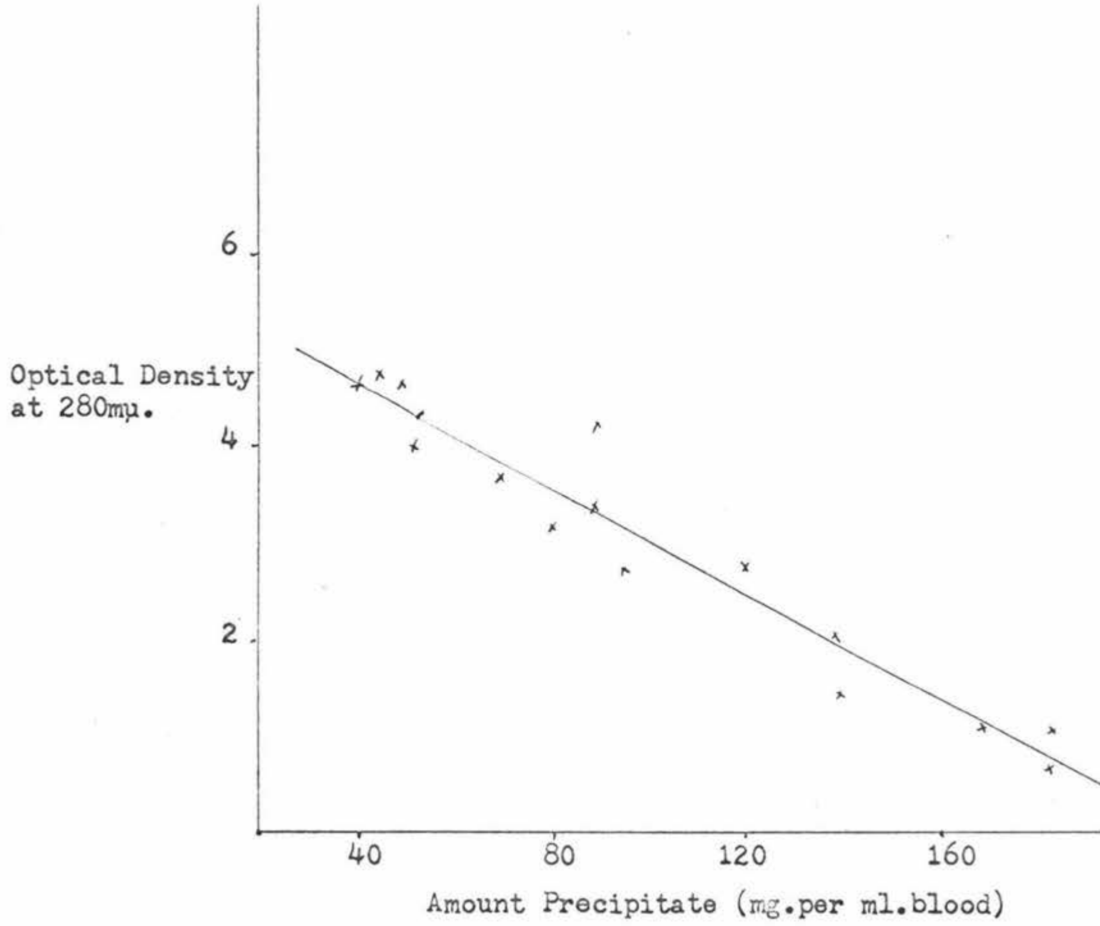


TABLE III.1 EFFECT OF HEATING TIME ON PARTICLE SIZE

Heating Time at 100°C (minutes)	Average Particle "Area" ($mm^2 x 10^{-6}$)
0	0
0.25	16
0.5	67
0.75	90
1.0	380
1.25	360
1.5	2360
1.75	1960
2.0	3750

There was a large variation of particle size within each sample, which is indicated by the fact that the standard deviations on the average particle "areas" presented in Table III.1. were equivalent to 20-25 percent of the average value. Despite this wide variation within each sample, the average particle "areas" showed that there was a definite increase in particle size as heating at 100°C continued.

The changes in the absorption spectra of the supernatants and precipitates of blood after heat treatment at 90°C for 0 to 3 minutes indicated the plasma proteins and the haemoglobin were precipitated from solution simultaneously. The electrophoretic results indicated this in more detail, and showed that some of the plasma proteins had been completely precipitated from solutions at an earlier stage in the heating process than other plasma proteins. Whether partial precipitation of all the proteins in the blood had occurred was not known, although it could be assumed that partial coagulation of albumin and haemoglobin had occurred as the amounts of these proteins declined during the heating process. The decrease in haemoglobin was observed in both the electrophoretic and absorption spectrophotometry results.

From these results it was concluded that the heat coagulation of blood occurred in several stages.

1. An initial "delay" period during which the temperature of the blood increased into the temperature range at which thermal coagulation took place. This stage was very short at temperatures above 80°C (less than 30 seconds). This stage was indicated by the lack of change (or occasional decrease) in the percent reflectance values and also the absence of change in the absorbance values.

2. The formation of small particles then occurred. This stage was represented by the initial increase observed in the reflectance values which coincided with an increase in the absorbance at 805mu up to 0.5. Until the absorbance values of the sample were above 0.8, only slight precipitation occurred (Figure III.19).

   It can be noted (Figure III.13) that the temperature of heat coagulation did not have any marked influence on the turbidity changes which occurred during this stage. This possibly indicated that this stage represented a rapid increase in the number of particles in solution and only small increases in the particle size.

3. A rapid increase in size and probably number of particles in the heated solution took place, resulting in the rapid increase of the optical density at 805mu and a rapid decrease in the percent reflectance values. The temperature of heat coagulation did influence this stage (Figure III.13) and it appeared that particles produced at higher temperatures had greater reflectance values than particles which had the same optical density but which had been produced at lower temperatures. This probably indicated that larger particles were formed at higher temperatures. During this process there was a rapid increase in precipitate formation and consequent decrease in the amount of soluble protein.

4. During this stage, the rate of precipitation of the protein declined until all the protein was precipitated. This stage was not shown in many of the experiments.

2. Influence of Species on the Heat Coagulation of Blood.

Because of the variability in the animals being killed at New Zealand meat-works, any study on the effect of species would have to be an extensive statistical experiment involving animals from different parts of New Zealand killed throughout the year. It was not possible to do this because it would have involved many thousands of analytical determinations. However, the results from two experiments in which blood from both cattle and sheep were heated and analysed could be used for comparison of these species. Figure III.21. shows the results obtained at a heating temperature of 90°C, on bloods from a Friesian bull and a hogget killed in early June. Similar results were obtained in the second experiment in July, in which blood from two bobby calves (pooled) was compared with blood from a hogget. As can be noted from Figure III.21, although slight variation in actual values occurred, the distinct changes in these properties occurred at approximately the same time in both species. A comparison of the times at which these changes occurred at the different temperatures used is presented in Table III.2. These figures do not indicate any definite species differences. The variation between these results was similar to the variation of results from the same species found in other experiments and so it was concluded that there was no difference between the heat coagulation of sheep and cattle bloods .

TABLE III.2 INFLUENCE OF SPECIES ON CHANGES CAUSED BY HEAT COAGULATION.

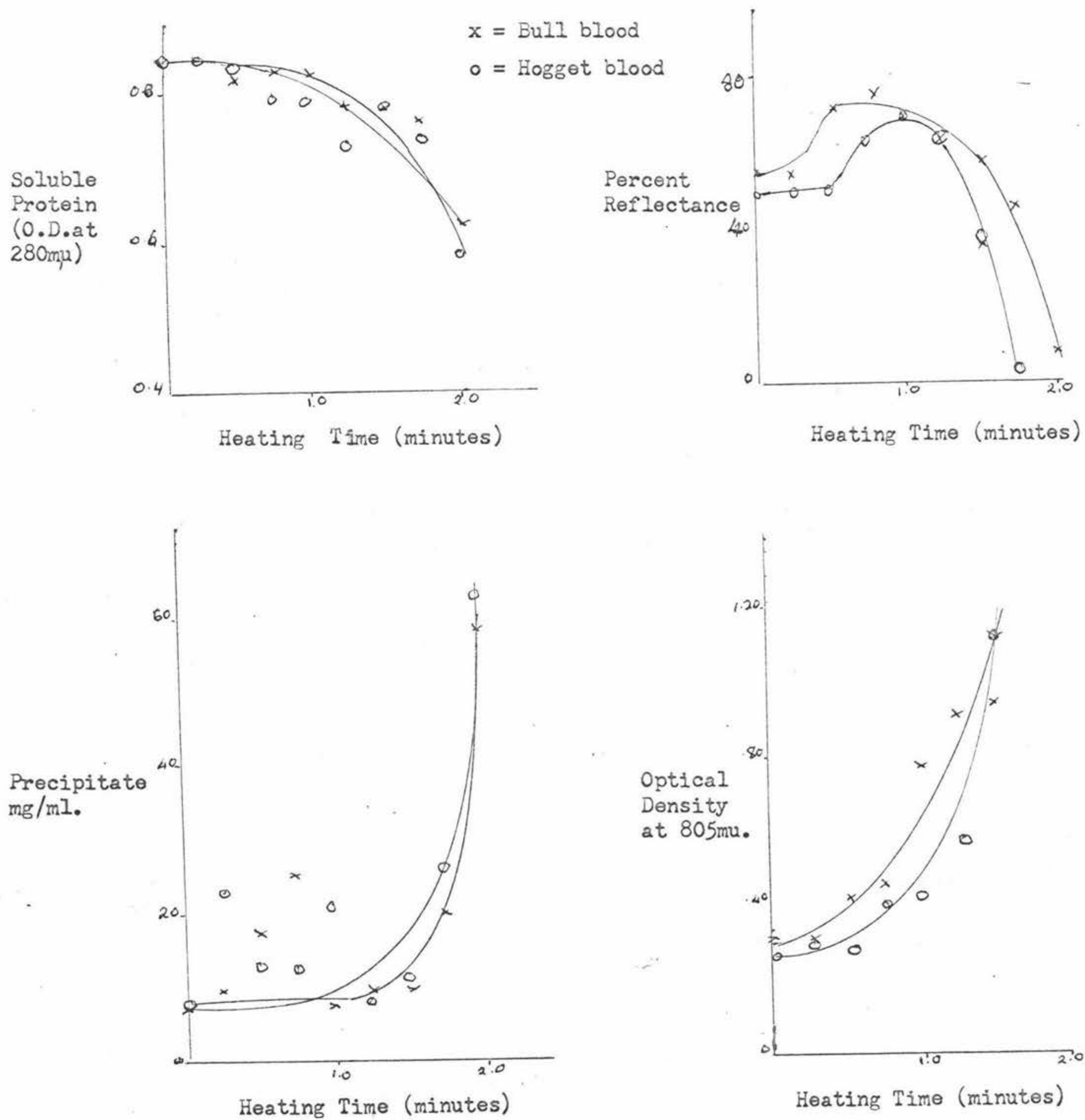
Heating Time at which specified value or change was observed (minutes)

Property		Maximum Percentage Reflectance		Optical Density at 805mu=1.0		Decrease in Soluble Protein		Increase in Precipitate		Precipitate = 0.1 g/ml.	
Temperature	Experiment*	Cattle	Sheep	Cattle	Sheep	Cattle	Sheep	Cattle	Sheep	Cattle	Sheep
70	a	4	4	16	15	12	15	12	14	18	6
	b	-	4	7	6	9	8	8	7	9	9
80	a	1.0	0.75	-	2.0	2.75	-	2.75	-	-	-
	b	0.75	0.5	1.5	1.5	2.0	1.75	2.0	2.0	2.25	2.0
100	a	0.5	-	0.75	0.75	1.0	-	1.0	-	1.25	1.25
	b	0.75	0.5	1.5	1.25	1.25	1.25	1.25	-	-	-

* a Cattle = Friesian bull; Sheep = Hogget

b Cattle = 2 bobby calves; Sheep = Hogget

FIGURE III.21 CHANGES OBSERVED ON HEATING CATTLE AND SHEEP BLOOD AT 90oC.



3. Effect of Age of Animal on the Effect of Heat on Blood.

There appeared to be no definite difference between cattle of different ages on the turbidity values during heat coagulation. The only observable variation amongst the group of steers, cows and bulls tested was the blood from an old Friesian bull. This produced very high reflectance values and also had a maximum absorbance value of about 0.9 when heated at 80°C. This variation could have been due to the age of the animal. The precipitate with the old bull blood also formed more slowly on heating at 80°C as compared with cow and steer blood, only starting to increase rapidly after two and three quarter minutes instead of one to one and a half minutes.

Calf blood, which had been held overnight after heating and before dilution, showed a slightly faster increase in absorbance on heating at 80°C than cow blood which had undergone similar treatment. There was not, however, a significant difference in the rate of precipitation.

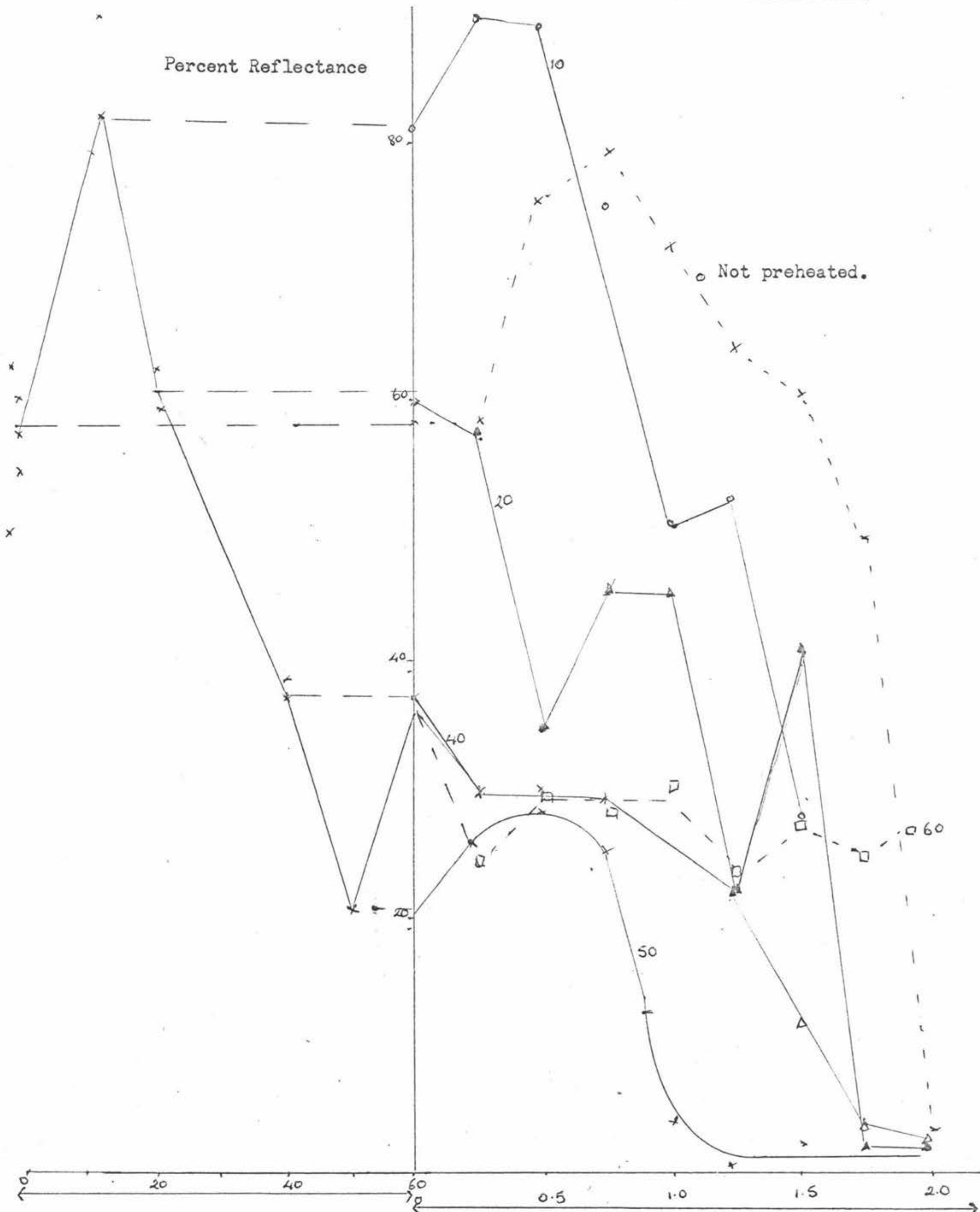
These results appeared to indicate that the age of the animal did not have a very significant effect on heat coagulation of blood, except with extreme differences in age - either very young or very old - which could cause increase in the rate of precipitation with very young animals and decrease in the rate of precipitation with very old animals. However many more tests would have to be done with a wide range of animals before this could be confirmed.

4. Effect of Preheating on the Heat Coagulation of Blood.

Preheating the blood at 60°C immediately before heating had quite a marked effect on the properties measured during coagulation. Preheating times of ten to sixty minutes were used.

After ten minutes at 60°C, the changes in percent reflectance were quite marked as can be seen in Figure III.22. The variation of the coagulation temperature also had a marked effect. At 90°C, the peak in the reflectance which was normally observed during heat coagulation and which was attained after ten minutes at 60°C remained for half a minute before the reflectance

FIGURE III.22 EFFECT OF PREHEATING ON CHANGES IN REFLECTANCE DURING HEAT COAGULATION.



Time of Preheating Temperature = 60°C.
(minutes)
Dotted line indicates the transfer of samples from preheating water-bath to heat coagulation bath.

Time of Heating Temperature = 90°C.
(minutes)

values decreased rapidly. At 95°C, however, no further increase in peak height was observed and the percent reflectance abruptly declined. Preheating for 10 minutes at 60°C, therefore, hastened the appearance of the maximum reflectance at 90°C and 95°C. (The maximum reflectance value was reached during the preheating period for the latter coagulation temperature).

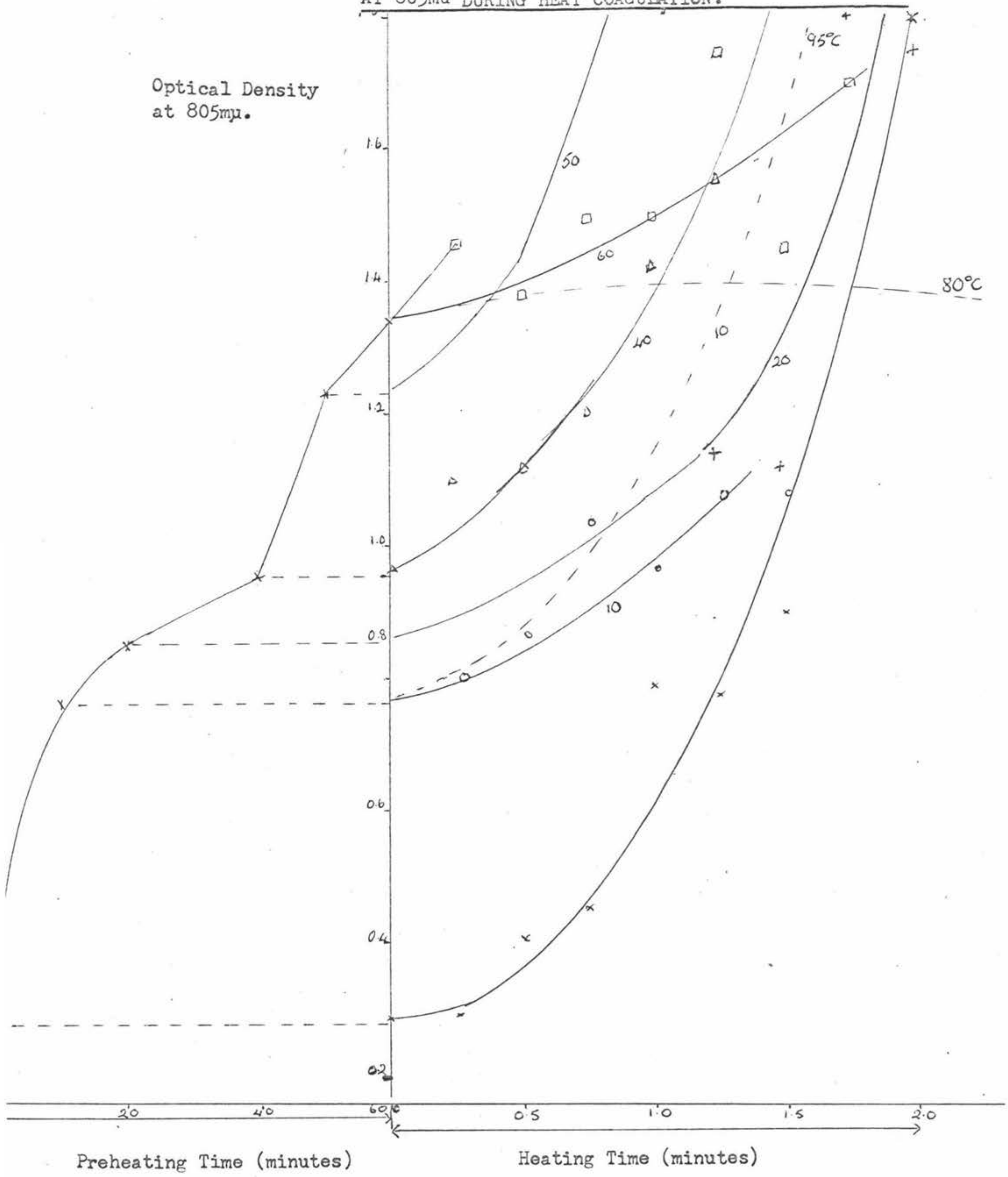
Preheating for longer times (20-50 minutes at 60°C) allowed the coagulation process to proceed further before the actual coagulation temperature was used. The succeeding heating then resulted in a decline in the percent reflectance at a much quicker rate than would have occurred without preheating.

After 60 minutes preheating, however, slight thermal resistance was indicated by the fact that heating at 80 or 90°C did not cause any further decrease in the percent reflectance value. Lower values had been obtained after on treatment following the shorter preheating times.

Preheating at 60°C eliminated the initial lag phase in the optical density at 805mu versus heating time curves, and therefore resulted in much higher optical density values of the heated samples. This effect is shown in Figure III. 23. The optical density continued to increase as the preheating time lengthened up to 50 minutes. The length of the preheating period merely influenced the OD_{805} value at commencement of heating. As the light absorbance at 805mu is due to the formation and aggregation of particles in the blood, this effect can be explained by the initiation of aggregation during the preheating process. After 60 minutes preheating, however, the particles appeared to acquire a slight resistance to the increase in aggregation on heating, and the rate of increase of OD_{805} declined markedly. When blood which had been preheated at 60°C for 60 minutes was heated at 80°C, no further increase in OD_{805} occurred, and after heating at 90°C only a slight increase was noted.

The effect of preheating at 60°C on the decrease in soluble protein and the amount of precipitate formed during heat coagulation also indicated that preheating periods up to 50 minutes enhanced the aggregation process, whereas

FIGURE III. 23 EFFECT OF PREHEATING ON CHANGES IN OPTICAL DENSITY AT 805mu DURING HEAT COAGULATION.



Temperature = 60°C. Broken lines in this portion of the graph are used to indicate the times at which preheated blood samples were transferred to a waterbath held at the heat coagulation temperature.

Temperature = 90°C for all solid line curves. Temperatures are noted on broken lines.

a sixty-minute preheating period inhibited this process. These results are presented in Table III.3.

TABLE III.3 EFFECT OF PREHEATING TIME ON THE CHANGES IN SOLUBLE PROTEIN AND PRECIPITATE FORMATION DURING HEAT COAGULATION.

Preheating temperature = 60°C.
Heat coagulation temperature = 90°C.

Preheating Time (minutes)	Heating Time after which a rapid decrease in soluble protein was observed. (minutes)	Heating Time after which a rapid increase in precipitate formation occurred. (minutes)
0	1.75	1.75
10	1.25	1.25
20	1.5	1.5
40	1.25	1.25
50	0.75	0.75
60	not observed in 2 minutes	-

It may be noted that ten-minute preheating at 60°C appeared to cause faster precipitation than a twenty-minute period.

5. The Effect of Aging on Heat Coagulation of Blood.

Aging of cattle blood at room temperature (16°C) for two days caused the changes during heat coagulation to occur at earlier times. The times taken to reach the maximum reflectance values and an optical density of 1.0 at 805mu during heat coagulation at 90°C are given in Table III.4.

TABLE III.4 EFFECT OF AGING ON HEATING TIME TO REACH SPECIFIC TURBIDITY LEVELS

(Time in Minutes)

Heating Temperature		60°	70°	80°	90°	100°
Time to reach Maximum OR2	Fresh	10	4.0	1.0	0.75	0.75
	Aged	10	2.0	1.0	0.5	0.25
Time to attain OD_{805}=1.0	Fresh	40	8	2.5	1.75	1 - 1.25
	Aged	30	5	2 - 2.5	1.0	0.5- 0.75

These indicate that aging promotes heat coagulation at 90°C. A similar effect was noted at other temperatures. The later stages of coagulation which resulted in precipitation of the protein also occurred faster in aged blood than in fresh blood on heating at temperatures between 60°C and 100°C. The rapid increase in precipitate formation occurred approximately 15 seconds earlier in aged blood than in fresh blood during heat coagulation at 80-100°C. This difference can be observed in Figure III.24.

6. Effect of Aging plus Preheating on Heat Coagulation of Blood.

Heat coagulation (at 90°C) of aged blood preheated at 60°C for up to 40 minutes occurred at a faster rate than heat coagulation of either fresh or aged blood. The maximum reflectance value was reached after 10 to 20 minutes at 60°C and therefore the reflectance values decreased during heat coagulation. The optical density at 805mu increased to values greater than 1.0 within the first 20 minutes of preheating. The decrease in soluble protein (which corresponded to the increase in amount of precipitate formed (Figure III.25)) occurred at an earlier heating time than in fresh or aged blood. At some preheating times (up to 40 minutes) the formation of the precipitate on heating appeared to occur at a slightly faster rate in aged blood than in fresh blood. This was, however, a small difference and was not very consistent between the various time-temperature combinations studied and more extensive experiments would have to be done before

FIGURE III.24 EFFECT OF AGING ON CHANGES IN OPTICAL DENSITY AT 805mμ AND PRECIPITATE FORMATION ON HEATING.

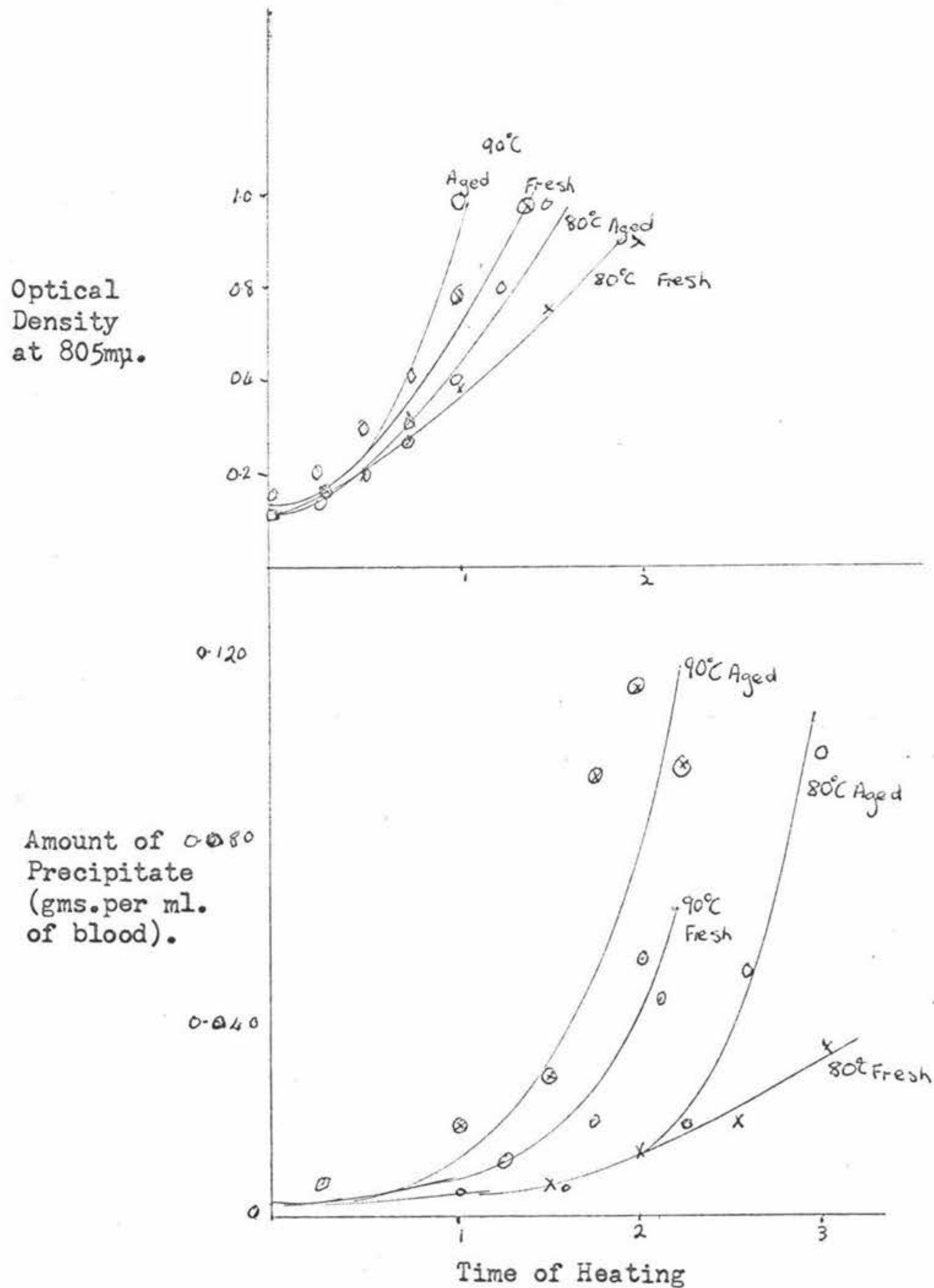
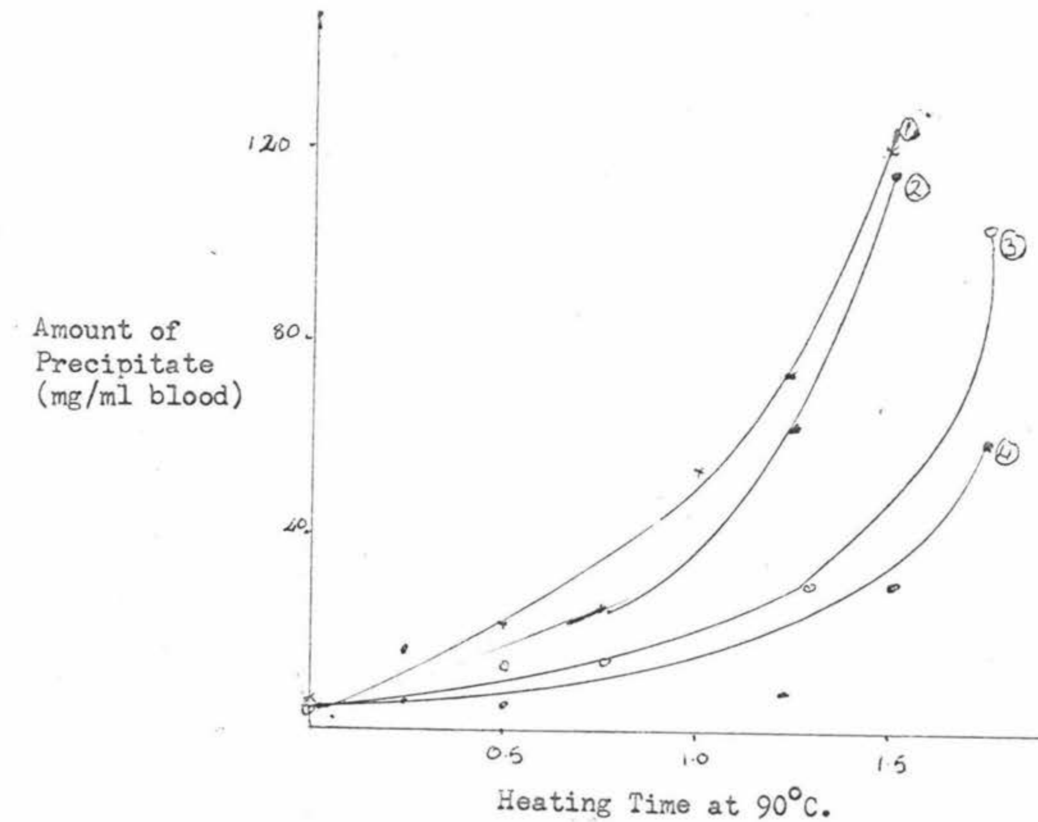


FIGURE III.25 EFFECT OF AGING AND PREHEATING AT 60°C ON PRECIPITATION CAUSED BY HEAT COAGULATION.



1. Blood aged two days, then preheated at 60°C. for 20 minutes.
2. Blood aged two days before heating.
3. Fresh blood preheated at 60°C. for 20 minutes.
4. Fresh blood.

this effect could be verified.

7. Effect of Other Variables on the Heat Coagulation of Blood.

The effect of dilution, addition of sodium chloride and the addition of blood solids on the precipitation of blood proteins were studied. The results of these investigations are presented in Table III.5.

TABLE III.5 AMOUNT OF PRECIPITATE FORMED ON HEATING.

Time of heating (minutes)	$1\frac{1}{2}$	$1\frac{3}{4}$	2	$2\frac{1}{4}$	$2\frac{1}{2}$	$2\frac{3}{4}$	3
Substance added to blood	mg.of precipitate per ml.blood						
-	35.6	53.5	60.7	135.6	159.7	167.5	141.3
10 percent Water	31.4	31.8	62.0	148.6	90.8	115.8	149.8
1 percent Sodium chloride	29.2	64.1	76.8	124.0	128.6	-	140.5
10 percent Sodium chloride	97.6	100.7	98.5	109.6	118.5	117.6	-
10 percent Sodium chloride, held for 2 hours before heating	27.3	50.4	51.4	76.1	152.8	168.2	170.7
10 percent Wet blood solids	101.5	83.1	114.1	-	-	-	-

These preliminary investigations indicated that the addition of 10 percent water did not influence the time of precipitation on heating at 80°C; whereas the addition of 2 percent sodium chloride did not have a marked influence on the heat precipitation of blood at 80°C, the addition of 10 percent sodium chloride caused the earlier precipitation of a large quantity of protein (after $1\frac{1}{2}$ minutes at 80°C as compared with 2 minutes for whole blood, plus 10 percent water and blood plus 1 percent sodium chloride), but the amount of precipitate did not increase markedly with further time. The addition of dried blood solids (1 percent moisture) did not have any influence on the heat coagulation. This was probably because these solids were fairly insoluble and rapidly sedimented

from the blood. The addition of 10 percent wet blood solids (80 percent moisture) appeared to increase the rate of precipitation of blood proteins, the stage of rapid precipitation commencing at $1\frac{1}{4}$ minutes.

8. The Effect of Heat on the Blood Fractions.

a. Effect of Heat on Plasma.

The reflectance values of plasma increased on heating. This increase occurred at a faster rate at higher temperatures as shown in Figure III.26. At heating temperatures of 60 and 70°C, the changes observed in the reflectance values during heating were not consistent between plasma samples. At 60°C, an apparent maximum percent reflectance was reached within the first 20 minutes of heating. The value of the maximum reached in sheep plasma was 60, whereas in cattle plasma it was 95, but as these were isolated experiments, the cause of this difference could not be determined.

Greater than 100 percent reflectance was obtained from plasma heated for three quarters of a minute at 90 to 100°C, two and a half minutes at 80°C, and four minutes at 70°C. A rapid decline in the reflectance values was noted after heating for one and a half to two minutes at 90 to 100°C, but was not observed at the lower heating temperatures. This decline is not shown in Figure III.26.

The optical density at 805mu of heated plasma increased slightly on heating at 60 to 100°C. After one and a half minutes at 80 to 100°C, the optical density at 805mu of plasma reached a maximum and began to decline slowly. At 60°C, this decline was not observed after 3 hours heating.

No change in the soluble protein of plasma occurred within 3 hours of heating at 60°C, but at higher temperatures a sharp decline in the protein content was observed. The heating time at which this decrease occurred was shorter at higher temperatures but the time at which the decrease occurred varied between the two experiments on sheep and cattle plasma.

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FIGURE III.26 INFLUENCE OF TEMPERATURE ON REFLECTANCE CHANGES DURING HEATING OF PLASMA.

100°C
90°C
80°C
70°C

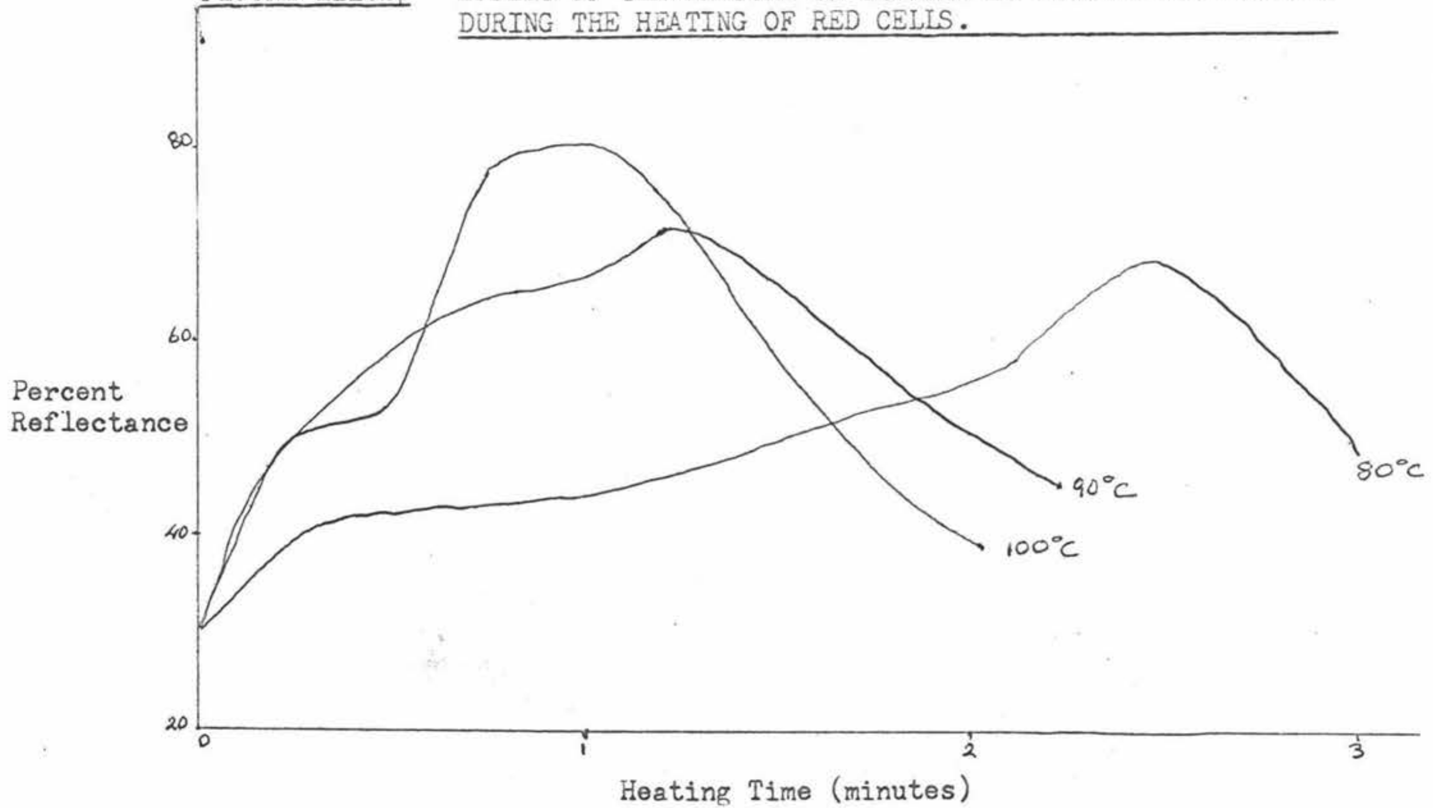
Percent Reflectance

x ⊗	100
•	90
o ⊙	80
□	70

100, 80, 60, 40, 20

0.5 1.0 1.5 2.0 2.5

FIGURE III.27 EFFECT OF TEMPERATURE ON CHANGES IN PERCENT REFLECTANCE DURING THE HEATING OF RED CELLS.



There was a small increase in the amount of precipitate formed during the heating of plasma. About 50 percent of the plasma proteins had precipitated in the unheated plasma. Approximately 0.06 g. of precipitate per ml.of plasma was obtained in these experiments after $1\frac{3}{4}$ to 2 minutes at 100°C, 2 minutes at 90°C, 3 minutes at 80°C, and 12 to 16 minutes at 70°C. This quantity of precipitate represents most of the plasma proteins (the protein content of plasma is 6 to 7 percent).

Some of the unheated plasma was examined by disc electrophoresis. A 5 ul. sample of plasma gave a clear electrophoretic pattern (Figure III.18); a 10 ul.sample produced a similar pattern but this was more blurred in crowded areas; whereas a $2\frac{1}{2}$ ul.sample resulted in a loss of the detail in the alpha-2 globulin region of the gel. Many of the bands in alpha-2 globulin area were only faintly recognisable with a 5 ul.sample.

Very little difference was detected between bovine and bovine plasma patterns. One band present in the alpha-2 globulin region of bovine plasma appeared to be absent from bovine plasma.

The supernatant from heated bovine plasma (80°C/2 minutes) did not show any well-defined differences from fresh plasma. At least one band in the alpha-2 globulin area disappeared while several decreased in intensity. Two bands in this alpha-2 globulin region increased in intensity.

The pattern from heated bovine plasma (80°C/2 minutes) was very similar to that from the unheated plasma, three bands decreasing in intensity.

Electrophoretic patterns of the proteins in the supernatant after further heating were not obtained.

b. <u>The Effect of Heat on the Red Cells</u>.

Heating the red cells did not have as marked effect on the reflectance values as the effect of heat on the reflectance values of blood or plasma. There was still, however, a temperature effect. At higher temperatures, the

maximum value was greater and reached within a shorter heating time (Figure III. 27). The basic shape of the reflectance versus time curve was similar to that obtained from blood as indicated in Figure III.28.

The light absorbance at 805mu of the red cells also followed a very similar trend to blood on heating, although the increase in absorbance was slightly slower (Figure III.29).

After an initial stage in which no change occurred, the soluble protein declined rapidly until approximately one quarter of the total protein remained in solution. The initial stage before this decrease was shortened by increasing the heating temperature.

The amount of precipitate formed during the heating of red cells increased gradually with the heating period (Figure III.30). At 80°C, there was a notable increase in rate of precipitation at 2 minutes of heating. It occurred about a quarter of a minute earlier at 100°C.

c. The Influence of the Blood Fractions - plasma and red cells - on the heat coagulation of blood.

The effect of the plasma fractions on the heat coagulation of blood could not be clearly defined from the changes in the heating curves of plasma and red cells.

An increase in light reflectance of blood, red cells and plasma occurred on heating. However, heated blood reached a maximum value much earlier (e.g. 0.5 minutes at 80°C) than did the red blood cells (e.g. 2.5 minutes at 80°C). The maximum in plasma at 80°C was not observed but was greater than 3 minutes. At 100°C and 90°C, the maximum value for the plasma was attained at a similar or later stage in the heating process than the red blood cells. (Figure III.28).

The changes in light absorbance at 805mu caused by heating were similar in blood and red cells. These values were determined on blood at a dilution of 1 ml. in 15 ml., and on red cells at a dilution of 1 ml. in 85 ml. Therefore,

FIGURE III.28 CHANGES IN REFLECTANCE DURING HEATING OF PLASMA, RED CELLS AND WHOLEBLOOD AT 80°C.

100
80
Percent Reflectance
60
40
20
Plasma
Red Cells
Whole Blood
1.0
2.0
3.0
Heating Time (minutes)

FIGURE III.29 CHANGES IN ABSORBANCE DURING THE HEATING OF PLASMA, RED CELLS, AND WHOLE BLOOD AT 80°C.

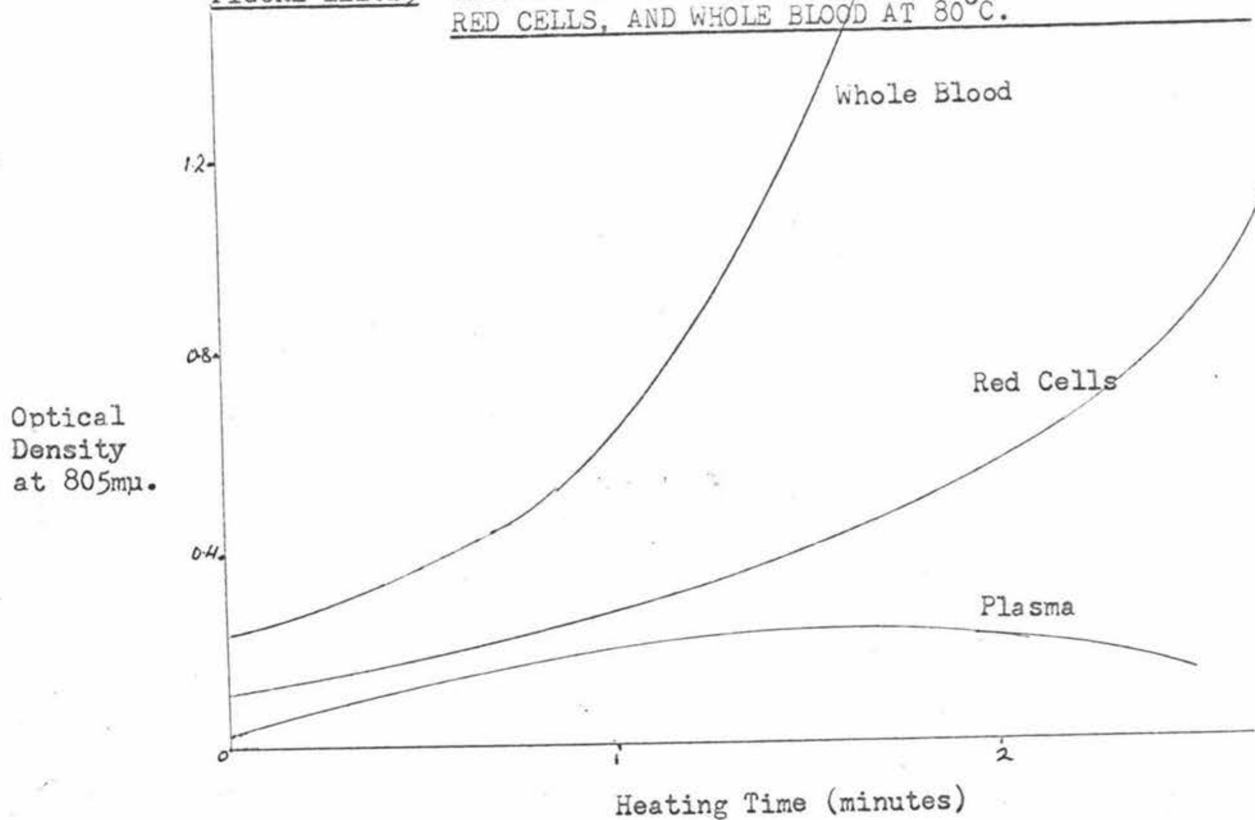


FIGURE III.30 PRECIPITATE FORMATION DURING HEATING OF PLASMA, RED CELLS, AND WHOLE BLOOD AT 80°C.

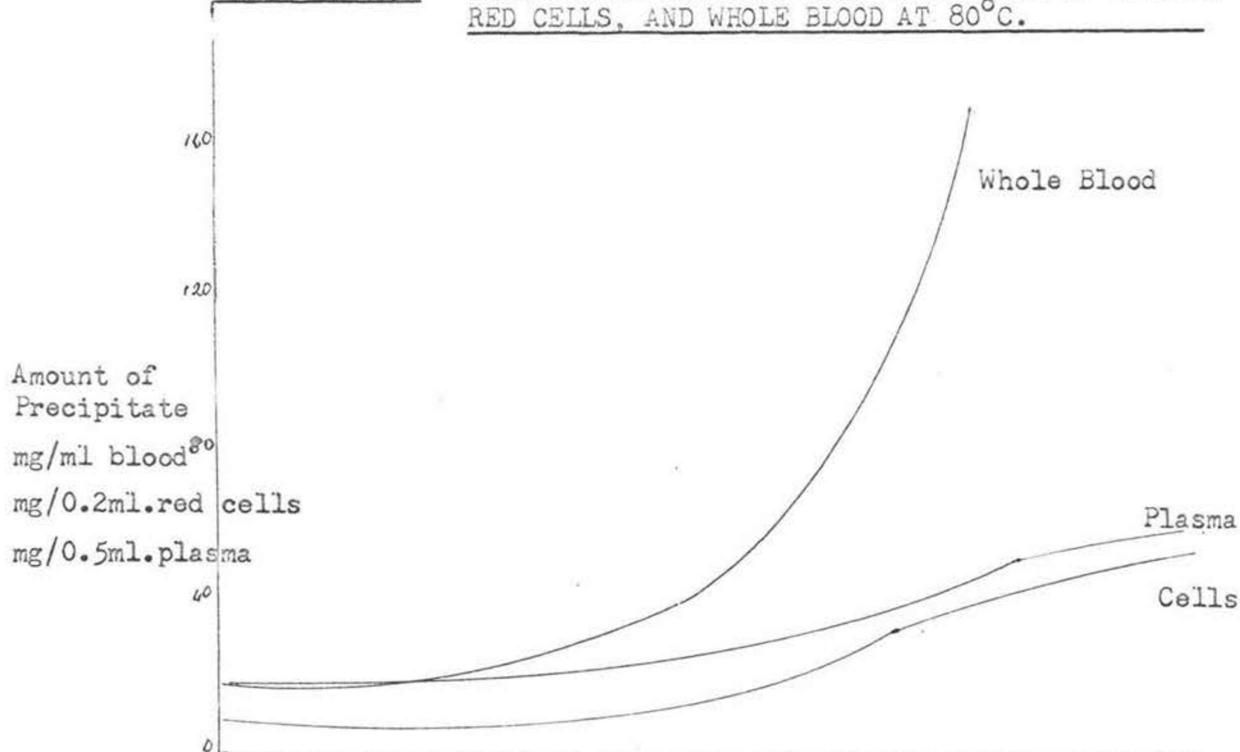


FIGURE III.31 CHANGES IN SOLUBLE PROTEIN ON HEATING PLASMA, RED CELLS AND WHOLE BLOOD AT 80°C.

1.0

0.8

Optical
Density
at 280mu.

0.6

Red Cells

0.4

Whole Blood

0.2

Plasma

1 2 3

Time of Heating (minutes)

it could be assumed that the rapid increase in the absorbance of blood samples on heating is quite markedly affected by the red cell fraction.

The low absorbance values of plasma showed little variation with heating and therefore did not have a great influence on the change in absorbance values of blood on heating.

The changes in the soluble protein from blood, red cells and plasma on heating were similar in all samples (Figure III.31). The results appeared to indicate that the protein in red cells decreased at a faster rate than the protein in blood or plasma. The more rapid decline observed on heating red cells was not solely due to the differences in protein concentrations in the heated solutions as blood and plasma samples exhibited a marked decrease in soluble protein after similar heating periods.

If the protein concentration had been the only factor which influenced the decreases in the soluble protein, the plasma proteins (which have less total protein) would have precipitated later than whole blood.

It has been noted above that the marked increase in percent reflectance of plasma on heating as compared to the red cell protein, haemoglobin, is probably due to the large change in conformation of the plasma proteins on denaturation (i.e. unfolding of the molecules), and as aggregation which leads to the precipitation of the protein follows this process it can be concluded that at least some of the plasma proteins are precipitated under the conditions of heating used in these experiments and therefore influence the heat coagulation of blood. This was verified by the electrophoretic experiments on the heating of plasma, and also by the amounts of precipitate formed in blood and plasma on heating (Figure III.30). All the plasma proteins had precipitated, while approximately 35 percent of the haemoglobin still remained in solution after heating for two and a half minutes at 80°C.

Pilot Plant Experiments.

1. Effect of Heat on Blood.

a. Indirect Heat Transfer.

Blood coagulated by heating in a tubular heat exchanger (Figure III.7) was examined by the methods used in the laboratory experiments described above. The heating rates obtained in these pilot plant experiments (indicated in Figure III. 8) were much faster than those obtained in the laboratory experiments (Figure III.2). When water at 80°C was circulated through the jacket of the heat exchanger in the pilot plant experiments, blood which was pumped through the apparatus attained a temperature of 76°C after a residence time of 26 seconds. In the laboratory experiments, the temperature of the blood in the centre of the test-tubes was only 46°C, 26 seconds after they were placed in a waterbath at 80°C, and did not reach 76°C until 2 minutes'heating time had passed. The faster heating rate in the pilot plant experiments was mainly due to the agitation of the blood as it was pumped through the tubes, although the smaller diameter of the tube of the heat exchanger (0.5 cm.) compared with the test-tubes (2 cm.) and the different heat-transfer materials (stainless steel and pyrex) would also have contributed to lower heat transfer coefficients in the pilot plant experiments.

The pump motor speed was used to control the residence time of the blood in the tubes of the heat exchanger to enable samples to be obtained after different times at the same processing temperature. The influence of the blood flow rate on the heat transfer coefficient was not determined in these experiments, but was assumed to be small.

The results of the pilot plant experiments are discussed briefly below. The stated temperatures are those of the heating medium (water or steam). The blood outlet temperature was generally 2 - 3°C lower than this temperature

depending upon flow rate.

(i) Optical Properties.

Turbidity by Reflectance. The percent reflectance of samples coagulated at 90°C reached a maximum after 22 seconds. During coagulation at 80°C the maximum percent reflectance was reached after 34 seconds. Further heating at both these temperatures caused a rapid decrease in the percent reflectance values, although they had not reached zero after 4 minutes. At 96°C and higher temperatures, the maximum percent reflectance was not detected as the shortest heating times after which samples were obtained was 10 seconds; the percent reflectance values obtained rapidly decreased to zero. Zero percent reflectance was reached after 31 seconds at 98°C and within 10 seconds at 102°C.

Turbidity by Absorbance at 805mu. The effect of various heating temperatures on the absorbance of the blood at 805mu is indicated in Figure III.32. At 80°C, the light absorbance at 805mu appeared to reach a maximum of about 0.55 within 50 seconds, and did not increase even after 4 minutes' heating. At 89°C and higher temperatures, the light absorbance increased very rapidly within 10 seconds rising to absorbances greater than 2.

(ii) Protein in Supernatant.

The influence of heating in the pipe heat exchanger on the soluble protein in the blood is shown in Figure III.33. At 80°C, the protein content did not decrease significantly even after 4 minutes'heating, although laboratory experiments at 80°C showed a rapid decline after 2½ minutes. At 89°C a rapid decline in soluble protein occurred within 1 minute of heating (cf. one and a quarter minutes in laboratory experiments at 90°C). This decline was more rapid at the higher temperatures - particularly with steam in the jacket of the heat exchanger. An apparent minimum value of .004 percent protein in the supernatant was obtained which was equivalent to 1.6 percent soluble protein remaining in the heated blood.

FIGURE III.32 INFLUENCE OF TEMPERATURE ON ABSORBANCE VALUES OF BLOOD AT 805mu.

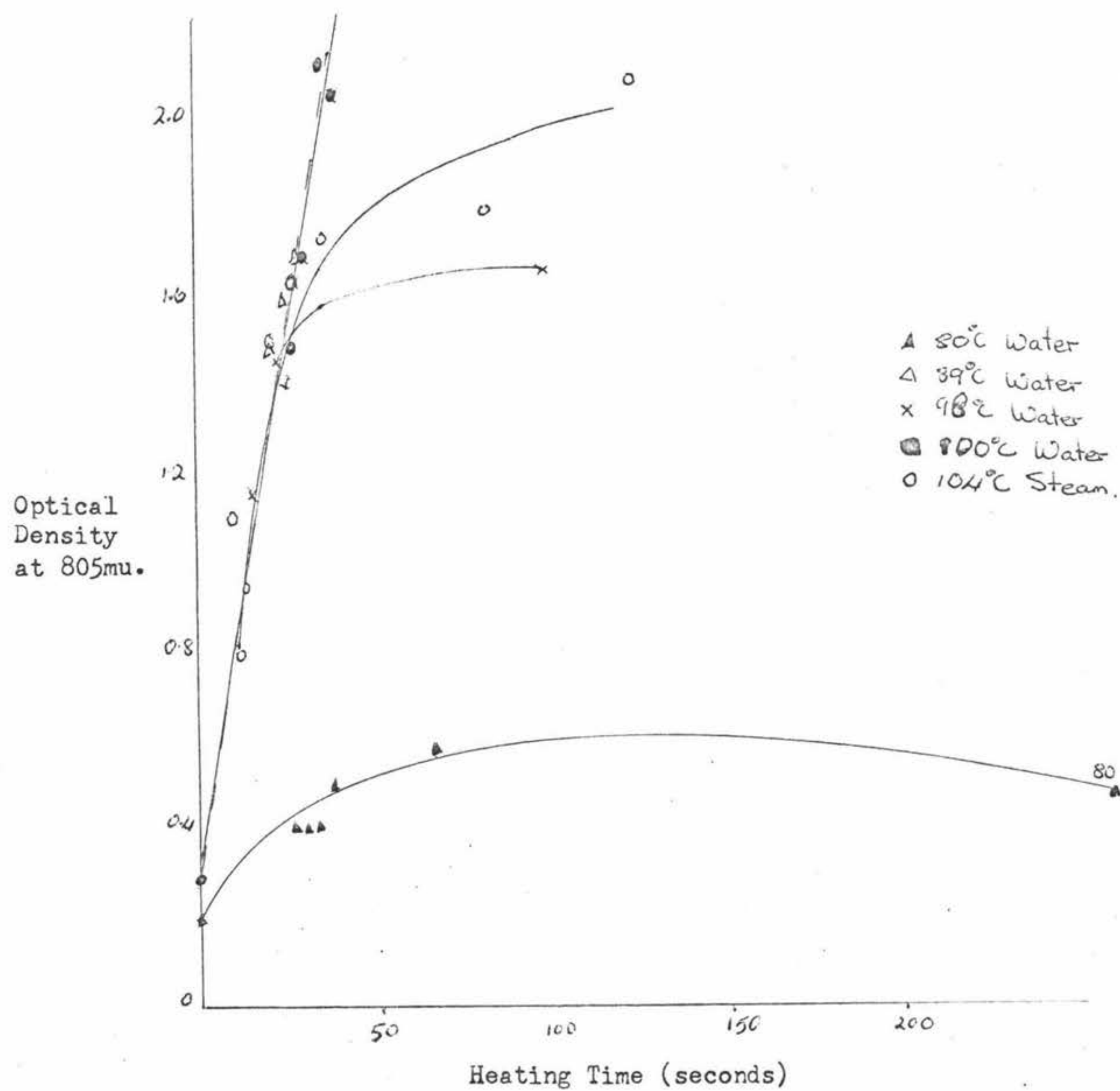
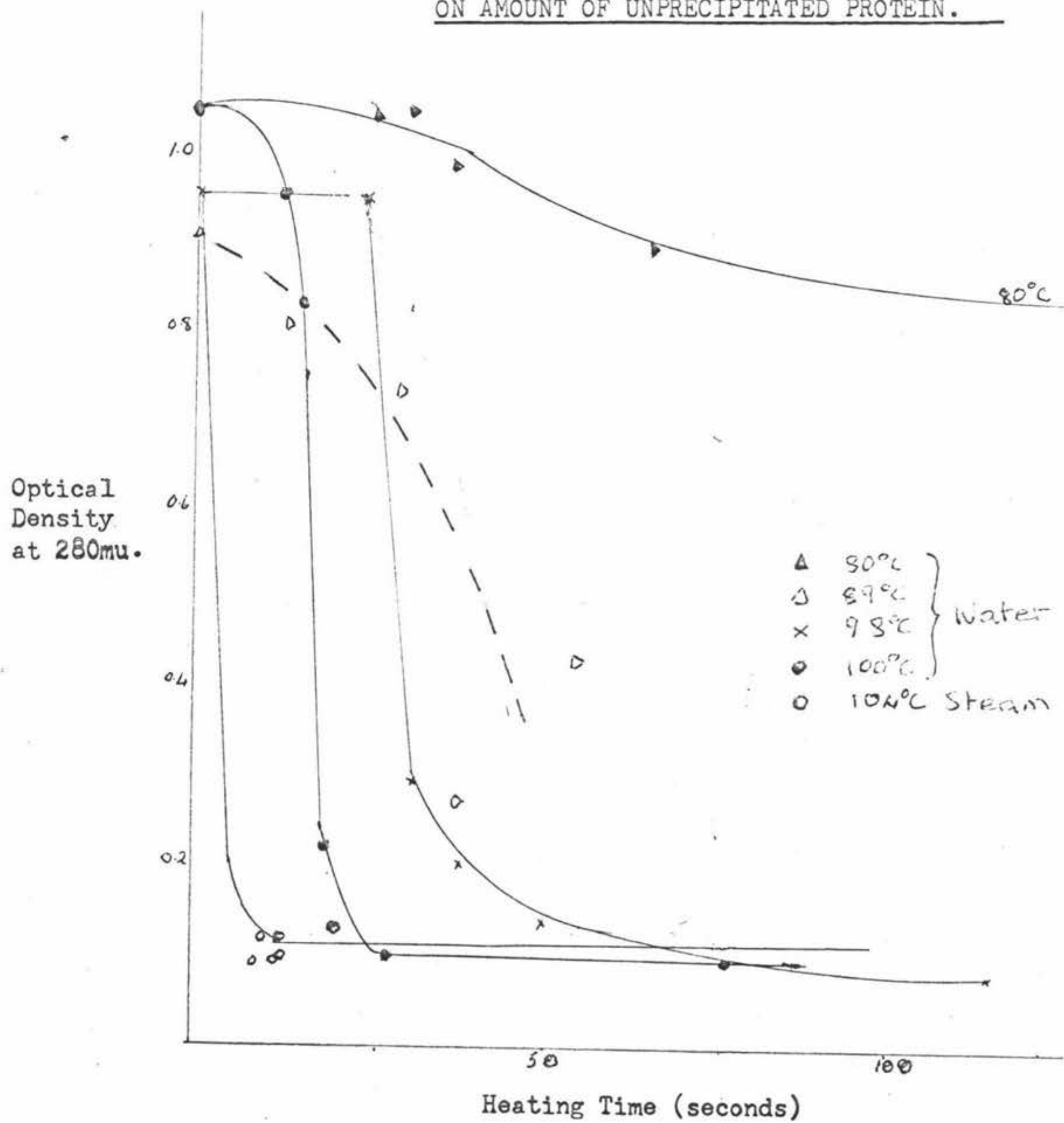


FIGURE III.33 EFFECT OF TEMPERATURE OF HEATING MEDIUM ON AMOUNT OF UNPRECIPITATED PROTEIN.



(iii) Precipitate Formation.

The increase in the amount of precipitate which occurred on heating at 80°C or above is shown in Figure III.34. At 80°C the rate of precipitation was very slow (30 mg.per ml. after 4 minutes) but at 89-104°C it increased very rapidly to a maximum of about 200 mg./ml., which is the same level that was reached in the laboratory experiments. This amount of precipitate was reached in the laboratory after 135 seconds at 100°C, compared with 1 minute in these pilot plant experiments.

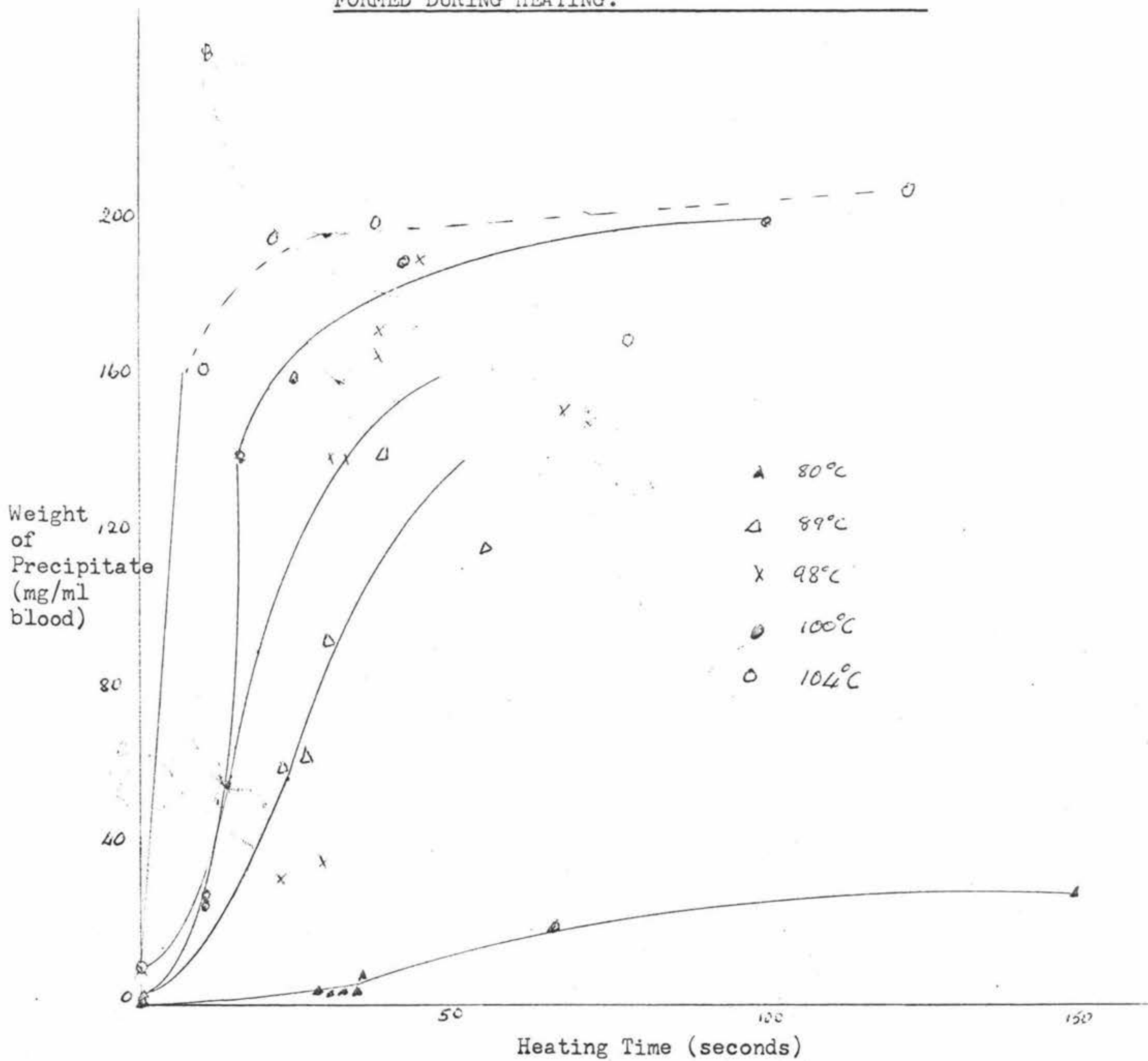
b. Steam Injection.

The extremely rapid rate of heat transfer obtained with the injection of steam at 3.8 pounds per hour directly into the blood flow was extremely inefficient in these preliminary experiments as the blood flow rate was very low, but this method can be used very efficiently for blood coagulation under more carefully controlled processing conditions. The experiments in which steam injection was used indicated that very short processing times (3-4 seconds) at temperatures in the 108-118°C range coagulated the blood to a similar extent as longer periods at temperatures near 100°C (i.e. reduced the soluble protein to 1-2 percent).

c. Discussion.

It may be noted that the changes which took place during the thermal processing of blood in the heat exchanger at 89°C and higher temperatures occurred much more rapidly than in the laboratory experiments at similar temperatures. This was attributed to the increased heat transfer rates in the pilot plant experiments. However, at 80°C the changes in blood treated in the pilot plant were slower than those observed in the laboratory experiments. Agitation influenced particle aggregation as was indicated by the fact that blood heated in a test-tube solidified more slowly if the tube was occasionally inverted during the heating process. This effect was probably inter-related with the effect of temperature but was not investigated in these

FIGURE III.34 EFFECT OF TEMPERATURE ON AMOUNT OF PRECIPITATE FORMED DURING HEATING.



experiments. The discrepancy between the laboratory experiments and the pilot plant experiments at the lower temperatures was probably due to the effect of agitation. It was assumed that at higher temperatures the rapid rate of heat transfer masked the other effects of agitation.

2. The Effect of Aging on Heat Coagulation of Blood.

Blood which had been held for two days at 16°C was coagulated in the tubular heat exchanger using water temperatures of 80 and 98°C. The results are shown in Figure III.35. The few results obtained did not indicate that aging had any marked influence on the changes caused by heat coagulation. A more extensive study of the effect of aging on heat coagulation would indicate whether the very slight influence noted in the laboratory experiments affected the heat coagulation under processing conditions.

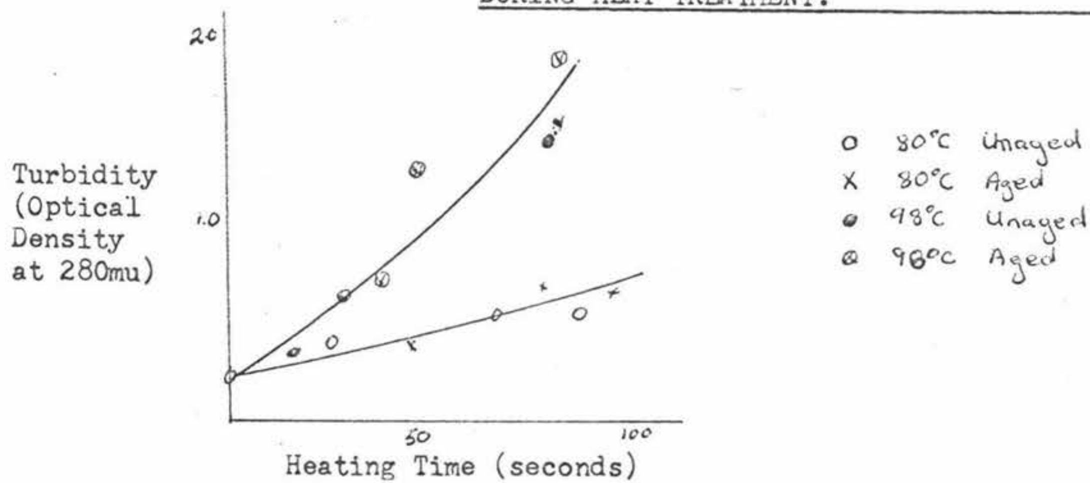
3. Effect of Dilution on Heat Coagulation of Blood.

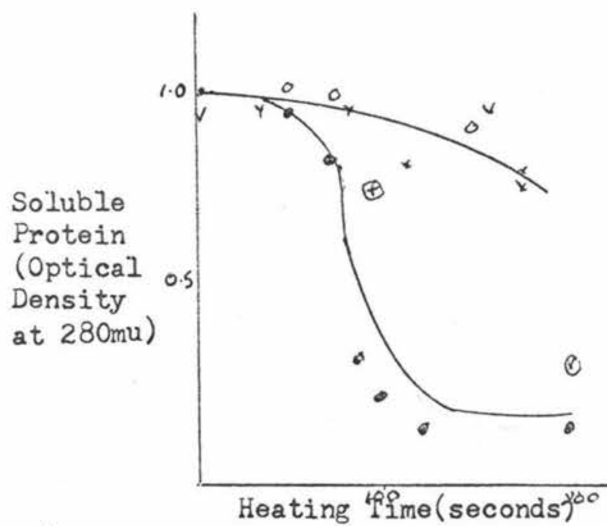
At a coagulation temperature of 98°C there was no difference in the change in optical density at 805mu between diluted and whole blood, although there appeared to be a slower decrease in the soluble protein content of dilute blood and, consequently, a slower rate of precipitation in diluted blood (Figure III. 36). Dilution of the blood with water (10 percent) did not appear to have any effect on these changes when a coagulating temperature of 102-106°C was used.

4. Effect of Sodium Chloride Addition on Heat Coagulation of Blood.

The few results obtained from an experiment in which sodium chloride was added to blood before treatment in the steam-jacketted three-pass tubular heat exchanger did not indicate any consistent trend with heating time, but this was attributed to the very short treatment times and the difficulty of cooling the samples rapidly. The range of the results obtained are presented in Table III.6, and indicate that there was no marked difference between fresh blood and blood to which 2 - 10 percent sodium chloride had been added.

FIGURE III.35 EFFECT OF AGING ON CHANGES IN TURBIDITY, SOLUBLE PROTEIN AND AMOUNT OF PRECIPITATE DURING HEAT TREATMENT.





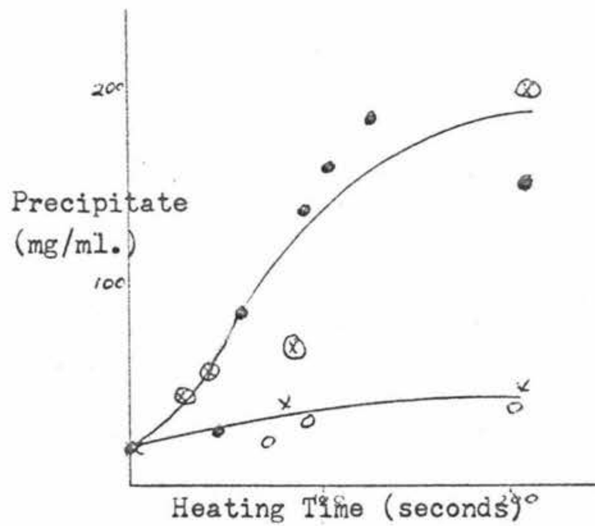
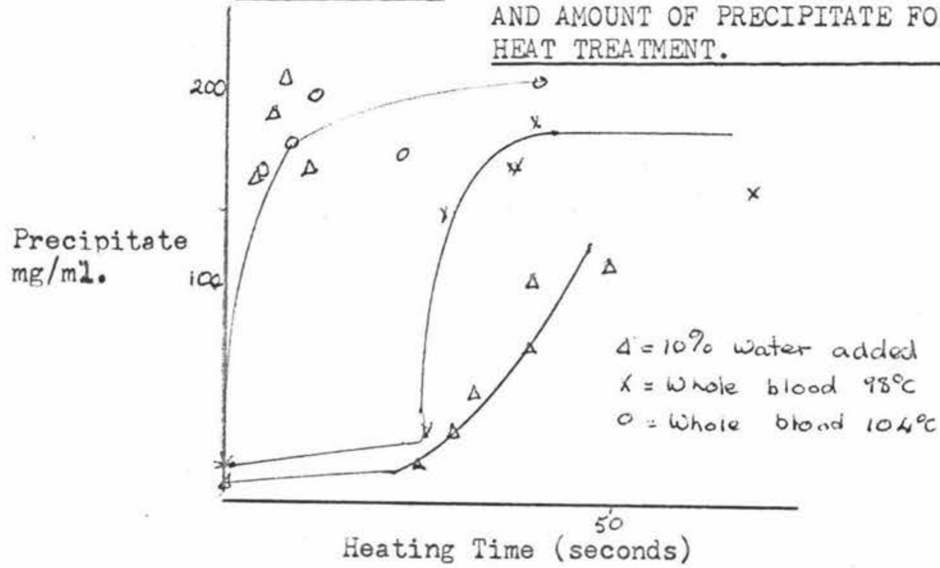


FIGURE III.36 EFFECT OF DILUTION ON SOLUBLE PROTEIN AND AMOUNT OF PRECIPITATE FORMED DURING HEAT TREATMENT.



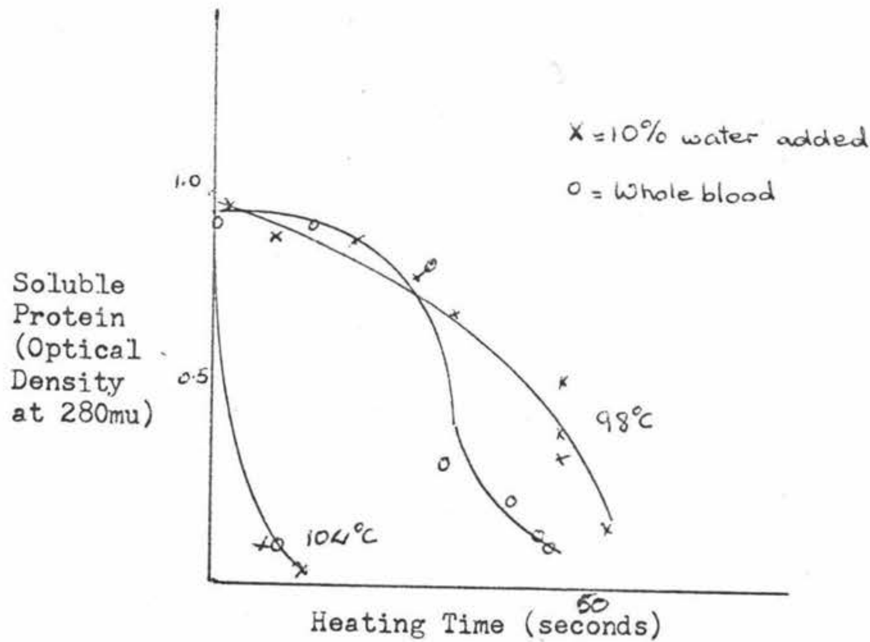


TABLE III.6 RANGE OF SOLUBLE PROTEIN IN THE SUPERNATANT AND ALSO THE PRECIPITATES OBTAINED AFTER 10 to 30 SECONDS AT 104°C.

	Percentage Soluble Protein	Precipitate (mg./ml.)
Fresh blood	0.02 - 0.03	0.16-0.22
Blood plus 1 percent NaCl	0.015- 0.02	0.12-0.18
Blood plus 10 percent NaCl	0.015- 0.04	0.15-0.19

C. Discussion

Heat coagulation is used by the meat industry throughout New Zealand for the recovery of solids from blood. This recovery of blood solids has presented many problems due to uncontrollable variability in the process. Although this study has been mainly confined to heating blood in test-tubes, it has indicated the stages of heat coagulation of blood which would probably occur under processing conditions.

Heating rapidly produced marked changes in the light reflectance and the light absorbance readings at 805mu of blood. These changes were due to the formation of light-scattering and light absorbing particles in the blood solution which was caused by the denaturation (unfolding and hydration) of the protein molecules and the subsequent aggregation of the denatured molecules. The heating temperature had a profound influence on the rate at which these changes occurred.

The initial increase in the reflectance values indicated the rate of unfolding of the protein molecules. The height of the peak observed in the reflectance versus heating time curves possibly was related to the extent of unfolding which occurred before the molecules aggregated. The peak height was influenced by the temperature, the maximum height being obtained by heating at 75°C. As the heating temperature increased above 75°C, the height of the peak decreased, but the peak appeared after shorter heating times and the rate of the subsequent decrease in the reflectance values increased. This decrease in reflectance value was directly related to a faster rate of precipitation. This indicated that at higher temperatures the molecules aggregated after less unfolding had occurred.

The early stages of aggregation were indicated by the increase in light absorbance at 805mu. The later stages of aggregation were observed in the rapid

formation of precipitate and decrease in supernatant protein.

Precipitation of all the blood proteins did not occur in these experiments as a small amount of soluble protein (1.6-4 percent) remained after the most extensive coagulation processes used (including steam injection). It was suspected that this soluble protein was small peptides, but further investigation on the nature of this uncoagulated protein material is necessary in order to determine the conditions under which it could be precipitated. It is interesting to note that blood processing plants generally are unable to reduce their effluent below 2-4 percent protein after much more severe heating than was undertaken in these experiments.

The precipitates formed on heating at 90°C contained proteins from all the major blood protein fractions detected in electrophoresis. An apparent increase in the beta globulin was observed. Many workers had previously reported that an increase in the alpha and/or beta globulin fractions occurred on heating serum due to the formation of complex proteins. These experiments have shown that some of the proteins precipitate as this complex. The effect of the individual protein fractions on the formation of this complex with the mobility of beta globulin was not determined, although it can be noted that some proteins from all fractions decreased on heating.

Although a large amount of protein precipitated out in a short period of time, the individual proteins appeared to precipitate out at differing rates. Although haemoglobin was precipitated out at an early stage in the heating process, haemoglobin was still present in quite large amounts when most of the plasma protein had been precipitated. This was probably due to the formation of one or more complexes with individual serum proteins as has been reported by some workers (Dimopoullos and Fellowes (1958)).

An investigation of the effect of heat on red cells and on plasma appeared to indicate that the plasma, which exhibited marked changes in reflectance but

relatively little change in the absorbance values, probably had an important effect on the denaturation stage. The changes observed in the aggregation stage - increase in 805mu and increase in amount of precipitate - appeared to be mainly due to the haemoglobin which is the major protein in blood.

In blood processing, preheating is often considered as a means of controlling excessive temperature fluctuations during the coagulation process and is usually carried out in the tank which controls the blood flow rate into the heat exchanger. Preheating at 60°C was chosen for these experiments,as blood which is held at this temperature for a few minutes immediately after collection does not undergo the natural clotting process which causes problems in blood processing plants. A more detailed study of the effect of various preheating temperatures would be useful.

Preheating at temperatures higher than 60°C during blood processing causes problems due to the development of a film of precipitated protein on the temperature control probes and also the accumulation of sludge in the bottom of the preheating plant. These problems are caused by the slow thermal coagulation of the proteins occurring during preheating. Obviously the critical temperature at which this begins to occur is highly dependent upon the time for which blood is held at the preheating temperatures. Precipitation of 40mg. of dry solids per ml. of blood was observed after two hours at 57°C, one hour at 60°C, 20 minutes at 64°C and 15 minutes at 65°C.

It is interesting to note that until a sixty-minute preheating period was attained, preheating at 60°C enhanced the heat coagulation of blood. After sixty minutes at 60°C, precipitation began to occur and it appeared that once this stage had been reached coagulation at higher temperatures was retarded. Kratochvil <u>et al</u>.(1962) found that heating human serum albumin at lower temperatures before increasing the temperature to cause aggregation often greatly retarded the aggregation process. They interpreted this effect as the

result of intramolecular modifications which altered the number and nature of aggregation sites on the molecules. It was found in the present experiments that shorter preheating times at 60°C modified the blood proteins in such a manner that coagulation at higher temperatures was enhanced, but after sixty minutes reactions had occurred which stabilised the proteins against rapid heat coagulation.

The few experiments in which the heat coagulation of cattle and sheep blood were compared did not indicate any marked differences. The age of the animal had a small effect on the changes which occurred on heat coagulation of blood. However, many more results would have to be obtained before the effects of species and age of animal on the heat coagulation process could be verified.

Holding the blood at room temperature for two days did not have any marked effect on heat coagulation in test-tubes or on the heat exchanger. This suggested that the holding of large volumes of blood in freezing works would not provide any great processing advantage.

These experiments were undertaken on blood to which anticoagulant had been added. Because of the difficulty of standardising and controlling clotting, the influence of the anticoagulant on these results was not determined, but it was assumed to have little effect on the changes which occurred on heat coagulation.

In conclusion, the following comments on heat processing of blood are made :

1. Blood should be processed soon after collection.
2. The natural clotting of blood will not occur if the blood is preheated at 60°C for five to ten minutes immediately after collection.

3. The temperature and time of preheating should be carefully controlled. Preheating is advantageous in that it reduces temperature fluctuations in the coagulator, and, under certain conditions, e.g. 60°C for less than 40 minutes, accelerates the thermal precipitation of blood proteins.

4. Prolonged preheating times should be avoided.

5. The time as well as the temperature of coagulation should be controlled. Temperatures above 90°C are recommended for heat coagulation, but the actual time/temperature relationship of the coagulation process should be determined for individual plants as heat transfer rates and other plant characteristics will vary.

6. The minimum heat process required for coagulation should be used in order to minimise possible deleterious effects of heat on the quality of the blood proteins.

7. It may be possible to recover more efficiently the small amount of soluble protein which remains after heat coagulation by treatment of the effluent from the blood coagulation plant by some other method, rather than extending the heat coagulation conditions.

8. Processing controls may be standardised by heating samples of the blood from the main holding tank in a test-tube for varying time intervals in a waterbath. The values obtained for light reflectance or light absorbance at 805mu can be calibrated to give the conditions required for coagulation in the plant.

9. Spectrophotometric determinations at 280mu of the protein in the effluent of the blood plant provides a rapid means of detecting variation in the processing conditions.

IV. SUMMARY

IV. SUMMARY

Heat coagulation is the most common method used to recover blood solids in New Zealand. The effects of many of the variables in this process have not been clearly defined and because of this it has been found difficult to control the recently introduced continuous coagulation processes. In an attempt to determine the influence of some variables on the heat coagulation, blood was heated in test-tubes or in a tubular heat exchanger.

Heat coagulation caused changes in the molecular conformation and intermolecular aggregation of the protein molecules. This resulted in the precipitation of the proteins. The rate at which these changes occurred was markedly influenced by the heating temperature. Above 90°C, the protein was precipitated very rapidly, but as the heating temperature decreased the rate of precipitation became slower until at 60°C complete coagulation was not observed after three hours' heating. The precipitation of the various plasma proteins and the haemoglobin appeared to occur at different rates, a portion of the haemoglobin remaining in solution until heat coagulation was almost complete.

The length of the preheating period influenced the changes which occurred during thermal processing of blood. At 60°C, the optimum preheating period was less than twenty minutes. Preheating at 60°C for periods longer than sixty minutes appeared to retard the heat coagulation.

Aging the blood for 48 hours at 16°C did not have any significant effect on the heat coagulation process. Preliminary experiments on some other variables - the age and species of animal, dilution with water and addition of up to 10 percent sodium chloride - did not show any increase in the rate of coagulation. However, addition of 10 percent wet blood solids caused a slight

increase in the rate of blood protein precipitation.

A small amount of protein material did not precipitate during the heat treatments applied in these experiments.

V. APPENDICES

1. The Effect of Variation in the Times of Dilution and of Reading on Turbidity Determinations.
2. Determination of Dilutions to be used in Turbidity Measurements.
3. Protein Determination - Kjeldahl Method.

APPENDIX 1. THE EFFECT OF VARIATION IN THE TIMES OF DILUTION AND OF READING ON TURBIDITY DETERMINATIONS.

Four different treatments, which were applied to blood after heating for different times at 85°C before their turbidities were determined, are listed below :

A. After heat treatment, the samples were immediately cooled by placing in a cold water bath at about 16°C, then diluted (1ml. in 15 ml.) with distilled water.

B. Immediately after heat treatment these samples were diluted (1 in 15) with distilled water and then cooled.

C. The heated samples were cooled, diluted and then held for 16 hours at 4°C.

D. The heated samples were cooled, then held at 4°C for 16 hours before dilution.

The effect of these treatments on the turbidity readings (both percent reflectance and optical density at 805mu) is shown in Figures V.1 and V.2.

Treatment A was used in most of the experiments and hence may be regarded as the normal treatment.

Dilution immediately after heating (Treatment B), which would cause more rapid cooling of the sample, had no effect on the turbidities of the samples heated for up to one minute at 85°C. However, during the second minute of heating the turbidity readings were slightly higher. The soluble protein content of the precipitates was higher after this treatment which indicated that the higher turbidity readings were probably due to the prevention of aggregation of denatured protein molecules during cooling in the undiluted sample. This treatment could not be used for a large number of samples as

FIGURE V.1 THE EFFECT OF VARIATION IN TIMES OF DILUTION & OF READING ON REFLECTANCE READINGS.

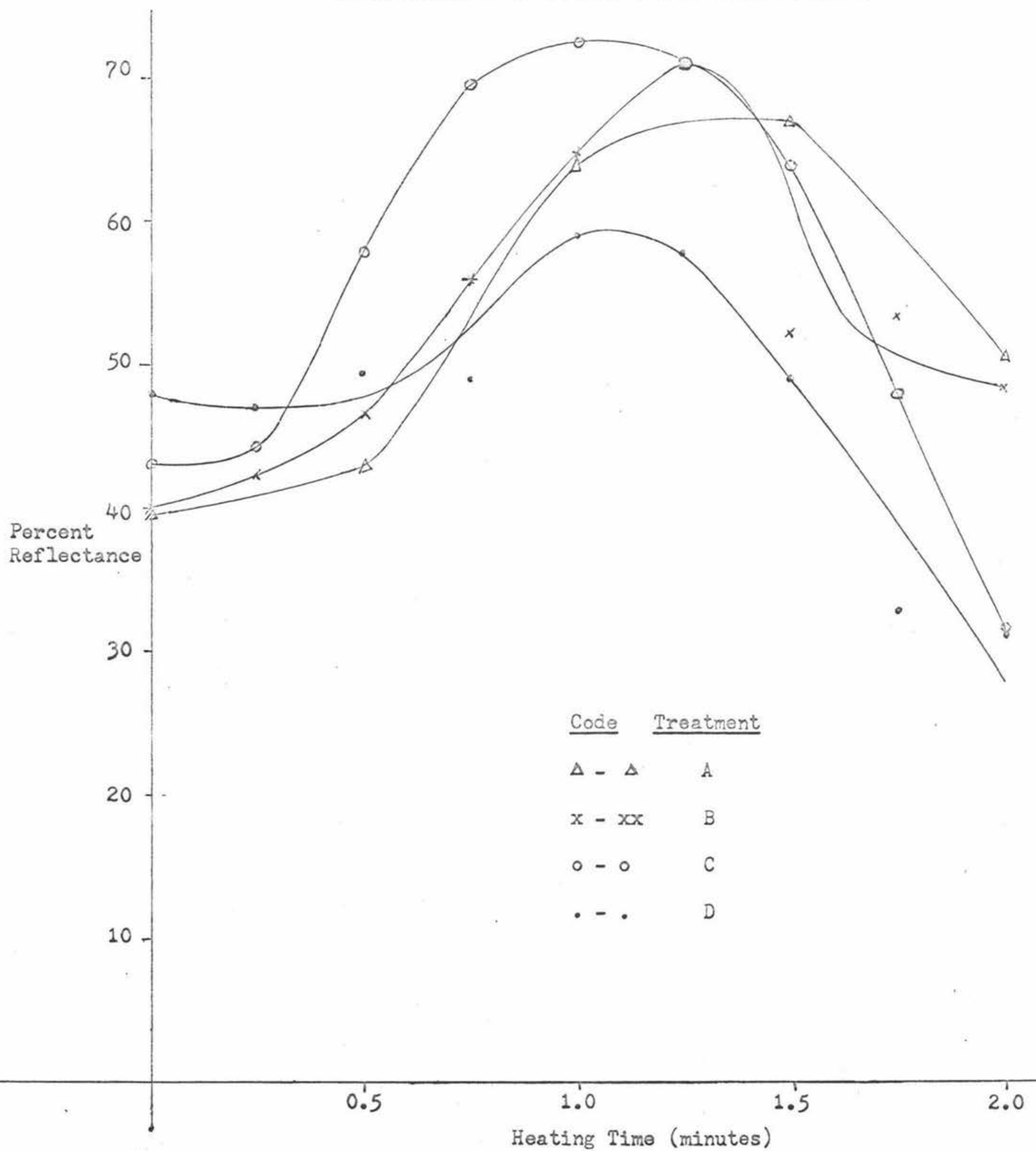
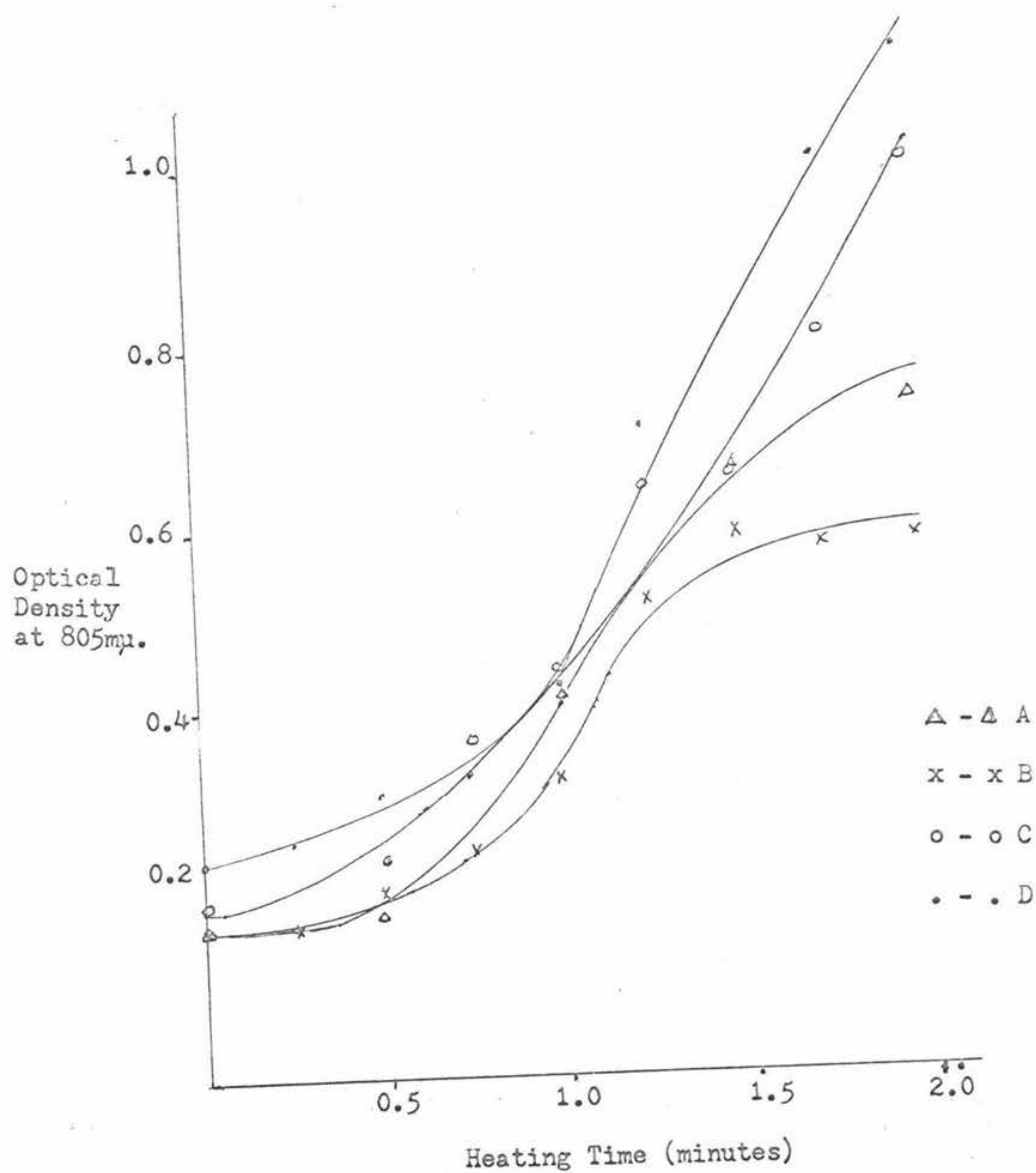


FIGURE V.2. THE EFFECT OF VARIATION IN TIMES OF DILUTION AND OF READING ON ABSORBANCE VALUES AT 805mμ.



careful (accurate) dilution could not be done very rapidly.

Holding the diluted samples overnight (16 hours), as in treatment C, did not make any difference to the turbidity of unheated blood, but possibly resulted in the slightly earlier appearance of the maximum percent reflectance reading.

Holding the undiluted blood samples before dilution resulted in a higher reflectance value for unheated blood and much lower values for blood that had been heated for longer than one minute. As the soluble protein in the precipitate was also lower it could be assumed that aggregation which leads to precipitation and insolubilisation takes place in undiluted samples on standing.

APPENDIX 2. DETERMINATION OF DILUTIONS TO BE USED IN TURBIDITY MEASUREMENTS.

The effect of dilution of the blood on the reflectance measurements is shown in Figure V.3. It can be noted that one inflection point, a maximum, exists on the curve. Because i) the percent reflectance decreased on heating (after an initial small increase) and, therefore, a high initial value would allow a wider range of readings to be taken, and

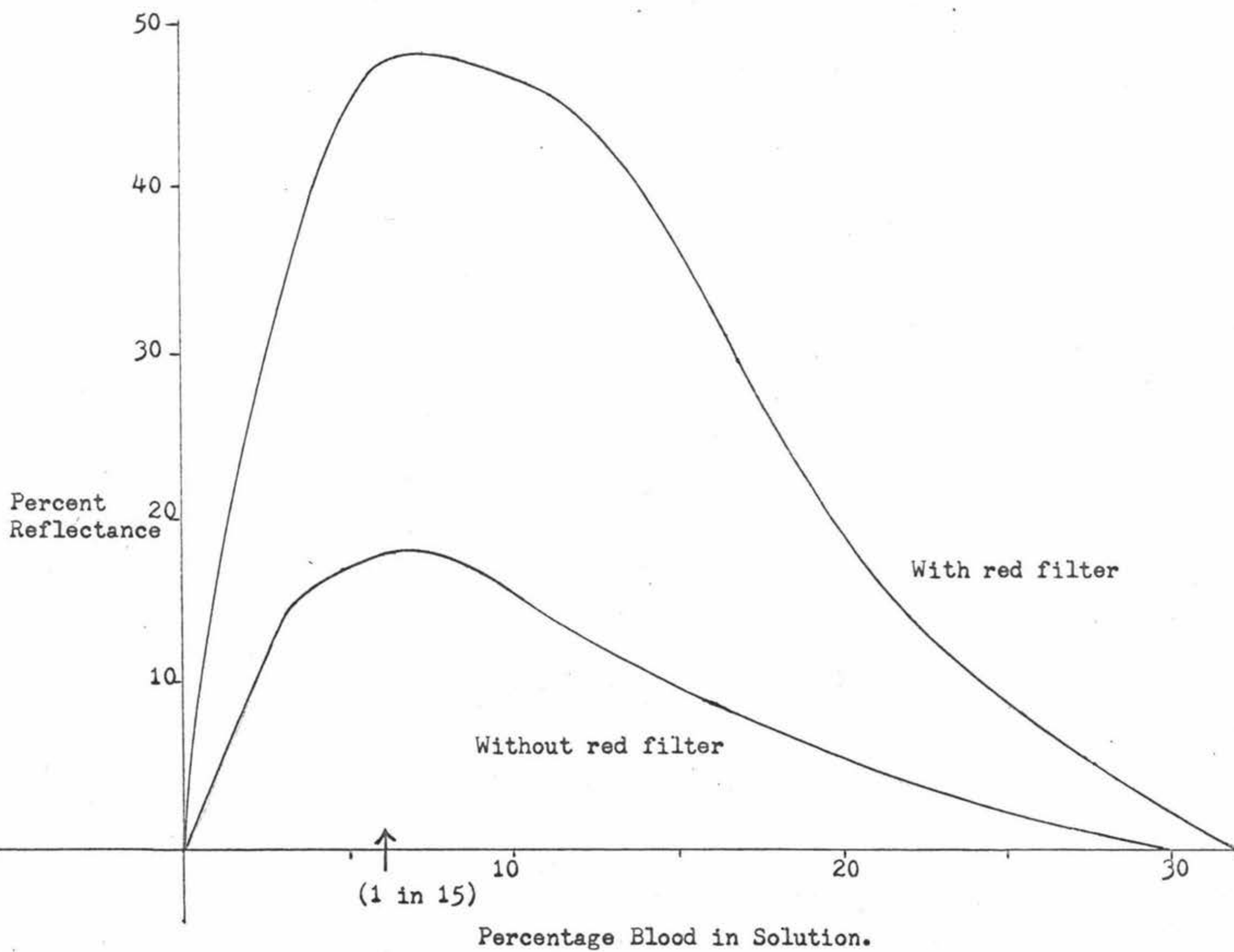
ii) the rate of change of the percent reflectance with concentration is lowest at the maximum and, therefore, dilution errors would have least effect on the readings determined at this dilution, it was decided to use the dilution at which the maximum occurred.

The optical density at 805mu of unheated blood at this dilution (1 ml.of blood in 15 ml.) was in the lower portion of the optical density range where the sensitivity of the spectrophotometer was greatest. However, on heating, the optical density of heated blood rapidly increased and, at this dilution (1 in 15), were in the less accurate range, above 0.8. However, it was felt that the samples which produced these high optical densities generally contained rapidly sedimenting particles which made accurate reading difficult and, therefore, the introduction of another time-consuming dilution step into the procedure would not have been of great benefit. For this reason the determinations of optical densities at 805mu were made on the 1 in 15 dilutions of blood.

The maximum percent reflectance of red blood cell solutions was obtained with a dilution of 1 in 95 and this dilution was used for turbidity determinations on red cells.

Maximum percent reflectance was obtained with a 1 in 8 dilution of unheated plasma but the marked increase in the turbidity of plasma on heating

FIGURE V.3. EFFECT OF DILUTION ON PERCENT REFLECTANCE READINGS



could not be followed at this dilution. A dilution of 1 in 32 was found to be suitable for determining the turbidity of a range of heated samples and was used in subsequent experiments.

This dilution effect was probably due to the variation of the number of light reflecting particles in the solutions. At high concentrations of blood all the light directed onto the solution was absorbed. At lower concentrations (e.g. 25-50 percent blood) the solutions were less opaque and the light passing into these solutions was partly transmitted, partly absorbed, and partly reflected. As the concentration was decreased further the amount of light transmitted through these solutions increased, the amount of light absorbed decreased,and the amount of light reflected initially increased. The increase in the amount of light transmitted on increased dilution was due to the separation of the light absorbing particles and was influenced by the number and size of the particles in solution. The initial increase of reflected light (which may be measured in one or several directions) was also due to the separation of these particles, as this exposed a greater surface area for light reflection. At these higher concentrations the "packing" of particles in solution influenced the surface areas exposed to the incident light and, therefore, determined the amount of light reflected. As the concentration decreased, a concentration was reached at which the maximum possible surface area for light reflection was exposed. Below this concentration the number of particles in solution determined the reflecting surface area available, and the amount of light reflected decreased as the concentration was lowered.

APPENDIX 3. PROTEIN DETERMINATION - KJELDAHL METHOD.

a) Determination of Total Nitrogen.

Digestion.

1. The samples were weighed (by difference) into 100 ml. Kjeldahl flasks.

2. Approximately two grams of sodium sulphate were added (to raise the boiling point of the mixture).

3. Five ml. of mercuric sulphate (catalyst), 20 ml.concentrated sulphuric acid and two glass beads (to prevent bumping) were added.

4. The flasks were then heated gently in a fume cupboard. When the frothing had ceased, the heat was increased and the solutions allowed to simmer for one and a half to two hours after they had cleared.

5. After the flasks had cooled,their necks were washed down with hot distilled water and the flasks then heated until fumes of sulphuric acid were visible.

6. The solutions were allowed to cool, the precipitate dissolved by swirling the contents of the flasks (to which a small amount of distilled water had been added), and the contents of the flasks transferred quantitatively to 100 ml. volumetric flasks and allowed to cool before dilution to 100 ml. (using dilution water to rinse out the Kjeldahl flask thoroughly).

Distillation

1. Five ml. aliquots were transferred to distillation flasks.

2. The distillation apparatus was then assembled and 5 ml. of 15 percent sodium hypophosphite added to the sample.

3. Immediately before commencing the steam distillation, 15 ml. of 60 percent sodium hydroxide were poured into the distillation flask.

4. The distillate was collected to 10 ml. of 2 percent boric acid.

5. Steam was passed through the apparatus for 5 minutes after the solution in the collection flask had turned blue.

6. After distillation the boric acid solution was titrated with 0.02N hydrochloric acid, using a mixture of one part 0.1 percent Methyl Red and five parts 0.1 percent Bromocresol Green in ethanol, as indicator, to a violet end-point.

7. Calculation :

$$\text{Percent Nitrogen} = \frac{(\text{Titre} - \text{Blank}) \times 0.02 \times 14.008 \times 100 \times 100}{\text{Sample Weight (mg)} \qquad 5}$$

$$= \frac{0.5603 \times (\text{Titre} - \text{Blank})}{\text{Sample Weight in grams}}$$

b) Determination of Non-protein Nitrogen.

1. Samples were weighed (by difference) into 50 ml. volumetric flasks and made up to volume with distilled water.

2. Five ml. aliquots of these solutions were pipetted into centrifuge tubes, then 5 ml. 50 percent trichloracetic acid added. The precipitated protein was then sedimented by centrifuging at 2000 r.p.m. for 20 minutes.

3. The nitrogen content of 7.5 ml. of the solution above the precipitated protein was then determined by the distillation procedure as outlined in the Total Nitrogen Method.

4. Calculation :

$$\text{Percent Nitrogen} = \frac{(\text{Titre - Blank}) \times 0.02 \times 14.008 \times 50 \times 10 \times 100}{\text{Sample Weight (mg)} \qquad 5 \quad 7.5}$$

$$= \frac{0.3736\ (\text{Titre-Blank})}{\text{Sample Weight in grams}}$$

c) Determination of Protein.

Calculation :

Protein Nitrogen = Total Nitrogen - Non-protein Nitrogen

Protein (percent) = 6.25 x Nitrogen (percent)

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