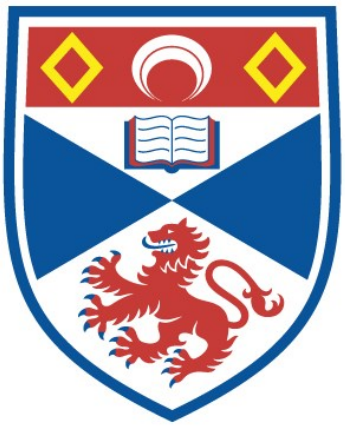
DYNAMIC AND STRUCTURAL STUDIES ON THE EGG WHITE PROTEINS

Frank Sinclair Steven

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



1955

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Being a thesis presented by

FRANK SINCLAIR STEVEN, B.Sc.(Hons. 1955)

to the University of St. Andrews in application for the

Degree of Doctor of Philosophy

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DECLARATION

I hereby declare that the following thesis is based on the results of the investigations carried out by me, that this thesis is my own composition, and that it has not previously been presented for a higher degree.

The research was begun in the Department of Biochemistry, St.Salvator's College and later continued in the Department of Biochemistry, Queen's College. Dr. G.R. Tristram has been the supervisor throughout this work.

CERTIFICATE

I certify that Frank Sinclair Steven has spent the equivalent of nine terms at research work in the Departments of Biochemistry, St. Salvator's College and Queen's College, University of St. Andrews, under my supervision, that he has fulfilled the conditions of Ordinance No. 16 (St. Andrews) and that he is qualified to submit the accompanying thesis in application for the Degree of Doctor of Philosophy.

ACADEMIC RECORD

I matriculated at the University of St. Andrews in October 1951, passed the examination for the Degree of Bachelor of Science and graduated in October 1954. I obtained a 1st Class Honours in Biochemistry in June 1955, and was then admitted as a research student to the Department of Biochemistry, St. Salvator's College. During the year 1955-56 I held a Carnegie Research Scholarship. From October 1956 I continued my research topic as an Assistant Lecturer in the Department of Biochemistry, Queen's College. I have been engaged in the present research throughout this time, the results of which are now submitted as a thesis for the Degree of Doctor of Philosophy.

CONTENTS

PART IV

N-TERMINAL ANALYSIS OF THE PURIFIED EGG WHITE PROTEINS

FOREWORD

The egg white proteins discussed in this work are those of the common chicken (Gallus domesticus) unless otherwise specifically stated. The word "pure" has been used throughout to describe proteins which have been rigorously purified within the limitation of modern preparative techniques. In many instances the product obtained has not satisfied all the conditions which indicate that the protein preparation was entirely composed of physically and chemically identical molecules. For these reasons the word "pure" has been used to imply a certain amount of reservation considering the achievements of the recent years in the sub-fractionation of proteins which were previously thought homogeneous.

For many years protein chemists have found the egg white proteins an attractive study, mainly because of the large quantities of first class protein which could be easily obtained free of carbohydrate and lipids. A further reason for research on the egg white proteins was the increasing number of "pure" proteins which were shown by modern analytical techniques to be heterogeneous. Although the complete amino acid composition has been accurately determined for the four major egg white proteins, only the partial sequential structure has been worked out for lysozyme.

One purpose of the present work has been to study the dynamic aspect of egg laying. This involved the question of establishing the origin of the egg white proteins, the classical theory being that the egg white proteins were synthesised in the oviduct. It is

suggested by the present author that the egg white proteins may be synthesised elsewhere in the body and transported by the blood to the oviduct. The results obtained from immunological and electrophoretic studies suggested that the second theory was possible, but did not disprove the classical theory.

The second part of this work has been the investigation of the structure of ovalbumin by chemical and physical means. From this study it has been possible to postulate a coiled "six-shaped" structure.

The experimental work carried out will be presented in three main sections, viz. the preparation of individual egg white proteins, dynamic studies on the origin of the egg white proteins, and structural studies on ovalbumin.

PART I

INTRODUCTION

1. Whole Egg White

The early literature on the study of egg white was well reviewed in "The Avian Egg" by Romanoff and Romanoff (1949). Two excellent reviews on the more recent work have been presented by Fevold (1951) and Warner (1954).

A survey of the present knowledge of whole egg white will be given before a more detailed review of the individual proteins.

Table I

Composition of egg white (Romanoff and Romanoff, 1949)

Component	% Composition
N	1.77
P	0.018
S	0.195
Water	87.9
Solids	12.1
Protein	10.6
Carbohydrate	0.9
Fat	0.03
Ash	0.6

The physical appearance of fresh egg white is heterogeneous. Romanoff and Romanoff (1949) showed that the egg white was made up of four physically distinct concentric layers, the "thin" layer being separated from the "thick" layer by allowing a shelled egg to stand on a plate, the "thin" layer flowing away from the "thick" layer. By piercing the "thick" layer a second thin layer, known as the "inner thin", separated. Finally the yolk was encased in a very thick layer called the "inner thick" or "chalaziferous" layer.

The relative proportions of these four physically distinct layers were given by Romanoff and Romanoff (1949) and are shown below:

Table II

Egg white	100% v/v
"Thin" layer	23.2
"Thick" layer	57.3
"Inner Thin" layer	16.8
"Chalaziferous" layer	2.7

Forsyth and Foster (1949) demonstrated by Tiselius electrophoretic analysis that the protein composition of the "thin", "thick" and "inner thin" layers was almost identical. (The chalaziferous layer was not examined.) It was presumed that these layers were formed during the deposition of egg white and differed from each other only in their

water content. The relative protein composition of each layer was identical with that obtained from homogenised egg white.

Table III

The distribution of proteins in the egg white

Protein	I	II	III
Ovalbumin	60.0	69.5	60.5
Conalbumin	13.8	9.0	16.0
Ovomucoid	14.0	12.9	12.0
Lysozyme	2.8	6.7	3.6
Globulins	8.9		
Ovomucin	-	1.9	-
Avidin	-	Trace	-

I - Longsworth et al. (1940). Data calculated as percent total protein, excluding ovomucin, obtained by Tiselius electrophoretic analysis.

II - Sørensen (1934). Data calculated as percent of total protein obtained by chemical methods.

III - Wetter et al. (1953). Data calculated as percent total protei obtained by immunological methods.

The figures obtained by the electrophoretic analysis using the Tiselius apparatus assumed that each electrophoretic peak represented a homogeneous protein. On more careful electrophoretic and chromatographic analysis of each of the purified proteins listed above,

a heterogeneous system has been demonstrated over a range of pH. The findings of these authors are tabulated below.

Table IV

Subfractions of egg white proteins

Protein	Components	%	Technique	Author
Ovalbumin	3,(A_1, A_2, A_3)	79:15:6	Tiselius electrophoresis	Cann (1949)
Ovalbumin	4		Chromatography	Tiselius et al. (1956)
Conalbumin	2,(C_1, C_2)	Variable	Tiselius electrophoresis	Longsworth et al (1940)
Ovomucoid	5,(O_1 -O_5)	30:45:17:6:2	Tiselius electrophoresis and ultracentrifuge	Bier et al. (1952)
Lysozyme	2 - 3	Variable	Chromatography	Tallan and Stein (1951)
Globulins	9		Immunoelectrophoresis	Kaminski (1957)
Ovomucin	Not examined			

The "pure" protein in each case has been shown to be a group of very similar proteins differing in the case of ovalbumin, for example in one phosphate residue per molecule (Perlmann, 1952). The ovomucoid subfractions were all closely similar electrophoretically and ultracentrifugally, and all had the same biological activity.

The analytical work has been carried out on the "pure" proteins and this may result in non-integral values for the number of amino

acid residues per molecule. This is why the phosphate analytical figure for ovalbumin is less than the theoretical two residues per molecule.

It should be remembered that more refined techniques may sho further subfractions in future work. The criterion of protein puri becomes increasingly more difficult to satisfy as techniques involvi more than one function of protein separation and detection are appli as for example in Kaminski's (1957) immunoelectrophoretic technique which identifies a protein fraction by its electrophoretic mobility and its specific antigenic activity. Even such a well recognised "pure" protein as insulin has now been shown to be heterogeneous (Harfenist, 1953). Colvin et al. (1954) sum up the situation in th following words; "It seems more correct to describe a native protei not in terms of a finite number of chemical entities, but as a population of closely related individuals which may differ either discretely or continuously in a number of properties".

A second complicating factor is the protein-protein interact which may take place during electrophoresis, causing abnormalities in the pattern (Longsworth et al. 1940).

The amino acid composition and physical constants of the fou main egg white proteins are tabulated below. The values have been compiled from the reviews of Tristram (1949), Fevold (1951) and Warner (1954). Ovomucin has not been extensively purified and is omitted from the table.

Table V

Physical and chemical analysis of egg white proteins

Protein	Ovalbumin	Conalbumin	Ovomucoid	Lysozyme
M.W.	45,000	87,000	27,000	14,800
pI	4.58	6.8	3.9	11.3
N%	15.76	16.3 - 16.6	13.3	18.6
Amide N%	1.04	1.04	1.0	1.8
Carbohydrate %	2.8	-	22 - 26	-
Amino acid residues/mole.				
Glycine	19	60	15	11
Valine	28	61	16	6
Alanine	25	43	11	10
Leucine	32	58	11	9
Iso-leucine	25	33	3	6
Proline	14	37	7	2
Phenylalanine	21	30	5	3
Half-cystine	2	26	16	5
Cysteine	5			
Arginine	15	38	6	11
Histidine	7	15	4	1
Lysine	20	60	12	6
Aspartic acid	32	87	28	20
Glutamic acid	52	70	15	4
Serine	36	52	11	10
Threonine	16	43	13	7
Tyrosine	9	22	5	3
Tryptophan	2.6	13	0 - 1	8
Methionine	16	12	2	2
N-terminal	None	Alanine	Alanine	Lysine
C-terminal	Proline	-	Phenylalanine	Leucine
No. of chains	?	1 - 9	1	1

The amino acid composition and physical constants for these four proteins are now comparatively well established. The interpretation of the N-terminal and C-terminal analysis as well as the determination of the number of peptide chains are not nearly so clear cut. The various possibilities in each case will be dealt with when the structure of the individual proteins is considered later. Some idea of the complexity of this type of analysis may be appreciated when it is reported by one author (Fraenkel-Conrat and Porter, 1952) that conalbumin contains a single chain, and another author (Fevold, 1951) maintains that there are nine chains.

From the analytical figures given in Table V, it will be seen that all the egg proteins are rich in the essential amino acids, and are thus an excellent source of nutritional protein. This factor is very important in the developing fertilised egg, the embryo depending on the egg yolk and the egg white proteins for its entire supply of raw materials. Throughout its embryonic life the young chicken lives in a closed system except for gas exchange. During the incubation period the rate of cell division and growth is enormous, the lipid in the yolk acting as an energy store for much of this metabolic activity. Unfortunately very little is known about the changes taking place in the egg white during incubation. Kaminski (1955, 1956) has studied the remaining egg white and the embryonic serum during growth and has clearly shown that some of the egg white

protein is incorporated into the serum and allantoic fluids.

The egg white contains very small quantities of enzymes; apart from lysozyme only tributyrinase, peptidase and catalase wer detected in small quantities by Lineweaver et al. (1948). The yo also showed a very low content of these three enzymes as well as a trace of amylase and phosphatase. This seems a remarkable state affairs since the developing embryo carries out a great deal of en activity in order to synthesise new tissue at the rapid rate at wh it grows. The presence of a tryptic inhibitor (ovomucoid) would suggest that trypsin is present during incubation for the enzymic breakdown of ovalbumin and other egg white proteins before these a metabolised by the young embryo for new protein synthesis. The dynamic changes taking place during incubation have not been studi enough at present to give a clear picture of what actually happens

The biological significance of the egg white proteins duri incubation has been a stimulus for further research, but the prese knowledge of the egg white proteins is still far from complete. Although ovalbumin contributes nearly 70% of the egg white protein specific biological function has been suggested, except the assump that ovalbumin acts as a store of first class protein which is metabolised by the embryo. Certain biological functions have, howe been proved to be associated with the other main proteins and thes are tabulated below:

Table VI

Biological significance of individual proteins

Protein	Biological function	Reference
Ovalbumin	Nutrition of embryo	
Conalbumin	Iron-binding	Alderton et al. (1946)
Ovomucoid	Tryptic inhibitor	Lineweaver and Murray (1947)
Lysozyme	Antibiotic	Fleming (1922)
Ovomucin	Haemagglutination inhibitor	Lanni et al. (1949)
Avidin	Biotin-binding	Eakin et al. (1941)

Conalbumin is thought to act in a similar manner to siderophil in its iron-binding and transporting function. It is also worth mentioning that the calcium required for the formation of bones is removed from the shell during incubation and transported in some way into the embryo's system. The mechanism of this transport is not understood, but calcium might possibly be bound to the proteins of the egg white in a similar way as the calcium in the serum is bound to the serum proteins (Sohjeide and Urist, 1956).

All the proteins of egg white so far examined (except ovomucin have been found to be immunologically active when injected as

antigens. By using the specific antibodies for the characterisatio of electrophoretically distinct components, Kaminski (1955-1957) has demonstrated the complexity of egg white and of the purer fractions obtained by crystallisation procedures. Further immunological cros reactions with laying hen's serum have demonstrated the presence of egg white proteins within the serum. This dynamic relationship between the egg white and serum proteins will be considered in a special section later.

Lysozyme has been found, apart from in the egg white, in blo serum, in tear ducts and in nasal secretion (Romanoff and Romanoff, 1949). Whether the lysozyme found in different parts of the body i in fact the same protein in each case or a slightly different protei but with the same biological activity is less certain. If the lysozyme of serum is identical with that of egg white, biosynthesis this protein cannot be confined to the oviduct alone. The functio of lysozyme in egg white is obviously an effective bacteriolytic agent. Meyer and Hahnel (1946) have shown that lysozyme enzymicall degrades the mucopolysaccharides of bacteria. The keratinous shell membrane also acts as a barrier for bacterial infiltration and, once this membrane is pierced, the egg soon becomes infected with bacteri demonstrating that the small quantity of lysozyme present is not sufficient by itself to prevent bacterial growth.

The egg white proteins of various species of birds have beer

examined by Bain and Deutsch (1947) employing Tiselius electrophoretic analysis. Although the same general composition was noted in all cases, it was found that the electrophoretic pattern obtained for whole egg white was characteristic for each species. Immunological analysis of the ovalbumins from one species of bird showed that, although the proteins were not immunologically identical within the species, they were closely similar. For example, it has been found that chicken ovalbumin was more closely related to turkey ovalbumin than it was to duck ovalbumin (Marrack, 1950). Hen ovalbumin cross-reacted with both duck and turkey ovalbumin. Generally speaking, proteins from species closely related phylogenetically will cross-reac more strongly than proteins of less closely related species.

Romanoff and Romanoff (1949) have drawn attention to the fact that the eggs of artrical birds, such as the pigeon, which have helple young at birth have much less protein and fat in the egg than the prococal birds which have young capable of looking after themselves at birth.

The mechanism of egg laying is a fascinating process even if it is not fully understood. A brief outline of the mechanism as describ by Romanoff and Romanoff (1949) will be given below. The question of whether the egg white proteins are synthesised in the oviduct or diffuse into the oviduct will be discussed in a later section describing the experimental work designed to investigate this problem.

The egg laying cycle

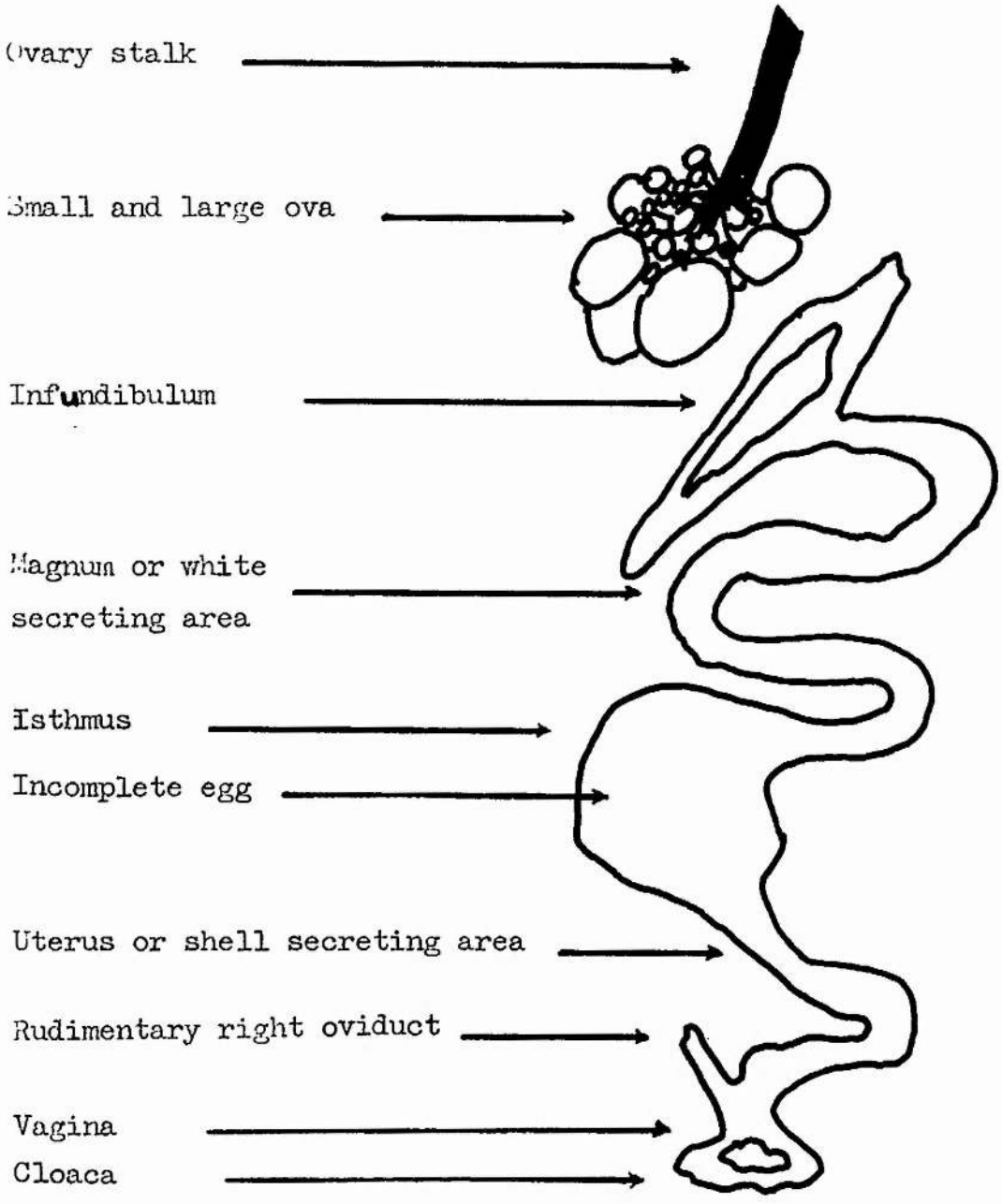


Fig.1

The hens oviduct and ovary during the egg laying cycle (Romanoff and Romanoff 1949).

At this stage the presence of the egg white proteins in the oviduct must be accepted, and the question of their origin disregarded. A diagrammatic representation of the structure of the ovary and oviduct is given in fig. 1.

The ovary contains a large number of small ova attached to the stalk of the ovary; at the same time a few larger ova are also present These large ova are in various stages of development so that each day the hen lays an egg, an ovum is fully developed and ready to leave the ovary. The proteins and lipid of the egg yolk are transported mainly in the blood to the ovary and deposited in concentric layers of "white" and "yellow" yolk to form an ovum. The yolk phosphoproteins, vitellin and lipovitellin, are rich in lipid and are held mainly as phospholipid as judged from the P:N ratio. These yolk proteins have been studied b Schjeide and Urist (1956) and found to be present in the blood of layin hens. Similar proteins were found in the serum of cocks injected with massive doses of oestrogens.

The first major event in the egg laying cycle is the cleavage of a fully developed ovum from the ovary stalk. The ovum falls into the infundibulum of the oviduct, and passes through the ostium into the magnum (white secreting area). The ovum leaves the ovary for the oviduct about twenty minutes after the previous egg has been laid.

The yolk remains for 2 - 3 hours in the magnum in which time the white is deposited. Conrad and Scott (1942) have demonstrated the

secretion of egg white from the goblet cells and the tubular cells which became enlarged before the yolk falls into the oviduct. The secretion of white is a reflex action initiated by the yolk falling into the magnum. If a marble is allowed to drop into the magnum just before the yolk normally enters, egg white will be secreted on the marble (Dr. D.J. Bell, Personal Communication). During the time the yolk remains in the magnum the secretion of the white takes place in a given order (Romanoff and Romanoff, 1949). Firstly a "mucin-lil chalaziferous layer is layed down, secondly a middle dense layer whicl is gradually converted to two distinct layers, the "inner thick" and "inner thin" layer, by rotation of the yolk and tightening of the strands of mucin during the passage through the magnum. This rotation twists the chalazae into their characteristic form.

The incomplete egg leaves the magnum and passes down the isthm to the uterus, taking about an hour to complete the journey. During this time the "outer thin" layer is formed by the infusion of a watery fluid.

The final 21 hours are spent in the uterus where the keratinous shell membrane and the shell are slowly deposited around the newly formed egg. It only remains for the hen to find a nesting place and deliver its egg, before starting the cycle again twenty minutes later. The effect of 24 hours of sunlight on battery hens appears to be an acceleration of the cycle with a more rapid accumulation of egg white in the oviduct.

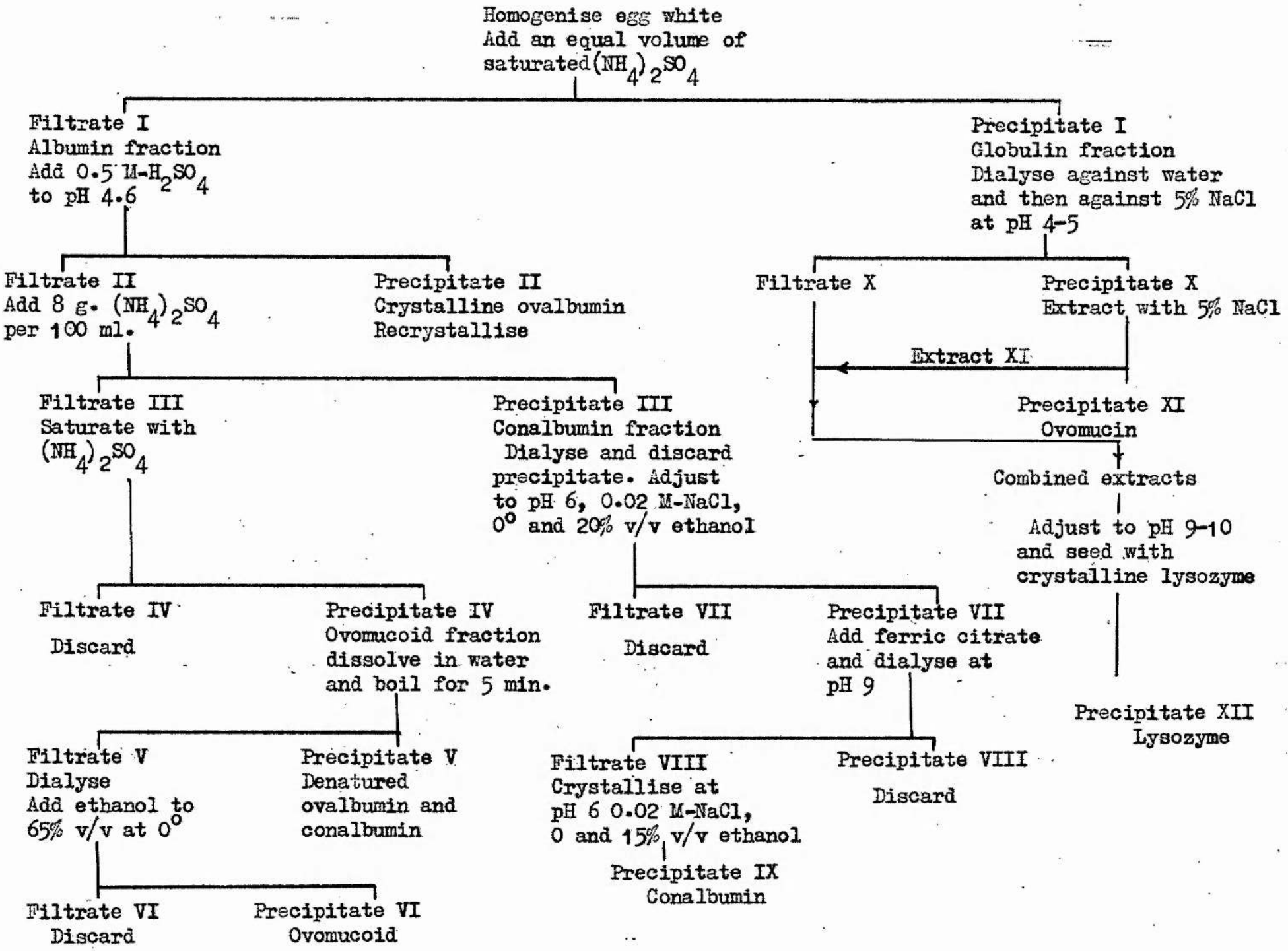

Homogenise egg white
Add an equal volume of
saturated $(NH_4)_2SO_4$
Filtrate I
Albumin fraction
Add 0.5 M-H_2SO_4
to pH 4.6
Precipitate I
Globulin fraction
Dialyse against water
and then against 5% NaCl
at pH 4-5
Filtrate II
Add 8 g. $(NH_4)_2SO_4$
per 100 ml.
Precipitate II
Crystalline ovalbumin
Recrystallise
Filtrate X
Precipitate X
Extract with 5% NaCl
Extract XI
Filtrate III
Saturate with
$(NH_4)_2SO_4$
Precipitate III
Conalbumin fraction
Dialyse and discard
precipitate. Adjust
to pH 6, 0.02 M-NaCl,
0° and 20% v/v ethanol
Precipitate XI
Ovomucin
Combined extracts
Adjust to pH 9-10
and seed with
crystalline lysozyme
Filtrate IV
Discard
Precipitate IV
Ovomucoid fraction
dissolve in water
and boil for 5 min.
Filtrate VII
Discard
Precipitate VII
Add ferric citrate
and dialyse at
pH 9
Precipitate XII
Lysozyme
Filtrate V
Dialyse
Add ethanol to
65% v/v at 0°
Precipitate V
Denatured
ovalbumin and
conalbumin
Filtrate VIII
Crystallise at
pH 6 0.02 M-NaCl,
0 and 15% v/v ethanol
Precipitate VIII
Discard
Precipitate IX
Conalbumin
Filtrate VI
Discard
Precipitate VI
Ovomucoid

Fig. 2.

The evidence obtained from electrophoretic analysis of the thre main physically distinct layers indicates that the egg white proteins are deposited all together in a homogeneous gel and not by different cells as individual proteins along the length of the oviduct (Forsyth and Foster, 1949). This was confirmed in the present work and will be described in the section of dynamic studies.

2. Physical Fractionation of Whole Egg White

For many years methods have been available for the physical fractionation of a single protein from whole egg white, however the other protein fractions had to be discarded. When the physical properties of the "pure" protein fraction obtained by these selective procedures had been studied, Warner (1954) was able to produce a comprehensive scheme for the fractionation of whole egg white into its five main protein fractions. The ammonium sulphate fractionation of egg white is given in fig. 2.

The protein fractions of ovalbumin, conalbumin and lysozyme obtained by this method may be further rigorously purified by special crystallisation procedures. The ovomucin fraction must be considered impure, since it becomes highly insoluble once the other proteins have been separated from it and cannot be further purified. This insolubl gel-like material has not so far been subjected to any critical analysis. Ovomucoid has not been crystallised, but the heat and

alcohol treatment effectively purify this fraction from the less stable proteins. As mentioned earlier, ovomucoid has been shown to be a complex of at least five components, all with tryptic inhibitor activity.

An alcohol fractionation procedure has been developed by Forsyth and Foster (1950) in which partially pure fractions are obtained. These fractions were then further purified as for the individual proteins. An outline of the main steps of the alcohol fractionation method is given in fig. 3.

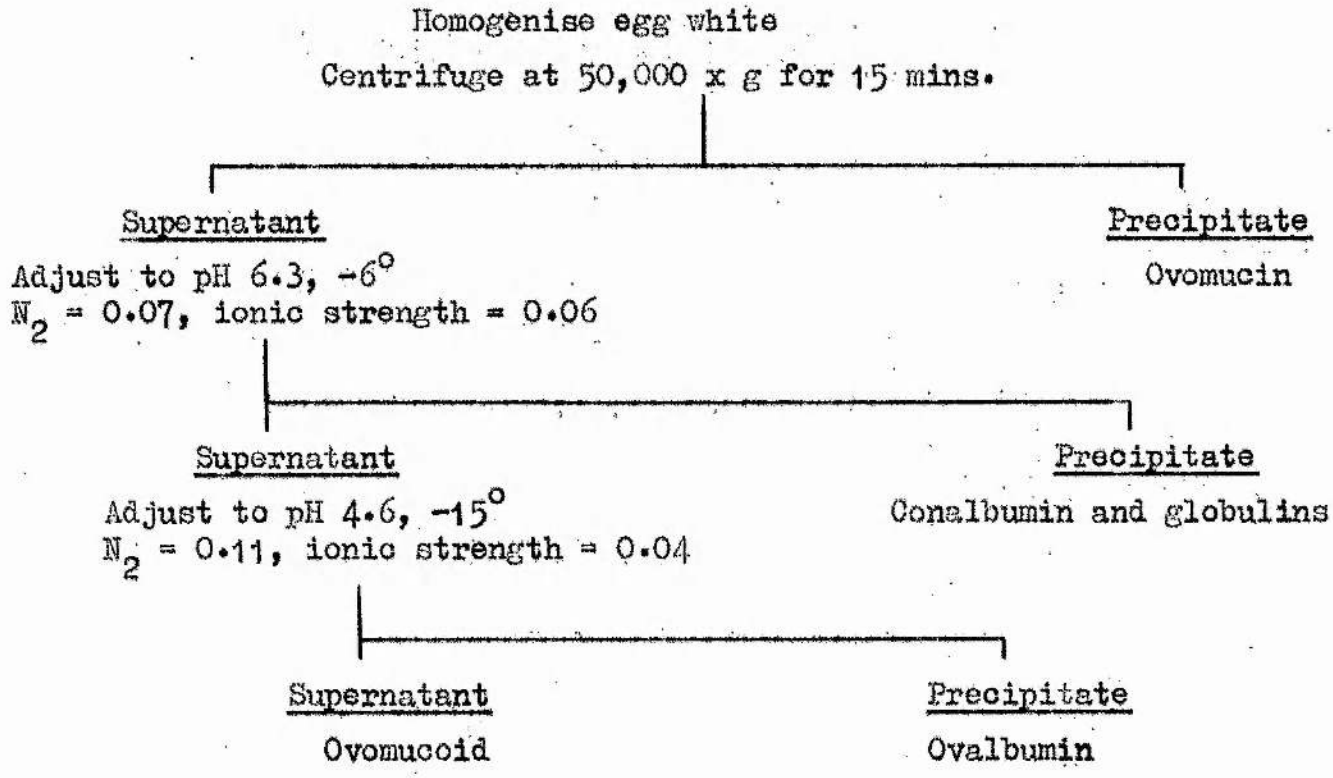


Figure 3.

N_2 indicates mole fraction of alcohol

Many attempts have been made to produce a suitable electrophoretic separation of protein mixtures for preparative purposes. The Tiselius moving boundary electrophoretic apparatus has found a very limited use as a preparative tool. Electrodialysis and the continuous paper electrophoretic technique appear to be the most useful methods so far developed. Two new methods may well be found to be of value. The first procedure has been developed by Porath (1954) using a vertical column packed with carbon or acetylated cellulose as support. The protein solution is applied at the top of the column, the electrophoresis carried out for a given time, after which the protein fractions are passively eluted by a buffer solution into a fraction collector. This method will be described in the experimental section.

The second fractionation technique has been worked out for the serum proteins by Sober et al. (1956). This method depends on the absorption of the protein fractions on a special ion-exchange resin prepared from cellulose. The protein fractions are eluted by buffers of increasing ionic strength, and collected in a fraction collector.

Both these methods have the great advantage of being able to cope with large quantities of protein for preparative purposes, but neither has so far been applied to egg white proteins.

The refined electrophoretic techniques employed by Smithies (1955) and Kaminski (1955-1957) will be described in detail in the

experimental section. These techniques have no preparative value except in cases where immunological analysis may be employed.

3. Purified Egg White Proteins

In this section the present state of knowledge of the individual egg white proteins will be reviewed. Throughout this section it must be remembered that a "pure" protein refers to a protein which has been purified within the limits set by modern preparation techniques.

3.1. Ovalbumin

3.1.1. Preparation. Ovalbumin was first crystallised by Hofmeister (1889) from homogenised egg white by salting out with ammonium sulphate. This discovery is the basis of all the modern methods employing ammonium sulphate. By allowing an aqueous solution of egg white containing 50% saturated ammonium sulphate to evaporate slowly, large crystals could be obtained. Purification of ovalbumin could be achieved by repeated crystallisation.

Hopkins and Pinkus (1898) and Hopkins (1899) improved the method by adding 10% acetic acid to the ammonium sulphate solution; crystallisation was then accelerated to a few days rather than the weeks of slow evaporation required by the previous method.

Sørensen and Høyrup (1915-1917) produced two papers remarkable for their detail and meticulous accuracy. This work has now become the classic preparation of ovalbumin. The pH of the solution was calculated

from the binding of NH_4^+ and SO_4^{--} to the ovalbumin molecule, the iso-electric point being 4.6 - 4.8 in which range no ammonium sulphate is bound. For crystallisation, sulphate ions were found to be essential in the ratio of 1:125 : : SO_4^{--} :protein N. The optimum conditions for crystallisation were pH 4.58 at half saturation with ammonium sulphate, after a little stirring rapid crystallisation took place. In order to avoid NH_4^+, the ovalbumin was repeatedly crystallised from sodium potassium sulphate, the total nitrogen estimation by Kjeldahl analysis being 15.62%. The crystalline ovalbumin contained 18% water, firmly bound within the molecule.

Cole (1932) modified Sørensen and Høyrup's method by using buffered ammonium sulphate pH 4.7 to initiate crystallisation. This method was quick and it was easy to control the conditions when the pH was maintained at 4.7 throughout.

Sodium sulphate was used by Keckwick and Cannan (1936) in order to avoid the use of ammonium salts. Precipitation was carried out at 30° and pH 4.6 with sodium sulphate followed by recrystallisation under similar conditions.

The practical details of all the methods described above, except Hofmeister's, will be given in the experimental section and their relative merits discussed.

The purity of ovalbumin as a homogeneous protein has been a classic claim of biochemists. Haemoglobin and ovalbumin have until

recently been accepted as the two proteins which could unquestionably be described as pure (Schmidt, 1932). Longsworth et al. (1940) demonstrated by Tiselius electrophoresis that ovalbumin contained at least two protein fractions under certain conditions of pH and ionic strength. The faster component was classified as A_1 and the slower, minor component as A_2. The proportions of A_1:A_2 varied with the preparation and the age of samples. Cann (1949) demonstrated the presence of a third electrophoretic component A_3, the proportions were A_1:A_2:A_3::76:18:6. Similar electrophoretic results were reported by Perlmann (1950), as well as by Kaminski (1957), who used agar gel immunoelectrophoresis. Four components have been described by Tiselius et al. (1956) using calcium phosphate gel chromatography. These fractions did not seem to correspond to A_1, A_2 and A_3 in their relative proportions.

Linderstrøm-Lang and Ottesen (1949) suggested that the non-integral number of phosphorus atoms per molecule might be accounted for by two components differing in the number of phosphate residues. This theory has been proved by Perlmann (1952) using prostate and intestinal phosphatases to hydrolyse the phosphate esters. At each stage in the enzymic dephosphorylation of ovalbumin one phosphate residue per molecule was removed. The relationship between the ovalbumins and their phosphate residues is given below in table VII.

Table VII

Distribution of phosphate residues in ovalbumins

(Perlmann, 1952)

Composition	Component A_1	A_2	A_3
% composition	76	18	6
PO_4 residues per molecule	2	1	0
PO_4 residues contributed per molecule ovalbumin	1.52	0.18	0
Calculated PO_4 residues per molecule ovalbumin		1.70	
Found PO_4 residues per molecule ovalbumin		1.70	

3.1.2. Composition. The amino acid analysis figures for ovalbumin are given in table V (p. 8). The analytical figures for the ionisable groups are in close agreement with the titration analysis carried out by Cannan et al. (1941). These figures are compared in table VIII.

Table VIII

Comparison of ionising residues per mole by analysis and titration

Component	Analysis	Titration
Arginine	15	14
Histidine	7	5
Lysine	20	22
Total basic groups	42	41
Aspartic acid	32	
Glutamic acid	52	
Amide	33-39	51
Free acidic groups	45-51	
Phosphate residues	1·7	
Total acidic groups	49-54	51

The titration figures given for the anions and the cations do not give any direct indication of any terminal α-amino or α-carboxyl residues. The analytical figure for the total basic groups is actually greater than the value obtained by titration. The uncertainty of the number of amide residues makes it impossible to determine whether the titration of free acidic groups includes a terminal α-carboxyl residue.

The presence of a terminal amino residue in ovalbumin has been the cause of considerable controversy. Porter (1950) showed that ovalbumin contained no N-terminal residue reacting with the Fluorodinitrobenzene (FDNB) reagent of Sanger (1945). Porter considered that there was either no N-terminal residue or the N-terminal residue was masked in some way, perhaps by a carbohydrate moiety. The absence of an α-dinitrophenyl (DNP) amino acid in the hydrolysate of DNP-ovalbumin has since been confirmed by many workers. It must be remembered, however, that certain DNP-amino acids are extremely labile in acid (Sanger, 1945 and Porter and Sanger, 1948). Acid lability will be discussed in greater detail in the experimental section.

Steinberg (1952) claimed that carboxypeptidase liberated a C-terminal residue from ovalbumin. Later the same author (1953) found that carboxypeptidase would only liberate alanine from ovalbumin after an initial enzymic reaction had taken place. This reaction was caused by a contaminating enzyme in the carboxypeptidase preparation. The claim of alanine as a C-terminal residue has therefore been withdrawn.

It was later found that carboxypeptidase did not attack proline as a C-terminal residue, a special enzyme, prolidase, being required for this amino acid (Anfinsen and Redfield, 1956). Niu and Fraenkel-Conrat (1955) demonstrated the presence of a C-terminal proline residue in ovalbumin using their hydrazinolysis method.

Since ovalbumin has a C-terminal residue, but no N-terminal

residue, the structure of the protein is difficult to describe with certainty. Anfinsen and Redfield (1956) suggested a "six-shaped" structure, but without giving any reason for their choice, except that it was similar to one of the two possible structures suggested by Linderstrøm-Lang (1952). An attempt has been made to verify this theory in the structural studies carried out in the present work.

The presence of a carbohydrate moiety in ovalbumin has long been known. Sørensen (1934, 1938) gave the carbohydrate composition as 1·4% glucosamine and 1·7% mannose. Neuberger (1938) found that ovalbumin contained a carbohydrate moiety which was an integral part of the protein molecule, 3·5% by weight, with a molecular weight of 1250. This residue was thought to be made up from four mannose and two glucosamine residues linked together. As mentioned in the previous paragraphs Porter considered the carbohydrate residue to be masking the true N-terminal residue. This would infer that the carbohydrate residue acted as a prosthetic group attached to the N-terminal end of the peptide chain, rather than situated within the backbone chain of ovalbumin. This view was opposed by Cunningham et al. (1957).

3·1·3. Transformation of ovalbumin to plakalbumin. Linderstrøm-Lang and Ottesen (1947) first showed that a salt-free ovalbumin solution contaminated with B. subtilis gave rise to a new protein, plakalbumin, by proteolytic cleavage of the ovalbumin molecule. The ovalbumin which had originally crystallised from ammonium sulphate as needles was

converted by the bacterial contaminant to plakalbumin which crystallised in large plates. The enzyme responsible for this cleavage was isolated and called subtilisin by Güntelberg and Ottesen (1952).

Plakalbumin appeared to be a protein closely similar to ovalbumin in chemical composition, molecular weight and electrophoretic mobility. The proteolytic cleavage involved the splitting of a hexapeptide or a quadrapeptide and a dipeptide from ovalbumin. The structures of these peptides were determined by Ottesen and Wollenberger (1952) and found to be as follows:

NH_2 - Ala - Gly - Val - Asp - Ala - Ala - COOH

NH_2 - Ala - Gly - Val - Asp - COOH

NH_2 - Ala - Ala - COOH

The loss in total nitrogen during this enzymic change was 1.4% of which 90% could be accounted for by the nitrogen of the dialysed peptides. No change was observed in the carbohydrate or phosphorus content. The molecular weight of plakalbumin was found to be approximately 44,700, i.e. 300 less than ovalbumin by osmotic pressure measurements (Güntelberg and Linderstrøm-Lang, 1949). The presence of plakalbumin in an ovalbumin solution greatly increased the solubility of the ovalbumin. This increased solubility has been used as an indication of the proportion of ovalbumin converted to plakalbumin (Linderstrøm-Lang, 1952). This phenomenon prevented the

fractional crystallisation of a mixture of plakalbumin and ovalbumin with ammonium sulphate solution. Preparation of plakalbumin was carried out after the complete enzymic degradation had taken place.

The electrophoretic mobility of plakalbumin was slightly more positive between the iso-electric point and neutral pH than that of ovalbumin (Perlmann, 1949). Electrophoretic studies by Perlmann (1952) showed that plakalbumin was heterogeneous containing three components corresponding to those found in ovalbumin. Dephosphorylation by phosphatases showed that the same sequence of events took place in ovalbumin and plakalbumin, this is diagrammatically shown in fig. 4.

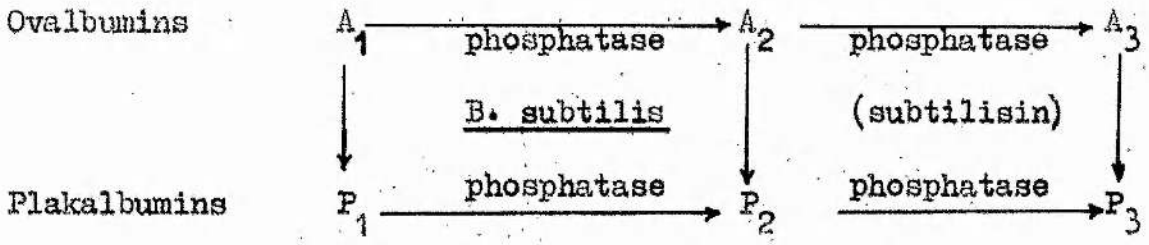


Fig. 4

Interrelationships of ovalbumins and plakalbumins.

The enzymic action of B. subtilis appeared to have at least three distinct phases. The first phase was the proteolytic cleavage of a peptide bond within the ovalbumin molecule to form a non-crystalline intermediate. The second stage was the liberation of the three peptides from the exposed peptide chain, the resulting protein being readily crystallised in the typical plates of plakalbumin. These first two ste

have been clearly demonstrated by Ottesen (1956) using crystalline subtilisin and following the reaction with the pH-stat. The first step was relatively rapid whilst the second was much slower. After prolonged exposure to the subtilisin, plakalbumin was further degraded in the third phase to an amorphous material. Photographs showing these three stages as obtained by the present author will be presented in the experimental section.

The conversion of ovalbumin to plakalbumin was not thought to be confined to B. subtilis. The action of subtilisin was confined to a few proteins which have a suitable peptide bond for attack. Linderstrøm-Lang (1952) pointed out the similarity between the ovalbumin - plakalbumin transformation and the action of trypsin or trypsinogen.

3.1.4. Configuration. The conversion of ovalbumin to plakalbumin raised several questions concerning the structure of ovalbumin. The main problem was to determine the original position of the hexapeptide within the protein molecule. The absence of an N-terminal residue in ovalbumin excluded the possibility of enzymic cleavage of the hexapeptide from the amino end of the molecule, this was supported by the evidence that no carbohydrate was released in the formation of plakalbumin. The C-terminal residue could not be involved since proline did not appear in the liberated peptides. These arguments would suggest that the cleavage took place within the peptide chain of ovalbumin. For such a cleavage two peptide bonds must be hydrolysed. The reaction studied by Ottesen

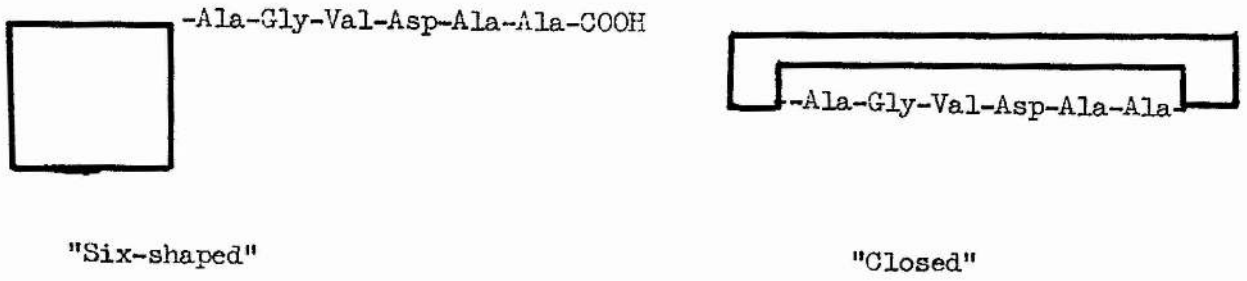
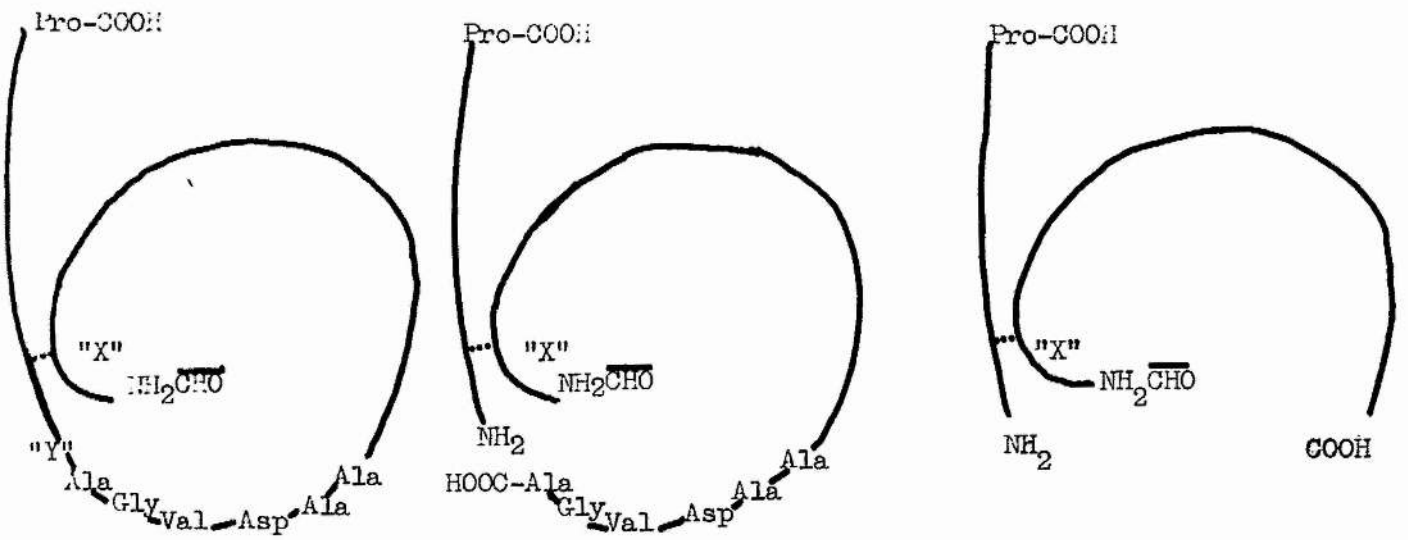


Fig.5

Postulated structures of ovalbumin, (Linderstrøm-Lang, 1952).



Modified "six-shaped" structure → Non-crystalline intermediate → Plakalbumin and peptides

Fig.6

Suggested action of B. subtilis on ovalbumin.

(1956) did in fact consist of two steps, in each case a peptide bond was hydrolysed.

Linderstrøm-Lang (1952) suggested two possible structures for ovalbumin, but the "six-shaped" structure was preferred by Anfinsen and Redfield (1956). These two structures are diagrammatically represented in fig. 5.

From the present work it will be suggested that a modified "six-shaped" structure accounts for the experimental observations very well. This structure is preferred by the present author since proline has recently been demonstrated as C-terminal residue (Niu and Fraenkel-Conrat, 1955). A possible sequence of events in the ovalbumin-plakalbumin transformation is suggested in fig. 6 using this modified structure as a model.

In the native ovalbumin the N-terminal residue is thought to be masked and an internal bridge ('X') thought to hold the cyclic structure in the "six-shape". If the reaction sequence in fig. 6 is correct, plakalbumin should be built up of two peptide chains linked together by the bridge 'X'. If this were in fact the case plakalbumin would have a free α-carboxyl as well as a free proline residue and a free and a masked N-terminal residue. The investigations of Ottesen (1956) and Steinberg (1954) seem to fit this hypothesis. Ottesen (quoted by Linderstrøm-Lang, 1952) detected DNP-serine as N-terminal residue in plakalbumin treated with Sanger's reagent. The yield was less than

one residue per molecule which might be accounted for by the acid lability of this derivative. During the reaction there was no exposure of the ovalbumin N-terminal residue, which would be expected to remain masked if this hypothesis was correct.

Steinberg (1954) found that pure crystalline carboxypeptidase did not attack ovalbumin. It will be remembered that C-terminal proline is not released by carboxypeptidase, and that proline is in fact the C-terminal residue. However, if a small amount of crystalline subtilisin was added to the preparation of carboxypeptidase and ovalbumin, one residue of C-terminal alanine was released. This evidence appears to indicate that the hexapeptide is situated in the closed ring of the "six-shaped" molecule of ovalbumin. In this reaction an intermediate "plakalbumin C" and alanine were found together. Plakalbumin C is thought to be ovalbumin with the peptide bond at 'Y' (fig. 6) opened by subtilisin. Alanine could then be liberated from the C-terminal end by carboxypeptidase.

3.1.5. Denaturation studies. The previous section described the enzymic modification of ovalbumin and its possible significance in connection with the structure of the molecule. Some idea of the configuration of a native protein may be obtained from observing the changes which occur during denaturation. Although a great deal of work has been carried out on the denaturation of ovalbumin, very little information has been obtained that can be used in postulating

a particular structure. The criteria of denaturation studied by many different workers have not all been justifiable, especially since it is now thought that denaturation of any protein by a variety of agents need not necessarily follow the same course.

Denaturation may be defined as a process which alters the structure of the native protein without breaking any primary covalent bonds. This definition gives no indication of the degree of denaturation. During the process of denaturation the protein usually uncoils with a concurrent increase in viscosity, laevorotation and availability of masked groups consequent upon a disruption of the helical structure. The protein usually becomes less soluble at the iso-electric point and loses any biological activity it may have possessed. There are of course exceptions, such as insulin which when fully extended in the denatured form retains its full biological activity. The denaturation process may be visualised as the breaking of hydrogen bonds between the peptide links within the protein chain (secondary bonds) and breaking interchain bonds, other than disulphide bridges (tertiary bonds). Since the secondary and tertiary bonds are the main source of structural rigidity in the native molecule, denaturation tends to cause uncoiling of the protein helix and randomization of the peptide chains. The uncoiling process enables selective reagents to react with certain masked groups which fail to react with the same reagents in the native protein.

The literature on denaturation is very extensive, two excellent reviews are presented by Neurath et al.(1944) and Putnam (1953). The helical structure of native proteins was developed from the study of denaturation by Mirsky and Pauling (1936) and Pauling and Corey (1951)

Hopkins (1930) demonstrated that ovalbumin gave a positive nitroprusside reaction when dissolved in urea solution, but gave no reaction for thiol residues in aqueous solution. The thiol group was thought to be masked in the coiled native protein, but after treatment with urea the protein uncoiled, exposing the masked residues. Various specific reagents were developed for thiol estimations such as p-chloromercuribenzoate, dichloroindophenol and porphyrindin. The reactivity of thiol groups in denatured ovalbumin was not necessarily the same for each method of estimation (Neurath et al., 1944). The steric hindrance of the amino acid side chains along the protein backbone prevented some of the larger reagents coupling with thiol residues which were reactive towards smaller molecules. Greenstein (1938) clearly demonstrated that guanidine hydrochloride was much more effective than urea, giving a greater total amount of thiol liberated, even at very low concentration of guanidine hydrochloride at which urea had no effect at all. Greenstein (1939) further showed that certain guanidine salts had differing ability in exposing thiol groups, guanidine hydrochloride was more effective than the corresponding bromide, iodide and thiocyanate respectively.

The complexity of protein denaturation may be appreciated when it

is realised that a protein denatured by different denaturants may yield products which have a varying number of exposed thiol groups when estimated by the same thiol reagent. Secondly, a single denatured protein preparation may have a different number of exposed thiol residues when measured by two different thiol reagents.

It would seem that although denaturation exposed masked groups suc as phenolic, thiol, disulphide and ε-amino groups of basic amino acid residues , the extent to which these groups became available to specific reagents is not necessarily the same degree to which denaturation has progressed. The availability of masked groups should be an indication rather than a criterion of the extent of denaturation (Bull, 1940). Perhaps the best criteria are obtained by physical methods such as the increase in viscosity and laevorotation, which are directly related to the uncoiling of the protein chain and not to the individual groups. It may be possible for a protein to contain all its masked thiol groups in close proximity to each other. A small amount of uncoiling in this area would expose all the thiol groups but not necessarily cause complete denaturation.

Mirsky and Anson (1936) claimed that heat denaturation of ovalbum was an "all-or-none" reaction. They found that partially denatured ovalbumin could be precipitated at the iso-electric point, the filtrate had no exposed thiol groups whilst the thiol groups of the precipitate were fully exposed. The protein was either native or fully denatured,

a partially denatured solution being made up of a mixture of the two forms. Anson (1940) decided that in heat denaturation of methaemoglobin, insolubility at the iso-electric point was not a good criterion of denaturation.

It is now considered that denaturation is unlikely to be an "all-or-none" reaction but probably a stepwise process (Putnam, 1953). The kinetic studies carried out by Gibbs (1952) suggested that a stepwise process took place in the acid and alkaline denaturation of ovalbumin. At each step a proton dissociated to give a labile intermediate in equilibrium with the other dissociation steps. Six dissociations have been suggested in the case of ovalbumin, each intermediate was thought to be converted to the denatured protein. The rates at which these intermediates denatured depended on their labilities. The thoery of absolute reaction rates (as developed by Steinhardt, 1937) was used by Gibbs to interpret his kinetic studies. This hypothesis explained the abnormally high energy values obtained in the analysis of protein denaturation kinetics. Gibbs claimed the dissociation steps explained the importance of pH and temperature in the denaturation of ovalbumin.

Simpson and Kauzmann (1953) - see also Schellman et al. (1953) and Frensdorff et al. (1953) - examined the change in laevorotation and viscosity of ovalbumin solutions dissolved in denaturants. Kinetic analysis of the graphs obtained by plotting the rotation observed in ure

and guanidine hydrochloride solutions against the time of reaction indicated that two distinct processes were taking place. The first process was a rapid uncoiling of the protein molecule which was complete in about three hours. The second slow reaction was apparent after twenty-four hours and was less marked, and this was shown to be the aggregation of the extended peptide chains. Thiol reagents inhibite aggregation. It was suggested that intramolecular disulphide groups exchanged with thiol residues in adjacent peptide chains, forming intermolecular disulphide bridges. The optimum pH for gel formation from concentrated solutions of ovalbumin in urea was demonstrated to be pH 9.0, far above the iso-electric point of the protein. The importance of thiol residues in aggregation would account for this high optimum pH, since the thiol residue ionises at pH 10. Gel and aggregate formation were found to be irreversible and showed a pronounced decrease in the number of thiol groups reacting with thiol reagents.

Kauzmann et al. (1953) used thiol reagents to dissociate the two processes and were able to study urea denaturation as an uncoiling of the ovalbumin molecule in the absence of the secondary reaction. Denaturation was found to be irreversible and inhibited by glucose. The rate of uncoiling, as measured by the viscosity and optical rotation was faster at 20° than at 0° or 30°. This rate was independent of the protein concentration and was proportional to the fifteenth power of

the urea concentration. The authors concluded that at 20° an unstable equilibrium mixture of complexes containing ovalbumin and urea existed. These complexes easily denatured, but at the same time dissociated into urea and native ovalbumin on raising the temperature above 20°. This theory is very similar to the earlier interpretation of Hopkins (1930).

Kauzmann and his colleagues considered that ovalbumin formed at least five unstable urea complexes, before becoming completely denatured These unstable intermediates may be similar to those described by Gibbs (1952) in connection with denaturation caused by extremes of pH.

Recent electrophoretic analysis of ovalbumin dissolved in various different buffer solutions indicated that there may well be an equilibrium mixture of several components in the native state (Cann and Phelps, 1957). These components do not differ in their chemical composition, such as a phosphate residue as in ovalbumins A_1, A_2, A_3, since a reversible equilibrium of this nature can hardly be visualised by merely changing the ionic strength. The significance of this observation is not understood, perhaps it is in line with the new theory of the "motility of proteins" (Linderstrøm-Lang, 1957; Ciba Foundation Lecture). This indication of a complex equilibrium between native protein molecules in aqueous solution further complicates the original idea that a pure protein must be homogeneous by all criteria and that the denaturation process is a well defined reaction between native and

fully denatured molecules.

Experimental studies on the increasing availability of ε-NH_2 groups of lysine during progressive denaturation of ovalbumin will be described, the results being in agreement with the "six-shaped" structure. The chemical analysis of the structure was confirmed by a study of the physical changes taking place simultaneously, viz. optical absorption and rotation and viscosity.

3.2. Conalbumin

Osborne and Campbell (1900) described conalbumin as a non-crystallisable albumin, soluble in 50% saturated ammonium sulphate and coagulating at a lower temperature than ovalbumin. The stability of conalbumin to surface denaturation enabled it to be purified from a mixture of this protein and ovalbumin.

3.2.1. Preparation. Preparation of conalbumin from egg white usually employed the "albumin" fraction obtained from half saturation with ammonium sulphate which contained ovalbumin, conalbumin and ovomucoid. Conalbumin was then selectively precipitated by lowering the pH to 4.0 and adding 1% w/v sodium chloride (Alderton et al. 1946). Longsworth et al. (1940) examined the acid-precipitated material by electrophoretic and ultracentrifugal analysis and found two components, C_1 and C_2. Whole egg white contained only C_1. C_2 was described as the acid modification of conalbumin which was stable below pH 4.0, whereas C_1 was stable above this range. The two conalbumins in the acid preparation were found to be present in varying proportions over a

range of pH the iso-electric points being 5.8 and 6.0 for C_1 and C_2 respectively. Bain and Deutsch (1948) used an alcohol fractionation procedure in which the solution was maintained above pH 4.0. The product obtained was electrophoretically homogeneous and identified as C_1, the material being then converted to a mixture of the two conalbumins by acidification. Cann and Phelps (1954) have demonstrated the presence of four electrophoretically distinct modifications of conalbumin.

Warner and Weber (1951) crystallised a pink iron-conalbumin comple: from alcohol at low temperature. The metal was removed from the iron complex by treatment with ion-exchange resins and citric acid, the iron-free conalbumin crystallising from cold alcohol.

3.2.2. Composition. The metal-binding power of conalbumin was first observed by Schade and Caroline (1944) in connection with the antibacterial activity of egg white. Warner and Weber (1953) examined the iron-binding activity of the protein and found that two iron atoms were bound per molecule. Copper and zinc were also bound to form metal complexes with the protein but each was less strongly held than iron. The metal required the presence of bicarbonate ions before it could be coupled to the protein in the same way in which siderophilin of human serum required these ions for the transport of iron. Warner and Weber suggested that the iron was attached to the phenolic

residues, since the absorption maximum at 280 mμ was altered when conalbumin was converted to the iron complex having a maximum at 470 mμ. The metal-binding capacity reached a maximum for one metal ion bound in a minimum molecular weight of 38,000. The molecular weight of the dimer (76,000 assuming two metal ions per molecule) was slightly less than the value of 87,000 obtained from diffusion and sedimentation analyses quoted by Fevold (1951).

The difference between the titration analysis and amino acid composition suggested that there were nine free α-amino groups (Fevold, 1951). Since the total nitrogen value (16.3 - 16.8%) was not very well established, the amide nitrogen (1.04%) may also be questionable. A small variation in the amide analysis would affect the number of free carboxylic acid residues and consequently give an inaccurate value for the number of peptide chains, as suggested from the difference between titration and amino acid data. Fraenkel-Conrat (1951) and Fraenkel-Conrat and Porter (1952) detected a single alanine residue per molecule in the N-terminal position. This indicated that the protein was made up from one peptide chain, a more acceptable structure than the nine chains originally suggested.

3.2.3. Biological activity. Hectoen and Cole (1928) studied the antigenic activity of conalbumin, and demonstrated the presence of this protein in the serum of fowls. Kaminski (1955, 1957) has used this antigenic activity to identify conalbumin which had been separated from

the other egg white proteins by agar gel electrophoresis. Kaminski and Durieux (1954, 1956) examined egg white, embryonic fluids and serum, as well as the serum of adult fowls by immunoelectrophoresis. In all cases conalbumin was found to be present.

The problem arises as to how the conalbumin passes from the egg white into the body fluids of the embryonic chick since it is the specific iron-binding protein which is essential in the embryo when the synthesis of haemoglobin begins to take place. It may be wondered how a huge molecule with a molecular weight greater than any other egg white protein can be selectively passed through the embryonic membranes. A similar problem is the presence of conalbumin in the serum of cocks as well as hens. Although the protein may be considered to pass from the oviduct to the hen's blood, this suggestion can not be accepted in the case of cocks. It would appear that some other site of conalbumin synthesis exists, apart from the oviduct.

3.3. Ovomucoid

3.3.1. Preparation. Mörner (1894) first demonstrated that a heat stable glycoprotein could be prepared from egg white by thermal coagulation of the other heat labile proteins. This protein, ovomucoid was further purified by alcohol precipitation in the cold.

Two methods were developed for the purification of ovomucoid. Lineweaver and Murray (1947) employed trichloracetic acid and cold alcohol precipitation; Fredericq and Deutsch (1949) used trichloracetic

acid and cold acetone in a similar fractionation procedure.

3.3.2. Composition. Fevold (1951) quoted the molecular weight to be 27 - 29,000. Sørensen (1934) estimated the carbohydrate moiety to be about 9.2%. The analysis of the sugars has been carried out by Dixon (1955) using ion-exchange resins instead of mineral acids for hydrolysis. The carbohydrate moiety was shown to contain glucosamin N-acetyl glucosamine, galactose and mannose. The amino acid analysis is given in Table V (page 8).

Very little is known about the structure of ovomucoid. Fraenkel-Conrat and Porter (1952) employing the DNP-technique showed that the protein had a single N-terminal alanine residue. Traces of an unidentified component were also found which may have been derived either from the degradation of DNP-alanine or DNP-amino sugars which are known to be unstable in the presence of carbohydrate during hydrolysis. The C-terminal residue was shown to be phenylalanine by Pénasse et al. (1952).

3.3.3. Biological Activity. Ovomucoid has been shown to be immunologically active and may be detected in the serum of a chick embryo (Kaminski and Durieux, 1954). The protein was found to remain in the embryonic serum for about 20 days, when it was replaced by a serum α-globulin having much the same characteristics as ovomucoid. No attempt has been made to identify the anti-tryptic protein of fowl serum with the α-globulin described by these authors.

Fraenkel-Conrat et al. (1949) have clearly shown that the inhibition of trypsin by ovomucoid was abolished by the acetylation of trypsin before incubation with ovomucoid. Acetylation was thought to protect trypsin from its inhibitor. Laskowski and Laskowski (1954) have reviewed the naturally occurring tryptic inhibitors, and it is interesting to note that Launoy (1918) found an inhibitor in fowl serum.

3.4. Lysozyme

Fleming (1922) described a bacteriolytic agent found in egg white which he named lysozyme; the same agent was reported to occur widely in body fluids. Alderton et al. (1945) showed that lysozyme and the globulin G_1 described by Longsworth et al. (1940) were identical. Epstein and Chain (1940) were able to show that the protein was an enzyme which lysed the specific carbohydrate present in the cell walls of susceptible bacteria.

3.4.1. Preparation. Alderton et al. (1945) purified lysozyme by the selective adsorption of the basic protein on bentonite. The other egg white proteins were eluted with 5% aqueous pyridine at pH 7.5, leaving lysozyme on the bentonite. The basic protein was eluted by lowering the pH to 5.0, followed by dialysis and crystallisation from 5% sodium chloride. Two distinct types of crystals were obtained, below pH 6.0 lysozyme chloride crystallised as tetragonal bipyramids whilst above this pH needle-shaped crystals were obtained. Tallan and Stein (1951, 1953) used cation-exchange resins to purify crystalline lysozyme and

separated three components all with the same biological activity. Raacke (1956) confirmed these results by starch electrophoresis. The relative amounts of the three components depended on the method of preparation and the storage time of the lysozyme. The similarity between lysozyme and ovalbumin in this respect is noteworthy.

3.4.2. Composition. The amino acid composition of lysozyme is given in Table V (page 8). The basic nature of the protein is at once apparent from the number of basic amino acids and the masking of the acidic residues by amide groups. The molecular weight has been determined by a number of methods (see Warner, 1954). The most acceptable figure derived from the analytical and osmotic pressure measurements seems to be 14,800.

3.4.3. Configuration. The structure of lysozyme has been extensively studied by sequential amino acid analysis. Two slightly different structures have been proposed by Thompson (1955) and by Thaureaux and Archer (1956). Both groups of workers agree in all but a few minor details. Fraenkel-Conrat (1951), Green et al. (1951) and Thompson (1951) have all shown that lysine is the N-terminal residue, and quantitative results were interpreted to signify one terminal residue per molecule (M.W. 14,800). The complexity of the hydrolytic products of DNP-lysozyme have been described by Schroeder (1951). At least four artifacts were found as well as di-DNP-lysine and corresponding DNP-lysyl peptides. Considerable degradation of di-DNP-lysine was

reported during the hydrolysis of the terminal di-DNP-lysyl-valyl residue. Similar observations will be described in the section dealing with the analysis carried out on DNP-lysozyme in the present work. Harris (1952) reported a C-terminal leucine residue.

A single chain is visualised for the lysozyme structure from the end-group analyses. Urea denaturation studies carried out by Fraenkel-Conrat (1949) and Leonis (1956) showed that the unreactive phenolic and disulphide groups of the native protein could be unmasked, but that the enzymic activity was unaltered by denaturation. Lysozyme was similar to insulin in its low molecular weight and retention of biological activity even though denatured.

3.5. Ovomucin

The main glycoprotein found in egg white was called ovomucin. The insoluble nature of this glycoprotein in the absence of other proteins prevented its purification. The literature contains very little information about ovomucin. Romanoff and Romanoff (1949) suggested that ovomucin was the main constituent of the chalazae.

3.6. Avidin

Avidin is the biotin-binding basic protein of egg white; the trace amounts of this protein make it an unattractive study for protein chemists.

PART II

THE FRACTIONATION AND PURIFICATION OF THE EGG WHITE PROTEINS

1. Whole Egg White

Warner's (1954) method was used to fractionate the five main egg white proteins from two batches of eggs. A diagrammatic representation of this fractionation technique is shown in Fig. 2. The whites were carefully separated from the yolks and chalazae, homogenised by squeezing through fine butter muslin three times, an equal volume of saturated ammonium sulphate added and the mixture gently stirred for two hours. The globulin fraction was centrifuged off (precipitate I) and dialysed against water. The albumin fraction which contained mainly ovalbumin, conalbumin and ovomucoid was separated as filtrate I.

1.1. Ovalbumin

The pH of the albumin fraction was lowered to 4.6 with small additions of 0.5 N-sulphuric acid and allowed to stand for 24 hours. The precipitate (II) obtained was redissolved and reprecipitated under the same conditions a further six times. In no case was crystallisation achieved since the purified material remained amorphous. The ovalbumin was dialysed free of ammonium sulphate and dried before a fan.

1.2. Conalbumin

The filtrate (II) was treated with a further 8 g./100 ml. of solid ammonium sulphate to precipitate the conalbumin fraction. The

heavy white precipitate (III) was centrifuged off and redissolved in water to give a pale brown solution. Purification of the conalbumin was achieved by dialysis and reprecipitation of traces of ovalbumin at pH 4.6 with half-saturated ammonium sulphate. Conalbumin was reprecipitated with an additional 8 g./100 ml. solid ammonium sulphate. The precipitate was then dialysed and dried to give a pale brown powder. This brown colour appeared to be due to the presence of bound iron. Crystallisation through the ferric complex was not attempted.

1.3. Ovomucoid

Filtrate III containing the ovomucoid fraction was precipitated (IV) by complete saturation with solid ammonium sulphate. Precipitate IV was redissolved in water and boiled for five minutes to precipitate heat labile proteins. The filtrate (V) was further purified by adding an equal quantity of 5% trichloracetic acid and the faint precipitate was removed by filtration through "Hyflo supercel". Alcohol was added to the filtrate to give 65% v/v and the mixture cooled to -5° for two hours to complete the precipitation. The precipitate was redissolved and again precipitated from 65% v/v alcohol. The precipitate was dialysed free of traces of ammonium sulphate and trichloracetic acid, and finally dried as a pale coloured powder.

The mother liquors from the 65% alcohol precipitations were

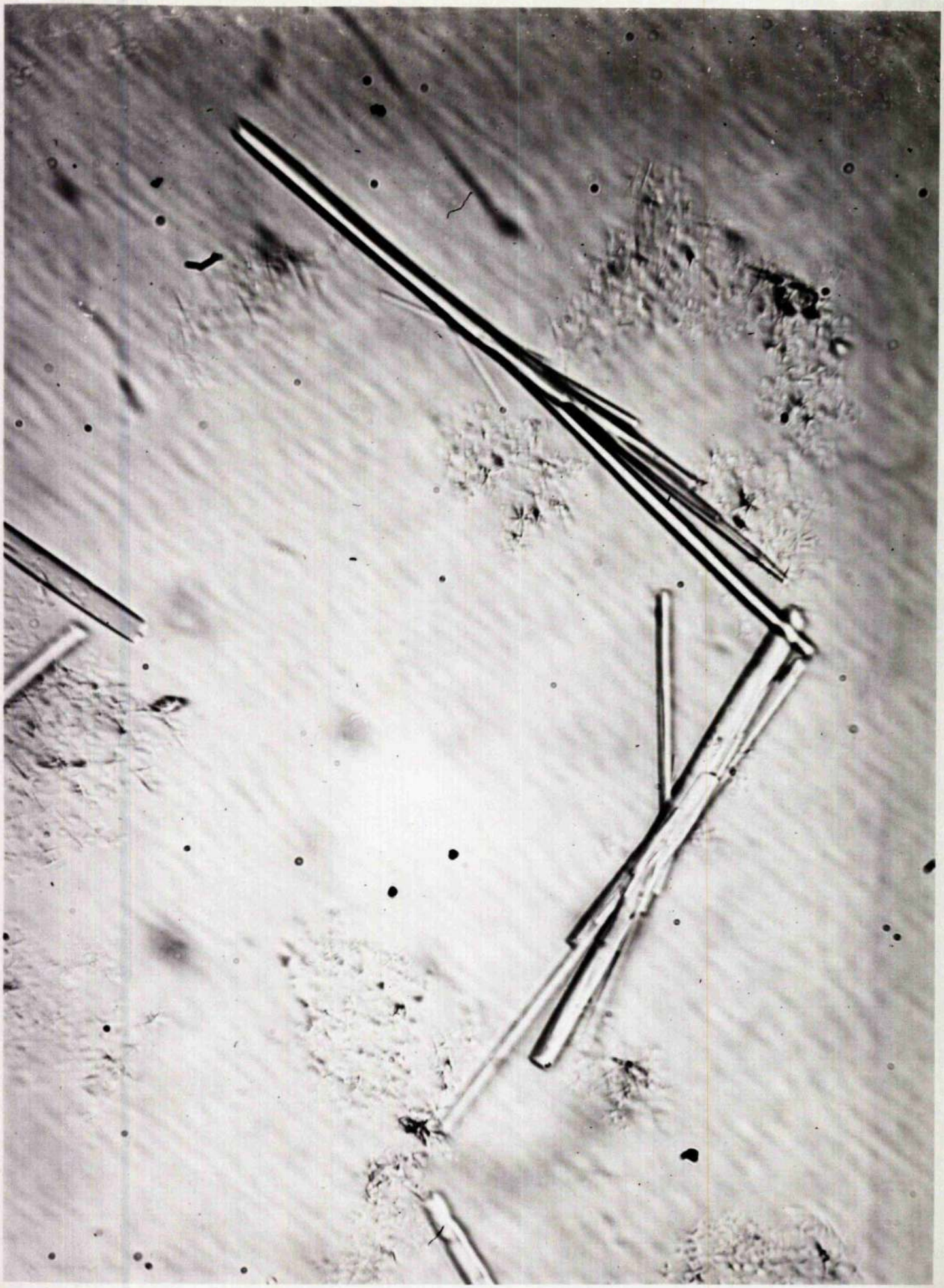


PLATE I

allowed to remain at -5° for a week. A small quantity of crystals appeared which were centrifuged off, dialysed and recrystallised from 70% v/v alcohol at -5°. A minute quantity of crystalline protein material was obtained and photographed (plate I). The material contained no free ammonium salts as tested by Nessler's Reagent, but contained organic nitrogen as shown by Lassaigne's test. On heating a small sample in a flame the typical smell of a burnt protein indicated that the material was in fact a crystalline protein. Unfortunately no refined electrophoretic technique was available for the further analysis of the material. Since ovomucoid is known to be non-crystalline and heterogeneous it seemed unlikely that the crystalline material was ovomucoid. It is suggested that the material may have been a very stable protein which is found in egg white in minute amounts.

1·4· Ovomucin

The globulin fraction was dialysed against water and finally against 5% sodium chloride at pH 4·2 for four days. During the dialysis against water both ovomucin and lysozyme were precipitated, the latter being redissolved in the dilute sodium chloride solution. A stringy precipitate (X) was centrifuged off and washed with 5% sodium chloride at pH 4·2. The final precipitate (XI) was a stringy gel-like protein, quite insoluble in water and considered to be fairly pure ovomucin. Any salt soluble globulins were removed by

extraction with 5% sodium chloride. The ovomucin was stored with a crystal of thymol in a plastic bottle at 5°. The stringy character of this protein closely resembled that of the chalazae.

1.5. Lysozyme

The filtrate (X) and the extract (XI) were combined and adjusted to pH 10. No crystalline lysozyme was available for seeding purposes, and no crystallisation could be induced on standing at 0° for a week. The solution was treated with an equal volume of saturated ammonium sulphate and the precipitate collected. After dialysis the lysozyme was freeze-dried to give a very fine white powder.

1.6. Analysis of fractions by electrophoresis

Analysis by starch gel electrophoresis (Smithies, 1955) indicated that the ovomucoid and conalbumin fractions were heterogeneous, as might be expected from the literature (see Table IV, page 6). Lysozyme was homogeneous, migrating towards the cathode at pH 8.6. Ovalbumin gave three distinct components corresponding to A_1, A_2 and A_3 of Cann (1949). Ovomucin was not examined because its insoluble nature prevented the application of electrophoretic techniques.

2. Selective Preparation of Ovalbumin

Four methods have been used to purify ovalbumin, only one of which has been found to be consistently good. Each method will be described in detail below.

2.1. Hopkins and Pinkus (1898) method

The homogenised egg white was mixed with an equal volume of saturated ammonium sulphate and gently stirred until all the globulin had been precipitated. The filtrate was then treated with a little more ammonium sulphate and acidified with dilute acetic acid until the solution became faintly turbid. After a week the precipitate of ovalbumin was examined and found to be amorphous. Hopkins and Pinkus pointed out that if slight excess ammonium sulphate was added before acidification, precipitation of the protein took place rather than crystallisation. The reason for this would appear to be that the crystallisation of a protein is a slow process, whereas precipitation is a rapid process requiring no special orientation of the molecules, the latter being favoured in excess salt concentrations.

2.2. Sørensen and Høyrup (1915) method

This method was similar to the previous one, except that acidification by 0.2 N-sulphuric acid was employed instead of acetic acid. The first precipitate was amorphous as described by these authors. The precipitate was redissolved in water and ammonium sulphate added until the solution was opalescent, on adding 0.2 N-sulphuric acid to bring the pH to 4.6 a strong precipitate was formed Even after repeated precipitations the product remained amorphous.

2.3. Cole (1932) method

This procedure was used on six batches of eggs and found to give

PLATE II

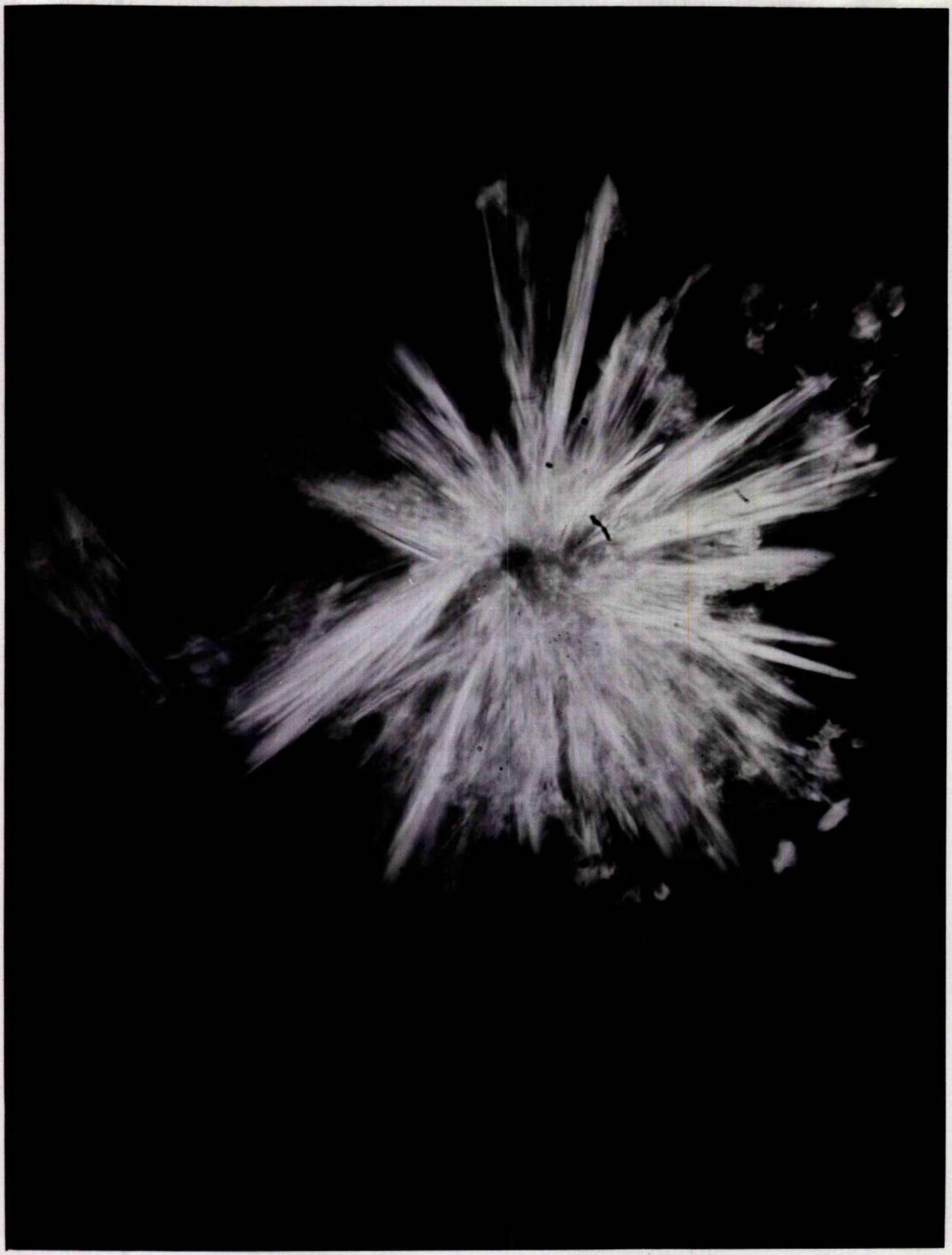


PLATE III

excellent results. The homogenised egg white was acidified with N-acetic acid (10% v/v) and the mucin removed by centrifugation. The globulin fraction, precipitated by adding ammonium sulphate solution until a final concentration of 40% saturation was reached, was removed by centrifugation and discarded. The supernatant fluid was cleaned by filtration through a pad of "celite" and saturated ammonium sulphate added until opalescence was reached at 47% v/v saturation. After gentl stirring for two hours an amorphous precipitate was collected. The ovalbumin was redissolved and buffered ammonium sulphate (M-acetate buffer pH 4.7:saturated solution of ammonium sulphate 1:9 v/v) added until opalescence was reached at 45% saturation with ammonium sulphate. After stirring for a few hours crystalline ovalbumin precipitated. The yield could be greatly increased by small additions of buffered ammonium sulphate over a period of a few days, bringing the final salt concentration to 55% v/v. Two distinct types of crystals were always found to be present. The first ovalbumin crystals to form were needle-like in appearance and on standing these needles branched into sheaflik clusters, large aggregates of the latter forming rosettes. The needlelike crystals are shown in plate II, a rosette in plate III.

The crystalline ovalbumin was purified by redissolving and recrystallising a further five times, dialysed free of ammonium sulphat and dried before a fan at 30°. The purity of the dried material was demonstrated by Smithies (1955) starch gel electrophoretic analysis which showed the presence of the three components, A_1, A_2 and A_3 in

approximately the same proportions as described by Cann (1949). Comparison with whole egg white showed that the albumin band of the latter also contained three discreet sub-fractions which corresponded exactly with those of the crystalline material examined on the same gel This indicated that the crystalline material was electrophoretically identical with the ovalbumin of whole egg white, and that the three fractions were not artifacts produced during crystallisation.

The dried protein remained quite soluble in water and had the following physical constants: Ash 2.8%, Moisture 9.3%, Ash and Moistur free Total Nitrogen 15.72% and Amide Nitrogen 1.04%. The figures for the nitrogen analyses were in good agreement with those in the literature (see Table V page 8). In the various samples of the crystalline protein analysed the nitrogen composition remained constant although the moisture and ash contents varied from 7 - 10% and 0.5 - 3.5% respectively. The ash content could be greatly reduced by prolonged dialysis.

Cole's method was found to be ideal for large-scale preparation of ovalbumin. With practice and very careful control of the ammonium sulphate concentration, crystallisation could be induced at the first step instead of the amorphous precipitate described originally by Cole An average of 1 g. of "pure" ovalbumin was obtained from each egg after six crystallisations (approximately 50% yield). The method has been used to crystallise ovalbumin from commercial egg albumin (obtained from B.D.H.). The crystallisation in this case was

much more difficult than for fresh eggs, and the resulting material was much less stable. Large quantities of the commercial protein precipitated after dialysis, even though it had previously been crystallised six times. In comparison hardly any insoluble material appeared during dialysis when ovalbumin was prepared from fresh eggs. The highly purified material from commercial ovalbumin was considered to be unsuitable for structural studies because of its instability.

An Evans Blue complex of ovalbumin was readily crystallised by Cole's method, the material separating as very large blue rosettes similar to those of pure ovalbumin. This dyed protein was used in the dynamic studies in an attempt to follow the fate of a labelled ovalbumin after intravenous injection into laying hens.

2.4. Keckwick and Cannon (1936) method

The preparation depended on the fact that the increased solubility of sodium sulphate at 30° was sufficient to precipitate ovalbumin from solution. An equal volume of sodium sulphate (400 g./litre) at 30° was added to the homogenised egg white and after standin for three hours in a water bath at this temperature the precipitated globulin fraction was removed by centrifugation. The supernatant was acidified with 0.1 N-sulphuric acid until the pH reached 4.7, the albumin fraction was then precipitated by the addition of a small volume of sodium sulphate solution. This crude ovalbumin was further purified by repeated precipitation under the same conditions; the

product remained amorphous.

2.5. Comparison of the methods

The present author found Cole's (1932) method to be the most simple and effective procedure for the crystallisation of ovalbumin. The success obtained with this method was possibly due to the fact that this was the last procedure to be attempted, and the experience gained in the previous failures contributed to the success in this case. As a result, Cole's method has been extensively used and without fail has given a fine crystalline product after the initial precipitation of amorphous material.

Both the methods of Hopkins and Sørensen were much slower and it was more difficult to obtain the exact concentration of salt and ac to induce crystallisation. In the procedure of Keckwick and Cannon it was awkward to control the salt concentration as crystallisation of a hydrated sodium sulphate took place rapidly if the mixture was cooled. This was inevitable if the precipitate formed at 30° was centrifuged at room temperature. The decrease in the strength of dissolved sodium sulphate was often sufficient to enable the ovalbumin precipitate to become partially redissolved. There was a possibility of bacterial action during the slow crystallisation procedure at 30° which could easily destroy a carefully prepared product.

3. Plakalbumin

3.1. Crystallisation

Two samples of crystalline ovalbumin were dialysed until salt-free and left in open flasks at room temperature for five days, after which time considerable bacterial growth had taken place. The solutions were filtered and brought to pH 4.7, Cole's method then being used to isolate ovalbumin in the hope that the unchanged ovalbumin could be fractionally crystallised from the plakalbumin. Large crystals of ovalbumin were formed in the usual way but at the same time some amorphous material and crystalline plakalbumin were also found to be present. The mother liquors yielded a precipitate of the mixed crystals on the addition of excess ammonium sulphate. Fractional crystallisation appeared to be impossible, even though at this pH plakalbumin is very much more soluble than ovalbumin. Linderstrøm-Lang (1952) drew attention to the fact that the solubility of a mixture of the two proteins was much greater than that of ovalbumin alone. This increased solubility due to protein-protein interaction has been used by the same author as a measure of the degree of conversion of ovalbumin to plakalbumin by subtilisin.

3.2. Attempted large scale preparation

Linderstrøm-Lang and Ottesen (1947) employed the purified enzyme subtilisin in the conversion of ovalbumin to plakalbumin. After the reaction had gone to completion the plakalbumin was easily

crystallised without fear of contamination with ovalbumin.

An attempt was made to carry out this enzymic reaction with B. subtilis (N.C.T.C. 6346). The bacteria were cultured in nutrient broth at 37° for twenty-four hours and the scum of floating organisms was separated from the culture fluid. Four large flasks were set up containing dialysed ovalbumin and bacterial scum added to two of these flasks, one being incubated at 20° and the other at 37°. The culture fluid was mixed with the other two protein solutions which were similarly incubated. Samples were removed from each of the reaction mixtures after 2, 4, 6, 24 and 48 hour intervals and crystallised by Cole's method. In all cases the typical ovalbumin crystals were readily obtained as well as a minute quantity of plate-like plakalbumin The mother liquors were treated with a further quantity of buffered ammonium sulphate which resulted in the crystallisation of the original protein only. Dr. Shepherd of the Bacteriology Department kindly helped with the bacterial culture preparation. He has suggested that the incubation of the culture medium at 37° may have inhibited the synthesis of subtilisin.

Although plakalbumin has been crystallised in the presence of contaminating ovalbumin, it was decided not to continue this investigation at this stage because of the complexity of the enzymic reaction. For ideal conditions a sample of purified subtilisin was necessary, as well as carrying the reaction to completion.

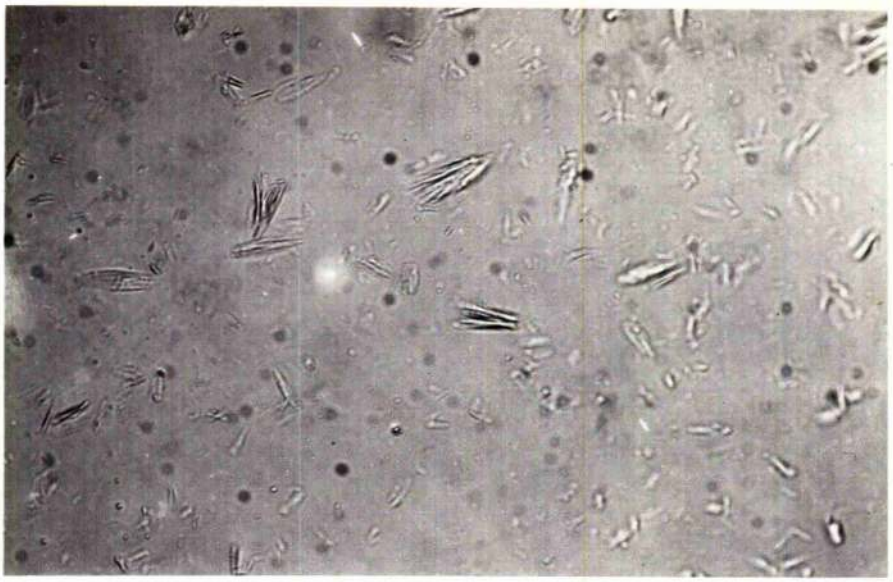


Plate IVa. Typical crystals of ovalbumin in 47% ammonium sulphate pH 4.7, crystallised six times according to Cole's method.

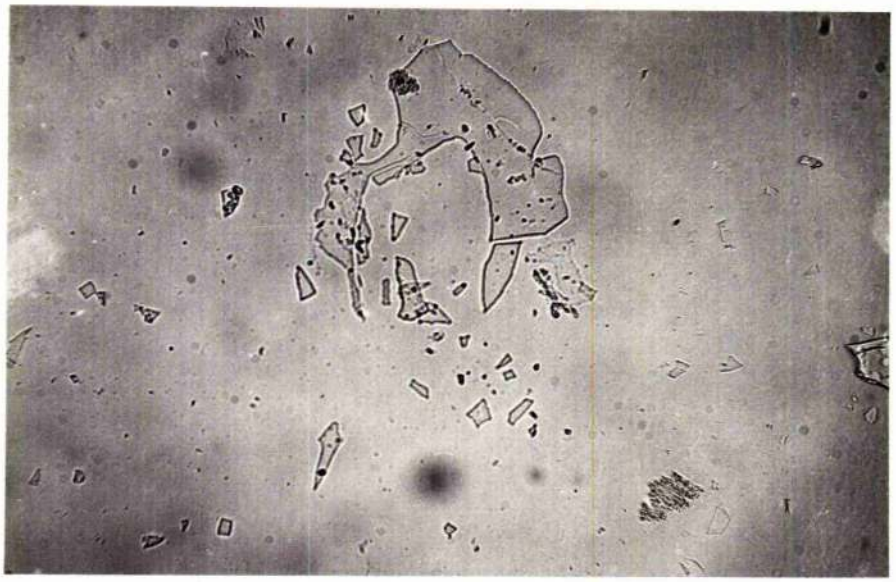


Plate IVb. Irregular plate-like crystals of plakalbumin.

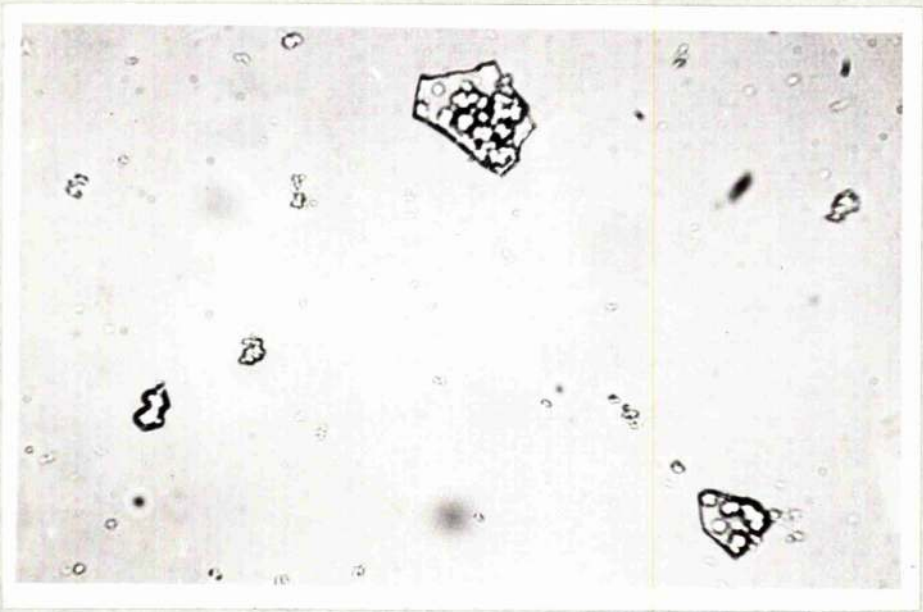


Plate IVc. Plakalbumin undergoing conversion to an amorphous material which retains the characteristic outline of the plakalbumin crystal. The final stage appears to be the breakdown of this amorphous material into clumps of globular material which have no structural similarity to crystalline plakalbumin. Plate IVc. was taken at the same time as Plate IVb, the two samples were obtained from the same reaction mixture.

3.3. Discussion

The complexity of this bacterial reaction is illustrated in plate IVa, b, c. At least three stages are clearly evident in the system containing ovalbumin and bacterial enzymes suspended in 47% ammonium sulphate at room temperature. The reactions are thought to take place in the following sequence.

Crystalline Ovalbumin $\xrightarrow{\text{Bacterial Enzymes}}$ Ovalbumin + Plakalbumin $\xrightarrow{\text{Bacterial Enzymes}}$ Plakalbumin + Amorphous materi

The changes in crystalline structure, which may be examined under the microscope, suggest that the enzyme responsible was still active in 47% ammonium sulphate. Samples from the mother liquors obtained from the above crystallisations were incubated with freshly dialysed ovalbumin in order to ascertain whether the enzyme was active. At hourly intervals separate aliquots of the incubation mixture were crystallised by Cole's method. In every instance the predominant crystalline material was ovalbumin with a few large plakalbumin crystal The possibility of bacterial infection during the incubation of the aliquots cannot be ignored since the conditions were not sterile. It would appear that the most likely explanation was that the mother liquors contained a small quantity of active enzyme which was shown to be extra-cellular in nature since samples of this solution containe no cells which could be cultured in nutrient broth.

PART III

DYNAMIC ASPECTS OF THE EGG WHITE PROTEINS

1. Introduction

The egg white proteins are well known to be deposited around the yolk in the magnum region of the oviduct. The site of synthesis of these proteins is much less certain, although it is often taken for granted they are actually formed in the oviduct.

The origin of the egg white proteins has been studied in the present work but no definite proof has been obtained that the synthesis of these proteins takes place in this tissue or in some other part of the body. The results indicate that synthesis of egg white proteins could take place in the liver for instance, as is the case with the serum proteins, in which case the oviduct might act as a collecting centre for these proteins.

Hectoen and Cole (1928) demonstrated that conalbumin obtained from egg white was immunologically identical with a protein found in the serum of laying hens. This serum protein was later shown by Marshall and Deutsch (1951) to be conalbumin and not ovalbumin as was originally thought. The ovalbumin used in the earlier experiments was contaminated with conalbumin which was strongly antigenic giving a precipitation reaction with rabbit anti-serum to laying hen serum.

Rochlina (1934) showed that the total serum protein of laying hens underwent considerable variation throughout the day, whereas the

serum of non-laying hens and cocks remained constant. This author suggested that the serum contributed to the formation of the egg. Romanoff and Romanoff (1949) concluded that the egg white proteins were derived at least in part from the serum of the hen.

Kaminski and her associates (1954-57) have studied the protein of the egg white in the fertilised egg, the embryonic fluids and the sera of the chicks throughout their development to adult fowls. The distribution of egg white proteins in these complex biological fluids was of interest. Cock and non-laying hen sera appeared to be almost identical in their protein distribution, both containing conalbumin. Laying hen serum contained conalbumin and traces of ovalbumin as well as a fast electrophoretic component which was found in non-laying hen but not in cock serum. Lysozyme and ovomucoid were only weakly antigenic proteins and were not detectable by immunological analysis. Kaminski and Durieux (1954) examined the "ovoglobulin" fraction of egg white (precipitated between 25 - 50% saturation with ammonium sulphate) which contained at least nine sub-fractions as shown by immuno-electrophoresis. Two of these ovoglobulins were shown to cross-rea with an α- and a γ-globulin of laying hen serum. The embryo fluids

were initially very similar to the egg white proteins in their serological and electrophoretic behaviour on agar gels. As the age of the embryo increased the pattern of the embryonic fluids changed to that of the newly hatched chick which contained four proteins found in whole egg white, namely ovalbumin, conalbumin and the two ovoglobulins mentioned above. The ovalbumin disappeared from the chick serum soon after hatching when the protein pattern approached that of the non-laying hen or cock serum.

Marshall and Deutsch (1951) demonstrated the presence of conalbumin and traces of ovalbumin in the yolk by immunological reaction. Since the yolk is formed in the ovary and is completed before entering the oviduct, these proteins could only have entered the yolk from the serum or been synthesised in the ovary itself.

Anfinsen and Steinberg (1951) claimed that the synthesis of ovalbumin took place in the oviduct. These authors incubated a homogenised oviduct suspension in a salt solution in the presence of radioactive carbon dioxide, after a given time interval they added excess ovalbumin as carrier and crystallised radioactive ovalbumin from the supernatant fluid obtained from the centrifuged suspension. The radioactivity was confined to the dicarboxylic amino acids. They concluded that the experiment proved that the biosynthesis of ovalbumin could take place <u>in vitro</u> in the oviduct.

The results presented by these authors might be accounted for

by an exchange reaction. It might seem remarkable that sufficient "de novo" synthesis could take place to give enough radioactive ovalbumin for analysis since no energy rich intermediates were added to the incubation medium. The "de novo" synthesis of ovalbumin would require sufficient energy to form 380 - 390 peptide bonds per molecule. The reaction might be thought to be the exchanging of carbon dioxide with the metabolic pool of dicarboxylic amino acids, the labelled amino acids then exchanging with aspartic and glutamic acids of the intact ovalbumin already present in the oviduct.

Hendler (1957) studied the incorporation of radioactive amino acids into the oviduct tissue. Although the significance of his results is not clear, he did demonstrate that these amino acids were first incorporated into the cell debris and later into the oviduct protein.

2. Theories Accounting for the Egg White Proteins in the Ovidu

Three possible sources of the egg white proteins may be considered.

(a) Synthesis of the proteins in the oviduct from which a small quantity of the four proteins, found to be common to both egg white and serum, diffuse from the oviduct into the blood stream.

(b) Synthesis of egg white proteins in some tissue other than the oviduct and transport via the blood to the latter. The oviduct would then act as a collecting centre for pre-formed egg white proteins.

(c) The carriage of intermediates in the serum to the oviduct which are then converted to egg white proteins. In this case the oviduct would act as a reconstruction centre.

The presence of conalbumin in cock serum indicates that even if the egg white proteins are synthesised in the oviduct, the presence of other sites for the synthesis of individual egg white proteins cannot be ignored. The evidence discussed above indicates that at least one of the proteins is in fact capable of being synthesised in a tissue other than the oviduct. The experimental work described in this section was carried out in an attempt to ascertain the source of the egg white proteins.

3. Electrophoretic Techniques Employed in the Separation of the Egg White Proteins

Some physical method was required which would give a clear picture of the individual protein fractions present in such complex mixtures as whole egg white and fowl serum. Electrophoretic techniques seemed to offer the greatest possibilities in this field and four different methods were attempted, only one of which was found to have sufficient resolving power to be of value in comparative analysis.

3.1. Tiselius Electrophoresis

Classical Tiselius electrophoretic analysis was carried out on whole egg white, with the help of Dr. Ellis of the Blood Transfusion

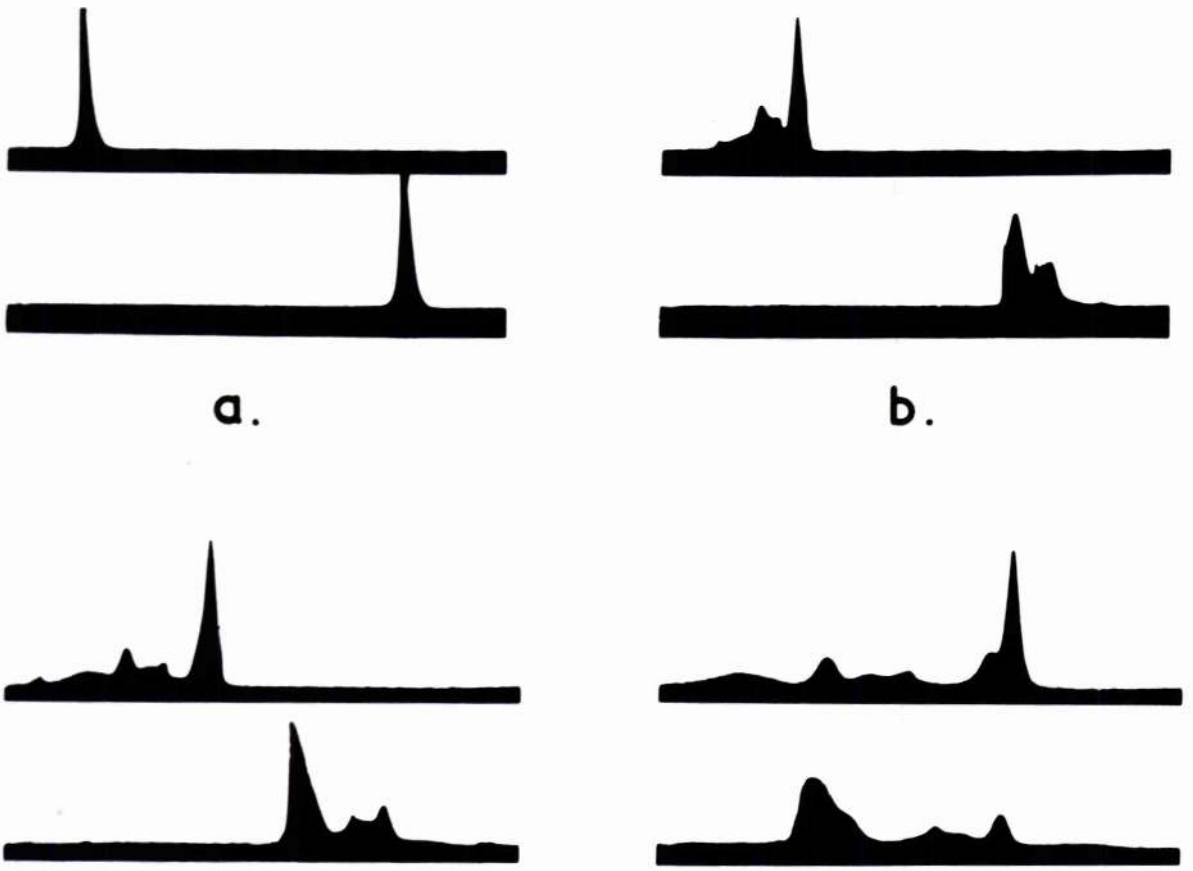
Centre, Edinburgh. The conditions used were those of Forsyth and Foster (1950). The egg white was homogenised by squeezing through muslin and a 2% v/v solution made up in 0.05 M-phosphate buffer pH 7.8 containing 0.15 M-sodium chloride. The protein solution was dialysed against a large quantity of the same buffer overnight before analysis and this buffer, which had been equilibrated with the protein, was employed in the actual experiment. Four photographs were taken at suitable times to record the appearance of the different electrophoretic fractions as shown in Plate Va-d.

The electrophoretic conditions were changed slightly during the experiment, these changes being shown in Table IX and correspond to the conditions at the time of taking the photographs.

Table IX

Tiselius electrophoresis of whole egg white using the conditions of Forsyth and Foster (1950). 2% protein solution in 0.05 M-phosphate buffer pH 7.8 containing 0.15 M-sodium chloride.

Plate V	Time (Min.)	Current mA.	Volts/cm.	Peaks
(a)	0	30	3.7	1
(b)	60	40	4.9	4
(c)	120	40	4.9	5
(d)	240	40	4.9	5



TISELIUS ELECTROPHORETIC ANALYSIS OF WHOLE EGG WHITE.

PLATE V

3.1.1. Analysis. The ascending pattern is represented in Plate V by the upper diagram. The last two plates are the most interesting. Plate V(c) shows four anodic peaks and a trace of a cathodic peak in the ascending limb, the order of the peaks being ovalbumin, ovomucoid, globulins (G_2 and G_3 of Longsworth et al. 1940), conalbumin and lysozyme respectively. The two globulins G_2 and G_3 have not completely separated. Plate V(d) was taken when the lysozyme had migrated too far towards the cathode to be recorded. The ovalbumin has been subdivided into two components A_1 and A_2, the globulins G_2 and G_3 remaining as a single electrophoretic peak.

Semi-quantitative analysis of the protein composition of egg white was carried out in the following manner. The area under the curve was cut out from the photograph, weighed and the percentage composition of each electrophoretic peak calculated from the weight of the area under the peak. Although this method is perhaps not as accurate as using a planimeter it does give a reproducible result which is in fair agreement with the values in the literature. The values given in Table X were obtained from the ascending limb, Forsyth and Foster's values being given for comparison purposes.

3.1.2. Results

Table X

Quantitative Tiselius electrophoretic analysis of whole egg white

Protein	Present Author percent.		Forsyth and Foster (1950) percent.
Ovalbumin A_1	47.5	60.0	64.9
Ovalbumin A_2	12.5		
Ovomucoid	8.4		9.2
Globulin G_2 / Globulin G_3	8.3		8.7
Conalbumin	17.6		13.8
Lysozyme	5.6		3.4
Ovomucin	Removed as precipitate before analysis		

No attempt has been made to calculate the protein distribution in the descending limb. Forsyth and Foster also found that the electrophoretic pattern obtained from the descending limb was less well defined than that of the ascending limb.

The globulins, G_2 and G_3 described by Longsworth et al. (1940), have not previously been mentioned in this work. These globulins appear in the literature in connection with electrophoretic analysis and have not been extensively studied. The recent immunoelectrophoret

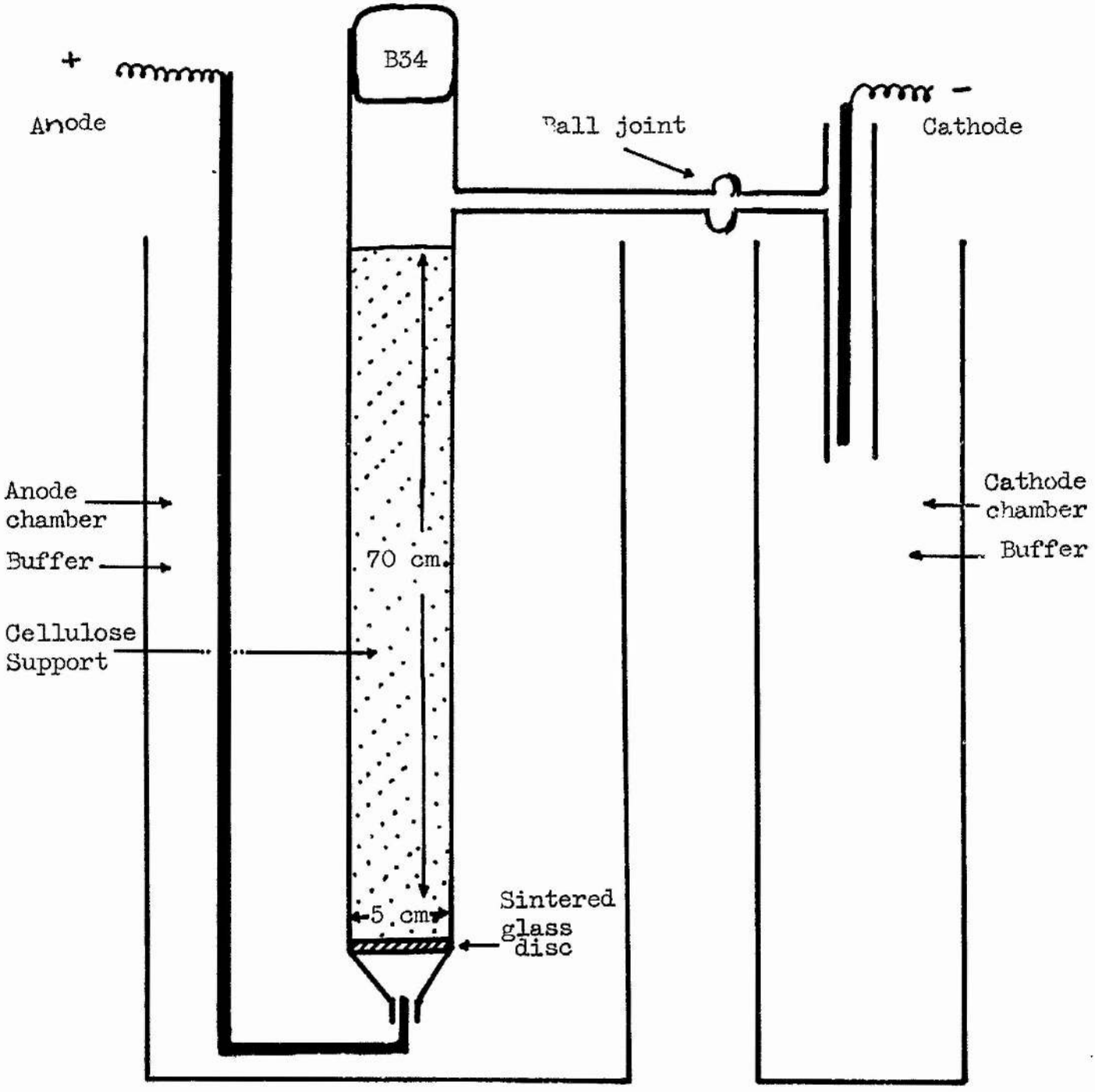


Fig. 7

Porath(1954) zone-electrophoresis apparatus.

studies of Kaminski (1956) on the "ovoglobulin fraction" suggest that G_2 and G_3 may be heterogeneous.

3.2. Porath zone-electrophoresis

Flodin and Porath (1954) and Porath (1954) described a zone-electrophoretic technique suitable for the isolation of individual proteins from a complex mixture. The technique depends on the fractionation of the mixture into discrete components during the application of a high voltage between the ends of a column of inert support on which the mixture has been absorbed. After completion of electrophoresis the protein fractions were passively eluted by allowing a buffer solution to percolate down the column, fractions being collected in a fraction collector.

3.2.1. Method. The support used in the present work was partially acetylated cellulose. This was prepared by refluxing 300 g. cotton wool with 4875 ml. ethyl alcohol and 375 ml. acetyl chloride for 20 hours. The product was filtered on a Büchner funnel and repeatedly washed in acetone until the material appeared quite clean, and then dried in a vacuum desiccator.

The apparatus was set up as shown in fig. 7. The buffer solution in the column and the electrode chambers was 0.03 M - acetate or phosphate with pH within the range 5.6 - 6.9. In all experiments 2 ml. of whole egg white was diluted with 8 ml. of buffer and filtered before applying to the top of the cellulose column. The protein

Conditions:

Sample volume: 2 ml.

Fraction volume:5 ml.

Buffer: 0.05 M acetate. pH 5.66

Voltage: 1000 V.

Current: 21 mA.

Duration: 5 hrs.

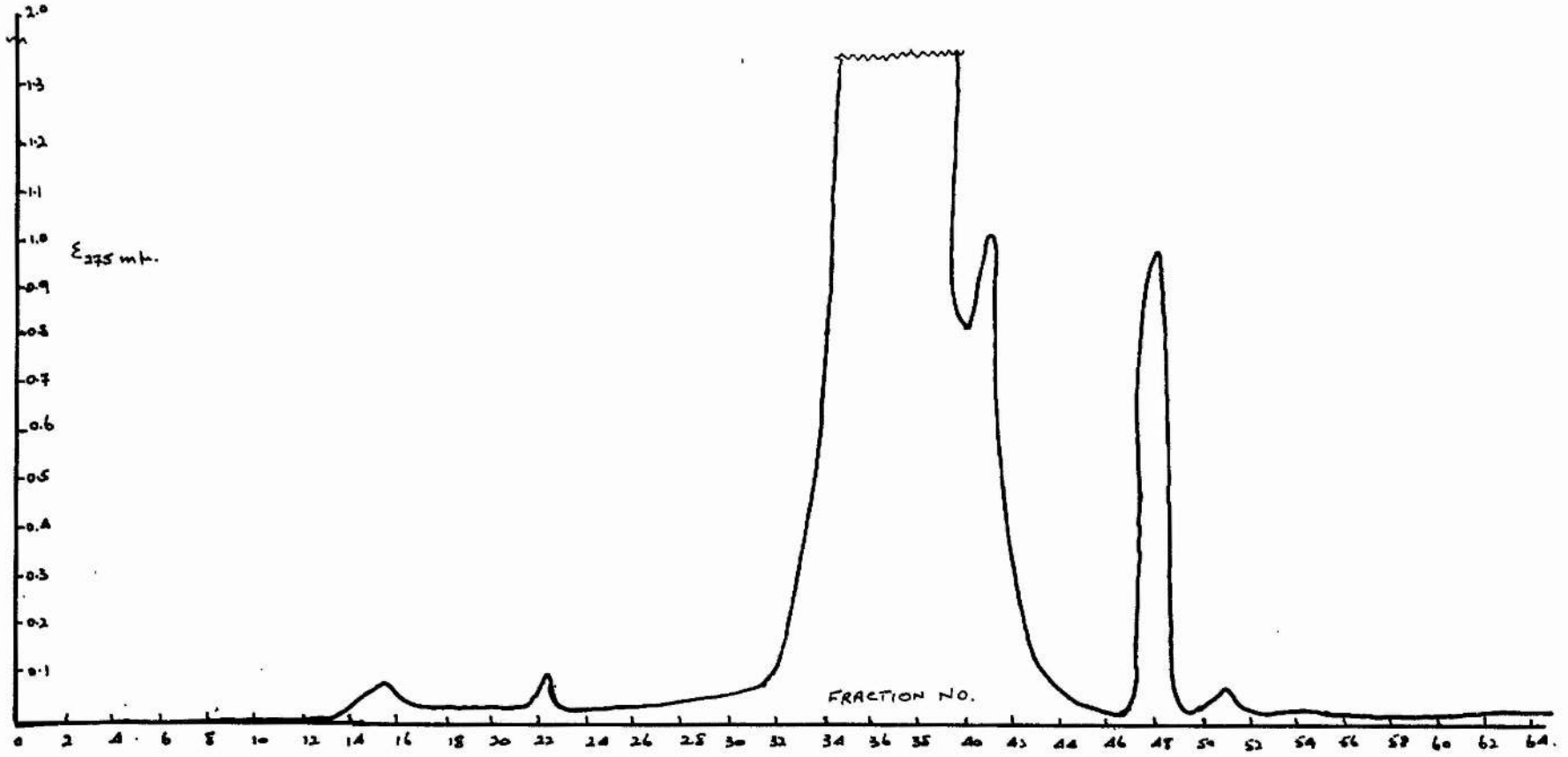


Fig. 8(a).

FRACTIONATION OF EGG WHITE ON PORATH(1954) COLUMN.

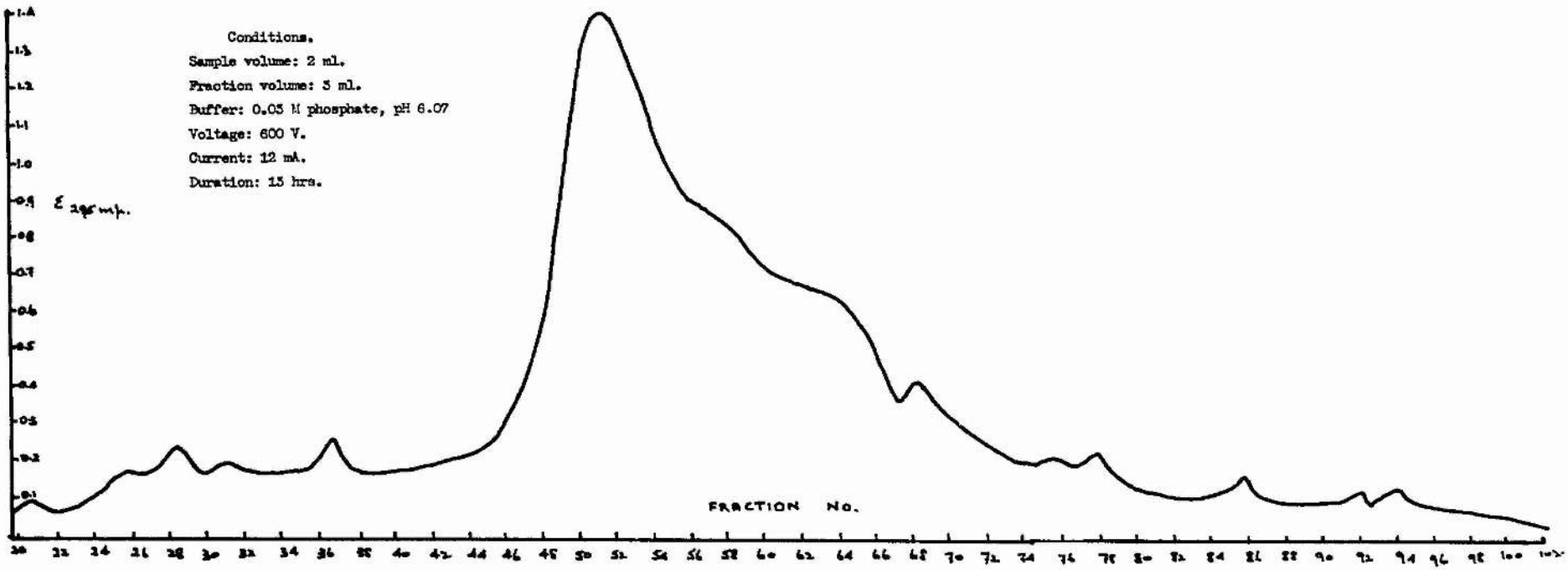
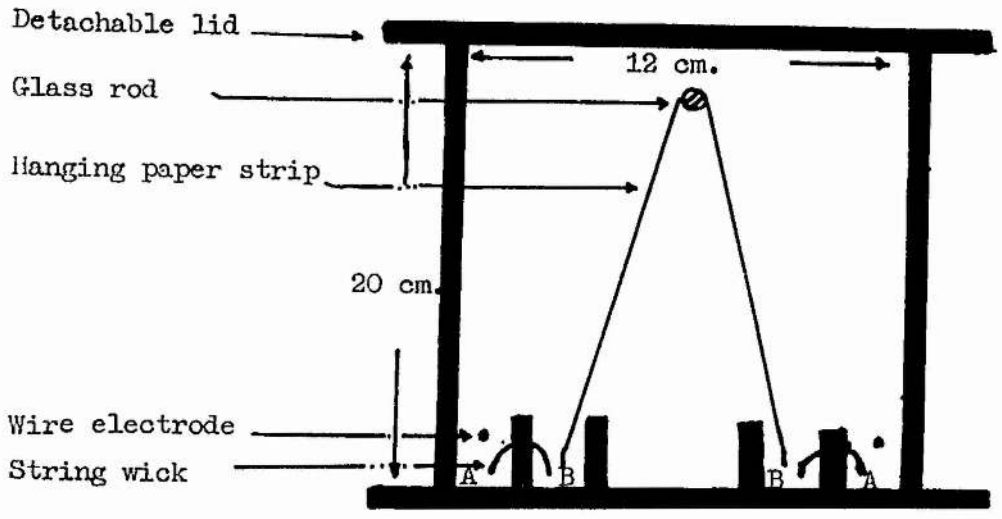


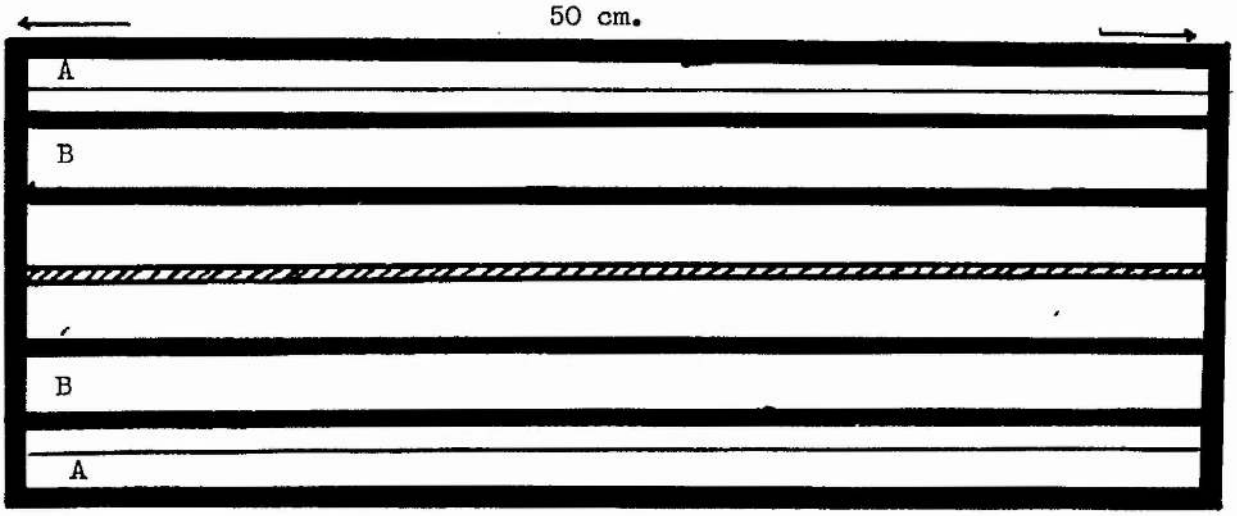
Fig. 8(b).

solution was washed into the support by the addition of 100 ml. buffer, the voltage applied for a given time and the fractions eluted. The actual conditions of experiment varied with the time of electrophoresis, a 10 hour run required 600 volts and about 18 - 20 mA whereas a 6 hour run required 1000 volts and about 23 - 25 mA. The current varied considerably and was greatly affected by the presence of gas bubbles which tended to accumulate in the ball-joint. In each experiment 600 - 800 fractions of 3 ml. were analysed by measuring the optical absorption at 275 mμ with the Unicam spectrophotometer. Typical electrophoretic patterns are given in fig. 8(a) and (b).

3.2.2. Discussion. This method could probably have been adapted and used with greater success if more time had been available for developing the technique to the fractionation of the egg white proteins Each run took over a week to complete and the result was not sufficiently promising to continue. One of the main advantages of this apparatus as described by Porath was that the support could be used for a series of analyses without being regenerated. In the present experiments it was observed that not all the protein was eluted with the buffer, some being irreversibly absorbed on the acetylated cellulose. Another disadvantage was that the elution rate became progressively slower due to the support packing harder with time This slow elution rate allowed more time for the diffusion of protein zones and reduced the resolving power of the technique.



Cross-section.



Longitudinal-section.

Fig. 9.

Flynn and de Mayo (1951) paper electrophoresis apparatus.

3.3. Paper Electrophoresis

The method employed was that described by Flynn and de Mayo (1951 An electrophoretic chamber of the "hanging strip" design described by Block et al. (1955) was constructed from perspex sheet (fig. 9).

3.3.1. Method. The conditions used throughout were as follows:

Paper: Whatman No. 1, six 5 cm. strips or twelve 2.5 cm. strips in parallel.

Buffer: Barbiturate pH 8.6. (10.3 g. sodium diethyl barbiturate and 1.8 g. diethyl barbituric acid per litre.)

Voltage: 110 V.

Current: 10 mA.

Time: 20 hours.

Fixation of proteins: The strips were dried in an oven at 100° for hal an hour to denature the proteins on the papter.

Staining procedure: (a) Proteins. Strips were stained with Naphthalene Black or Amido Black 10 B dissolved in water: methanol:glacial acetic acid (5:5:1). After an hour the papers were removed and the gross excess dye washed off with water, the residual dye being removed by exhaustive washing with the dye solvent mentioned above. When the background dye had been completely removed the strips were washed in ethanol and hung up to dry. The solvent was cleaned by filtration through charcoal and re-used.

(b) Lipoproteins. Acetylated Sudan Black dissolved in methanol was employed. The time of staining was approximately twenty-four hours since the dye must be allowed considerable time to dissolve in the lipoprotein. The excess dye was washed out with water.

Scanning: (a) Proteins. Strips stained with Naphthalene Black were made translucent with liquid paraffin and scanned with the EEL Scanner using a green filter.

(b) Lipoproteins. Strips stained with the lipid dye were soaked in water before scanning.

Analysis of protein distribution: The areas under the individual peaks were calculated by cutting out and weighing, an approximate estimation of the percentage composition was then possible.

3.3.2. Materials. The materials examined by paper electrophoresis were egg white, oviduct white (i.e. the egg white at a stage before it is layed), and fluid obtained from the oviduct of a pathological hen which was unable to pass its eggs beyond the magnum. Sera from laying, non-laying hens and cocks, as well as the yolk and ova proteins were examined. Comparative analyses were carried out on various combination of these materials, one mixture being run alongside the other on the same paper strip.

3.3.3. Results. (a) Whole egg white. Seven fractions were clearly shown to be present as well as a trace of material remaining at the

Paper electrophoretic analysis patterns.

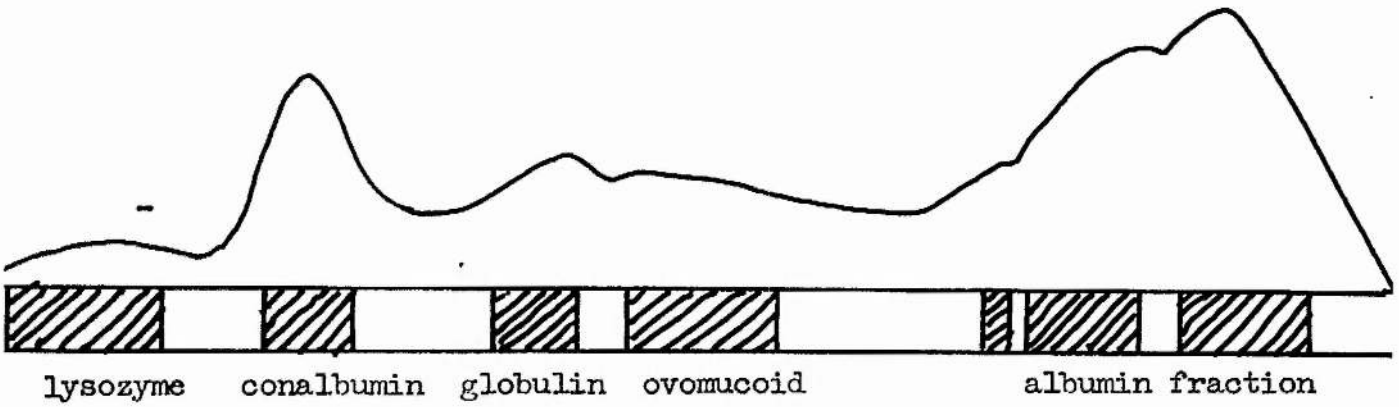


Fig 10 (a).

Fractionation of whole egg white.

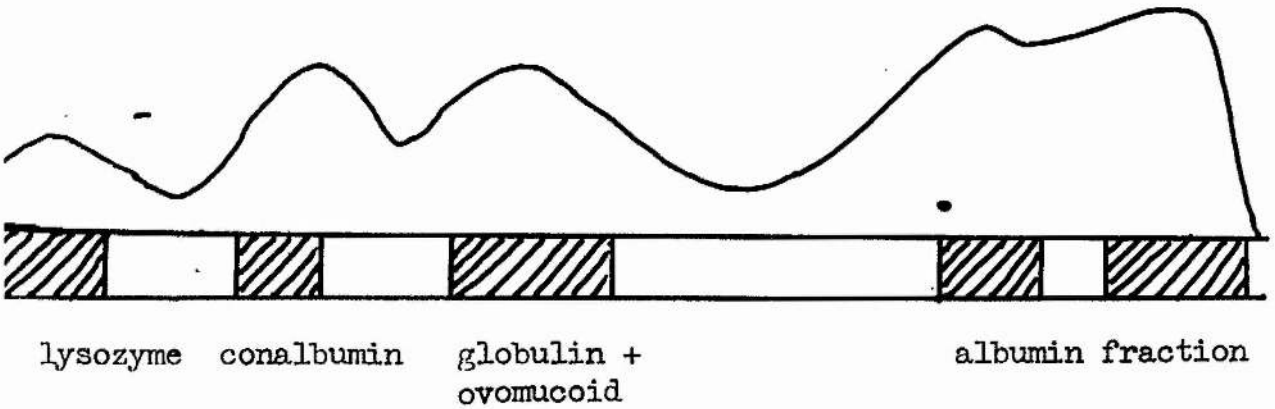


Fig. 10 (b).

Fractionation of oviduct white.

origin, which was probably ovomucin. The fractions were identified by comparison with the purified proteins obtained in the work described earlier in this thesis. The sequence in which these protein migrated on paper was identical with that found in the Tiselius analysis. The ovalbumin fraction was partially resolved into three components which may be more clearly observed from the scanned diagram of the stained paper shown in fig. 10 (a). No lipoprotein was detected in egg white.

(b) Oviduct white. Laying hens were killed and the material deposited around the yolk in the oviduct removed. In seven hens examined the pattern was exactly the same as for whole egg white; see fig. 10 (b). The water content of the oviduct white was much less than that of the fully formed egg but the relative distribution of the protein fractions remained closely similar, at least within the experimental errors incurred in estimation. The oviduct white was examined at three different stages of development, an early, an intermediate and a late stage when the oviduct white was just leaving the magnum. At all three stages the electrophoretic patterns appeared to be identical in their relative protein distribution.

(c) Fluid from the oviduct of a pathological hen. The oviduct of one hen was obstructed in some way below the magnum during its normal laying cycle. Two almost fully developed eggs were found above the obstruction. At the same time a small quantity of a rather watery fluid was obtained. The two oviduct whites from the nearly completed

Paper electrophoretic analysis patterns.

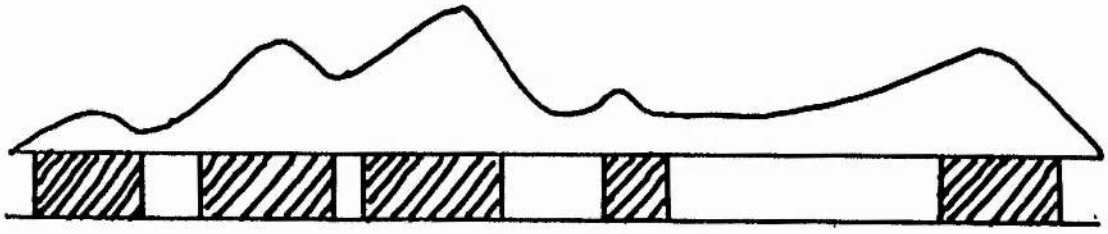


Fig. 10 (c)

Fractionation of oviduct fluid.

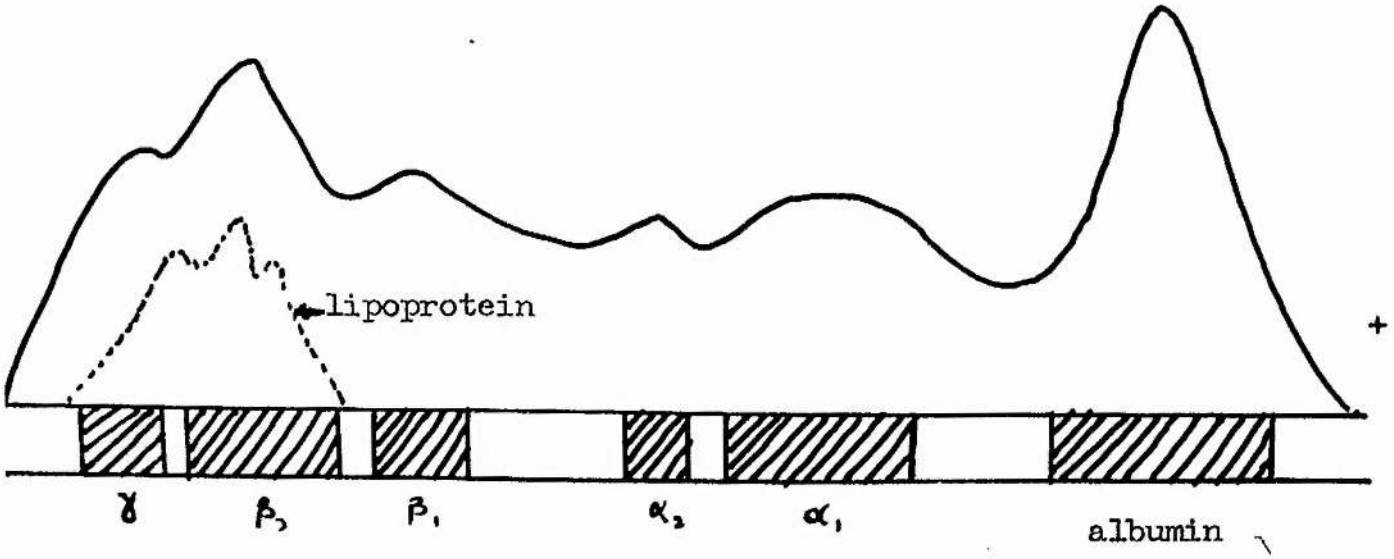


Fig 10 (d)

Fractionation of laying hen serum.

eggs appeared quite normal in their electrophoretic behaviour. The oviduct fluid was shown to contain five electrophoretically distinct fractions (see fig. 10(c)). The fluid was compared with whole egg white run on the same paper strip, the components common to both mixtures being ovalbumin, ovomucoid, globulin fraction, conalbumin and lysozyme. In this fluid the ovalbumin and ovomucoid appeared to be much reduced whereas the conalbumin and the globulin fractions were larger than the corresponding components in whole egg white.

(d) Laying hen serum. Laying hen serum was found to contain six major electrophoretic components. These protein fractions were tentatively classified after the corresponding human nomenclature as serum albumin, α_1-, α_2-, β_1-, β_2- and γ-globulins respectively. No purified serum proteins obtained from fowls were available for comparative studies. The serum albumin fraction seemed to be poorly defined and may well be heterogeneous. Recently King and Craig (1957) have published similar observations on human and bovine serum albumin, three components being detected in each case. The tailing of the albumin gave an increased background stain and probably increased the percentage composition of the slower fractions. This tailing was most likely caused by surface denaturation of the albumin during electrophoresis. Three distinct lipoprotein fractions were demonstrated in laying hen serum (see fig. 10(d)).

(e) Cock serum. Cock serum was separated into six main fractions, the albumin being clearly subdivided into three minor fraction, fig. 10(

Paper electrophoretic analysis patterns.

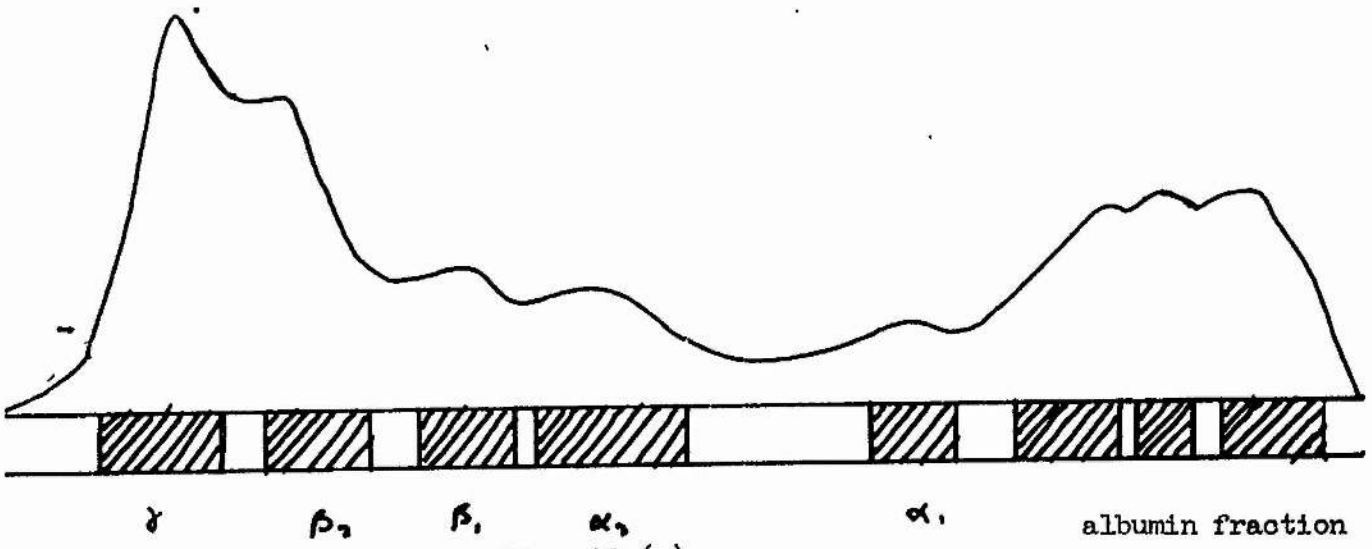


Fig. 10 (e)

Fractionation of cock serum.

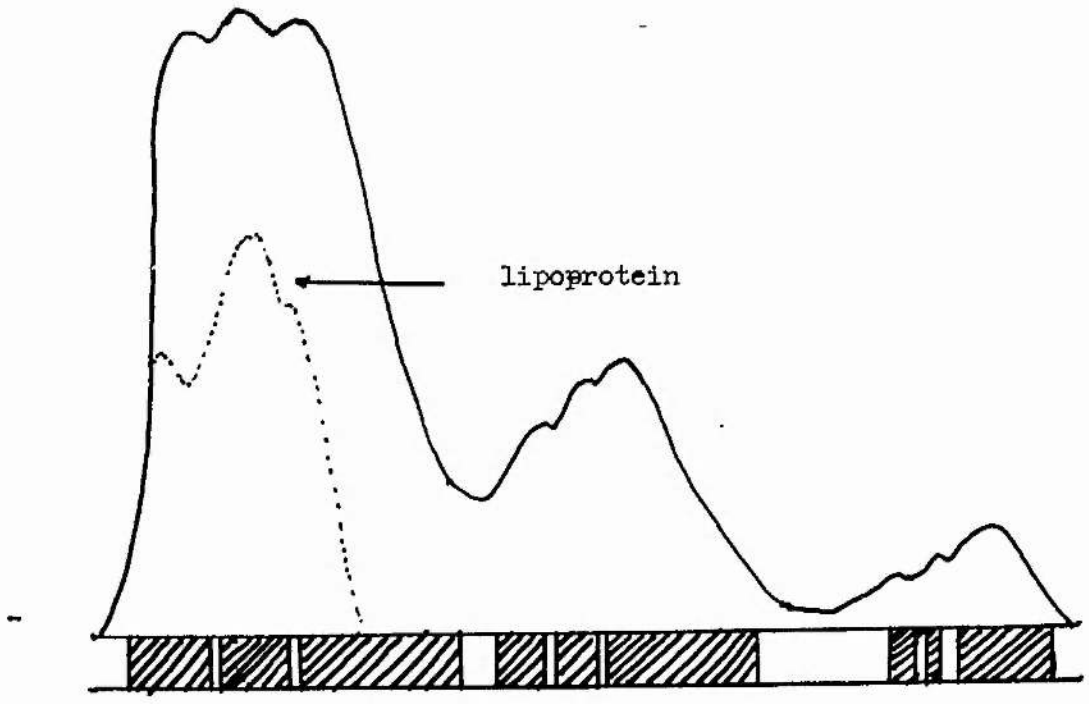


Fig. 10 (f).

Fractionation of egg yolk.

By scanning a trace component was detected which migrated immediately behind the serum albumin but it could not be seen by eye on the stained paper due to the tailing of the albumin. The protein fractions were similar in electrophoretic mobility to those found in hen serum.

(f) Non-laying hen serum. The protein fractions appeared to be qualitatively identical with those of laying hen serum, however the lipoprotein fractions differed markedly. Non-laying hen and cock sera had only a small trace of lipoprotein whereas laying hen serum was rich in three lipoproteins.

(g) Comparative examination of protein mixtures run on the same paper. Whole egg white, oviduct white and the oviduct fluid were compared by taking two of these mixtures and subjecting the pair to electrophoresis side by side on the same paper strip. Under these conditions two protein fractions should migrate as parallel bands with the same mobility if they are electrophoretically identical proteins or as staggered bands if they are different proteins. All three mixtures were shown to contain the same major electrophoretic components; viz. ovalbumin, ovomucoid, "globulins", conalbumin and lysozyme.

The α_2- and β_2- globulin components of laying hen serum were electrophoretically similar to ovomucoid and conalbumin respectively when compared under the conditions previously described. Cock serum appeared to have only a trace of α_1-globulin which was much increased in hen serum.

(h) Egg yolk and ova proteins. The egg yolks were removed and carefully washed free of the white proteins; the ova were removed directly from the ovary where there could be no possible contamination from the egg white proteins. The materials were compared and found to be electrophoretically identical. The mixture contained three major protein fractions, each of which was further subdivided into three smaller fractions, as shown in fig. 10(f). These main fractions were classed according to their relative mobilities as lipoprotein, globulin and albumin. The largest fraction stained with acetylated Sudan Black indicating that the three protein subfractions corresponded with three distinct lipoproteins. These three lipoproteins were shown to be electrophoretically identical with three lipoprotein staining fractions of laying hen serum (fig. 10(d)).

The protein distribution in these mixtures was obtained from the scanned diagrams and the results are presented in tables XI and XII. It has been assumed that the dye is equally bound by each protein fraction.

Table XI

Protein distribution expressed as percentage

(a) Egg white proteins	Ovalbumin	Ovomucoid	"Globulin" (G_2, G_3)	Conalbumin	Lysozyme
Egg white (Tiselius)	60	8.4	8.3	17.6	5.6
* Egg white (Paper)	57	10.2	9.7	18.9	4.5
* Oviduct white (Paper)	54	22.4		16.7	6.7
Oviduct fluid (Paper)	39.4	6.3	30	19.2	5.3

(b) Serum proteins	Albumin	α_1	α_2	β_1	β_2	γ
Laying hen (Paper)	23.4	18.4	10.4	16.8	18.1	12.9
Cock (Paper)	33.8	6.1	10.8	4.0	21.8	23.8

(c) Yolk and ovum proteins	"Albumin"			"Globulin"			"Lipoprotein		
	1	2	3	1	2	3	1	2	3
Yolk (Paper	2.1	2.4	4.2	7.0	5.6	12.8	30.8	20.4	14.
Total		8.7			25.4			65.9	

* Corrected for albumin tailing.

Table XII

Lipoprotein distribution expressed as percentage

Mixture	Lipoprotein 1	2	3
Yolk	23.8	57.6	18.6
Laying hen serum	43.6	39.8	16.6
Non-laying hen serum	Trace only		
Cock serum			
Egg white proteins	None		

3.3.4. Discussion. The quantitative figures are only given as an indication of the approximate proportions of the main protein fractions. The method used for calculating the percentage composition was not very reliable and too few paper strips were examined to permit a comprehensive analysis. The tailing of the albumin fraction made the estimation of the smaller fractions extremely difficult since the presence of a small amount of albumin greatly affects the area under a small peak. In Table XI the figures have been calculated on the assumption that each peak corresponded to a single protein fraction, except in the cases where the albumin tailing has been accounted for as indicated.

It may be noted that the β_2- and γ-globulin fractions are relatively much greater in fowl than in human serum as observed by

Brandt et al. (1952) and also McKinley et al. (1953). The latter authors demonstrated the presence of lipid material staining with Oil Red O in the albumin fraction, but this was not confirmed with acetylated Sudan Black in the present work.

The considerable difference in the relative compositions of laying hen and cock serum is mainly due to the very large increase in the α_1-globulin in the former. It is not known whether this increased α_1-globulin has any relationship to the formation of egg white proteins. The scanned diagrams of these two sera appear to be quite different from each other in their albumin fraction, the latter being much more compact in laying hen serum. The relative proportions of the β_2- and γ-globulins in these sera are also of interest as the large increase of serum lipoproteins in laying hens would account for the relative increase of the β_2- as compared to the γ-globulin. In cock serum the γ- was greater than the β-globulin.

3.3.5. Summary. (1) The three egg white mixtures examined contained the same five major electrophoretic components, but differed in their water content.

(2) Oviduct whites examined at different stages of development appeared to have the same protein pattern and the same relative composition as whole egg white.

(3) Two egg white proteins had identical electrophoretic mobilities as two serum proteins examined on the same paper strip;

these were ovomucoid and conalbumin corresponding to the α_2- and β_2-globulins respectively.

(4) Three lipoproteins found in egg yolk were shown to have the same electrophoretic mobilities as three corresponding lipoproteins of laying hen serum. Cock and non-laying hen sera contained very little lipid. The egg yolk lipid was probably derived from the serum lipid.

(5) Paper electrophoresis indicated that the serum albumin fractions were almost certainly mixtures of closely similar proteins and were not strictly homogeneous.

3.4. Smithies starch gel zone-electrophoresis

Smithies (1955) developed a most sensitive electrophoretic technique which employed a starch gel as a supporting medium. The technique had the advantage that it resolved protein fractions, not only by their charge difference but also by differences in their size and shape. A specially hydrolysed starch was prepared which had approximately the same particle size as protein molecules, so that during electrophoresis a molecular filtration process took place as well as a migration due to the applied voltage. Using this method two proteins with the same net electrostatic charge at a given pH but differing in their size and shape could be distinguished, whereas the Tiselius technique applied under identical conditions would detect a single electrophoretic component. The apparatus was very simple and could easily be made in the laboratory at low cost.

Perspex troughs were made with the following internal dimensions 24 x 2 x 0.9 cm. A cutting block was made with the same dimensions as the troughs except that the height was half that of the latter (0.45 cm.) This enabled a gel placed on the cutting block to be cut horizontally into two equal halves with a piece of thin wire like a cheese cutter. A suitable power pack gave a range 0 - 400 volts and 0 - 30 milliamps. The set-up of the apparatus is shown in fig. 11.

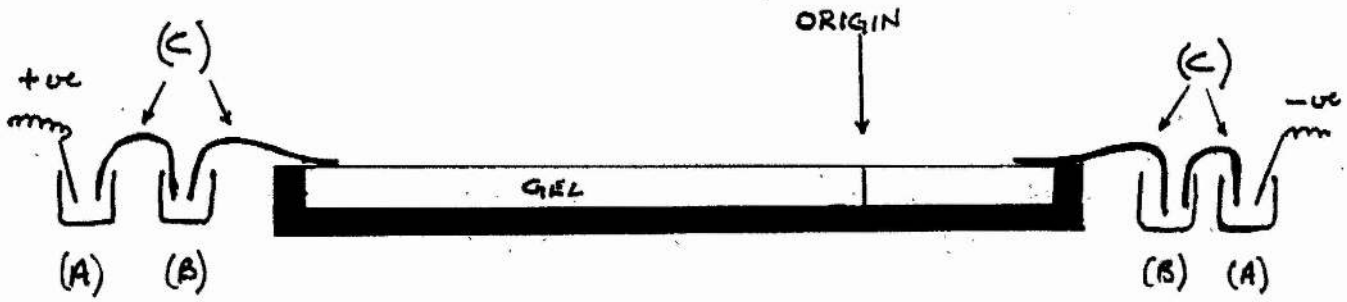


Fig. 11.

Starch gel zone-electrophoresis apparatus.

Carbon electrodes were placed in beakers (A) containing a 2% sodium chloride solution. The current was led into the buffer vessels (B) containing a borate buffer by means of thick wads of filter paper (C). Filter paper bridges also carried the current onto the gel from (B). The complete apparatus was placed in a cold room at 0° during electrophoresis to prevent overheating and cracking of the gel.

3.4.1. Method. The starch was prepared from B.D.H. Soluble Starch, large quantities being prepared at a time so as to give reproducible gels. Soluble starches varied considerably in their gelling properties and the hydrolysis conditions were varied accordingly so as to give a material which gave the same gel strength at 15% concentration.

The only criterion of a suitably hydrolysed starch is a clear reproducible electrophoretic pattern. Prepared starches are now commercially available from Dr. Smithies' laboratory. All batches of hydrolysed starches prepared by the present author were made according to Smithies (1955) procedure except that the time of hydrolysis was varied. Starch (400 g.) was suspended in 800 ml. of a mixture of acetone and concentrated hydrochloric acid (99% acetone, 1% acid) and incubated at 37° for a period of time (Smithies used 45 minutes). The reaction was stopped by the addition of 200 ml. M-sodium acetate and the starch quickly filtered on a large Büchner funnel. The residual acid and acetate were removed by exhaustive washing with water and finally the material was allowed to remain overnight in water before filtration and careful drying in acetone. Acetone washing was continued until the starch appeared like silk to touch, after which the product was dried by spreading on trays in a current of warm air. The drying process was found to be critical. If the starch contained a little too much moistur or was dried at too high a temperature the resultant powder contained very small hard granules which caused irregularities in the final gel.

As each starch preparation had slightly different gelling properties the starch concentration was altered to give a gel of the correct strength for electrophoresis, i.e. 15 - 17% or 19 - 21%. These two ranges of gel strength were used for the short and the long times of electrophoretic runs respectively. The reason for a stiffer gel being used on a long run was that less fluid was lost from the anodic end of the gel under these conditions. Electro-endosmosis caused the dehydration of the anodic end which cracked the gel and distorted the electrophoretic pattern.

The conditions of electrophoresis were rather variable and were worked out to suit each mixture examined. This was necessitated by variations in starch and by the duration of the run. The conditions were not those of Smithies (1955) since a longer time was found to be essential for the fractionation of the egg white proteins than for serum. This may have been due to the massive quantities of ovalbumin which tended to mask other fractions, unless this protein was drawn far along the gel towards the anode. The most satisfactory conditions are given in the accompanying table (XIII). The times of runs were so arranged that the apparatus was used during the day on short runs and overnight for long runs. Although the patterns obtained were almost identical in the relative position of their protein staining bands, the actual distance migrated was greater in the long run. The higher voltage and less rigid gel used in the shorter run seemed to have approximately the same resolving power as the conditions used in the

longer run.

Table XIII

Starch gel zone-electrophoresis conditions

Condition	Short run	Long run
Duration	8 hr.	18 hr.
Gel composition	15 - 17%	19 - 21%
Voltage	400 v.	250 - 300 v.
Current	10 mA.	7 mA.
Gel buffer	0.03 M-Boric acid, 0.012 M-Sodium hydroxide, pH 8.48	
Bridge solution	0.3 M-Boric acid, 0.06 M-Sodium hydroxide	
Electrode solution	2% Sodium chloride	
Electrodes	Carbon rods	
Temperature	0°	
Stain solvent	Methanol:Water:Glacial acetic acid: (5:5:1)	
Stain	Naphthalene Black or Amido Black 10B dissolved in stain solvent	
Washing solvent	Stain solvent	

The preparation of the gels was carried out under standard conditions in all experiments. The correct quantity of hydrolysed starch was suspended in the gel buffer and heated over a Bunsen burner with continuous shaking until the starch granules burst and a clear gel

formed, this taking place just as the mixture began to boil. The gel was allowed to stand for two minutes so that the bubbles rose to the surface. If the gel was poured at once the bubbles were trapped in the rapidly solidifying gel and interfered with the electrophoretic separation. The perspex containers were prepared by placing a fine strip of polythene sheet in the base and then greasing the whole trough with a little liquid paraffin or silicone grease. The gel solution was then poured into the troughs and a second greased polythene strip placed on the top so as to prevent evaporation. The gels were cooled in an ice-box at -5° for twenty minutes when they were quite firm and could be used for electrophoresis. Gels prepared and left overnight were found to be satisfactory, provided that no cracking due to loss of moisture had taken place.

Samples to be examined were inserted on small pieces of filter paper in a slit cut in the gel near the cathode end. Two samples could be compared by inserting the samples side by side in the same slit with a small piece of filter paper soaked in water acting as a diffusion barrier in between the samples. The suspension method described by Smithies was found to be less satisfactory than the filter paper insertion method, the former method giving indistinct patterns due to protein diffusion. The suspension method employed starch as a support for the insertion of the protein mixture. Finally the polythene top cover was replaced under a series of microscope slides to ensure contact between the gel and the cover.

The gels were transferred to the cold room and set up as shown in fig. 11. Three gels could be run in parallel, or four in a combination of two pairs connected together in series.

After completing the electrophoretic run the gels were lifted out of their troughs and transferred to the cutting block. Each gel was cut horizontally along its length and immersed in the stain solvent, the two halves were then carefully separated by flotation with the help of a spatula pushed gently between the cut surfaces. The upper half of the gel was turned upside down so as to expose the cut surface for staining, the solvent removed by a syphon system and the dye solution poured round the gels. The strength of gel was critical at this stage since the gels were extremely fragile and required care in handling. After remaining in the solvent the gel became harder and more easily managed.

Staining with a saturated solution of dye was carried out for half an hour, excess stain being washed out from the gel background by washing with solvent for twenty-four hours using several changes of solvent. The solvent was recovered after cleaning with charcoal. The time of staining described by Smithies was half a minute in the saturated dye solution and with such a short period the excess dye was easily eluted from the gel in a few hours. However, clearer patterns were obtained in the present work by using the longer period of staining. This was particularly noticeable in the

case of minor components. The stained gel was examined and the protein bands recorded by laying the gel on a piece of filter paper and marking off the position of each band on the paper, the pattern was then transferred to a sheet of graph paper on which all gel pattern were recorded. No satisfactory photographic technique was available because of the thinness of the bands and also the difficulty in handling gels. The gels were stored in boiling tubes with well fitting corks in the dark, but in spite of all precautions the dye faded within a few months.

In all experiments, no two gels could be guaranteed to give identical patterns in respect to the mobilities of a given band such as the albumin. The relative mobilities of the bands were nearly constant, and the electrophoretic pattern was readily reproduced using the same conditions.

3.4.2. Results. The results may be best described by examining the patterns obtained from various protein mixtures which are diagrammatically shown in figs. 12 - 14.

(a) Whole egg white. Egg white was examined after a twenty hour run and sixteen protein fractions were clearly separated. Nine of the were major fractions and seven were sub-fractions. The numbering system employed describes all anodic fractions as positive and cathodic as negative. Sub-fractions are indicated by adding a subscript to the number of the major component having slightly lower

mobility. For instance 5_1 will be the minor fraction which migrates between the major components 5 and 6. It was not possible to characterise all the fractions. A single electrophoretic component migrated at a different rate from that at which the same component moved in a complex mixture such as egg white or serum. As protein-protein interaction is a real danger in the interpretation of electrophoretic patterns obtained in this type of analysis, it is not advisable to draw rigid conclusions as to the identity of protein fractions. For example when two protein mixtures such as egg white and serum are compared on the same gel, certain protein bands may appear to be in phase on both halves of the gel. These fractions with corresponding electrophoretic mobility should not necessarily be assumed to be an identical protein, common to each complex mixture. This technique is very suitable for the comparison of two mixtures closely similar in protein composition such as different sera or egg and oviduct whites.

Fractions 6, 7 and 8 were shown to be ovalbumins appearing in approximately the proportions given by Cann (1949). Six times crystallised ovalbumin prepared by Cole's (1932) method also contained the same components, which migrated with the same mobilities alongside the ovalbumin fractions of egg white. These three components of egg white were eluted, concentrated and compared with a sample of the crystalline protein on a second gel, the three components from each side of the gel being electrophoretically identical.

Fraction -1 was shown to be lysozyme. Components 1, 1_1, 1_2, 2, 3, 4 and 4_3 were shown to be similar to those derived from a sample of "ovoglobulin" prepared according to Kaminski (1956). This fraction was precipitated between 25 - 50% saturation with ammonium sulphate and probably contained traces of the more soluble proteins, since the crude material from the first precipitation was shown by paper electrophoresis to be similar to whole egg white except that the globulins were enriched.

"Thick", "thin" and "inner thin" layers of white were shown to be identical to whole egg white in all sixteen protein fractions, although the "thick" layer had to be diluted before electrophoretic analysis.

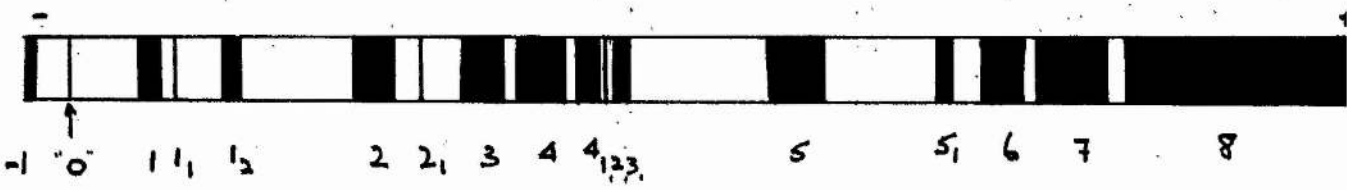


Fig. 12.

Protein fractions from egg white and oviduct white obtained by starch gel analysis. "O" indicates the origin.

(b) Oviduct white. Oviduct white and egg white were shown to be electrophoretically identical. By comparison on the same gel the two mixtures each gave a pattern after staining of sixteen protein bands in phase with each other along the length of the gel. Oviduct whites

at different stages of development were compared and all found to have the same electrophoretic distribution. The material obtained from the upper end of the magnum was so viscous that it had to be diluted with gel buffer before analysis. It was obvious that the oviduct whites differed from egg white and among themselves only in their water content, as was found in the paper electrophoretic analysis. The diagram of the starch gel fractionation of oviduct white is given in fig. 12.

(c) Laying hen serum. Sera from laying hens were found to vary slightly between individual birds and this was confined to the three protein fractions numbered 2, 3 and 4, which appeared to be greatly increased in some birds. This difference may well be an indication of several genotypes in fowls similar to those demonstrated in humans by Smithies (1955) and in cattle by Ashton (1957). In this case hen serum was shown to contain at least thirteen or fourteen distinct protein fractions. The cathodic component usually appeared as a thick diffuse band but was occasionally subdivided into two minor components. Three distinct prealbumins, similar to the two prealbumins of human serum, were easily separated from the main serum albumin fraction (5). The electrophoretic pattern, which could be obtained by either the short or long run, is diagrammatically shown in fig. 13.

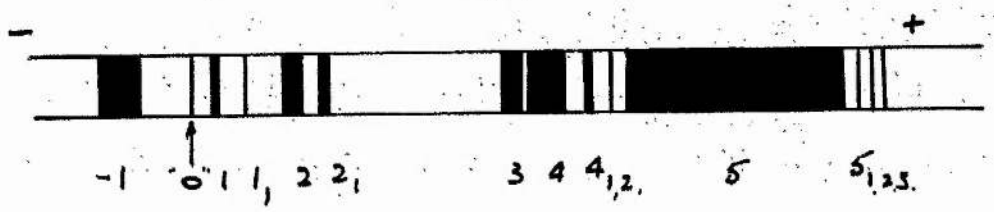


Fig. 13.

Starch gel fractionation of laying hen serum. "0" indicates the origin.

(d) Non-laying hen serum. This seemed to be very similar to laying hen serum in the distribution of protein fractions, again thirteen or fourteen components were demonstrated.

(e) Cock serum. With cock serum it was found that poor resolution occurred of the components which migrated at a slower rate than the serum albumin but these were always indistinct and blurred. The quantitative differences shown between laying hen and cock sera on paper electrophoresis could not be supported by the starch gel analysis attempted. These may be real differences which could not be shown on gels because of the diffuse patterns obtained or they might equally well be due to genetic differences between the two cocks examined and the more numerous hens. It will be remembered that three of the major hen serum protein fractions were shown to be greatly increased in some birds.

(f) Yolk proteins. Yolk proteins obtained from washed egg yolks and ova removed from the ovary, were shown to be electrophoretically identical in their protein composition, four groups of fractions

present containing twelve sub-fractions. The nine protein bands obtained on paper were further subdivided by the more refined gel technique. In this case no surface denaturation was likely to occur and gel electrophoresis was preferable to paper when examining lipoprotein mixtures. Acetylated Sudan Black was used in an attempt to detect the lipoproteins but after immersion in an alcoholic solution of the dye for twelve hours no staining was observed. The failure of lipid staining was probably due to the fact that the protein on the gel could not be fixed prior to staining and that the lipid was extracted by the solvent rather than the fixed lipoprotein adsorbing the dye. It is porbable that the two largest fractions numbered 3 and 4 were lipoproteins since the largest components of yolk were shown to be lipid-containing by paper electrophoresis. The protein pattern obtained with yolk proteins is given in fig. 14.

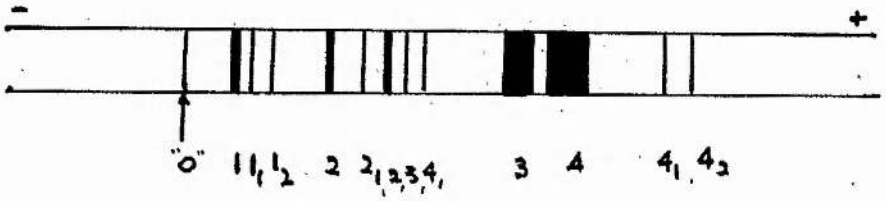


Fig. 14.

Fractionation of yolk and ova proteins on starch gels. "O" indicates the origin.

3.4.3. Discussion. Comparative studies on egg white and serum proteins were not carried out using starch gels because of the

uncertainty in interpreting the resultant electrophoretic patterns. The effect of protein-protein interaction has already been mentioned and an example of this phenomenon can be given to illustrate this point. A small sample of hen serum albumin was prepared by ammonium sulphate fractionation using Smithies technique as a guide, and by a process of trial and error the fraction which precipitated in the range 33 - 35% saturation was found to be mainly serum albumin. This albumin enriched fraction was shown to contain a major component which migrated with the same mobility as serum albumin of whole serum, when examined on the same gel. On further purification of this material by reprecipitation a single component was obtained which migrated at a rate considerably greater than serum albumin. This component could not have been a prealbumin since the enriched serum albumin fraction had given no trace of a prealbumin band. The final purified material was mixed with whole serum and examined and the serum albumin found to be increased but not the prealbumin. This was a clear case of protein-protein interaction which changed the electrophoretic behaviour of one protein in the presence of a mixture of other proteins. Similar observations have been described by Kaminski (1957) for agar gel electrophoresis.

The resolution achieved on starch gels was far greater than was possible with the conventional Tiselius or paper electrophoresis, even so it was obvious that the gel technique could be made to give still greater fractionation if the length of gel along which the fractions

migrated was increased. For example, if the conditions suggested by Smithies were employed, the albumin band would move only about 6 cm. and the major fractions could then be clearly detected. If the conditions suggested in the present work were used the distance moved by the albumin would be approximately trebled and the minor fractions become distinct entities. It is suggested that the resolving power of Smithies technique can be increased and that the major fractions will be further fractionated. Poulik and Smithies (1958) have achieved greater resolution with two-dimensional gel electrophoresis.

3.4.4. Summary. (1) The three physically distinct layers of egg white examined were demonstrated to have an electrophoretically identical protein composition, each containing sixteen fractions.

(2) Oviduct white was found to have the same protein composition during its formation as whole egg white.

(3) Hen serum was shown to contain fourteen protein fractions, three of which may be associated with different genotypes within the species.

(4) Yolk and ova proteins were found to be identical, each having twelve components.

(5) No definite conclusion could be reached concerning the origin of the egg white proteins.

4. Intravenous Injection of Dyed Ovalbumin

It was thought that injected labelled ovalbumin might filter through the oviduct wall and appear in the eggs. Ovalbumin was reacted with excess Evans Blue (T 1824) and finally crystallised as the dyed protein. The absorption spectrum indicated a broad maximum at 580 mμ, and this was used for assay purposes. Two hens which were in the middle of their laying cycle were given intravenous injections through the wing veins of the concentrated dialysed material. The eggs layed after injection were examined for absorption at 580 mμ but in neither case was any dyed protein detected.

The dyed protein was found to be irreversibly adsorbed on dialysis tubing, filter paper and other materials, probably due to the number of free ionised groups on the dye molecules. It is suggested from these observations that the dyed ovalbumin was irreversibly bound to the walls of the blood vessels and could not pass into the oviduct. Radioactive ovalbumin would have been a more suitable protein to follow in the blood stream, but facilities for this type of work were not available.

5. Immunological Studies

This work was carried out by Miss Wakefield (Honours B.Sc. Thesis, St. Andrews, 1956), who used the proteins prepared as described earlier in this work. The anti-sera were obtained from

rabbits sensitised according to the method described by Porter (1955).

5.1. Results

The results of this study are summarised below.

(1) Rabbit anti-sera against ovalbumin cross-reacted with laying hen serum at a dilution of antigen of 1 g. in 8,000 ml.

(2) Anti-sera against laying hen serum cross-reacted with both conalbumin and ovalbumin using a dilution of antigen of 1 g. in 8,000 ml.

(3) Lysozyme and ovomucoid were found to be very weakly antigenic and gave no cross-reaction with anti-sera against hen serum.

5.2. Discussion

These results are in good agreement with those of Marshall and Deutsch (1951), who examined the quantities of ovalbumin and conalbumin in fowl serum by immunological means. Their results are presented in Table XIV.

Table XIV

Immunological analysis of egg white proteins in fowl serum (Marshall and Deutsch, 1951)

Serum	Ovalbumin	Conalbumin
Laying hen	7.6 mg. %	170 mg. %
Non-laying hen	1.4 mg. %	110 mg. %
Cock	0.25 mg.%	170 mg. %

Marshall and Deutsch were able to precipitate ovalbumin from laying hen serum by adding anti-ovalbumin. When the antigen-antibody complex was separated and the serum ovalbumin compared electrophoretically with whole egg white, the former was found to be identical with egg white ovalbumin. Similarly conalbumin from hen serum was shown to be identical, in immunological activity, iron-binding power and electrophoretic mobility, with conalbumin obtained from egg white.

The results of these authors and Miss Wakefield are in agreement with the theory that the egg white proteins are not dependent for their synthesis entirely on the oviduct. Some of these proteins are capable of being synthesised elsewhere. The other sites of synthesis may not contribute greatly to the egg white, but this problem has not yet been fully examined.

6. Conclusion

The experimental work carried out on the dynamic aspects has not definitely proved whether the egg white proteins are synthesised in the oviduct or elsewhere. The immunological studies of Miss Wakefield and the evidence in the literature do give the impression that these proteins may be derived at least in part from some source other than the oviduct. Kaminski's immuno-electrophoretic techniques should prove of value in this work. As the techniques employed in the present study were not capable of solving this problem the dynamic aspects have been laid aside in order to study the structure of native ovalbumin.

PART IV

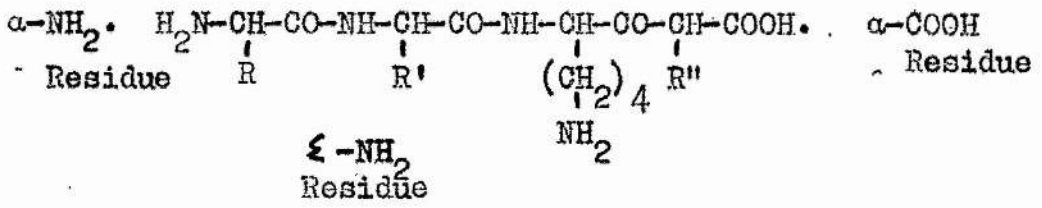
N-TERMINAL ANALYSIS OF THE PURIFIED EGG WHITE PROTEINS

1. Introduction

The most popular chemical method for the analysis of the N-terminal residues of protein chains is the DNP technique initiated by Sanger (1945). 1-Fluoro-2:4-dinitrobenzene (FDNB) is coupled to the free α-NH_2 residues and also to any side chain NH_2 groups (usually ε-NH_2 groups of lysine) to form the dinitrophenyl derivative or DNP-protein. Acid hydrolysis of the DNP-protein yields free amino acids; ε-DNP-lysine and δ-DNP-ornithine from the side chains and α-DNP amino acids from the N-terminal residues. The DNP-amino acids are usually fairly stable and have a characteristic yellow colour which is a great convenience in their analysis by chromatography.

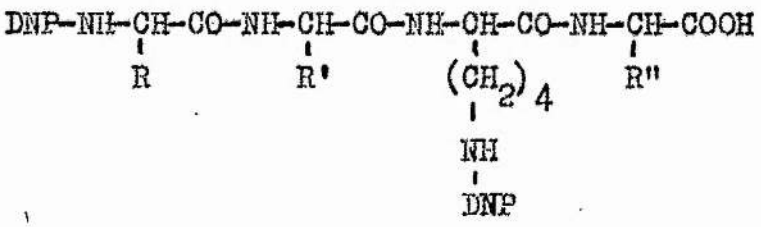
Sanger (1945) originally carried out the reaction in 65% ethanol FDNB dissolved in ethanol was added to a 2% protein solution dissolved in 2% sodium bicarbonate and the mixture shaken at room temperature for a few hours to complete the reaction. The pH was lowered to stop the reaction with the simultaneous precipitation of the DNP-protein. The precipitate was washed free of excess reagent with ethanol, with water to remove salts and finally with ethanol and ether before drying.

Levy (1955) used the pH-stat to keep the pH constant throughout the reaction, this modification enabling the reaction kinetics to be



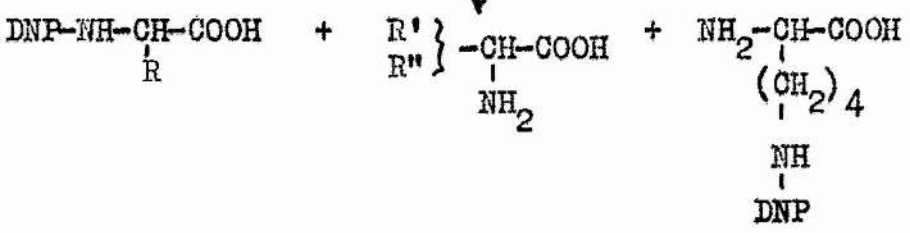
PROTEIN IN 2% $NaHCO_3$ SOLUTION

FDNB IN ETHANOL



DNP-PROTEIN PRECIPITATED IN ACID

6.N-HCl HYDROLYSIS FOR 18 HOURS



α-DNP-Amino Acids Amino Acids ε-DNP-Amino Acids

ETHER FRACTIONATION

Ethereal fraction	Aqueous fraction
α-DNP and Di-DNP-Amino Acids	Free Amino Acids
	ε-DNP-Amino Acids

Fig. 15.

Sanger (1945) DNP technique.

studied. The number of DNP groups introduced per molecule of protein was calculated from the kinetic data. Other modifications of the reaction conditions have been made by Courts (1954) and Levy and Li (1954) in which the presence of ethanol is not required. The method of Courts will be described in another section dealing with the structure of native ovalbumin.

Senger hydrolysed the DNP-protein in 6N-hydrochloric acid for a period up to eighteen hours under reflux. The hydrolysate was then extracted with ether to separate the ether soluble α-DNP-amino acids from the water soluble ε-DNP-basic amino acids. Analysis of the two fractions was carried out on silica gel columns.

The reaction is best described diagrammatically as shown in fig. 15.

The reaction products are unfortunately not always simple to fractionate and characterise. The complexity of the hydrolysis products is due to the fact that other side chains apart from ε-NH_2 residues of basic amino acids also react with FDNB as well as the artifacts formed from the acid degradation of some less stable DNP-derivatives. The amount of degradation of DNP-amino acids varies from protein to protein; this might be expected from the different lability of a given DNP-amino acid when linked by peptide bond in the DNP-protein to different amino acids. The presence of carbohydrates in the protein hydrolysate is also thought to cause degradation of DNP-derivatives.

The amino acid residues given in the following table react with FDNB under the conditions used by Sanger (1945). Those which are acid labile are indicated along with the artifacts produced (where these are known). The two main artifacts 2:4-dinitrophenol and 2:4-dinitroaniline will be abbreviated throughout this work to DNPOH and $DNPNH_2$ respectively.

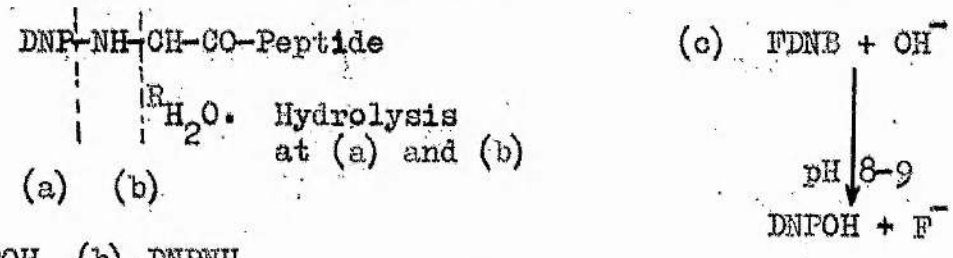
Table XV

Amino acid residues in the intact protein reacting with FDNB and their acid labile products formed during hydrolysis at 105° with 6N-HCl.

Class of Amino acid	Amino acid	DNP product	Fraction	Artifact
1 Terminal				
α-NH_2	All	α-DNP-amino acid	Ether	-
	Proline	α-DNP-proline	Ether	DNPOH, $DNPNH_2$
	Glycine	α-DNP-glycine	Ether	DNPOH
	Cysteine	α-DNP-cysteine	Ether	DNPOH
2 Side chain				
ε -NH_2	Lysine	ε-DNP-lysine	Water	-
δ -NH_2	Ornithine	δ-DNP-ornithine	Water	-
-SH	Cysteine	S-DNP-cysteine	Water	DNPOH
-OH	Tyrosine	O-DNP-tyrosine	Water	DNPOH
-Im	Histidine	Im-DNP-histidine	Water	DNPOH, $DNPNH_2$
$-NH_2$	Amino sugar	N-DNP-amino sugar	Water	? ?

The presence of a basic amino acid as an N-terminal residue will lead to disubstitution with FDNB, for example α-ε-di-DNP-lysine as found in DNP-lysozyme (Thompson, 1951) and in DNP-casein (Mellon et al. 1953). Arginine is the only basic amino acid which will not substitute in the side chain ε-NH_2 position at a reaction pH 8 - 9, since the guanidine group is completely charged below pH 12. Arginine was found to be substituted only in the α-NH_2 position by the present author in earlier work. This has been confirmed by Dr. Porter (personal communication).

The two main artifacts DNPOH and $DNPNH_2$ in the hydrolysates of DNP-proteins may lead to confusion during the chromatographic analysis unless great care is taken. These compounds are both found in the ether extract and may be distinguished from true DNP-amino acids by their ultraviolet spectra in acid and alkaline solution. DNPOH is readily decolourised in acid and this fact is used to detect it on paper chromatograms which may be suspended in acid vapour, the yellow colour of the DNPOH spot being rapidly bleached. The artifacts are probably formed from the degradation of acid labile DNP-derivatives as shown in the diagram below. DNPOH is formed very slowly in the initial reaction and is bound to the DNP-protein (see Fig. 16(c)).



(a) DNPOH (b) $DNPNH_2$

Fig. 16.

Formation of artifacts during DNP analysis.

2. Preparation of DNP-amino Acids

Several DNP-amino acids were prepared by Sanger's (1945) technique. In some cases selective substitution was carried out by using suitable blocking agents such as the copper chelates and carbobenzoxy residues attached to the α- and ε-NH_2 residues respectively. The melting points of these derivatives are given in the accompanying table, which also includes comparative figures obtained from the literature.

Table XVI

Melting point data for DNP-amino acids prepared

DNP-derivative	Recrystallising solvent	Mp. found	Mp. quoted	Author
DNP-glycine	Methanol	200-202	195-202	Sanger (1945)
DNP-phenylalanine	Methanol	179-186	186	Sanger (1945)
	Ethanol	187-188		
DNP-threonine	Ethanol/water	137-140		
DNP-alanine	Methanol	176	178	Abderhalden & Blumberg (1910)
α-DNP-arginine HCl	20% HCl	168		
ε-DNP-lysine HCl	20% HCl	186	186	Sanger (1945)
di-DNP-lysine	Ethanol	124-126		

The melting points of DNP-derivatives are not always sharp and tend to vary with the solvent employed in crystallisation. The melting points are not considered to be of great value in the characterisation and estimation of purity in these derivatives. No mention was found in the literature of the melting points of DNP-threonine, α-DNP-arginine and di-DNP-lysine. In many cases the purity and identity of these derivatives has been deduced by comparison of the suspected and authentic derivative on chromatographic behaviour and elementary analysis of C.H.N. and O. These methods were used by Katchalski et al. (1948) in the characterisation of di-DNP-lysine.

3. Preparation of DNP-proteins

Sanger's (1945) conditions were used to determine the N-terminal residues. The reaction was carried out at room temperature with constant shaking using a 2% protein dissolved in 2% sodium bicarbonate solution and adding an ethanolic FDNB solution until approximately 65% v/v ethanol. The reaction was usually continued for eighteen hours before stopping by acidification. The DNP-protein was precipitated with ethanol and washed free of excess reagent with ethanol and ether before drying under vacuum. In some preparations the precipitation with ethanol at acid pH did not take place unless the pH was brought to the isoelectric point.

3.1. Hydrolysis conditions

Duplicate samples of the DNP-protein (ca. 100 mg.) were hydrolysed in 6N-hydrochloric acid under reflux for eighteen hours. The acid was found to cause degradation of DNP-derivatives unless previously distilled. Constant boiling point hydrochloric acid was found to give the most reproducible results.

3.2. Extraction of DNP-amino acids

The hydrolysates were extracted four times with peroxide-free ether. The presence of peroxides in the ether caused rapid degradation of DNP-derivatives, especially in the aqueous layer in which hypochlorite was produced by reaction with the acid. Hypochlorite rapidly decolourised the yellow DNP-amino acids. The ether was purified before extraction by shaking with a solution of acidified ferrous sulphate. The ether extract containing the α-DNP-amino acids, di-DNP-amino acids as well as the artifacts, was concentrated and analysed by chromatography on columns and paper.

The aqueous fraction containing ε- and δ-DNP-amino acids, any other water soluble derivatives such as S-DNP-cysteine and unsubstituted amino acids was concentrated by vacuum distillation six times until all traces of excess acid had been removed. The concentrated material was then ready for analysis.

3.3. Analysis of DNP-amino acids

Paper and columnar chromatography were the main analytical method

used, all techniques being carried out in the dark where possible since DNP-derivatives are known to be light sensitive to a varying degree.

3.3.1. One-dimensional paper chromatography. Whatman No. 1 and No. 20 papers were used throughout and the results were confirmed using several of the solvent systems. These systems are given below with their respective abbreviations.

(a) n-Butanol-Acetic Acid-Water : 4:1:5 (BAW)

(b) s-Collidine-Water saturated (CW)

(c) Tetrahydrofurfurylalcohol-Water-Urea: 80:20:20:(w/v/w) (THFA)

(d) Tertiary Amyl Alcohol buffered at pH 6.0 (BTA) described by Blackburn and Lowther (1951).

(e) n-Propanol-Petrol Ether (bp. 80 - 100): 30:70 buffered with 0.05 M-phosphate pH 6.0 (BPPE).

The filter papers were soaked in the appropriate buffer and allowed to dry before a warm fan when the THFA and BPPE systems were used. The latter system was a modification of the solvent described by Blackburn and Lowther (1951) and was found to give excellent results. In particular, DNPOH and DNP-alanine were well separated from each other whilst $DNPNH_2$ travelled near the solvent front. Most other solvent systems were found to give a single spot when both these compounds were applied to the paper as a mixture.

3.3.2. Two-dimensional paper chromatography. A limited number of two-dimensional chromatograms were carried out using the conditions of Biserte and Ostrieux (1951). Large sheets of Whatman No. 1 paper were

used, the first direction was run in a toluene mixture (ascending) and the second developed by descending chromatography using phosphate buffer. The toluene mixture was prepared from a mixture of toluene-pyridine-glycol monochlorohydrin (5:1:3 v/v). To this solution were added three volumes of 0.8 N-ammonium hydroxide and the two phases equilibrated for an hour before separation. The toluene layer was cleaned of water droplets by filtration whilst the aqueous layer was mixed with 300 ml. of 0.8 N-ammonium hydroxide and equilibrated in the chromatographic tank with the paper before running with the organic solvent. After the completion of the ascending run the paper was dried in a hot air blast and finally developed in the second direction with phosphate buffer (M-sodium phosphate and 0.5 M-sodium dihydrogen phosphate). This system also had the advantage of fractionating DNPOH and DNP-alanine, although the single dimension separations were more easily carried out.

3.3.3. Columnar chromatography. This was employed for the analysis of larger quantities of DNP-derivatives. The advantages of columnar compared to paper chromatography were that the fractions could be re-examined on paper, analysed by their ultraviolet absorption spectra or degraded to the free amino acids by Lowther (1951) ammonolysis. The application of a second analytical technique to the individual fractions was considered to be essential in the characterisation of DNP-derivatives.

Columns were packed with a variety of supports, viz. cellulose,

talc and celite. The conditions described by Courts (1954) for celite 545 (Johns Mansville and Co.) were found to be satisfactory. The celite was equilibrated with M-sodium dihydrogen phosphate pH 4.0 and dried before use, mixtures of ether and chloroform being used as solvents (Courts, 1954).

The best results were obtained with columns packed with cellulose buffered with M-sodium dihydrogen phosphate pH 4.0, using n-propanol-petrol ether (30:70 v/v) buffered with the same phosphate buffer as solvent. This system will be abbreviated to BPPE pH 4.0. Remarkable fractionations were possible; for example a 6 x 0.5 cm. column gave a clear separation of a mixture the DNP-derivatives of the following, amino acids, alanine, aspartic acid, glycine, leucine, phenylalanine, serine and threonine. The only disadvantage of the technique was the degradation of a small quantity of the DNP-derivatives to $DNPNH_2$. This may have been due to light sensitivity of certain DNP-amino acids. A mixture of DNPOH and DNP-alanine applied to the column gave a trace of a fast component which was identified as the artifact previously mentioned. For qualitative analytical purposes the cellulose columns were preferred to the celite columns of Courts. This system was developed from the modification of Blackburn and Lowther (1951) solvent used for paper chromatography.

4. Results

The results of the analyses carried out on the DNP-egg white

proteins will be given for each protein separately.

4.1. DNP-ovalbumin preparations

The results of the analyses of several preparations are presented in Table XVII.

Table XVII

Analysis of DNP-ovalbumin hydrolysates

Technique	Fraction	Solvent	DNP-derivative	Commen
(a) Paper chromatography	Ether	BAW	DNPOH	Bleached acid and spectrum
	Ether	THFA	DNPOH	
	Ether	BPPE	DNPOH	
	Ether	"Toluene"	DNPOH	
	Aqueous	BAW	ε-DNP-lysine	Positive ninhydrin reaction
	Aqueous	BPPE	ε-DNP-lysine	
(b) Celite 545 columns	Ether	Chloroform-ether, pH4.0 M-NaH_2PO_4	DNPOH	U.V. spectrum
	Ether	BPPE pH 4.0	DNPOH	
(c) Cellulose columns	Ether	BPPE pH 4.0	DNPOH	
	Aqueous	BPPE pH 4.0	ε-DNP-lysine	

Spectra of D.TOH in various solvents.

Solvents: (a) Water, (b) 0.1N HCl, (c) 1 % $NaHCO_3$.

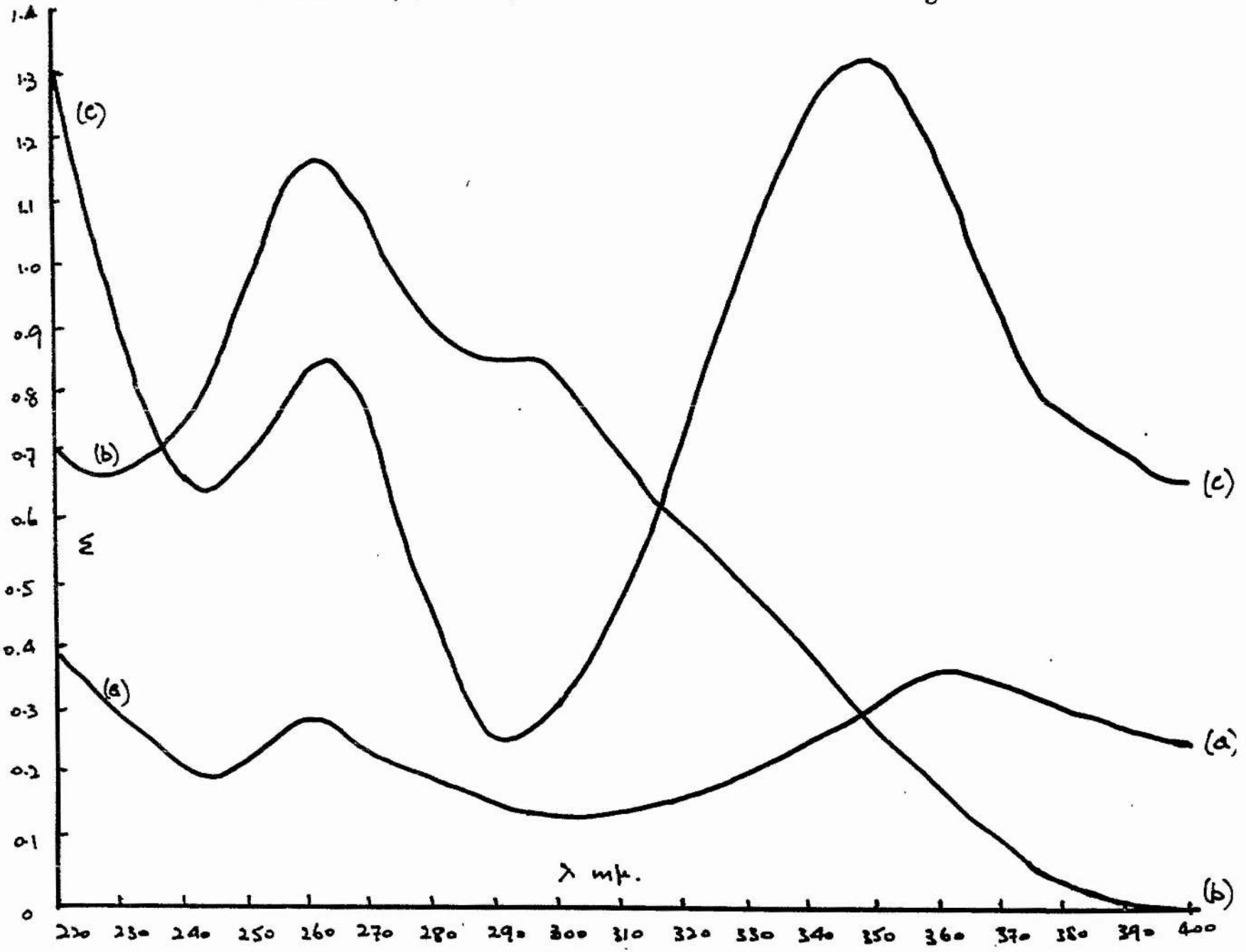


Fig. 17

The chromatographic analyses were checked by comparing a mixture of the authentic DNP-derivative and the fractions isolated from the columns. In every case the mixture was shown to run as a single fraction having the same R_f value as the isolated fraction alon Added confirmation of the presence of DNPOH in the ether extract was obtained from the Lowther degradation of this fraction since no free amino acid could be detected. Acid bleaching of DNPOH was readily shown on paper chromatograms as well as the positive ninhydrin reaction given by ε-DNP-lysine.

Ultraviolet absorption spectra were obtained from the fractions eluted from columns and papers. These analyses indicated the importa of employing more than a single technique for the characterisation of DNP-derivatives. From an examination of the graphs given in figs. 17 - 19, it will be seen that the DNP-ovalbumin ether extract had a spectrum quite unlike free DNPOH. The spot obtained from paper chromatography of this extract was shown to be identical with the spot from an authentic sample of DNPOH run on the same paper, the spectra of the two eluates were identical. The conditions used for the spectral analyses are given in the figures, quartz cells being use throughout.

The graphs in fig. 17 show the change with pH in the absorption spectrum of DNPOH in aqueous solution. In fig. 18 the spectra of the ether extracts of DNP-ovalbumin and the corresponding

Spectra of ether extracts dissolved in water.

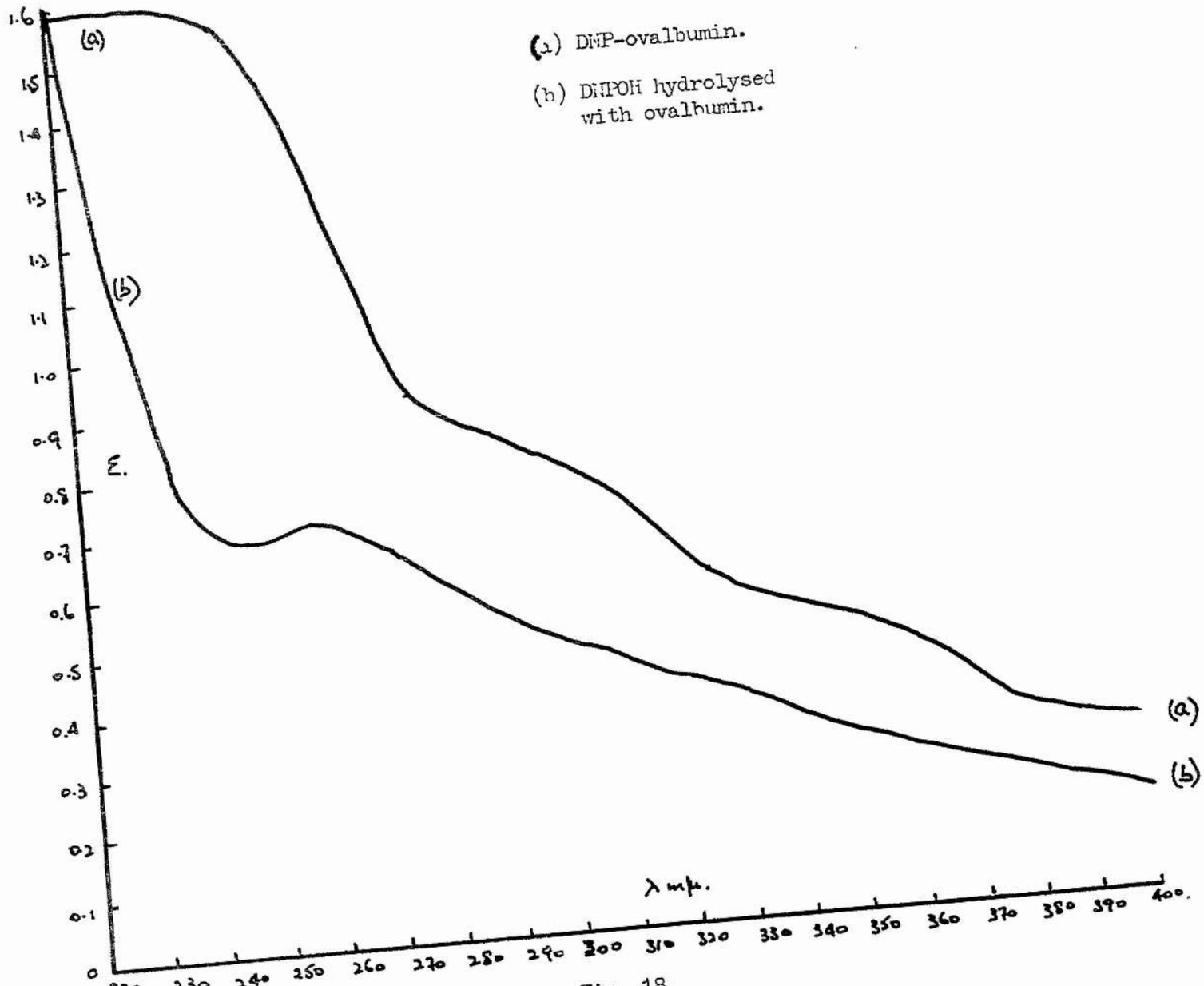


Fig. 18.

Spectra of eluates from paper chromatograms using PIA solvent.

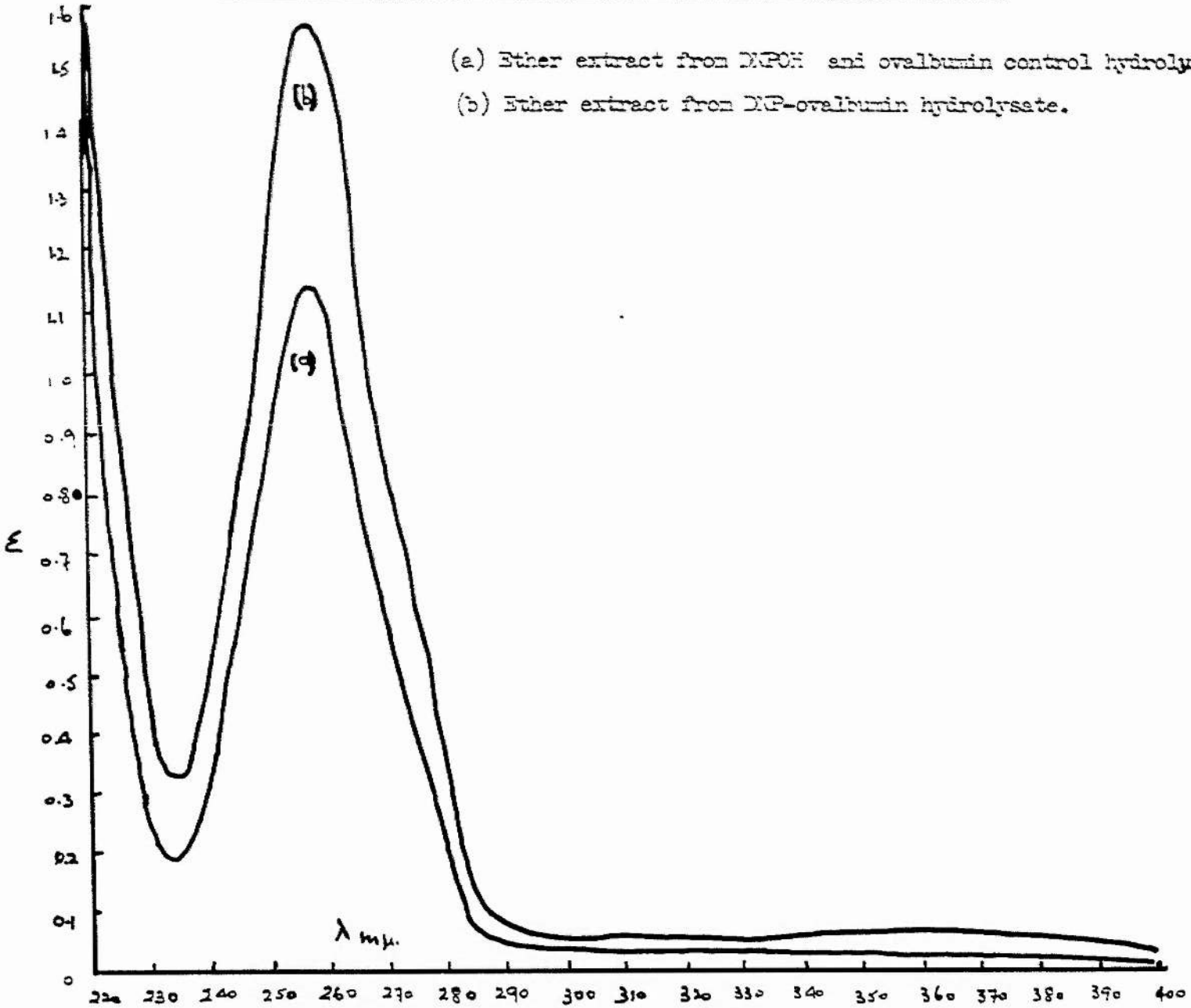


Fig. 19

extract from a control hydrolysate of DNPOH in the presence of ovalbu are compared in aqueous solution; these two curves show a certain degree of similarity but show little resemblance to the curves in fig 17. These two extracts were compared by paper chromatography, the spots eluted and their spectra compared in acid solution as shown in fig. 19. In this final figure the two extracts can be seen to be identical demonstrating that the only ether soluble DNP-derivative obtained from DNP-ovalbumin was the artifact DNPOH. Similar confirmation was obtained from examining these two ether extracts aft chromatography on a celite column.

It may be concluded that ovalbumin contains no N-terminal residue which yields a stable α-DNP-derivative, the hydrolytic produc being DNPOH and ε-DNP-lysine. These results are in agreement with the literature (Porter, 1950). It is suggested that the artifact ma be derived from the degradation of Im-DNP-histidine or from the bindi of DNPOH formed in the initial reaction to the protein (Carstein and Eisen, 1953).

4.2. DNP-conalbumin preparations

The results of the N-terminal analysis of conalbumin were more complicated to interpret than in the case of ovalbumin. The ether soluble fraction contained a number of components which depended on the method of preparation, the quality of the hydrochloric acid, as well as the prior denaturation of conalbumin before reaction with

FDNB. The analytical methods were the standard techniques employed for ovalbumin. The results are given in Table XVIII.

Table XVIII

Paper chromatographic analysis of DNP-conalbumin hydrolysates (ether extracts)

No.	Preparation method		HCl used	Solvent used	DNP-derivative
	Prior denaturation	FDNB reaction			
C_1	-	Sanger (1945)	A.R.	BPPE	$DNPNH_2$
C_2	-	Sanger (1945)	A.R. distilled	BPPE	$DNPNH_2$, DNPOH
C_3	0.75 M. Guanidine HCl	Sanger (1945)	A.R. distilled	BPPE	"Slow" component DNP-alani
C_4	"	Sanger (1945)	A.R. distilled	BPPE	$DNPNH_2$, DNPOH

The results obtained on paper were confirmed using the cellulose columns and BPPE pH 4.0 solvent system. The fraction corresponding to DNP-alanine obtained from C_3 and C_4 was re-run as a mixture with authentic DNP-alanine and found to give a single fraction.

The presence of impurities in the analar (A.R.) hydrochloric

Spectra of $DNPNH_2$.

Solvent: (a) 1 % $NaHCO_3$, (b) 0.1 N HCl.

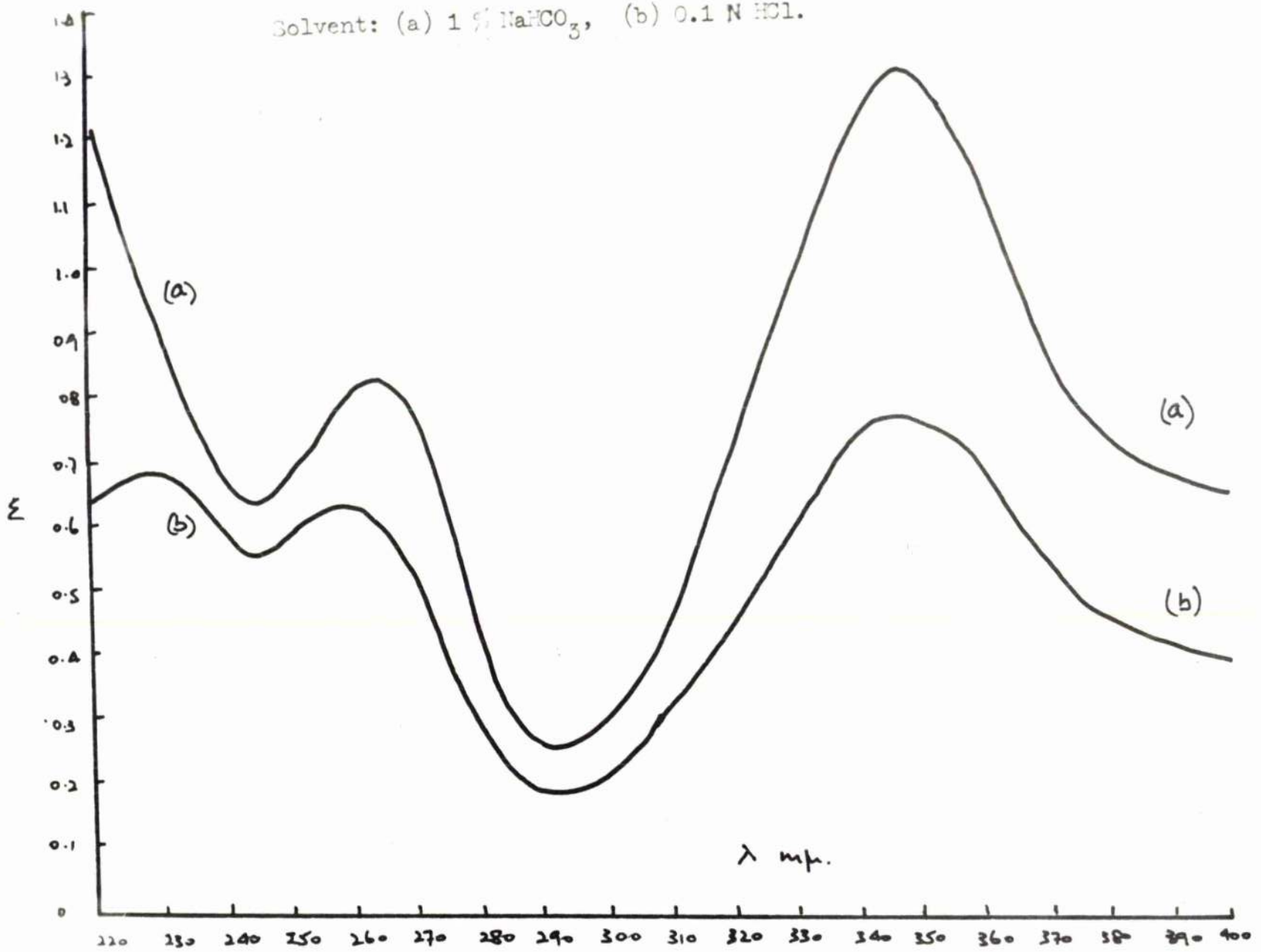


Fig. 20

Spectra of eluates from celite columns using BPPE pH 4 solvent.

Conditions: 0.1 N HCl

Graphs: (a) Fraction $(50)_1$ obtained from DNP-conalbumin C_4

(b) Fraction $(49)_1$ obtained from authentic DNPHH$_2$

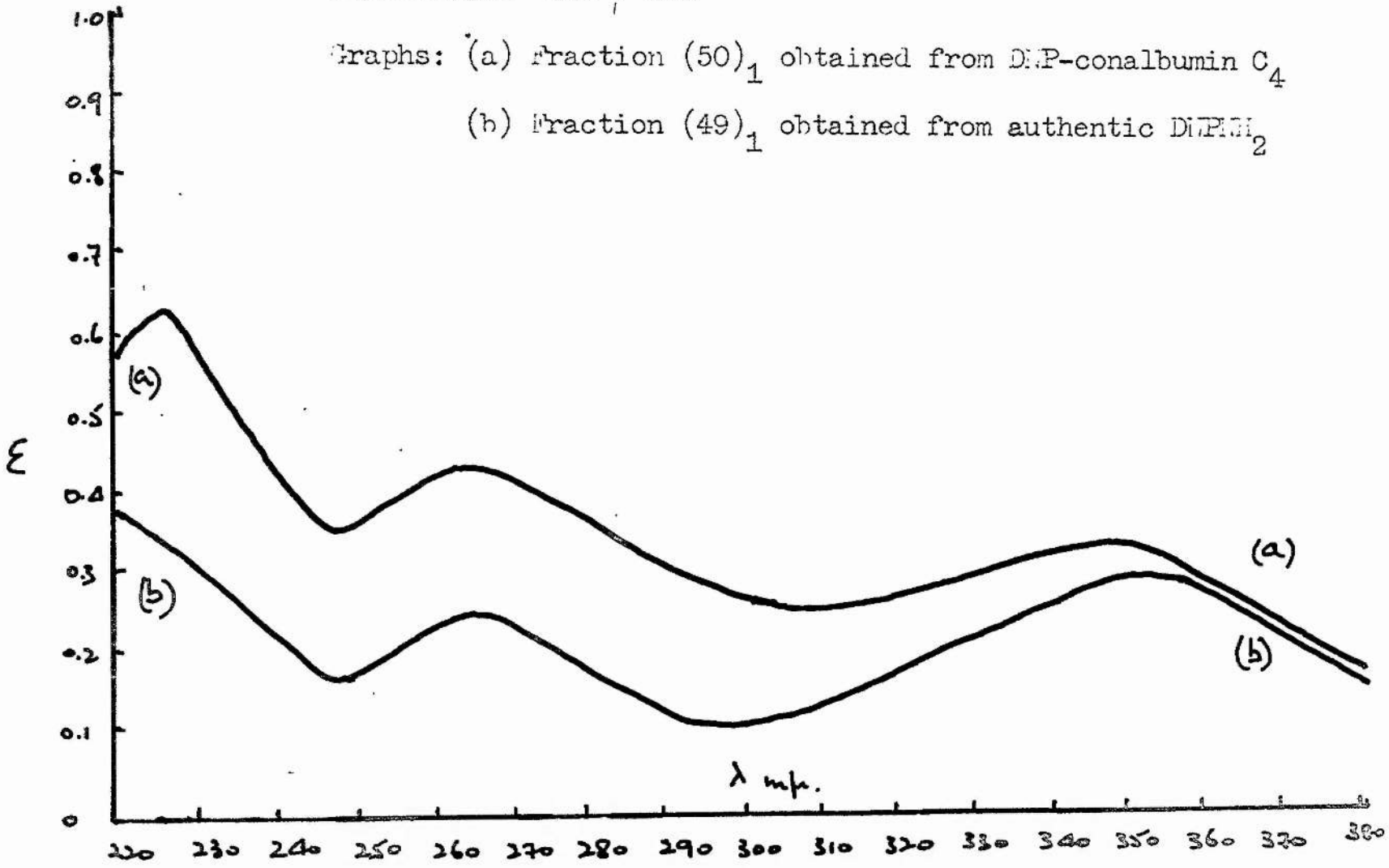


Fig. 21.

acid are thought to have caused the conversion of DNPOH or other DNP-derivatives such as DNP-alanine to $DNPNH_2$. This was confirmed by adding a small quantity of the concentrated impurities obtained from the residue left after distilling A.R. hydrochloric acid to a sample of distilled acid. This mixture was used to hydrolyse C_3 and the only DNP-derivatives formed were the artifacts whereas distilled acid yielded DNP-alanine and the "slow component", as well as the artifacts. The lability of DNP-alanine has already been mentioned in connection with the use of cellulose columns where it was shown to be converted to a mixture of DNP-alanine and $DNPNH_2$. The ultraviolet spectra of $DNPNH_2$ in acid and alkaline solution are given in fig. 20 and it can be seen that under these conditions the spectra are similar to each other unlike those of DNPOH given in fig. 17.

Fig. 21 compares the spectra of fractions obtained from chromatography on celite columns of the ether extract of DNP-conalbumi C_4 and authentic $DNPNH_2$. The fast running fraction from C_4 (fraction 50_1) appears to be closely similar to that of authentic $DNPNH_2$ (fraction 49_1). These two fractions were combined and re-examined on a second celite column and also by paper chromatography and found to travel as a single fraction in each case.

Guanidine hydrochloride denaturation prior to FDNB reaction clearly demonstrated the presence of DNP-alanine as well as a trace of a "slow component" which may have been DNP-serine in the ether

extract. The denaturation is thought to have facilitated the reaction of FDNB with the N-terminal residue, by exposing this group after the molecule had been completely uncoiled. DNP-alanine was confirmed by comparison on paper and columns of the suspected DNP-alanine fraction and the ether extract obtained from a control hydrolysate of DNP-alanin and conalbumin. The control gave a single fraction with identical R_f value to the suspected fraction in BPPB solvent systems, a mixture of the two fractions travelling as a single component. It was observed that free DNP-alanine which had not been subjected to the control hydrolysis and extraction ran at a slightly slower rate than th DNP-alanine in the control.

This control hydrolysate indicated that the free DNP-alanine was not degraded to artifacts during hydrolysis but DNP-alanine bound in a peptide bond may well be less stable.

The aqueous fractions contained only ε-DNP-lysine. It may be concluded that conalbumin contains an N-terminal alanine residue which reacts more readily with FDNB after denaturation. A trace of a "slow component" is also present and this may be due to impurities in the protein preparation or else to a labile DNP-residue. The possibility of serine undergoing acyl-migration in the protein during preparation may also be considered since the "slow component" had a similar R_f to DNP-serine.

4.3. <u>DNP-ovomucoid preparations</u>

The results of paper chromatography of the ether extracts of

hydrolysed DNP-ovomucoid are presented in Table XIX.

Table XIX

Paper chromatographic analysis of DNP-ovomucoids (ether extracts)

No.	Preparation method	Solvent	DNP-derivative
O_1	Sanger	CW DEA BPPB	DNP-alanine DNPOH $DNPNH_2$
O_2	Courts	CW DEA BPPB	DNP-alanine DNPOH $DNPNH_2$
O_3	Prolonged Sanger (48 hrs.)	BPPB	"Slow component" DNP-alanine DNPOH $DNPNH_2$

The analysis of hydrolysed DNP-ovomucoid preparations was found to give very similar results to those described for DNP-conalbumin. The ether soluble fractions all contained the three main components, DNP-alanine, DNPOH and $DNPNH_2$. A trace of the "slow component" was also detected in the case where the FDNB reaction was prolonged - this component was tentatively suggested to be DNP-serine, which may arise

from acyl-migration during the initial reaction. Two other trace components were observed after the ether extract had been allowed to stand in day-light for a few days due to the light sensitivity of the extract. ε-DNP-lysine was found to be the only water soluble derivative by paper chromatography.

The ether extracts were fractionated on celite and cellulose columns, the results obtained confirming those found on paper. The three main fractions were isolated and compared with authentic DNP-alanine, DNPOH and $DNPNH_2$ on cellulose columns, in each case a single fraction being demonstrated. The presence of a small amount of the "slow component " was confirmed in preparation O_3. These results are in excellent agreement with the work of Fraenkel-Conrat and Porter (1952).

It was concluded that ovomucoid contained alanine and possibly a trace of serine as N-terminal residues as shown by DNP analysis, but the serine may not be a true N-terminal residue.

4.4. DNP-lysozyme preparations

Lysozyme was obtained from Armour and Co. since the amount prepared from egg white by the author was insufficient for the number of analyses carried out. The results were the same as far as demonstrating the presence of di-DNP-lysine were concerned. The method of preparation determined the composition of the ether soluble fraction as found in the previously described analyses. Di-DNP-lysine

was confirmed to be the true N-terminal residue by Lowther degradation, ultraviolet spectra and chromatographic analyses. The results of the analyses carried out on paper are given in Table XX. The solvent system used throughout was BPPE which was found to be ideal for this particular mixture. The water soluble fraction contained ϵ-DNP-lysine.

Table XX

Paper chromatographic analyses of DNP-lysozyme preparations (ether extracts)

Preparation No.	Reaction time	HCl used	DNP-derivative
1	18 hrs.	A.R.	DNPOH, $DNPNH_2$
		A.R. distilled	di-DNP-lysine, DNPOH
2	2 weeks	A.R.	DNPOH, $DNPNH_2$ (di-DNP-lysine trace)
3_G	18 hrs.	A.R.	DNPOH, $DNPNH_2$
		A.R. distilled	di-DNP-lysine only
4_U	18 hrs.	A.R.	di-DNP-lysine, DNPOH
		A.R. distilled	di-DNP-lysine only
5_A	18 hrs.	A.R.	di-DNP-lysine, DNPOH
		A.R. distilled	di-DNP-lysine, DNPOH

Sanger reaction conditions were used throughout, the subscript indicat 18 hrs. prior denaturation, G = guanidine hydrochloride, 0.75 M, U = Urea, 8M and A = Alcohol, 80% v/v. BPPE was the solvent in all experiments.

In some cases the ether extracts contained a fast running artifact which was not identified. This component had an R_f value slightly greater than $DNPNH_2$, travelled at the solvent front and was only found in trace amounts when A.R. hydrochloric acid was used in the hydrolysis.

Analar hydrochloric acid used without prior distillation caused the degradation of some labile DNP-derivative in the same way as previously mentioned in the analyses of DNP-conalbumin and DNP-ovomucoid. This would indicate a common DNP-derivative in the hydrolysates, and it is suggested that this might be Im-DNP-histidine which is known to be acid labile (Porter, 1950).

The number of artifacts formed during the hydrolysis of di-DNP-lysine are given in Table XXI. It will be seen from the control hydrolysate with added iron that this impurity in bottled hydrochloric acid was not responsible. Prior denaturation with guanidine hydrochloride or urea appears to reduce the amount of artifact formed in the hydrolysis of the DNP-protein.

Table XXI

Artifacts identified in the control hydrolysates of di-DNP-lysine and lysozyme.

HCl used	DNP-derivatives identified	Fracti
(a) A.R. distilled	di-DNP-lysine only	Ethe
(b) A.R.	di-DNP-lysine	Ethe
	α-DNP-lysine	Wate
	ε-DNP-lysine	Wate
	DNPOH	Ethe
	$DNPNH_2$	Ethe
(c) A.R. distilled plus 4×10^{-3}% Fe^{++}	di-DNP-lysine only	Ethe

Spectra of ether extracts dissolved in N HCl.

Graphs: (a) DNP-lysozyme 4_U

(b) di-DNP-lysine and lysozyme control hydrolysate

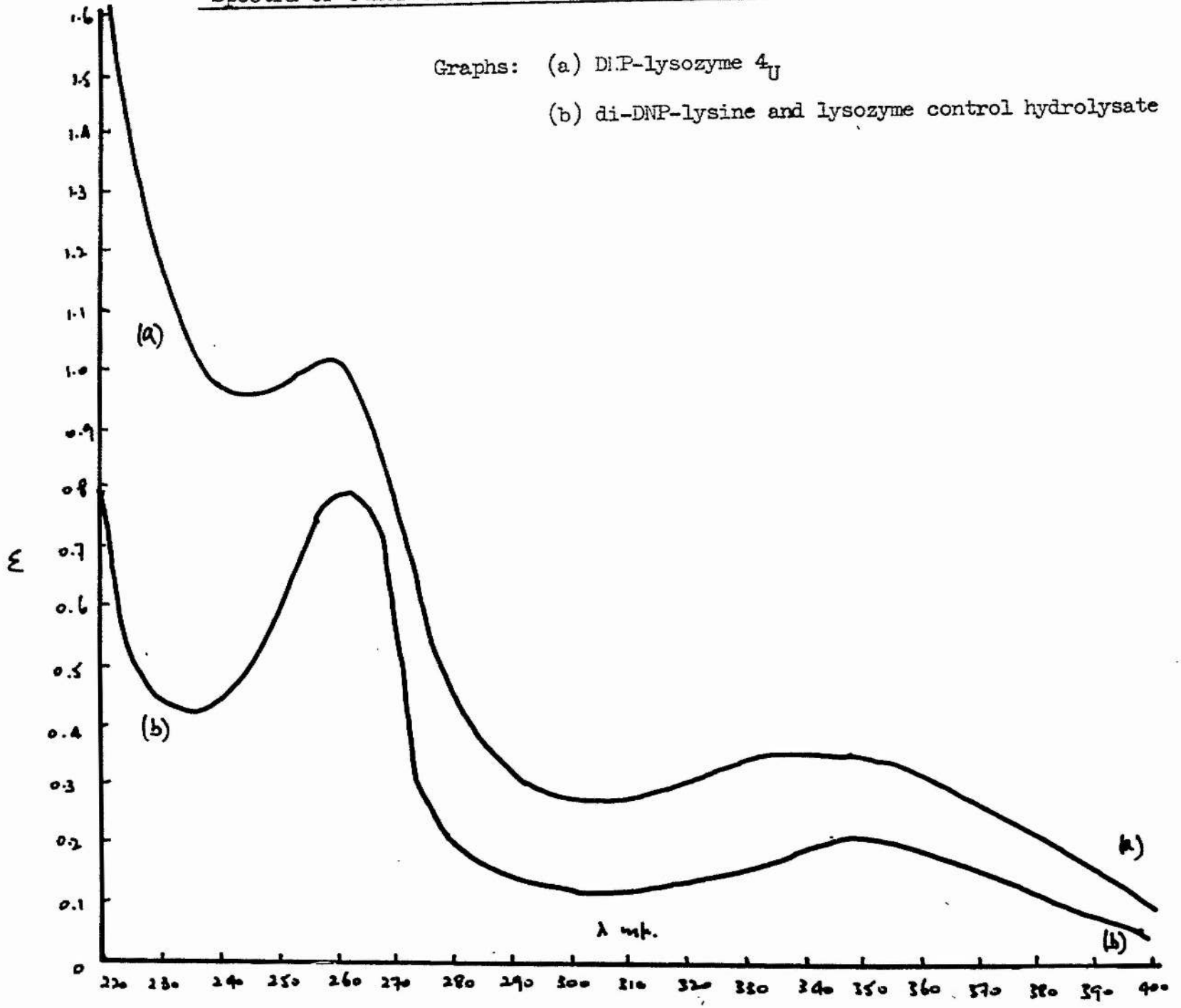


Fig. 22

The graphs in fig. 22 compare the ultraviolet absorption spectra of the ether extract from the hydrolysed DNP-lysozyme 4_U and that of a similar extract obtained from the control hydrolysate containing lysozyme and di-DNP-lysine. The two curves show a good fit and confirm the presence of lysine as the N-terminal residue in this protein. The di-DNP-lysine was confirmed by Lowther degradation since free lysine was detected among several unidentified derivatives.

4.5. DNP-ovomucin preparations

The aqueous fractions contained ε-DNP-lysine. The ether extracts of DNP-ovomucin hydrolysates were shown to contain only trace amounts of DNPOH and no true DNP-amino acid derivative. In some preparations there was no trace of DNPOH which suggested that the presence of this artifact in certain preparations was caused by the binding of the artifact to the DNP-protein rather than the degradation of acid labile derivatives. The large quantity of carbohydrate in this protein may mask the N-terminal amino acid residue. There appears to be no literature on the structure of ovomucin.

5. Summary of the DNP analyses of egg white proteins

1. Ovalbumin and ovomucin contained no N-terminal residue.
2. Ovomucoid and conalbumin both contained alanine and a trace of an unidentified "slow component" as terminal residues. The "slow component" was tentatively suggested to be derived from serine which

may appear as an N-terminal amino acid due to acyl-migration during the preparation of DNP-proteins.

3. Lysine was found as the N-terminal residue in lysozyme.

4. In all DNP-protein hydrolysates ε-DNP-lysine was found to be the only water soluble derivative.

5. The ether extracts of DNP-protein hydrolysates often contained the two artifacts DNPOH and $DNPNH_2$.

6. These results are in good agreement with the literature which has been quoted in the introduction of this work.

PART V

STRUCTURAL STUDIES ON NATIVE OVALBUMIN

1. Introduction

The structural configuration of a native protein may often be surmised by a critical analysis of the changes which take place during denaturation. The work to be described in this section will be presented in two parts, firstly a chemical approach to the problem and secondly, a physical investigation of the same problem. The latter confirmed the hypothesis put forward from the results of the chemical investigation.

Mirsky and Anson (1936) considered the heat denaturation of ovalbumin to be an "all-or-none" reaction. The present work can only be interpreted in the form of a stepwise denaturation process, which is opposed to the "all-or-none" theory but seems to be in agreement with modern trends of thought (Warner, 1954; Putnam, 1953).

The problem has been studied by examining the chemical and physical changes which take place during a series of partial denaturation reactions. These changes were the chemical reactivity of lysine residues within the protein towards selective substitution, the increase in viscosity, laevorotation and optical absorption. In every case the change in the property studied plotted against the concentration of the denaturing agent indicated a sudden steep rise in

the curve before it finally levelled off at the limit of denaturation.

2. Material

Two samples of ovalbumin were used in this work, one for chemical and the other for physical studies. The confirmation obtaine by these two different approaches to the problem is evidence for the uniformity of the two batches of ovalbumin. Cole's (1932) method was used in the preparation of six times crystallised ovalbumin which had Amide Nitrogen 1.04% and Total Nitrogen (by Kjeldahl) 15.7%. Smithies (1955) electrophoretic analysis demonstrated the presence of three components, probably A_1, A_2 and A_3 as described by Cann (1949).

3. Chemical Study

3.1. Methods

3.1.1. Preparation of DNP-proteins. Several methods were used to couple FDNB to ovalbumin. The method of Sanger (1945) was carried out in the presence of 65% ethanol. The ethanol caused considerable denaturation with the consequent exposure of ε-NH_2 residues of lysine. This technique was obviously unsuitable for the analysis of these groups exposed during the sequential denaturation with a reagent such as urea.

The mild reaction, used by Courts (1954) was found to be of value in this type of work. The reaction with FDNB was carried out

in 5% sodium bicarbonate at 37° after prior denaturation of the protein and stopped by acidification or by dialysis against water followed by acidification. Dialysis removed excess FDNB and bicarbonate which caused frothing and possibly surface denaturation when the acid was added. The preparation of the DNP-proteins was carried out exactly as described in the section on the N-terminal analyses of egg white proteins.

3.1.2. Amide Nitrogen Analysis. For a quantitative analysis of the number of ε-DNP-lysine residues found in a DNP-protein preparation, some reliable method for the estimation of the protein content was essential. Micro-Kjeldahl estimations of DNP-ovalbumins were of no value, since the total nitrogen varied with the degree of substitution. The nitro-groups of DNP-derivatives did not completely react during digestion with the copper-selenium catalyst employed, for example, ε-DNP-lysine was shown to give only 75% recoveryof total nitrogen. The tyrosyl hydroxyl groups in ovalbumin also react with FDNB to form an unstable O-DNP-derivative and for this reason the protein content could not be assayed by measuring the optical absorption at 280 - 195 mμ.

The amide residues in a protein are not attacked by FDNB. Bailey (1937) developed an analysis of amide nitrogen which was modifie in the present work for semi-micro estimations of DNP-proteins. The method consisted of hydrolysing the DNP-protein, followed by Kjeldahl

distillation as described by Bailey, the actual estimation of amide nitrogen being carried out by Nesslerisation rather than by titration.

The procedure was as follows: five aliquots of DNP-protein of varying weight were taken (e.g. 0.5 - 5.0 mg.). These samples were hydrolysed under reflux for three hours with 2N-hydrochloric acid, using glass distilled water to dilute the distilled acid (ca. 6N). After cooling the hydrolysates were made up to 100 ml. with glass distilled water with enough N-sodium hydroxide added to colour thymolphthalein blue (pH 11). The hydrolysates were then distilled in a Kjeldahl apparatus and 20 ml. of distillate collected in 2 ml. N/100 hydrochloric acid. The distillates were made up to 25 ml., two 10 ml. portions were then using for Nesslerisation. 2.5 ml. Quantitative Nessler reagent (B.D.H.) was added to each 10 ml. aliquot and the optical density immediately read on an EEL photoelectric colorimeter with the blue filter No. 303. A standard ammonium sulphate solution was always used in duplicate as a control.

The optical densities were plotted against the weights of DNP-derivative used. In all cases the straight line graph obtained passed through the ordinate above the origin. The small reading after extrapolating to zero protein concentration gave the blank value, which varied daily according to the quality of the glass distilled water used. Water distilled from a metal container gave a much greater blank value and was consequently not used for this work. The concentration of ammonia in the solution was calculated from the slope

Nessler calibration curve.

Direct reading of Amide N in hydrolysate.

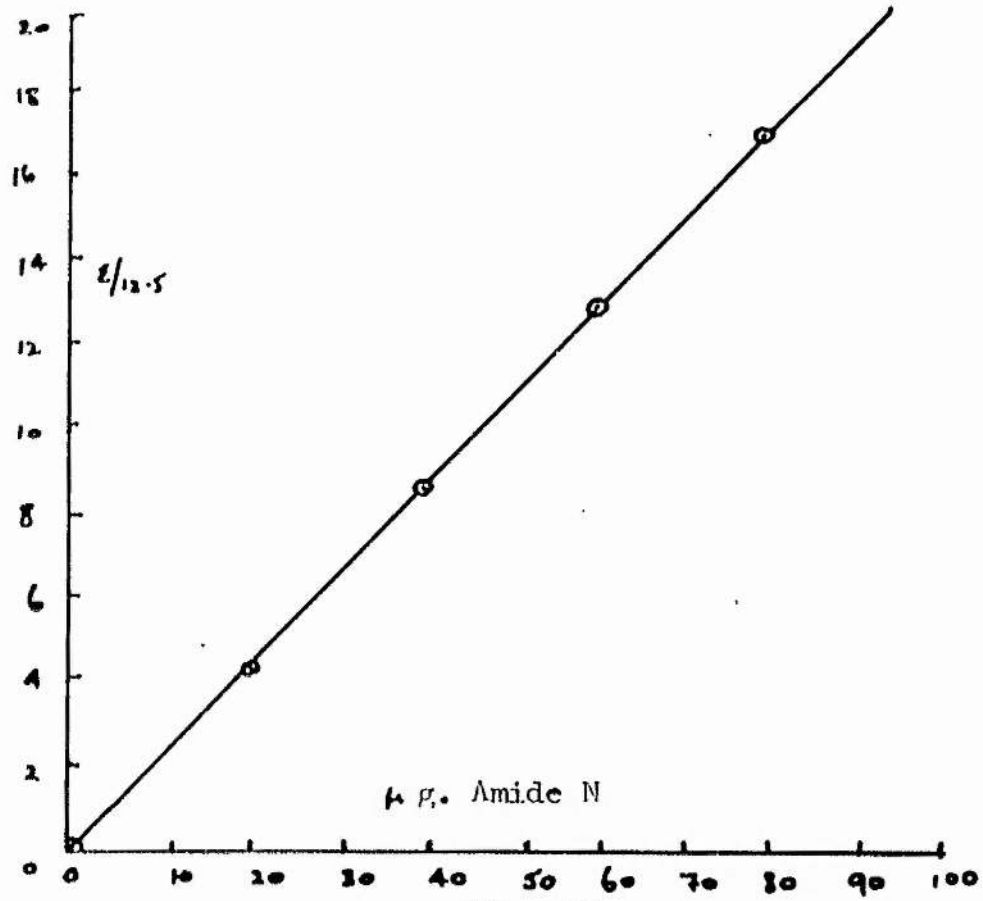


Fig. 23.

Amide N in DNP-protein hydrolysate.

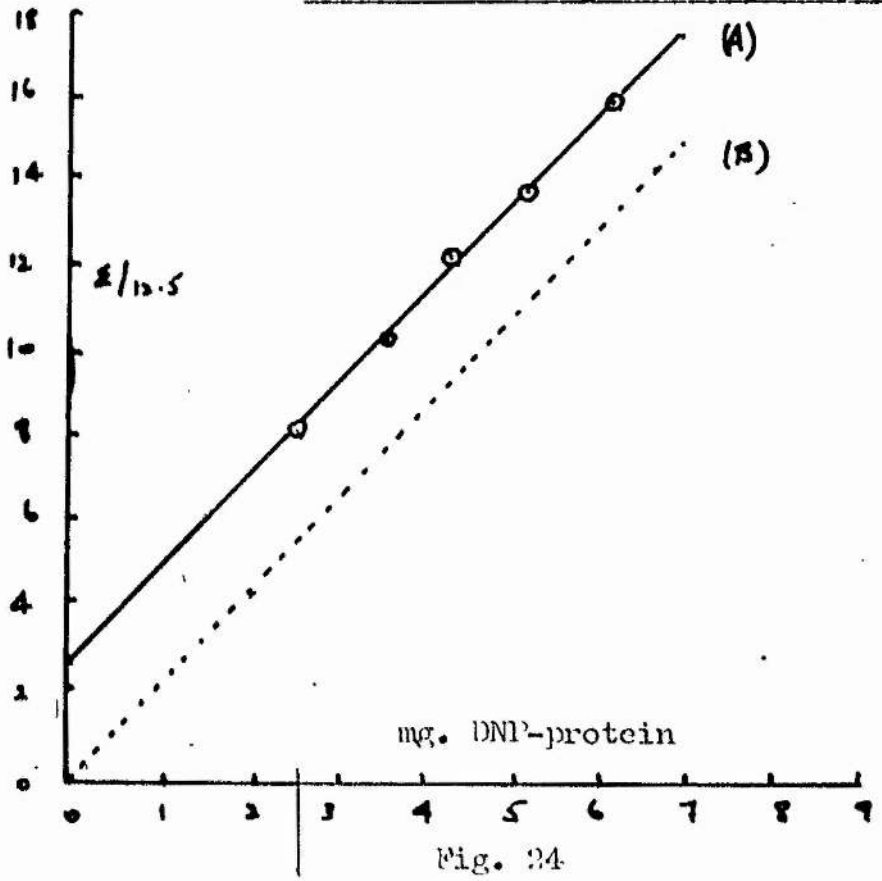


Fig. 24

of the graph and from this figure the amide nitrogen determined. The percentage amide nitrogen found in the DNP-protein compared with 1.04% of pure ovalbumin determined the protein composition. A calibration curve drawn from the estimation of ammonia liberated from a standard ammonium sulphate solution which had been subjected to the same hydrolysis and estimation conditions is given in Fig. 23. The calibration curve takes into account losses involved in hydrolysis and distillation and is a direct reading curve. The absorption given by the 12.5 ml. reaction volume is plotted against the amide nitrogen found in a 25 ml. aliquot of distillate obtained from the hydrolysed protein.

A typical analysis of the amide content of a DNP-protein is shown in Fig. 24. The actual graph is shown in Fig. 24(A), the blank value in this case was 2.6. The dotted line (B) takes the blank value into account. The calculation is as follows: in Fig. 24 the absorption corresponding to 6.3 mg. DNP-ovalbumin is 13.4 which is equivalent to 6.3×10^{-2} mg. amide nitrogen in Fig. 23. Hence the amide nitrogen in this DNP-protein is 1.00%. The ovalbumin content corresponds to $\frac{1.00 \times 100}{1.04}$ i.e. 95.5%.

3.1.3. <u>ε-DNP-lysine analysis.</u> Five aliquots of DNP-ovalbumin (1.0 - 5 mg.) were hydrolysed for eighteen hours under reflux with 6N-distilled hydrochloric acid. These hydrolysates were ether-extracted four times, each extraction being washed through the

ε -DNP-lysine calibration curve.

Direct reading of ε-DNP-lysine in hydrolysate.

Conditions: solvent 1% $NaHCO_3$, volume 50 ml., λ 360 mμ

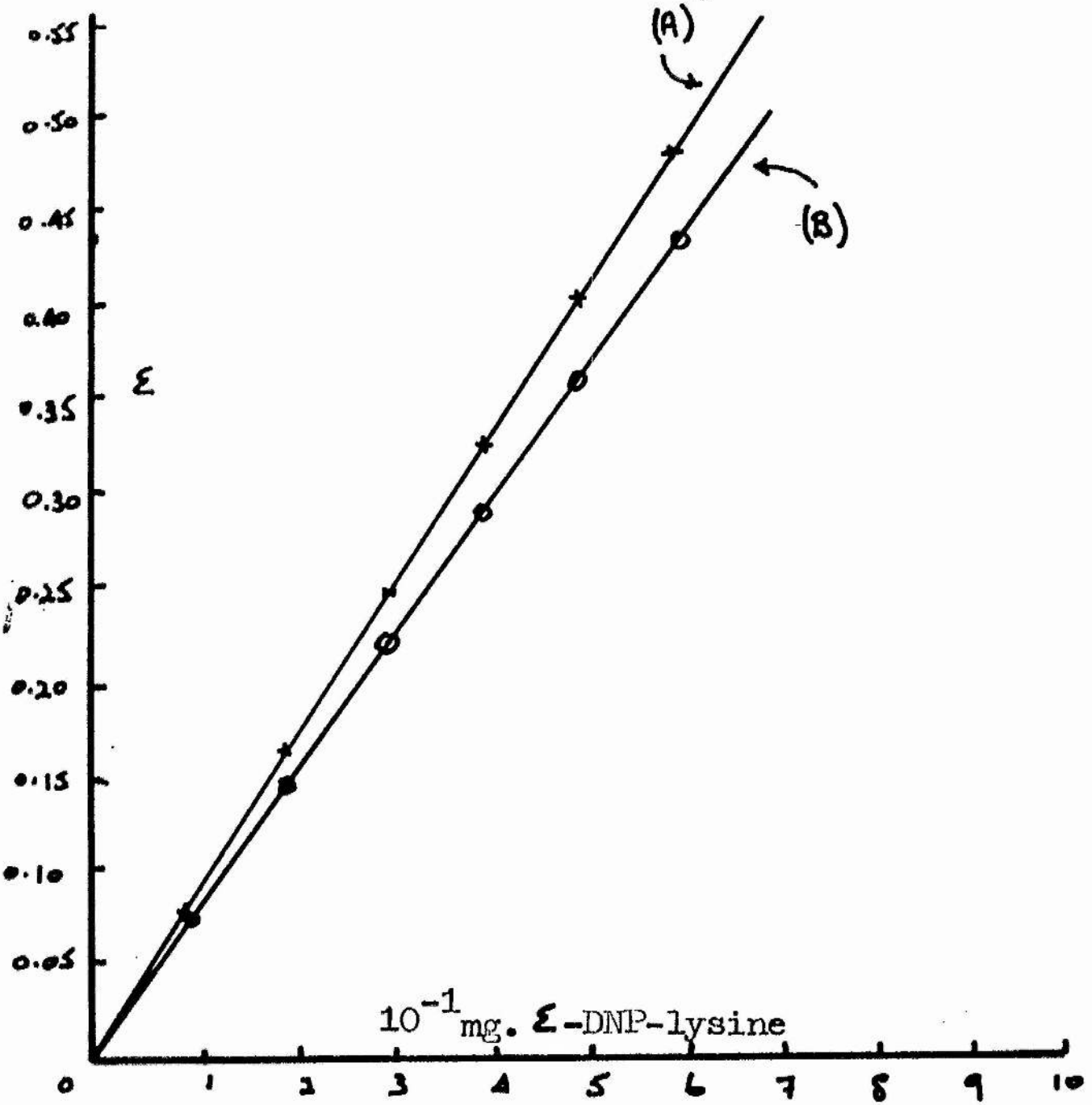


Fig. 25.

separating funnel with a little distilled water to ensure that the water dissolved in the ether layer contained no ε-DNP-lysine. The aqueous extracts were each taken to dryness six times by vacuum distillation to remove all traces of free acid. The concentrated extracts were then washed into a 50 ml. volumetric flask with 25 ml. 2% sodium bicarbonate, water being added to bring the final volume to 50 ml. The extracts were filtered and their optical densities measured at 360 mμ with the Unicam spectrophotometer using 1 cm. glass cells. The optical densities were plotted against the quantity of DNP-protein hydrolysed and the straight line produced was found to pass through the origin.

The quantity of ε-DNP-lysine was calculated from a calibration curve drawn from the results of a control hydrolysate of samples of ε-DNP-lysine in the presence of ovalbumin. The recovery of ε-DNP-lysine was 92% after taking into account losses involed in hydrolysis, extraction and concentration. Fig. 25 shows the theoretical (A) and actual (B) calibration curves for the analysis of ε-DNP-lysine measured at 360 mμ. The actual calibration curve demonstrates the 8% loss. The figures appear to be accurate for the degradation of free and peptide bound ε-DNP-lysine since in the fully denatured ovalbumin twenty residues were substituted which is in fact the theoretical maximum for the molecule with molecular weight 45,000 (Warner, 1954).

3.2. Results

3.2.1. Partial denaturation with various reagents. Tables XXII and XXIII give the results obtained from the study of the chemical reactivity of the ε-NH_2 groups of lysine in DNP-ovalbumins prepared under different conditions. The first table compares the number of preparations of DNP-ovalbumin prepared under different conditions of denaturation. The results obtained from serial denaturation of ovalbumin are presented in the second table.

Table XXII

Denaturation by various reagents and exposure of ε-NH_2 residues in ovalbumin

Prior denaturation at 37°				Reaction with FDNB			%	ε-NH_2 residues
No.	Reagent	hr.	T°	% Ethanol	hr.	T°	$NaHCO_3$	per mole (MW 45,000)
D	—	-	-	—	18	37	5	3
N	0.5 M-Guanidine HCl	4	37	—	18	37	5	7
C	0.5 M-Guanidine HCl	0.5	37	50	18	20	2	11
O	0.75 M-Guanidine HCl	18	37	—	18	37	5	20
Q	2.5 M-KI	18	37	—	18	37	5	11
R	2.5 M-KCNS	18	37	—	18	37	5	12
E	—	—	—	35	18	20	2	9
B	—	—	—	50	18	20	2	12
H	—	—	—	60	18	20	2	20
J	8 M-Urea	18	37	—	18	37	5	20
!M	(D) + 8M-Urea	18	37	—	18	37	5	20
"P	8 M-Urea	18	37	—	48	37	5	3

! (D) signifies that preparation D was used in place of crystalline ovalbumin in this experiment. "P indicates M-glucose incubated with the protein before adding the urea, glucose inhibits denaturation.

Before considering the effect of serial denaturation, certain points will be made in connection with the results given above in Table XXII. It is at once obvious that the reaction conditions control the amount of FDNB substitution. The normal Sanger (1945) conditions will cause full substitution to take place when the ethanol concentration exceeds 60% v/v. If less ethanol is used then incomplete substitution may take place. The high ethanol content used by Sanger has two functions; primarily to denature the protein and secondly to increase the concentration of dissolved FDNB. These functions could be achieved by carrying out the reaction in two stages in the absence of ethanol. The protein was first denatured to expose the lysine residues to FDNB and then reacted according to the procedure employed by Courts (1954) in which 5% sodium bicarbonate at 37° increased the solubility of FDNB.

Glucose inhibits denaturation (Warner, 1954) and also inhibits the exposure of ε-NH_2 groups to FDNB in strong urea solution. Porter (1948) considered that FDNB stabilised the protein on forming a DNP-derivative, so that further denaturation became more difficult. In the case of ovalbumin this has not been found. In preparation D only three ε-DNP-lysine residues were substituted but in the presence of 8M-urea this preparation reacted with FDNB to yield a further seventeen residues, the theoretical maximum.

It is of interest that no preparation gave less than three ε-DNP-lysine residues per molecule. This would indicate a minimum

value which was obtained only in the mildest reaction conditions (D) and in the case of glucose inhibition (P). It appears that nine lysine residues are exposed one by one between the states of denaturatio at which three and twelve residues are available to FDNB. In no preparation was a value of between twelve and twenty obtained. From these considerations it was thought that a serial partial denaturation reaction would show three distinct groups of lysine residues within the protein molecule. These stages would be a minimal one at which three residues reacted, an intermediate stage where up to twelve reacted and a final stage where all residues reacted. Such a serial analysis was carried out using ethanol as prior denaturant.

3.2.2. Serial ethanol denaturation. Ovalbumin was dissolved in sodium bicarbonate and warmed to 37° in an incubator. To each of a series of flasks containing the dissolved protein, aliquots of ethanol at 37° were added so as to give a range of ethanol concentration The mixtures were kept at this temperature for three hours before the ethanol was removed by aeration at room temperature. The partially denatured ovalbumin preparations were then reacted with FDNB in 5% sodium bicarbonate at 37° for eighteen hours before the reaction was stopped by acidification and the DNP-proteins prepared in the manner already described. The results of this analysis are given in Table XXIII.

Table XXIII

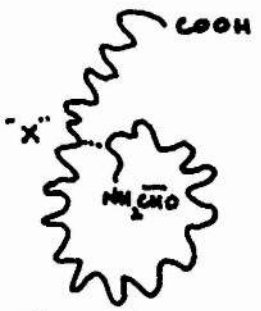
Serial ethanol denaturation of ovalbumin prior to FDNB reaction at 37°

DNP-ovalbumin Preparation No.	% w/v Ethanol	ε-DNP-lysine residues per mole (MW. 45,000)
1	0	3
2	10	5
3	20	6
4	30	8
5	40	10
6	50	12
7	55	20
8	60	20
9	70	20

The results of the serial denaturation with ethanol prior to FDNB reaction are in good agreement with the theory expressed in the previous discussion of the results in Table XXII. The results given above indicate that a point is reached at 50 - 55% ethanol when the remaining eight lysine become simultaneously available for substitution with FDNB. It is suggested that three types of lysine residues are found in ovalbumin.

(a) Three residues which react under the mildest conditions, requiring no denaturation for exposure to the substituting reagent.

Reactivity of ε-NH_2 groups towards FDNB during serial ethanol denaturation of ovalbumin.



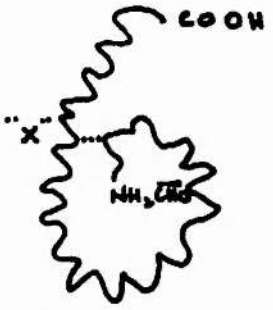
Stage 1

Native protein

Ethanol: 0% v/v

FDNB absent

Substituted NH_2: 0



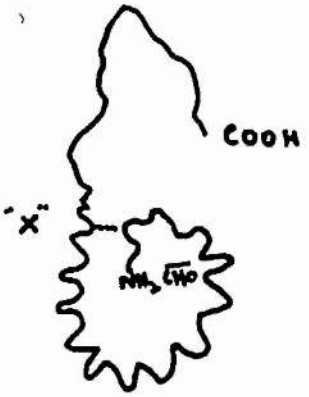
Stage 2

Native protein

Ethanol: 0% v/v

FDNB present

Substituted NH_2: 3



Stage 3

Partially denatured

Ethanol: 50% v/v

FDNB present

Substituted NH_2: 12

Stage 4

Fully denatured

Ethanol: 55% v/v

FDNB present

Substituted NH_2: 20

Fig. 26(a).

(b) Nine residues which react one after another during partial denaturation.

(c) Eight residues which react simultaneously but only after a certain degree of denaturation has been exceeded.

3.3. Discussion

The absence of a true N-terminal residue in ovalbumin has already been described. Even after complete denaturation with urea no α-DNP-residue could be detected. This would indicate that the α-NH_2 group in ovalbumin is masked and that denaturation does not expose it. For this reason it seems likely that the carbohydrate moiety may be involved in masking the α-NH_2 group. There is, however, a C-terminal residue, proline, as shown by Niu and Fraenkel-Conrat (1955). The present work would suggest a "six-shaped" structure of the type first postulated by Linderstrøm-Lang (1952) and later modified by Anfinsen and Redfield (1956). The structure suggested involves the hypothesis that the native ovalbumin molecule contains some form of covalent internal bridge which does not employ the N-terminal residue as such. If this residue were involved in such an internal link, on complete denaturation the N-terminal residue would be exposed to FDNB reaction. This, however, has been shown not to take place.

The progressive denaturation and concurrent exposure of ε-NH_2 groups towards FDNB is diagrammatically shown in Fig. 26(a).

The "six-shaped" structure postulated is the only possible configuration which would account for the analytical results presented.

In Fig. 26(a) the N-terminal residue is masked and the hypothetical internal bridge is marked "X". Stage (1) shows the proposed structure of native ovalbumin. In Stage (2), native ovalbumin reacted with FDNB under the mildest conditions, three lysine residues are available for substitution. These must be situated at easily accessible sites on the surface of the native molecule. Stage (3) shows the effect of gradual ethanol denaturation during which nine residues are exposed in a stepwise fashion. These residues are thought to be situated in the coiled "tail" of the molecule, and require some uncoiling by denaturation to become available for FDNB substitution. The uncoiling produced by ethanol up to 50% is thought to involve this "tail" only, presumably beginning from the free C-terminal end. The internal link remains intact at this concentration of denaturant.

Finally at stage (4) the "tail" is fully uncoiled but still protrudes from the closely coiled inner cyclic portion, held in place by the internal link. After exceeding 50% ethanol some disruptive force then breaks this link with the immediate uncoiling of the cyclic residue into an extended chain exposing simultaneously the residual eight lysine residues towards FDNB substitution. The disruptive force might be visualised as the effect of the uncoiling of the "tail" to expose the tightly coiled cyclic structure to stresses which break the internal link. This link is fairly stable as can be seen from the

Reactivity of ε-NH_2 groups towards FDNB during serial ethanol denaturation of ovalbumin.

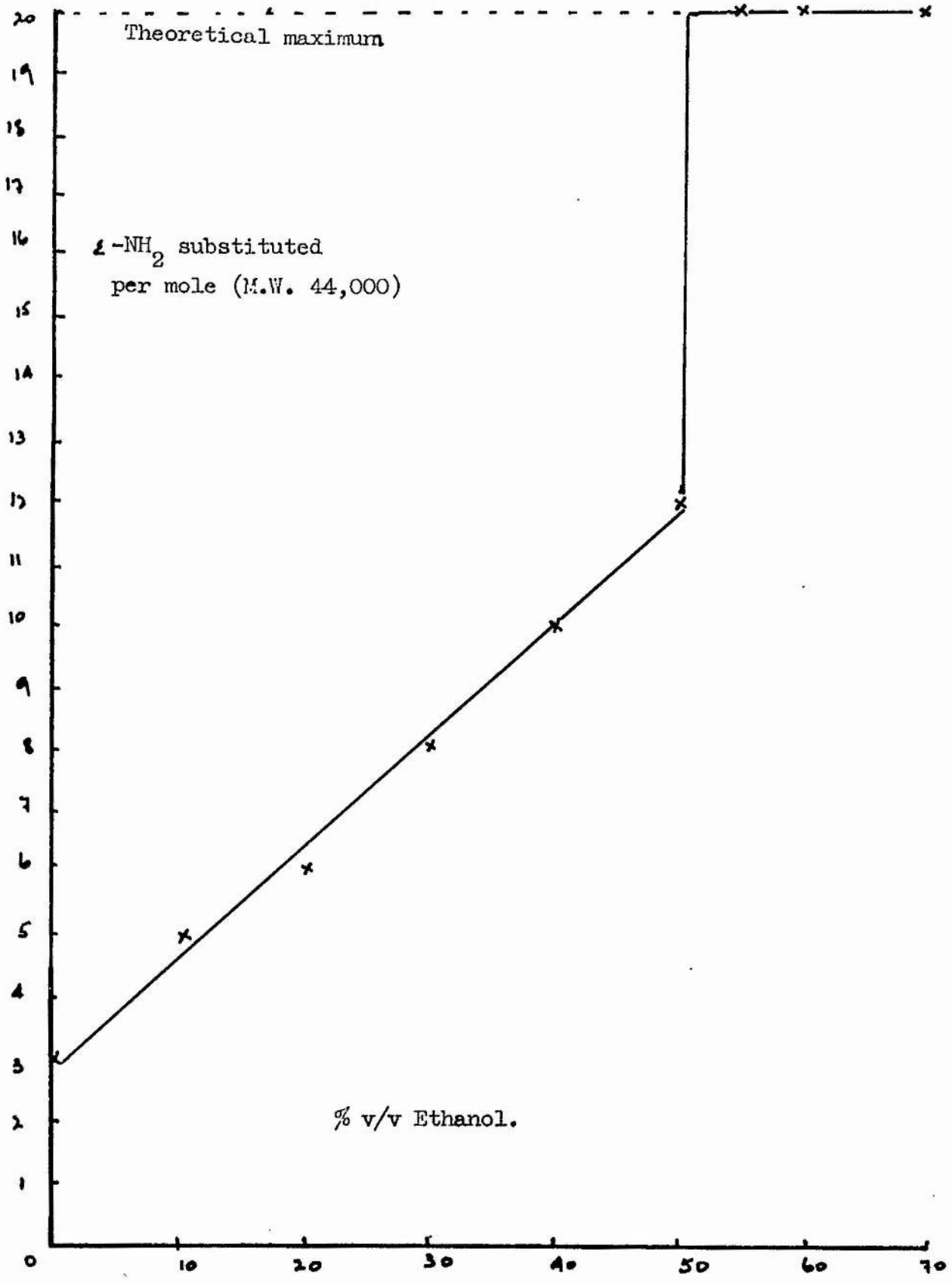


Fig. 26(b).

number of DNP-ovalbumins prepared which had twelve lysine residues substituted with FDNB.

As already stated the original theory of Mirsky and Anson (1936) considered that a partially denatured ovalbumin solution was made up from a mixture of native and fully denatured ovalbumin, at least in the case of heat denaturation. The theory of an "all-or-none" reaction is incompatible with the results obtained in the present work. If an "all-or-none" reaction were involved in the ethanol denaturation process, then a partially denatured ovalbumin solution should yield a precipitate at the isoelectric point containing completely denatured protein and a soluble fraction of native ovalbumin. After fractionation and carrying out the FDNB reaction on the two fractions the precipitate should yield twenty and the supernatant fluid three ε-DNP-lysine residues per molecule respectively. A partially denatured preparation of ovalbumin was prepared, fractionated and the two DNP-proteins prepared. On analysis these preparations yielded almost exactly the same number of substituted lysine residues per molecule, a value intermediate between the two extremes suggested by the "all-on-none" theory.

A second indication that ethanol denaturation is actually a stepwise process is given from the graph obtained by plotting the number of lysine residues substituted against the percentage ethanol content of the reaction mixture, Fig. 26(b). The graph appears to be linear from 0 - 50% ethanol but then rises steeply to a theoretical

maximum at 55% ethanol. If the "all-or-none" reaction took place in ethanol it would indicate that the reaction went smoothly until 50% v/v ethanol was reached when there would be 47% native and 53% denatured protein present which would give the average figure of twelve ε-DNP-lysine residues per molecule. At ethanol concentrations greater than 50% v/v the remaining 47% native protein would then become suddenly denatured. This situation seems most unlikely to the present author. It may be pointed out that a solution of ovalbumin in 50% ethanol certainly does not contain a soluble fraction at the isoelectric point amounting to 47% of the total protein concentration.

4. Physical Studies

4.1. Introduction

In the previous section the course of ethanol denaturation of ovalbumin was studied by measuring the increase in reactivity of the ε-NH_2 groups of lysine. In the present section certain physical properties were examined during serial denaturation in the hope that the postulated "six-shaped" structure of the native molecule could be confirmed. Certain physical changes take place during denaturation due to the uncoiling of the protein molecule, for example increase in viscosity, optical absorption and laevorotation. These changes have been studied where possible during denaturation by ethanol, urea and guanidine hydrochloride. Ethanol denaturation was found to

be extremely difficult to follow by changes in viscosity since two reactions took place, each influencing this property. The increase in viscosity caused by the primary uncoiling reaction was masked by a secondary process of gel formation. For this reason the ethanol denaturation study will be described at the end of this section.

4.2. Urea Serial Denaturation

Method. 10 ml. aliquots of an ovalbumin solution at pH 11.6 were added to solid urea in a series of flasks, the quantity of urea being varied to give concentrations from 1 - 10 M. The reaction mixtures were allowed to equilibrate for eighteen hours and then examined for the change in physical properties.

4.2.1. Optical absorption. Crammer and Neuberger (1943) demonstrated that the optical absorption maximum changed from 280 to 295 mμ at pH 12 - 13 on denaturation of ovalbumin. At the same time the magnitude of absorption at these two wavelengths increased. These authors considered that there were probably six tyrosyl residues held in hydrogen bonds in the native state which were converted to the quinone form, so increasing the intensity and wavelength of maximum absorption. However, no quantitative analysis of this protein was possible by optical means due to the absorption of other residues such as phenylalanine and tryptophan. The change taking place is diagrammatically shown in Fig. 27.

The effect of pH and denaturation on the spectra of ovalbumin.

Graphs: (1) Native protein, pH 12.6

(2) Native protein, pH 8.6

(3) Heat denatured protein, pH 8.6

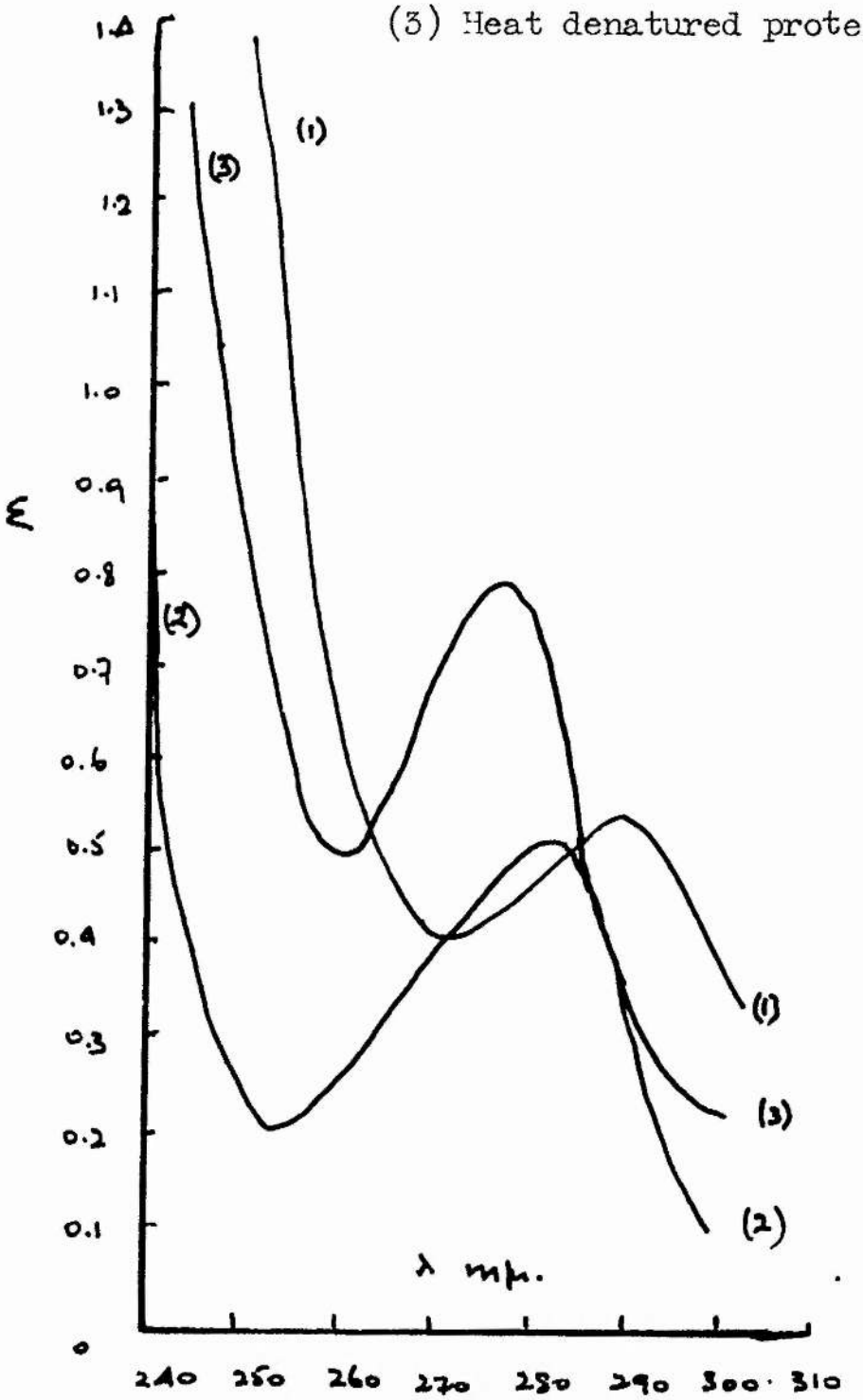


Fig. 28.

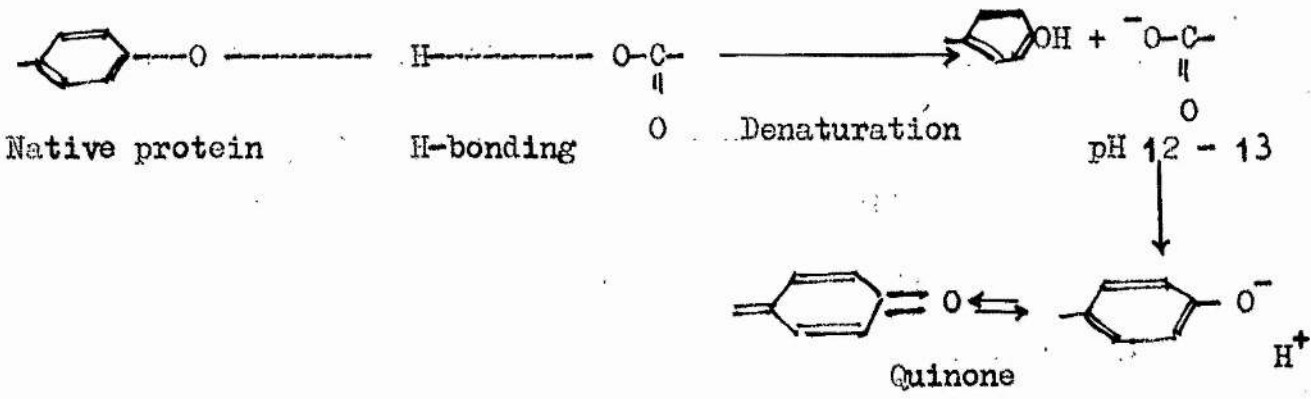


Fig. 27

Diagrammatic representation of the changes taking place in tyrosyl residues during protein denaturation.

Glazer et al. (1957) examined ovalbumin in urea solution and found that the absorption maximum occurred at 277 mμ with the greatest difference between native and denatured ovalbumin solutions at 285 mμ. At first sight this recent work would seem to contradict Crammer and Neuberger (1943). The present work indicates that both these groups of authors are in fact correct and that the urea denaturation process has been over simplified.

Before describing the serial denaturation studies in detail, the ultraviolet spectra of ovalbumin and tyrosine in urea solution will be given in order to show the effect of pH and denaturation.

Fig. 28 shows the effect of changes in pH on the ultraviolet absorption spectrum of native ovalbumin. The absorption maximum shifts from 280 to 290 mμ with a slight increase in magnitude when the pH is raised from 8.6 to 12.6. This increase in the wavelength of

The effect of urea on the spectra of ovalbumin at pH 8.6.

Graphs: (1) Native protein

(2) Protein in 10M urea after deducting graph (3)

(3) 10M urea

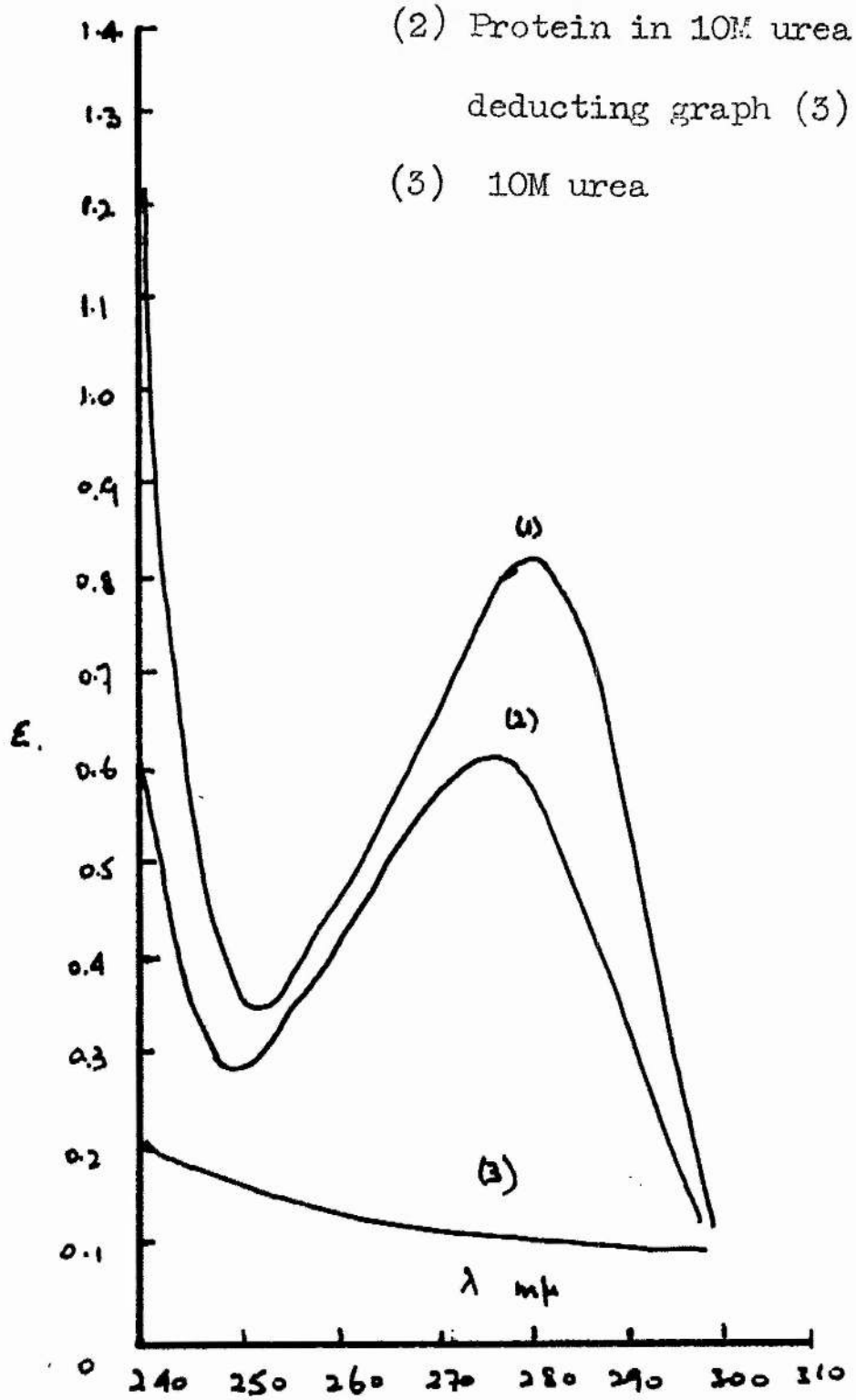


Fig. 29.

maximum absorption is due to the change in pH and not to denaturation as can be seen from the third graph in this figure, that of heat denatured ovalbumin at pH 8.6 which has a maximum at 277 mμ. Three conclusions may be drawn from this figure, in confirmation of Crammer and Neuberger (1943) and Glazer et al. (1957).

(1) Denatured ovalbumin at pH 8.6 has ε_{max} at 277 mμ (Glazer et al. 1957).

(2) Native ovalbumin at pH 8.6 has ε_{max} at 280 mμ (Crammer and Neuberger, 1943).

(3) Native ovalbumin at pH 12.6 has ε_{max} at 290 mμ (Crammer and Neuberger, 1943).

The effect of adding solid urea to ovalbumin at pH 8.6 is shown in Fig. 29. In this case the actual figures are recorded for 10 M-urea, a deduction having been made for the absorption of the urea in the mixed solution. The spectrum for urea solution alone is also given. The curve for the mixed solution is unexpected as it is in fact less absorbing than the original solution of ovalbumin and this anomaly will be explained below. Two conclusions are drawn from this figure.

(1) Urea denaturation at pH 8.6 caused the ε_{max} to fall from 280 mμ to 277 mμ as was found in the case of heat denaturation at this pH (see also Glazer et al. 1957).

(2) Denaturation with solid urea depressed the absorption at pH 8.6 over the whole spectrum.

The apparent depression of the absorption spectrum of ovalbumin

Molecular extinction coefficients of tyrosine

pH 11.6 in the presence of urea.

Graphs: (1) Wavelength 295 mμ

(2) Wavelength 280 mμ

Key: 0----0, calculated value

x----x, observed value

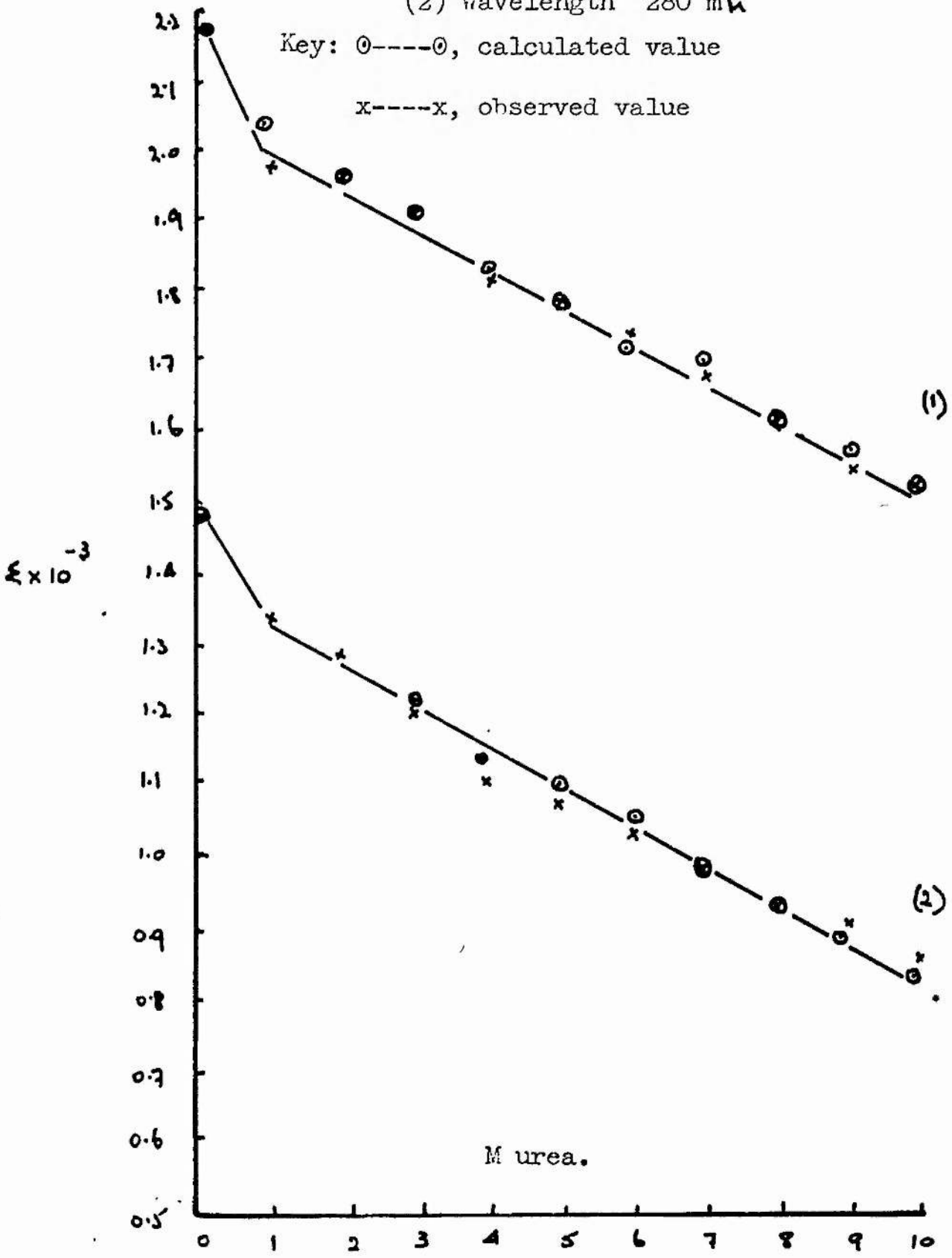


Fig. 30.

Effect of urea on the spectra of ovalbumin after allowing for the volume change.

Conditions: pH 8.6, 10M urea, 18hrs. 20°

Graphs: (1) Ovalbumin alone

(2) Ovalbumin with added solid urea, after deducting urea absorption and allowing for the increase in volume

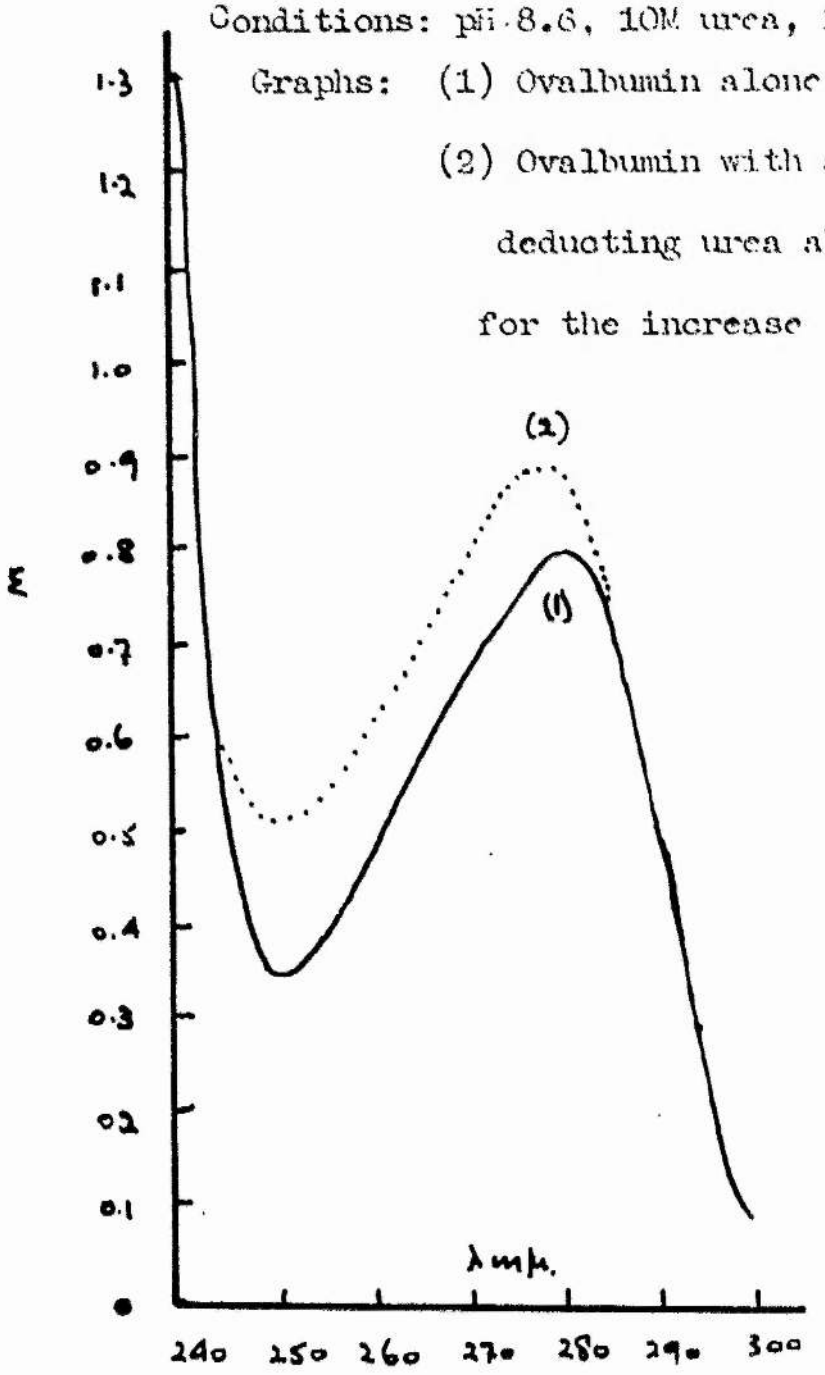


Fig. 31.

denatured with solid urea at pH 8.6 may be accounted for by examining the spectra of tyrosine solutions prepared with urea in the same way as the ovalbumin previously described. Fig. 30 shows the actual extinction coefficients of tyrosine in urea solutions at 280 and 295 mμ. The actual values were depressed as the urea strength increased. On the same figure the theoretical depression of the extinction coefficient as calculated from the densities of the solutions is also given, the two curves being almost identical. The densities of the solutions were determined and the actual volumes calculated from the weight of urea and tyrosine solution used. The increase in volume due to the solution of the solid urea caused a corresponding decrease in the tyrosine concentration, which accounted for the depression of the optical density measured. The spectrum of ovalbumin in 10 M-urea pH 8.6 is given in Fig. 31 after allowing for the volume change and for the absorption of the urea. The urea denatured protein is compared under the same conditions with the native protein. The effect of the urea denaturation of ovalbumin is to increase the intensity of the absorption maximum as well as causing a shift of ε_{max} from 280 to 277 mμ. This is what would be expected if denaturation breaks hydrogen bonds involved in links with tyrosyl hydroxyl groups. After denaturation these masked phenolic residues become exposed and cause an increased absorption at wavelengths in the region of 280 mμ.

The conditions used for the serial denaturation of ovalbumin

Molecular extinction coefficient of ovalbumin in urea solutions.

Conditions: pH 11.6, 18 hrs., 20°

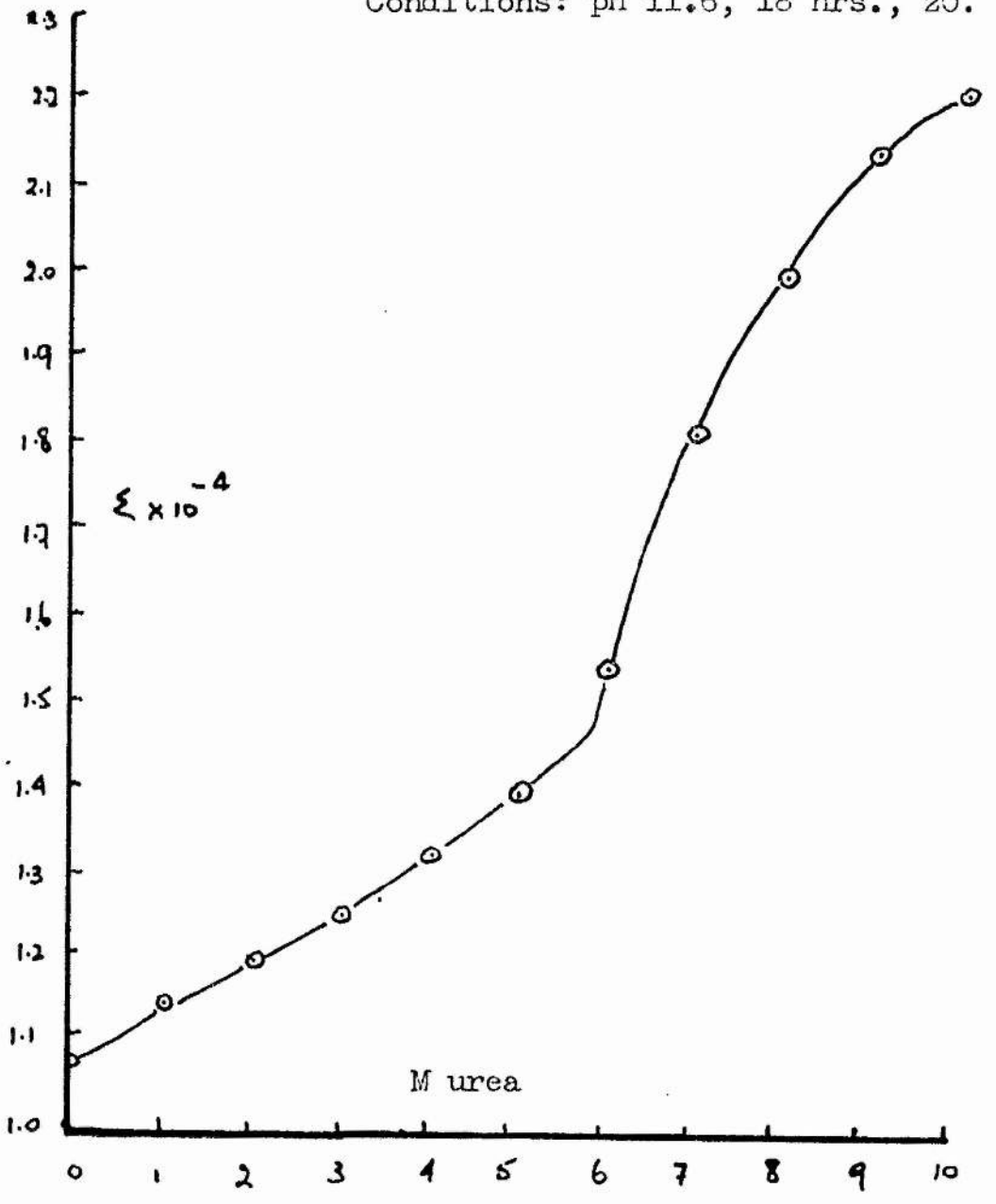


Fig. 32.

were pH 11.6 and the absorption measured at 295 mμ over a range of urea strength from 0 to 10 M. This pH was chosen for two reasons, firstly, the absorption is enhanced at pH greater than 10 whilst the alkali has no denaturing effect below pH 12.0. Secondly, the viscosity and optical rotation measurements required a strong solution of protein and were therefore carried out at this pH. The denaturation of ovalbumin in urea solutions can thus be compared under identical conditions by three different physical criteria.

According to the theory of the chemical denaturation of ovalbumin previously presented, the graph of the extinction coefficients plotted against the urea molarity should show a steep inflection if there is an internal bond broken at an intermediate stage between native and denatured protein. The graph is given in Fig. 32 and actually does show the expected inflection at about 6 M-urea (the volume changes have been taken into account).

4.2.2. Optical rotation. The optical rotation of a protein solution is well known to increase during denaturation due to the uncoiling of the helical structure (see Kauzmann, 1957). Ovalbumin solutions (1 - 1.5%) were made up at pH 11.6 and added to solid urea, the rotations were measured after eighteen hours and corrected for the volume changes. Fig. 33 shows the increase in optical rotation of ovalbumin in urea, the predicted inflection again taking place at 6 M. This would indicate that the uncoiling process is accelerated at this concentration of denaturant.

Optical rotation of ovalbumin in urea solutions.

Conditions: pH 11.6, 18 hrs., 20°.

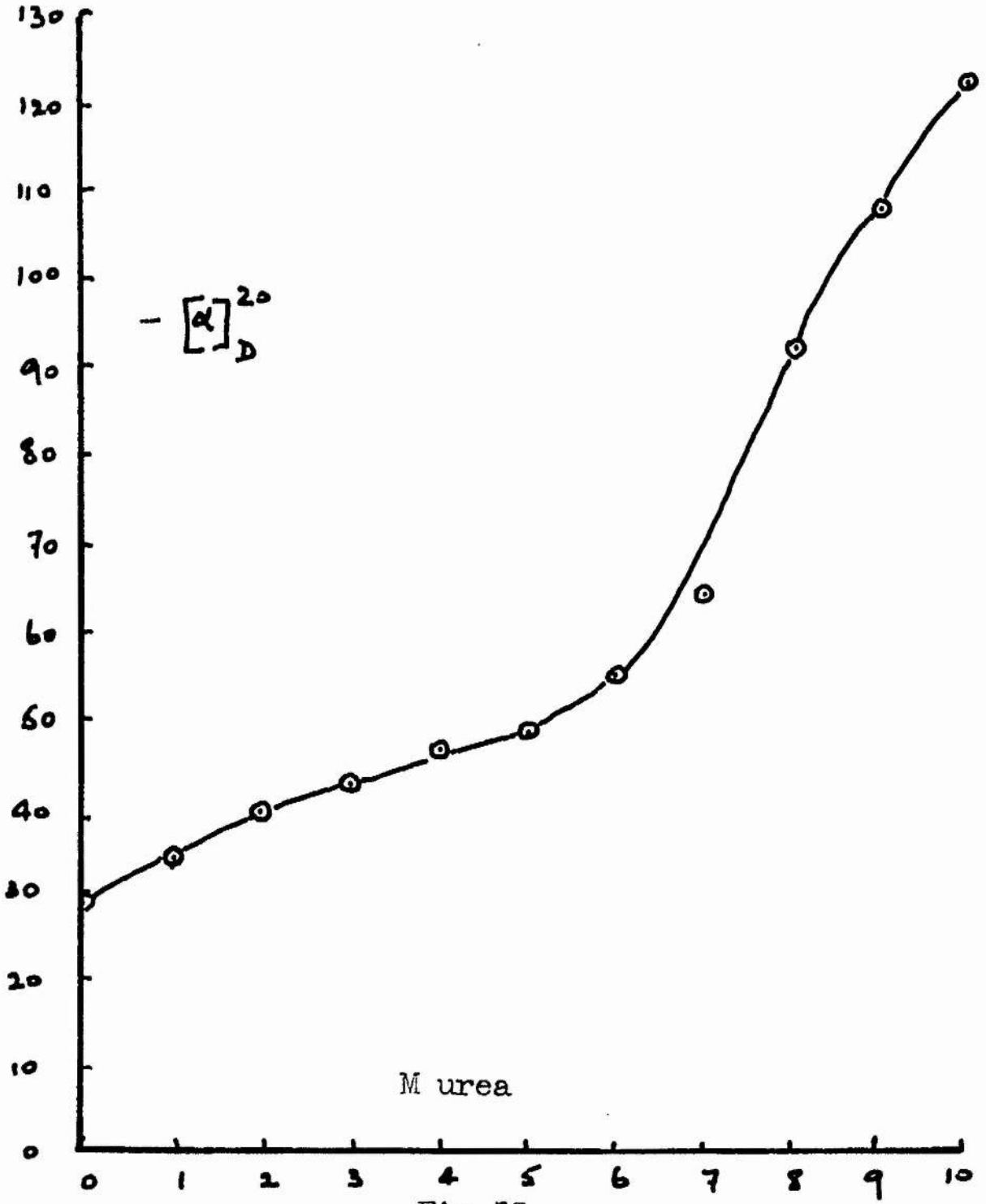


Fig.33.

4.2.3. Viscosity. The rupture of an internal link in the denaturation of ovalbumin would be expected to cause the molecule to uncoil and the viscosity of the solution to increase greatly.

Viscosities were measured with an Ostwald viscometer and the values calculated from the known viscosity of water at the same temperature (viscosities of water for a range of temperatures are given in "The Handbook of Physics and Chemistry"). The symbols used throughout this work were as follows:

η_1 = Viscosity of protein solution in millipoise.

η_o = Viscosity of pure solvent, in millipoise, in most cases this is the solution of denaturant.

$\frac{\eta_1 - \eta_o}{\eta_o} = \frac{\Delta\eta}{\eta_o} = \eta_{sp}$ = Specific viscosity.

$\frac{\eta_{sp}}{c}$ = Reduced viscosity. c = Concentration of protein (g.%)

$\frac{\Delta\eta}{c}$ = Change in viscosity per gram of dissolved protein.

mp. = Millipoise, the units of viscosity employed.

Viscosity measurements are usually presented as the reduced viscosities. However, in the present work the viscosity changes taking place during serial denaturation were found to be more conveniently demonstrated by plotting the values of $(\frac{\Delta\eta}{c})$ against the concentration of denaturant. The viscosity changes brought about by two different denaturants may be compared without difficulty using this method. If a protein solution shows an equal increase in $\Delta\eta$ during sequential

denaturation in urea and guanidine hydrochloride, then the value of $(\frac{\Delta n}{c})$ will be the same for both denaturation processes, whereas the values of the reduced viscosities will not be equal since the viscosities of the two denaturants in solution differ greatly. Secondly, the viscosities of urea and guanidine hydrochloride solutions increase with the concentration of solute whilst the viscosity of ethanol-water mixtures first increase and then decrease with increasing ethanol concentration. Thus a fully uncoiled protein in solutions of urea or guanidine hydrochloride will show a maximum value of reduced viscosity and then this value will decline as the change in protein viscosity remains constant and the viscosity of the denaturant solution continues to increase. The position would be reversed during ethanol denaturation where the viscosity of the solvent decreases at higher concentrations.

For each temperature, a given protein will have a constant value for $(\frac{\Delta n}{c})$ which will vary inversely with temperature. Native ovalbumin was found to have $(\frac{\Delta n}{c})$ values of 0.5 and 0.4 mp. at 20° and 30° respectively. These values correspond to a reduced viscosity of 0.06 mp.

Serial urea denaturation was carried out at pH 3.0 and 11.6. The protein solutions (1 - 1.5%) were made up to the required pH and added to the appropriate quantity of solid urea and left at 20° for eighteen hours before the viscosities were measured at this temperature.

Change in viscosity of ovalbumin in urea solutions

Conditions: pH 11.6, 18 hrs., 20°

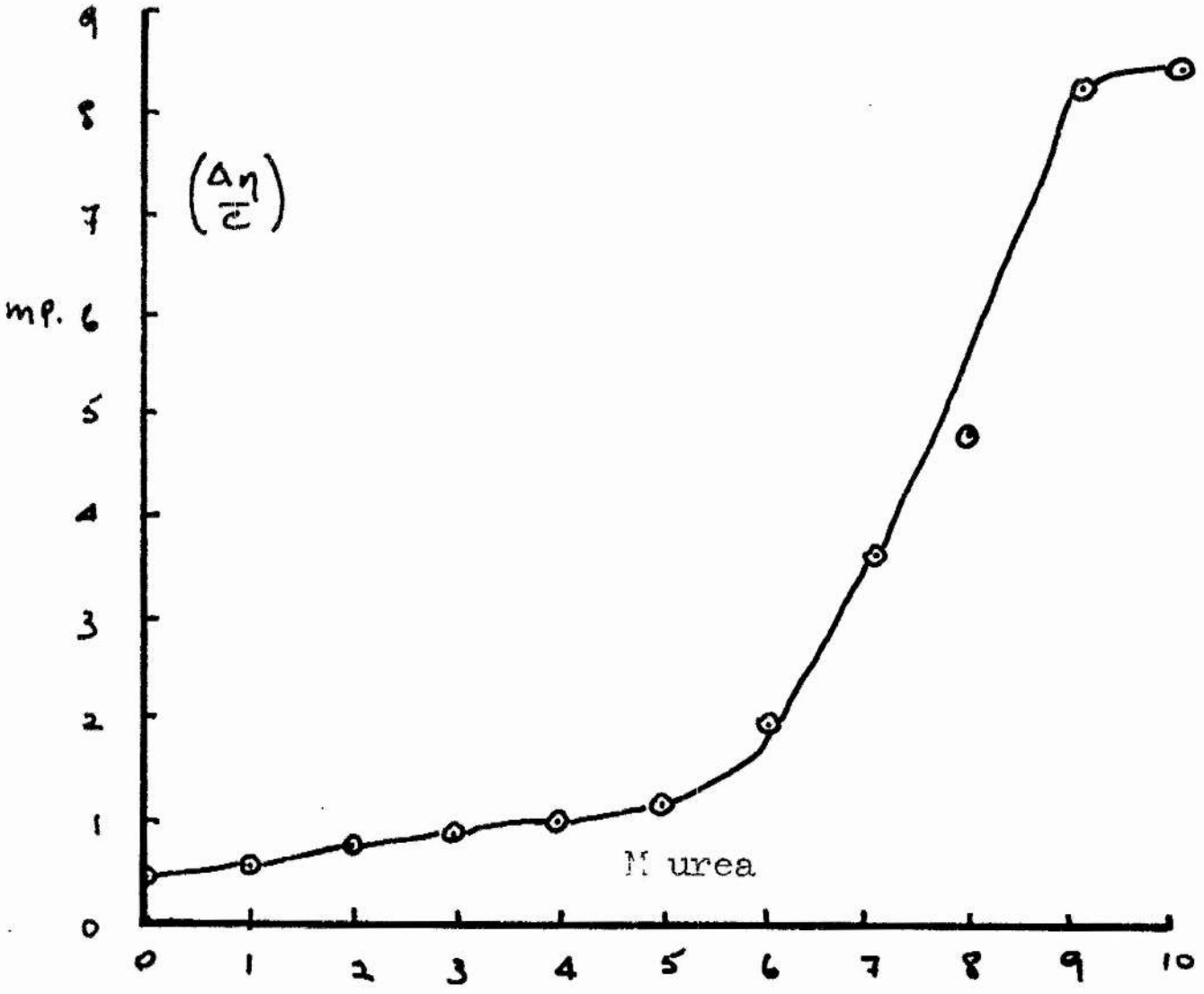


Fig.34.

Physical changes in ovalbumin in urea solutions.

Conditions: pH 11.6, 18 hrs., 20°.

Graphs: (1) Molecular extinction coefficient
(2) Optical rotation
(3) Viscosity

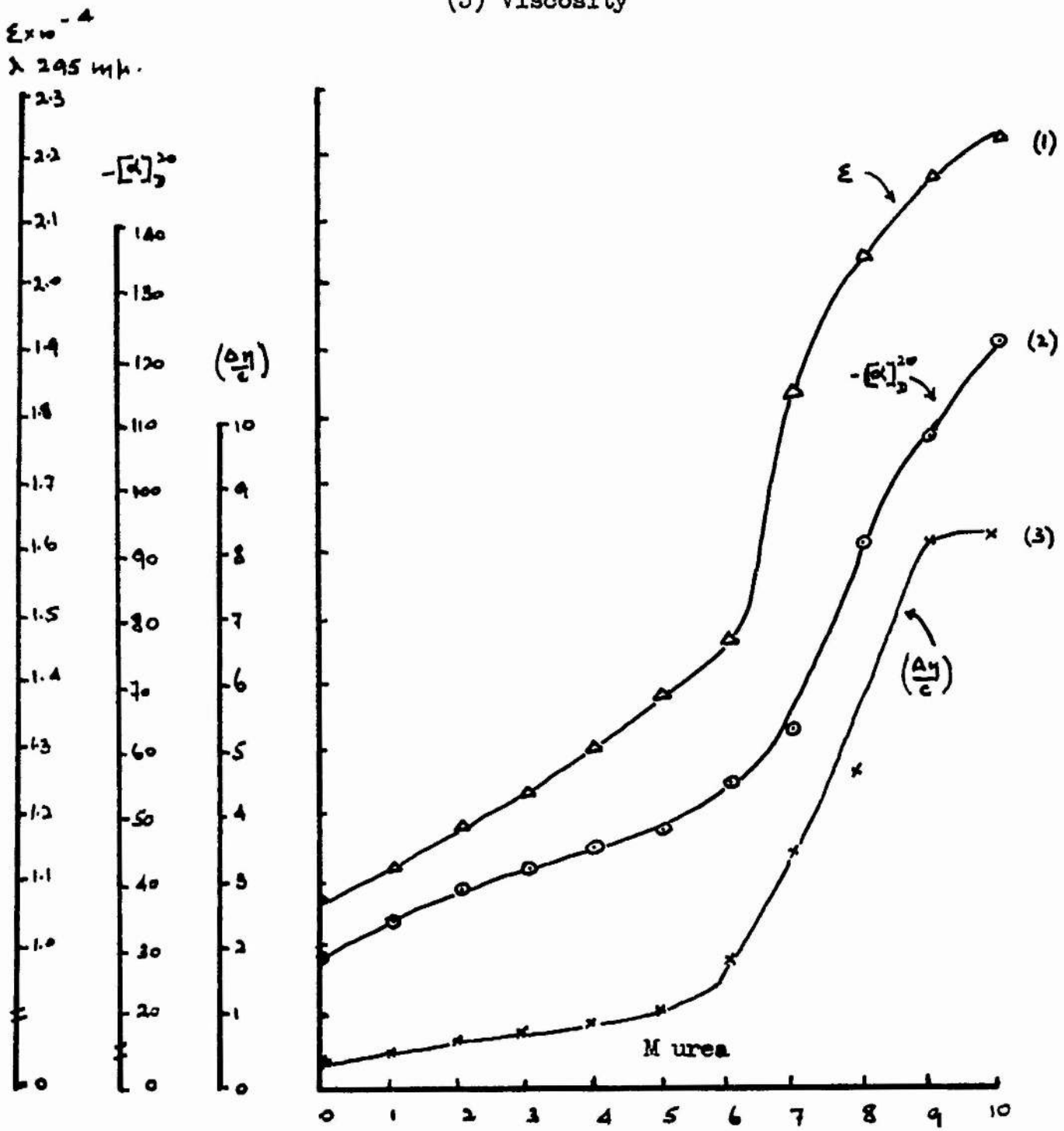


Fig. 55.

Effect of pH on the viscosity of ovalbumin in urea solutions.

Conditions: 18 hrs., 20°.

Graphs: (1) pH 11.6
(2) pH 3.0

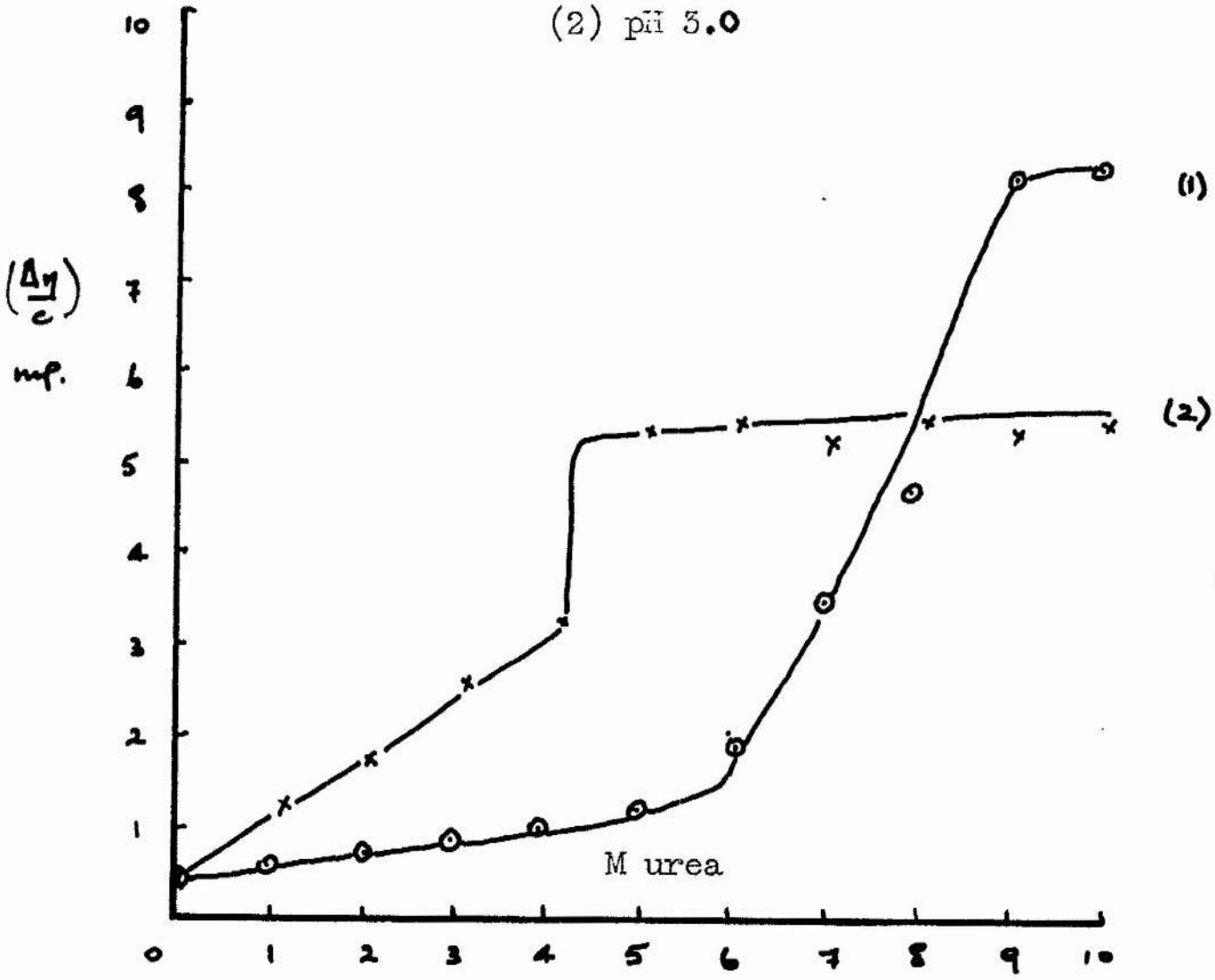


Fig. 36.

The results were corrected for increase in volume and are presented in Figs. 34 - 36. The viscosity change at pH 11.6 is shown in Fig. 34. The effect of pH on urea denaturation is shown in Fig. 36. At each pH the graph shows an inflection and in alkaline solution this takes place at 6 M-urea whereas in acid solution this takes place at 4 M-urea. In acid solution the viscosities were slightly inconsistent at the higher urea concentrations due to the presence of strands of denatured protein which interfered with the taking of measurements.

Two conclusions are drawn from these results.

(1) The changes in viscosity in alkaline solution are almost parallel to those obtained under the same conditions for the optical rotation and optical absorption. The three curves are compared in Fig. 35 and in each case the inflection occurs at 6 M-urea. This is in support of the chemical evidence obtained with ethanol for a hypothetical internal bridge.

(2) At acid pH urea denaturation is completed at a much lower concentration of denaturant than in alkaline solution. This might be taken to indicate that the internal link was more easily ruptured in acid solution by hydrogen bond breakers. The results for the guanidine denaturation support this view.

4.3. Guanidine Hydrochloride Serial Denaturation

4.3.1. Viscosity. It was not found possible to follow the

Effect of pH and time on the viscosity of ovalbumin in guanidine solutions.

Graphs: (1) 37°, pH 3.0, 2 hrs.
(2) 37°, pH 3.0, 1 hr.
(3) 37°, pH 11.6, solid guanidine HCl, 1 hr.
(4) 37°, pH 11.6, free guanidine pH 10, 1 hr.

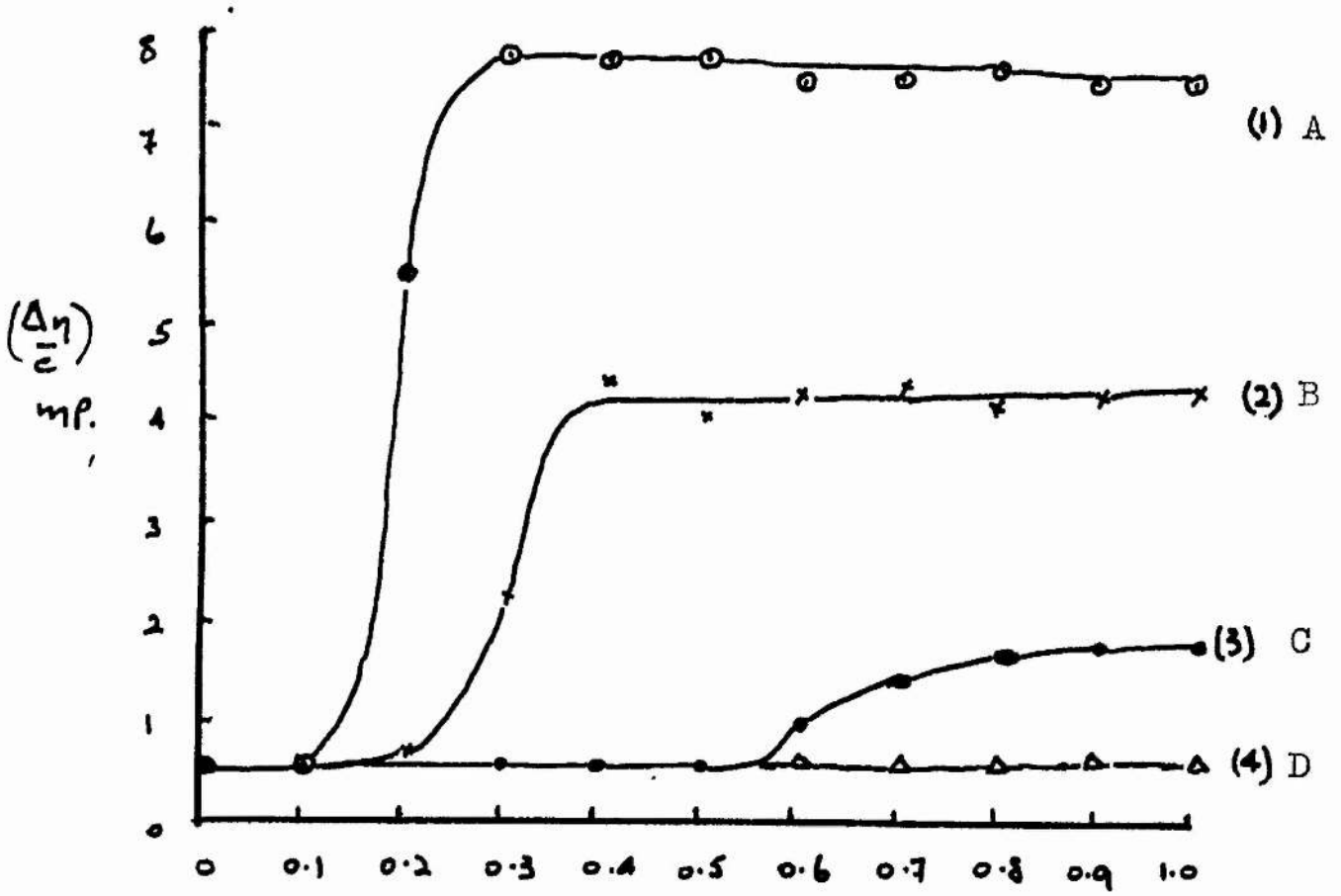


Fig. 37.

denaturation process in guanidine hydrochloride solution by measuring the changes in optical absorption and rotation because of the opacity and precipitation which took place in acid solution. Although the solutions often contained very fine precipitates the viscosity measurements were carried out at the lower concentrations since the mixture acted as a true solution and the inflection could be clearly determined. All measurements were made at 37°.

The viscosities of the reaction mixtures were examined under various conditions, the results are given in Fig. 37. In all experiments except (D) the ovalbumin was made up to the pH indicated in the graphs and added to the appropriate quantity of solid guanidine hydrochloride and the viscosity measured after a given time of incubation at 37° without bringing the pH back to the original value of the protein solution. In all three cases the viscosities have been corrected for the volume change due to the solution of the solute. In experiment (D) the guanidine hydrochloride was neutralised and made up to pH 10 with sodium hydroxide before adding to the ovalbumin solution at pH 11.6. This enabled the denaturing action of the free base to be studied in the absence of the hydrochloride salt. In this case the protein remained completely dissolved but showed no change in viscosity, indicating that the free base had no denaturing action, unlike urea which was effective at this pH. It may be inferred that the guanidine must be in the form of one of its salts to be an effective denaturing agent. This is very similar to the earlier observations

of Greenstein (1939) who showed that the degree of denaturation indueed in the ovalbumin molecule by guanidine salts varied with the individual salts, the hydrochloride being the strongest.

The effect of time on the guanidine hydrochloride denaturation process is shown in graphs (A) and (B) in Fig. 37. It may be seen that a two hour interval of incubation before measuring the viscosities produced the maximum denaturation effect at lower concentrations of denaturant than the usual hour interval. In both cases the inflection takes place at 0.1 - 0.2 M whereas in the slightly alkaline reaction mixture (C) the inflection takes place at 0.5 - 0.6 M. The latter conditions are nearly the same as those used for the FDNB analysis, which indicated an internal link in the range 0.5 - 0.75 M-guanidine hydrochloride. This again indicates that the chemical studies are confirmed by the physical studies.

4.4. Ethanol Serial Denaturation

4.4.1. Viscosity. The optical methods were not used to study the reaction mechanism because of the light scattering caused by the opalescence produced on mixing the protein solution with a high concentration of ethanol. The graphs were complex to interpret because of the formation of gels and aggregates. As may be seen from Figs. 38 - 41, the values of $(\frac{A_{sp}}{c})$ are very much greater than would be expected from the previous work on the urea and guanidine denaturation reactions. This can be accounted for by two processes taking place at

Change in viscosity of ovalbumin in ethanol.

Conditions: 37°, pH 11.6, 3hrs.

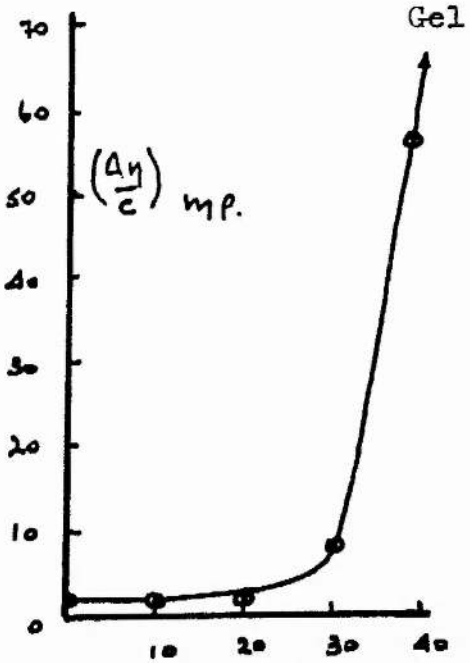


Fig. 38.

Conditions: 37°, pH 3.0, 3 hrs.

Values for primary reaction obtained from kinetic data in fig.41.

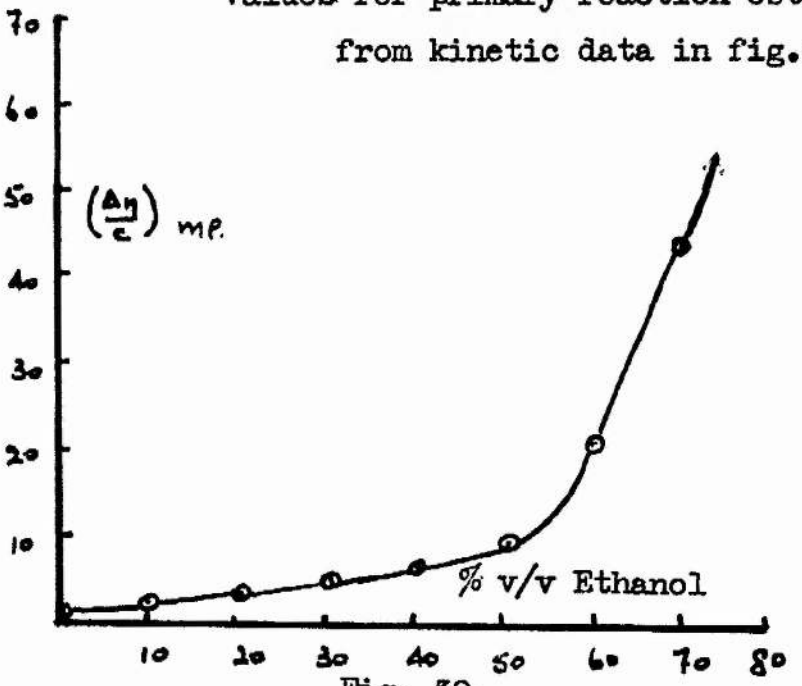


Fig. 39.

the same time, the primary uncoiling process and the secondary aggregation reaction. The latter is thought to involve the cross-linking of the extended peptide chains by hydrogen bonding and van der Waal's interactions.

In each experiment the ovalbumin was made up to the required pH, 2 ml. of this solution was then mixed with the appropriate quantities of water and ethanol. The reaction mixtures were then incubated for a given time at 37° and the viscosities measured at this temperature.

Fig. 38 shows the viscosity changes in an ovalbumin solution at pH 11.6 after three hours' incubation with ethanol at various concentrations. This graph indicates a very rapid increase in the viscosity change which occurs at 30% v/v ethanol. This was due to gelation, since the solutions at a higher concentration were almost solid. The inflection in the graph corresponding to the sudden uncoiling of the cyclic residue of the ovalbumin molecule has been masked by the great increase in $(\frac{\Delta\eta}{c})$ contributed by gelation.

Some method had to be devised to overcome the difficulties introduced by gel formation in ethanol solutions in order to attempt to correlate the physical studies with the chemical investigation of serial ethanol denaturation. Kauzmann and his associates (1953) were able to dissociate the two processes by adding a small quantity of p-chloromercuribenzoate. This reagent reacted with thiol radicals

Change in the viscosity of ovalbumin in ethanol. Kinetic studies in the presence of 0.01 iodoacetate, pH 11.6, 37°.

Graphs: (1) 60% v/v ethanol
(2) 50% v/v "

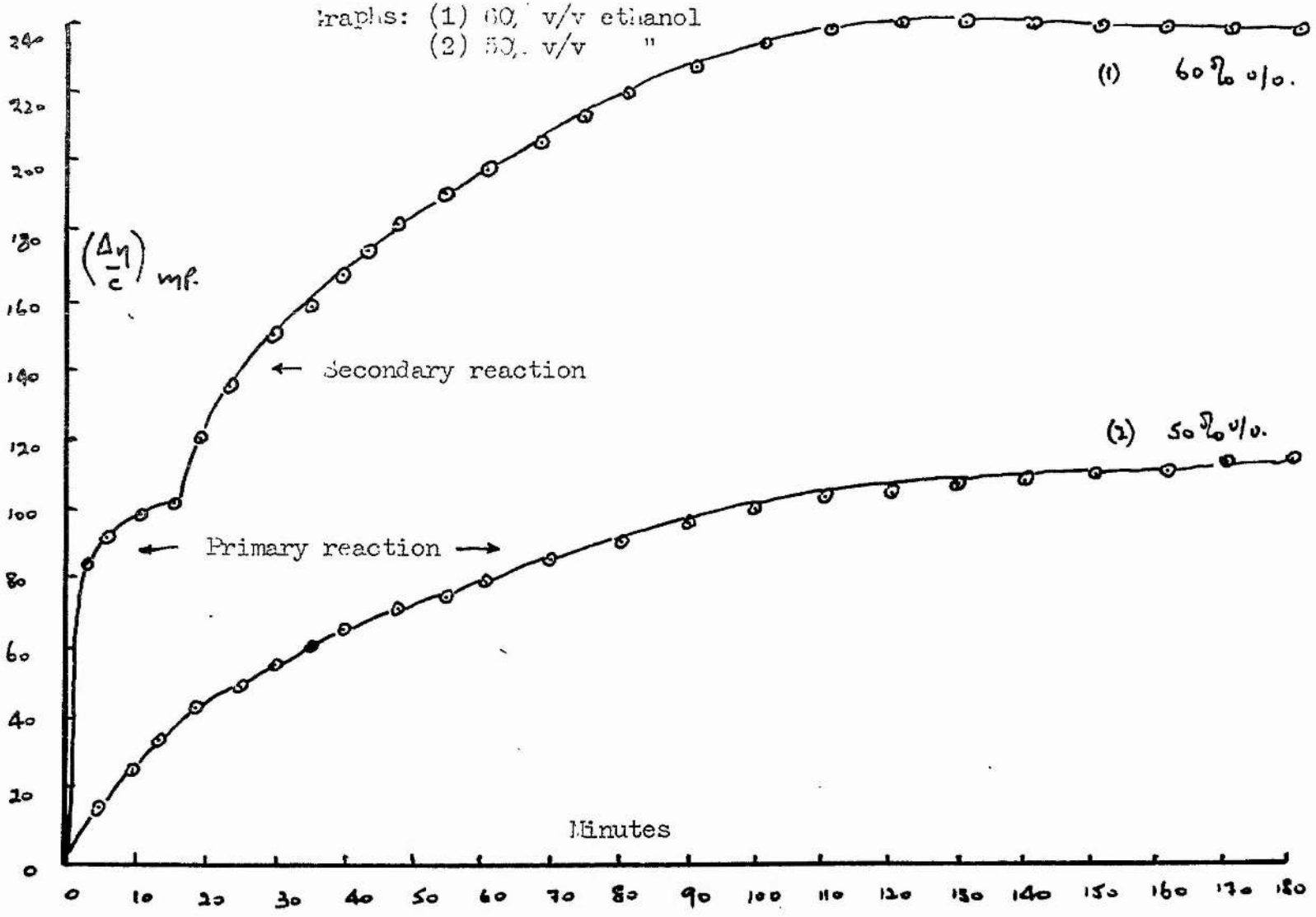


Fig. 40

which were temporarily formed during the conversion of intra-molecular disulphide bridges into inter-molecular bridges. Gelation was thus inhibited and the primary reaction studied independently. In the present work iodoacetate was employed for the same purpose since it is well known that thiols react with this reagent. Iodoacetate was foun to suppress gelation but not entirely inhibit the formation of gels at high ethanol concentrations. This may be seen from the kinetic experiments carried out with iodoacetate at pH 11.6 (see fig. 40). Several conclusions may be deduced from these results.

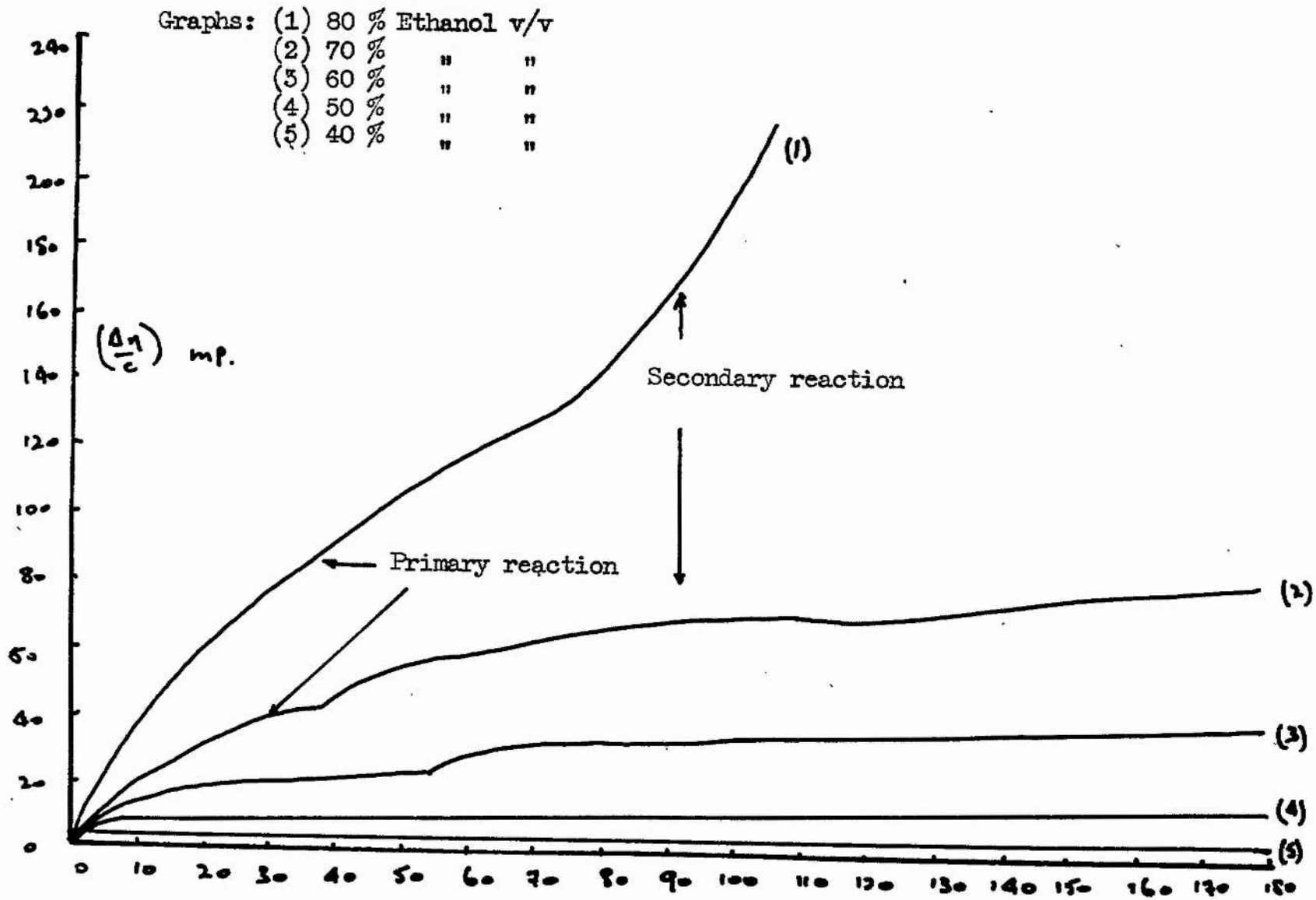
(1) Iodoacetate suppresses gelation, indicating the importance of disulphide bridges in gel formation at high pH. In this case, after three hours at 37°, no gel formed in 60% v/v ethanol which is an ethanol concentration nearly twice that at which gels formed in the absence of iodoacetate at the same pH. On standing for two days exposed to air a gel was formed. This was probably due to the oxidation of thiol reagent to the inactive disulphide.

(2) The secondary reaction does take place after the primary reaction even in the presence of iodoacetate at 60% v/v ethanol, but does not occur at concentrations below 50% v/v ethanol.

(3) The two graphs in Fig. 40 indicate that a large change in the viscosity due to the primary (uncoiling) process takes place between 50 - 60% v/v. This is what would be expected from the investigation of the uncoiling process by chemical means (i.e. using FDNB in slightl

Change in the viscosity of ovalbumin. Kinetic studies

in acid solution, pH 5.0, 37°.



alkaline solution).

A second approach to this problem was to employ an acid pH. Under these conditions thiol and disulphide groups are unlikely to react due to the unfavourable pH, thus preventing gelation. The kinetics of ethanol denaturation are given for the range 40 - 80% v/v ethanol at pH 3.0 in Fig. 41. The lower ethanol concentrations are not included in this figure for simplicity. However, the complete reaction sequence is given in Fig. 39. The graphs in Fig. 41 indicate that, although the disulphide bonds are chemically unreactive, gel formation does take place. The secondary reaction appears to be similar to that occurring at pH 11.6 in the presence of iodoacetate, except that 80% ethanol at the acid pH has approximately the same effect as 60% in the alkaline solution. Gelation could take place without the formation of inter-molecular disulphide links, although the formation of these cross-links enabled a gel to form at a lower concentration of denaturing agent.

The kinetic experiments allow the primary and secondary reactions to be differentiated. The changes in viscosity due to the primary reaction alone obtained from these studies are presented in Fig. 39. It may be seen that the primary reaction shows a steep inflection at 50 - 60% v/v ethanol. A similar observation might be predicted from the kinetic experiments with iodoacetate inhibition (Fig. 40). In this case the pH does not affect the concentration of ethanol required to produce the inflection in the primary reaction.

4.5. Discussion

The ethanol denaturation of ovalbumin as followed by the changes in viscosity taking place with increasing time and ethanol concentration support the chemical evidence for an internal link which is broken between 50 - 55% v/v ethanol. The physical studies carried out during urea and guanidine denaturation also support this hypothesis.

Some indication has been obtained from these viscosity measurements that this link is more easily ruptured by urea and guanidine in acid pH than in alkaline pH. The pH was found to have no noticeable effect on the concentration of ethanol which was required to break this link. It is therefore suggested that ethanol has a different mechanism from the hydrogen bond breakers in breaking the postulated link. The low pH favouring the rupture of this link precludes the possibility of disulphide bridges acting as intra-molecular bridges. Anfinsen and Redfield (1956) considered that there were two disulphide groups in the molecule and these acted as a cross-link between the coiled peptide chains. The structure suggested by these authors is compared in Fig. 42 with the modified "six-shaped" structure postulated in the present work.

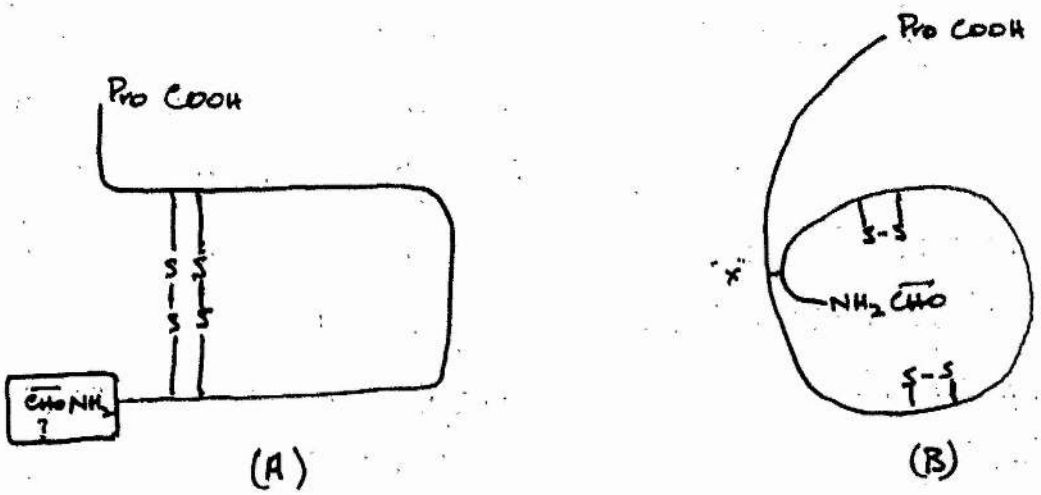


Fig. 42

A = Anfinsen and Redfield (1956). B = Suggested "six-shaped" structure. "X" indicates the hypothetical internal link.

The only difference between these two structures is the type of intra-molecular link, two disulphide bridges or "X". Strong evidence for the disulphide links would be obtained if an increase in viscosity and laevorotation were shown to take place in an ovalbumin solution after the specific reduction of these groups to thiol residues. Thioglycollic acid and other mild reducing agents have been demonstrated to carry out this reduction without affecting any other groups (Stern and White (1937), Fraenkel-Conrat et al. (1942) and Olcott and Fraenkel-Conrat (1947)). Reduction with

thioglycollate was carried out on ovalbumin using bovine serum albumin (BSA) as a control, since the latter is thought to contain seventeen disulphide groups involved in intra-molecular bridges (Anfinsen and Redfield, 1956). The effect of thioglycollate on the viscosity and optical rotation was measured at given time intervals after mixing the protein solutions at pH 11.0 with an equal volume of 0.1 M-thioglycollate at this pH. The results are presented in Table XXIV.

Table XXIV

The effect of thioglycollate on the viscosity and optical rotation of ovalbumin and bovine serum albumin solutions. Conditions: 0.05 M-thioglycollate, 20°, pH 11.0.

Property	Time	Ovalbumin		BSA	
		plus Water	plus Thioglycollate	plus Water	plus Thioglycollate
$-[\alpha]_D^{20}$	0 hr.	30	-	58.5	-
	18 "	30	30	58.5	73.5
	42 "	30	30	58.5	75.8
$(\frac{\Delta n}{c})$	42 "	0.5	0.5	3.7	7.3
Change in configuration		None		Uncoiling of helix	

It is inferred from the results obtained that the disulphide groups in both proteins were reduced. It is assumed that the thioglycollate was capable of reaction with ovalbumin as well as BSA.

The disulphide groups in BSA have been shown to be important in stabilising the native configuration, since once these were reduced the protein helix uncoiled.

The ovalbumin molecule was not dependent on the presence of disulphide bridges for the maintenance of the coiled configuration found in the native state. It is suggested from this evidence that the cyclic residue in the ovalbumin molecule is not cross-linked by disulphide bridges as described by Anfinsen and Redfield (1956) but rather by some other type of intra-molecular link, which has been called "X" in this work for lack of a precise knowledge of its chemical nature. It seems more likely that the two disulphide residues are situated close together as cystine residues, as has been demonstrated for the intra-molecular cystine bridge in the A chain of insulin.

6. Conclusion

1. The chemical and physical studies of the changes taking place during the denaturation of ovalbumin suggest a "six-shaped" structure for the native molecule.

2. Disulphide links are not thought to be important in the maintenance of the coiled configuration of native ovalbumin.

3. Denaturation is thought to be a stepwise process rather than an "all-or-none" reaction.

SUMMARY

1. The literature on the egg white proteins has been reviewed.

2. The individual egg white proteins have been isolated and the methods used described.

3. The possible origin of these proteins has been discussed. An attempt has been made to elucidate this problem by refined electrophoretic techniques. No definite conclusion could be reached with the methods available.

4. The N-terminal residues of the individual proteins were examined and the results found to be in good agreement with the literature.

5. The structure of the native ovalbumin molecule has been examined by both chemical and physical means during progressive denaturation in alcohol, urea and guanidine hydrochloride. The results of the two investigations were shown to be in agreement with one another.

6. A "six-shaped" structure has been proposed for the configuration of the native ovalbumin molecule.

7. This structure involves the hypothesis of an intra-molecular link since there is known to be a C-terminal but no N-terminal amino acid residue. The disulphide bridge suggested by Anfinsen and Redfield (1956) has been shown to be unlikely. This link has not been identified as a chemical entity although it has been suggested that low pH enables hydrogen bond breakers to rupture the molecule more easily.

FUTURE WORK

It is hoped to continue investigations on the following:

1. The chemical reactivity of lysine residues in ovalbumin during serial denaturation in urea and guanidine solutions.
2. The chemical nature of the internal link.
3. The structural changes in the ovalbumin-plakalbumin conversion.
4. Possible species differences in ovalbumins from other birds.
5. Other proteins.

Note added in preparation

Rhodes et al. (1958) have recently employed the cellulose ion-exchangers described by Sober et al. (1956) and Peterson and Sober (1956) in the analysis, fractionation and purification of the egg white proteins. Egg white was separated into fourteen protein fractions each of which could be prepared on a large scale.

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X indicates that the original paper was not consulted.

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