

STUDIES ON EGG WHITE PROTEINS

A thesis submitted by

MARK DAVID MELAMED

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Abstract

Two egg white glycoproteins, both with known biological activities were investigated in this study.

1. Avidin assays on single eggs demonstrated a wide variation in its content from one egg to another, even when they were from the same commercial chicken breeder. Two large scale (up to 1000 eggs) preparations of crude avidin were conducted by batchwise adsorption onto carboxymethyl cellulose and salt gradient elution. Conditions were devised for chromatography on Amberlite CG 50 which yielded a very pure avidin and fractionated it into essentially two types which differed primarily in their content of basic amino acids. Purification of crude avidin by two further CM-cellulose columns yielded a preparation more than 90% pure. Virtually all the weight and nitrogen of the preparation was accounted for in terms of amino acids, carbohydrates, amides. Ultraviolet extinction spectra and $E_{1cm,282m\mu}$ were determined.

2. From the behaviour of ovomucoid on gel filtration it was concluded that it has a more expanded or hydrated structure (probably because of its carbohydrate content) than normal globular proteins of equal molecular weight. Attempts to purify and/or fractionate ovomucoid into electrophoretically different molecular forms on DEAE-Sephadex or DEAE-cellulose were unsuccessful. On CM-cellulose a better purification was obtained but fractionation was erratic. The β -aminoethylcysteinyl derivative of a fully reduced preparation of crude ovomucoid was prepared and yielded a number of discrete glycoprotein peaks on CM-cellulose chromatography. Amino acid analyses were performed on two of these. By the use of starch gel two electrophoretically distinct fractions of ovomucoid were isolated. Sialic acid accounted for only

part of this heterogeneity. Amino acid, carbohydrate and antitryptic activities were determined on the fractions. It was shown that in the presence of 6 M urea ovomucoid and trypsin do not form a complex.

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PART I - A BRIEF SURVEY OF EGG WHITE PROTEINS

For over a hundred years hen's egg white has been used as starting material for the isolation and study of proteins and especially glycoproteins. Together with the yolk it supplies all the materials necessary for development of the fertilized egg cell to the hatched chick. On the average the 33 ml of white comprise 60% of the total weight of the egg. The white consists almost exclusively of a suspension or solution of proteins and glycoproteins (10.2% by weight). In addition to free sugar (0.4%) and fat (0.05%) small amounts of the common minerals are found in the white; S (0.2%), K (0.17%), Na (0.16%), Cl (0.15%), P (0.1%), Ca (0.01%) and Mg (0.01%) as well as a few vitamins (in μg per egg white, average values) choline (400), riboflavin (123), pantothenic acid (100), vitamin B₆ (68), nicotinic acid (29), folic acid (0.5) and B₁₂ (0.025), (Bolton, 1961). The pH of egg white is usually between 8 and 9 and appears to increase on storage.

Free boundary electrophoresis when applied to egg white by several groups (see Warner 1954 for review) yielded ovalbumin, conalbumin, ovomucoid, lysozyme and one or two minor globulins as the constituents of the protein present. Fractionation of egg white on CM-cellulose (Rhodes et al. 1958) demonstrated also the presence of a flavoprotein. Including the multiple peaks of ovalbumin and the five uncharacterized minor globulin components a total of 15 separate peaks were eluted by this procedure. From this work the contents of the individual proteins in egg white appears to be as follows:- ovalbumin 60%, conalbumin 15%, ovomucoid 10%, flavoprotein 8%, lysozyme 4%, globulins plus avidin 3%.

The values given for ovomucoid and flavoprotein are possibly too high as the isolated products may not be pure. In addition there is about 2% of ovomucin which is usually precipitated by dilution of the egg white before electrophoresis or fractionation of the remaining proteins.

Starch gel electrophoresis of egg white has yielded 19 separate bands (Lush, 1961; Feeney et al., 1963). These include all the major constituents although they are possibly not all resolved from each other. The different ovalbumins and the ovomucoids run in the same region under the conditions used by these authors but their method of staining may not have revealed the ovomucoid. At least one minor constituent, avidin, does not appear on the gels. Taking all the evidence together, therefore, present methods of protein fractionation indicate a total of twenty-five to thirty molecular species of protein and glycoprotein in the egg white. Many of these occur only in very small amounts and they are not necessarily all present in any one egg. The presence or absence of some of the minor components is genetically determined and a few studies have been devoted to their occurrence (Lush, 1961; Baker and Marwell, 1962; Feeney et al., 1963; Baker, 1964).

A brief account will now be given of the main components of egg white not dealt with in the present study.

1. Egg Albumin. The major constituent of egg white is the glycoprotein egg albumin or ovalbumin. It has occupied a central position in investigations of the properties of proteins since its first crystallization by Hofmeister (1890). The single amino acid chain is probably cross-linked by one or two disulphide bonds and has one small heterosaccharide

attached (3.2% of the total weight). The molecular weight of the glycoprotein is 45,000 and the chain is terminated at the amino end by an acetyl-glycine and at the carboxyl end by a proline residue. The identification of the terminal amino acids, in common with the elucidation of many other features of the chemistry of ovalbumin (such as the content of sulphydryl groups and disulphide bonds) has been beset by a number of unusual difficulties. The isoelectric point of ovalbumin is 4.6 and its heterogeneity on free boundary electrophoresis is due to the presence of varying numbers (0 to 2) of phosphate esters. This protein has been used frequently for studies on denaturation, but it appears to be rather unusual in that it has not yet proved possible to reverse the denaturation brought about with reagents like urea or guanidinium chloride. The general properties of egg albumin have been reviewed by Warner (1954) and Neuberger and Marshall (1965).

The carbohydrate of egg albumin has been investigated since Neuberger (1938) showed conclusively that it was an integral part of the protein (see Neuberger and Marshall, 1965 for review). The single heterosaccharide moiety is usually found to consist of 5 residues of mannose and 3 of N-acetyl glucosamine although recent work suggests that different amounts of either or both of these sugars may occur in some cases (Cunningham et al., 1963). The carbohydrate is attached by a glycosylamine bond of β -anomeric configuration between an N-acetyl glucosamine and the side chain carboxyl group of an aspartic acid residue within the chain. Besides the position of the N-acetyl glucosamine reducing end in the heterosaccharide, it is also known that there is one branch point and two non-reducing ends. One of these is

N-acetyl glucosamine and the other mannose.

2. Conalbumin. The iron-binding protein in egg white, conalbumin, has been extensively purified and crystalized both in the iron-free form and as the metal complex. It consists of a single amino acid chain of molecular weight about 80,000 with isoelectric point in the region 6 to 7 and contains 2.2% carbohydrate (Warner, 1954; Williams, 1962). In its metal binding properties it strikingly resembles the serum protein transferrin. In fact, transferrin from hen's serum (as well as that from egg yolk) has been shown to be largely identical with egg white conalbumin in amino acid and amino sugar composition but the two glycoproteins differ markedly in their hexose content (Williams, 1962).

The ferric-conalbumin complex is reddish in colour with an absorption maximum at 470 mμ and is considerably more stable to urea denaturation than the colourless metal-free protein (Glaser and McKenzie, 1963). Each molecule of conalbumin binds two atoms of Fe^{3+} and the two sites appear to be fully independent of each other. The protein is not totally metal specific as Cu^{2+} and Zn can also be bound. Two recent studies on the nature of the binding have both implicated 3 tyrosyl residues and 2 nitrogen atoms (most probably imidazole side chains) as the chelating site (Aasa et al, 1963; Windle et al, 1963). Many workers have noted the electrophoretic heterogeneity of conalbumin particularly when starch gels are used, but nothing is known of the nature of the differences between the various protein bands (see e.g. Williams, 1962; Parker and Bearn, 1963; Clark et al, 1963).

3. Lysosyme. This is the only protein in hen's egg white at present known to have definite enzymic activity. It is classified as N-acetyl-

muramido glycanohydrolase E.C. 3.2.1.17, and has a β -(1 $\rightarrow$ 4) glucosaminidase activity with the ability to hydrolyse a monopolysaccharide component of some bacterial cell walls (e.g. Micrococcus lysodeikticus). The bond cleaved appears to be β (1 $\rightarrow$ 4) N-acetylmuranyl N-acetyl glucosamine (Salton, 1956). Lysozyme will also hydrolyse the 1 $\rightarrow$ 4 linear N-acetyl glucosamine polymer, chitin (Berger and Weiser, 1957). The physical parameters of lysozyme have been investigated in detail (Champagne et al, 1961) and the full primary amino acid sequence determined independently by two groups (Jolles et al, 1963; Canfield, 1963). The protein consists of single amino acid chain of molecular weight 14,386 containing four disulphide bridges with lysyl amino terminal and leucine carboxyl terminal, and an unusually high isoelectric point of about 11.

The three-dimensional structure of crystalline lysozyme at 2 Å resolution has recently been completely elucidated (Blake et al, 1965) which makes it the best characterized of all the hen's egg white proteins. There is about 40% α -helix in the molecule and the disulphide bridges are all associated at least on one side with a helical region. Although the lysyl and arginyl side chains are all external and there is a hydrophobic interior of the molecule consisting mainly of 6 tryptophanyl residues, the molecule does not fall totally into a case of external hydrophilic residues and internal hydrophobic ones. Three tryptophan side chains protrude from the surface and on the other hand one serine and one glutamine are completely shielded from the surrounding liquid.

4. Ovomucin is a poorly characterized heterogeneous fraction from egg

white which is highly gelatinous in alkaline solution and insoluble in acid solutions (see Warner, 1954 for review). It is precipitated by dilution of egg white with water or, together with the globulins, by half-saturation with ammonium sulphate. Ovomucin has never been satisfactorily purified due largely to its progressively increasing insolubility during preparation. Prior reduction with mercaptoethanol may be useful in this connection (Robinson and Monsey, 1964), although the choice of iodoacetic acid as alkylating agent produced a derivative which was too acidic to be chromatographed. Crude ovomucin preparations contain approximately 10% hexose (Lanni et al, 1949) 11% hexosamine (Brooks and Hale, 1961) and 2.6% sialic acid - which accounts for 40% of the total sialic acid of the egg white (Feeney et al, 1960).

An interesting property of ovomucin is its ability - in common with other sialic acid rich mucins - to inhibit the haemagglutination of red cells by influenza virus (Lanni et al, 1949; Gottschalk and Lind, 1949a). As may be expected the removal of sialic acid from ovomucin by viral neuraminidase destroys the anti-haemagglutinating ability of the ovomucin (Gottschalk and Lind, 1949b). Ovomucin may play an important role in determining the physico-chemical properties of egg white in which regard it has been suggested that it forms cross-linked chain-like complexes with lysozyme (Brooks and Hale, 1961).

5. Others. The flavin-binding protein from egg white has been purified chromatographically (Rhodes et al, 1959). It is a glycoprotein containing 0.5% sialic acid with a molecular weight of about 33,000 and an isoelectric point of 4. The flavin moiety of the isolated flavoprotein

is riboflavin and all the riboflavin of egg white is apparently bound in this way. An approximately equal quantity of apoprotein is also present in egg white. Acidic conditions (below pH 4) as well as urea (8 M) cause dissociation of the complex, but heating the apoprotein in 0.05 M tris pH 7.0 at 100° for 15 minutes brought about no loss in flavin binding capacity. The protein is unusual amongst flavoproteins in that riboflavin itself is more firmly bound than the phosphate or the adenine dinucleotide.

An interesting protein isolated from egg white, "ovoinhibitor", was found to inhibit proteases from B. subtilis and an unidentified Aspergillus (Matsushima, 1958a,b). Preparations of ovoinhibitor have also been shown to be effective against trypsin and chymotrypsin as well as an A. oryzae protease. The inhibition appeared to be equally effective against trypsin and chymotrypsin and could inhibit the two simultaneously (Rhodes et al, 1960; Feeney et al, 1963).

A glycoprotein has been isolated which accounts for about 1% of the total egg white protein (Ketterer, 1962 and 1965). It contains 13.6% hexose (mannose and galactose), 13.8% glucosamine and 3.0% sialic acid. Its isoelectric point is about 4 and it is not heat coagulable. No specific biological property has been found associated with it.

PART II - AVIDIN

A INTRODUCTION

Early studies on nutrition by various workers between 1920 and 1940 repeatedly revealed that when egg white was used as a source of dietary protein the animal suffered injury. Various foodstuffs had

the power of counteracting these ill effects; however, for a number of reasons, the condition did not appear to be one of a simple deficiency and in some respects had the appearance of an actual toxicity (see e.g. the lengthy and meticulous studies of Boas, 1927). The situation was clarified in the late 1930s with the discovery that two factors were involved. Biotin was shown to be the essential growth factor in which the animal was showing a deficiency, the "egg-white injury" factor making the biotin unavailable to the animal even if small quantities of this growth factor were present in the diet. The finding that egg white rendered biotin unavailable for the growth of microorganisms provided a convenient and sensitive assay procedure for the injurious factor, which was soon shown in fact to be a protein. Eakin, Snell, Gyorgy and co-workers devised a microbiological assay using yeast; and concentrated the protein by acetone precipitation followed by ammonium sulphate fractionation (Eakin et al, 1941; Gyorgi et al, 1941; Gyorgi and Rose, 1941; Gyorgi et al, 1942; Pennington et al, 1942). They named the protein "avidin". Their best preparations required 350 parts by weight to combine with one of biotin. Simultaneously Woolley and Longworth (1942) showed that Clostridium butylicum could not utilise biotin in the presence of egg white and these workers also obtained avidin in a highly concentrated form. In their case 200 parts by weight of protein was sufficient to inactivate one of biotin. A certain proportion of the avidin in the preparations of both groups was present in an insoluble form.

In a reinvestigation of the isolation and properties of avidin,

Fraenkel-Conrat et al (1952a) showed that these insoluble fractions of avidin were in fact complexes with nucleic acid or with an uncharacterized acidic glycoprotein. These complexes could be dissociated (e.g. by electrophoresis) and the free soluble avidin was recovered. The best preparations combined with biotin in the ratio of about 120:1 by weight. Avidin was shown to be a glycoprotein containing mannose and hexosamine and its amino acid composition was determined. Chemical modifications of various side chains in the avidin did not yield much useful information about the binding site of the biotin (Fraenkel-Conrat et al, 1952b). A convenient large-scale method of concentrating avidin was subsequently devised (Dayse, 1954). Although this renders the material largely in an insoluble form and in a relatively low state of purity, it was considered suitable for commercial production of avidin (Nutritional Biochemicals). In the course of a complete fractionation of egg white proteins on CM-cellulose (Rhodes et al (1958) isolated a small amount of avidin, but it was not characterized in detail. The peak which contained the avidin represented a far larger fraction of the egg white than the known avidin content had indicated and was thus presumably of relatively low purity.

Interest in avidin in this department was initiated by the finding that when the photosynthetic bacterium Rhodospseudomonas spheroides was grown in a biotin deficient medium the concentration of bacteriochlorophyll and of a yellow carotenoid were both reduced per unit dry weight (Gibson, Neuberger and Tait, 1962). In addition the activity of the first enzyme in the biosynthetic pathway of the porphyrins, amino-

lævulinic acid synthetase, was markedly decreased under conditions of biotin deficiency. Commercial avidin (Nutritional Biochemicals) strongly inhibited this enzyme, but preincubation of this avidin with biotin did not abolish the inhibition. This avidin was made by the method of Dhyse (1954) and had an activity of 2.5 µg biotin/mg. However chromatographically prepared avidin (N.M. Green) even when still impure no longer inhibited the enzyme. Because of its interesting and unusual properties avidin warranted further study in its own right and the investigation described below was undertaken.

B. ISOLATION OF AVIDIN FROM EGG WHITE

1. General Outline

Avidin comprises 0.05% of the protein of egg white. Early methods of concentrating and isolating it invariably yielded largely insoluble products. These were later shown to be complexes between avidin (I.E.P. 10.5) and DNA or an acid glycoprotein. Free avidin is readily water soluble and fully active against biotin and efforts were concentrated, therefore, on its isolation in this form. As avidin is not removed from egg white by centrifugation it seemed likely that it occurred naturally largely dissociated from the acidic components. Dissociation of the complex would be facilitated in the native egg white because the pH is about 9 (where the avidin does not have its maximum positive charge) and because the avidin is present in very low concentration (0.005% or about $10^{-6}M$). Any manipulation which might promote precipitation by association of oppositely charged proteins such as drying the egg white, precipitating all the proteins by acetone, removal of electrolytes by dialysis, or lowering of the pH should be avoided unless the avidin and the acidic macromolecules are no longer in a position to combine.

2. Assay of Avidin

a) Radioactive Biotin Assay

The biotin binding activity of avidin solution was determined by the methods of Green (1963a). The principle of the radioactive biotin assay is to add to an avidin solution a known amount of ^{14}C biotin in excess of that needed to saturate the avidin. The avidin-biotin complex is then removed by adsorption onto CMC^{*} leaving the excess ^{14}C biotin in solution and this is then counted. The accuracy and reliability of the method depend on the distribution coefficient of the avidin-biotin complex between free solution and CMC, and this is virtually entirely in favour of the CMC. Adsorption of avidin or its biotin complex onto CMC is practically quantitative around pH 5 at ionic strength below 0.4. A pH value much lower than this is not suitable as the CMC becomes progressively less charged in acid solutions. A pH of 5 is thus used for the determinations. Since the avidin-biotin complex is adsorbed onto CMC as readily as avidin and since avidin when adsorbed on CMC is virtually as good as free avidin at combining with biotin, the order of addition of avidin, biotin and CMC does not affect the final result. The rate limiting step is the adsorption of the protein onto CMC and the half-time for this process is about 3 seconds.

Method A suspension containing 10 - 12 mg. dry weight of CMC/ml was prepared from well washed CMC filter cake (see page 21) in 0.1 M pyridine-acetate buffer, pH 5.0 (25 ml. 2 N acetic acid and 4.25 ml. pyridine diluted to 500 mls.) Radioactive biotin was added to a concentration of 1000-2000 cpm/ml. Samples (1 ml) of this suspension were

^{*} Carboxymethyl cellulose.

dispensed from a tuberculin syringe without a needle into small test-tubes containing the avidin samples (0.2 ml). The CMC was prevented from adhering to the walls of the tubes by rendering them non-wettable with a suitable silicone agent. After standing with occasional shaking for 2 to 3 minutes the CMC was centrifuged and the excess of ^{14}C biotin was determined by plating and counting a sample (0.2 - 0.4 ml) of the supernatant. In the early stages of the work the solution containing biotin plus 0.1 ml of 0.2% cetyltrimethylammonium bromide was evaporated on 2 cm^2 polythene planchets in an oven at 60° . The biotin was then counted at infinite thinness with an end window geiger counter. In subsequent experiments a Nuclear Chicago Corporation automatic gas flow counter with a Microsil window was employed. Samples up to 1 ml were dried down in the centre of 4 cm^2 aluminium planchets and counted. The relative efficiencies of the two counters were compared and the gas flow counter was found to be 4.4 times more efficient than the Geiger counter. All the data from the early work was thereafter multiplied by this factor and activities recorded are given in terms of the gas flow counter. The absolute efficiency of the counter was not determined accurately but is about 25-30%

The useful limits of the method were investigated by adding increasing amounts of avidin to a series of similar samples of ^{14}C biotin. Where less than 20% or more than 80% of the biotin was adsorbed a significant departure from linearity was noted. Where almost all the biotin was removed from the solution the very low counts recorded were liable to error as the small amount of "soluble CMC" always present in

the CMC (see page 20) binds a significant amount of radioactive avidin-biotin complex. For this last reason too the ratio of CMC to avidin has an optimum value. As the amount of avidin to be assayed affects the requirements both of ^{14}C biotin and of CMC in the same way the two reagents were prepared together in a fixed ratio to each other. With biotin of specific activity 14.8×10^6 cpm/mg, 0.01 μg . biotin or 1 μg avidin in 0.2 ml of solution could be readily detected and 5 μg avidin could be determined with a standard deviation over 4000 cpm of $\pm 3.5\%$ measured on 12 replicate planchets at 60% adsorption of the added biotin.

b). Spectrophotometric Assay

A more accurate, although less sensitive method, for assay of avidin was developed by Green (1963b) from the discovery (Green, 1962) that addition of biotin to a solution of avidin brings about a shift in the ultraviolet absorption spectrum of the protein towards longer wavelength (red shift). This is the opposite effect to that produced on denaturation of the protein which brings about a shift to shorter wavelength (blue shift). If the extinction of a solution of avidin-biotin complex is measured in a double beam spectrophotometer against a solution of avidin at the same molar concentration the resulting difference spectrum has a sharp maximum at 233 m μ (ΔE_{233}) almost equal to the E_{280} of the avidin solution ($\Delta E_{233}/E_{280} = 0.94$). Biotin has virtually no extinction at this wavelength and excess biotin does not affect the difference spectrum. The avidin assay is best performed as a difference spectrophotometric titration with biotin.

Method: A Unicam SP 700 double beam recording spectrophotometer was used. To a matched pair of 10mm cuvettes was added an equal volume of avidin solution in 0.01 M phosphate buffer at an approximately neutral pH. [Actually the method is remarkably independent of pH giving the same result over the pH range 2 to 13). A light path of 10 mm was always used either with full width cells which had a minimum capacity of 1.5 ml and gave the most accurate results, or with narrow cells which had a minimum capacity of 0.35 ml. Avidin concentration was such that the E_{280} was about 0.4. This gives an E_{233} of 1.4. Higher avidin concentration introduces significant stray light errors at this wavelength. After checking the baseline at this stage, biotin solution equivalent to about 10-20% of the avidin was added to the sample cuvette in a volume of 10 or 20 μ l with a Carlsberg constriction pipette, and an equal volume of buffer was added to the reference cuvette. After mixing ΔE_{233} was measured. Between 5 and 10 more additions were performed in this way until the difference extinction at 233 m μ showed no further change. ΔE_{233} was plotted against biotin added (see fig. 1). The readings should be corrected for dilution for higher accuracy. The point of intersection of the two straight line portions of the plot represents the amount of biotin corresponding to the avidin in the cuvette. As the $E_{1\text{cm}}^{1\%}$, 282m μ of avidin was determined on a weight and nitrogen basis the absolute weight of avidin present in the cuvette could be obtained from the volume and the E_{282} .

Note on Biotin-binding activity units: The amount of biotin bound by a preparation of avidin can be expressed in a number of different units.

Biotin was measured in terms of radioactivity and for purposes of comparison the most direct procedure was to use cpm to indicate the amount of biotin. As the specific radioactivity of the biotin preparation was known, the biotin determined by radioassay could be expressed on a weight or a molar basis. In the case of the spectrophotometric titration biotin ^{was} recorded directly on a weight or molar basis. Protein concentration in column effluents was recorded in terms of $E_{280\text{ m}\mu}^{1\text{ cm}}$. It was most convenient therefore in this case to compare specific biotin binding activities with 1 ml. of protein solution of $E_{280\text{ m}\mu}^{1\text{ cm}} = 1$ as a standard amount of protein. In later work, weight of air dried protein or true dry weight of protein could be used. Of the various possible combinations, $\mu\text{g biotin/ml } E_{280} = 1$ and $\mu\text{g biotin/mg air dry protein}$ will be used.

3. Avidin Assays on Single Eggs - Selection of "Breeds"

(a) Development of the Method

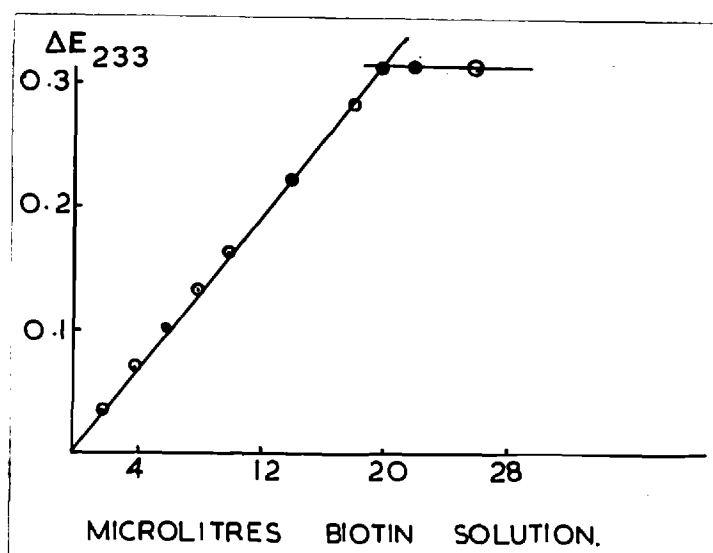
In the early work eggs were obtained from a dealer without regard to their origin in terms of "breed" of chicken. Later, eggs were obtained from chickens described by the supplier as being of one given "breed". However no detailed enquiries were made into the genetics of these "breeds", but they are probably not fully homozygous. As differences in diet between one breeder and another may also affect the results, eggs were classified in terms of the sender rather than the name of the "breed". To ascertain which sender would be the most suitable source for large scale preparations the variation in avidin content between individual eggs from the same sender and between one sender and another was measured. Total biotin-binding capacity of egg white had

previously been determined by microbiological assay (Eakin et al, 1941- Woolley and Longworth, 1942). In our case an overall biotin-binding figure could have been obtained by adding excess ^{14}C biotin to the egg white, and precipitating all the proteins. However, this may have led to high results due to non-specific binding and trapping of biotin in the very bulky precipitate. As the final yield of avidin was the main concern, a method more directly comparable with that used for the isolation was developed to evaluate individual eggs.

The first series of experiments were directed at finding the best conditions for the assay. The white of an egg was diluted with an equal volume of ion-free water and homogenised. To one-fifth of this solution (usually 12-13 ml) 1 g. CMC filter-cake was added and the pH brought to 7 by addition of 2 N acetic acid with constant stirring. The suspension was briefly centrifuged and the supernatant (A_1) was removed. The CMC was washed twice by resuspension in 10 ml of 25 mM ammonium acetate buffer pH 9, both washings being saved for assay of avidin (assay A_2 and A_3). Supernatant A_1 was stirred with a fresh batch of CMC (1 g.), which was likewise recovered by centrifugation and washed with the same buffer. Both CMC batches were pooled and made up to 10 ml with distilled water. One ml of this suspension (B) was centrifuged and the CMC was assayed for total bound avidin (Assay B). The remaining 9 ml of suspension B were centrifuged and the CMC resuspended in 10 ml of 1% $(\text{NH}_4)_2\text{CO}_3$ to elute the avidin. After centrifuging, the supernatant was neutralised and assayed for avidin (Assay C_1) and the CMC was washed a second time in 1% $(\text{NH}_4)_2\text{CO}_3$ as before (Assay C_2). The CMC was finally assayed for residual biotin-binding capacity (Assay D).

TABLE I THE FATE OF TOTAL CMC-BOUND AVIDIN

Assay	cpm for one-fifth of an egg white	
	Experiment 1	Experiment 2
A ₂ 1st ammonium acetate pH 9 wash	12,100	6,800
A ₃ 2nd ammonium acetate pH 9 wash	11,500	2,600
B Total CMC-bound avidin after washing	59,200	15,200
C ₁ 1st ammonium carbonate elution	50,000	15,200
C ₂ 2nd ammonium carbonate elution	14,800	2,000
D Residual CMC activity	600	nil

**Fig. 1 Spectrophotometric Titration of Avidin (D 31)**

Avidin solution ($E_{282} = 0.352$; 1.6 ml) titrated with biotin (250 $\mu\text{g/ml}$). Activity of avidin = 8.9 $\mu\text{g biotin/ml } E_{282}=1$.

Two typical series of results are presented in Table I. It is clear that washing by suspension in a relatively large volume of 25 mM ammonium acetate pH 9 buffer removes a considerable proportion of the avidin. For routine determination of the avidin content of single eggs, therefore, 20 mM ammonium acetate pH 7 was used for washing and this did not remove significant amounts of avidin. As direct determination of the biotin binding capacity of the protein on the CMC appeared to reflect fairly accurately the amount of recoverable anti-biotin activity the following procedure for assaying single eggs was finally adopted:

(b) Method

The egg white was homogenised with an equal volume of ion-free water. One-fifth of the solution was stirred with 1 g CMC filter-cake and the pH was adjusted to 7 with 2 N acetic acid. The suspension was centrifuged briefly to bring down the CMC with adsorbed basic proteins; the supernatant was discarded. The CMC was washed twice by suspension in 10 ml 20 mM ammonium acetate (pH 7), centrifuged, and finally suspended in 50 ml of distilled water. To 1 ml of this suspension was added 0.3 ml of a solution containing 3000 cpm of [14 C] biotin in 0.1 M pyridine acetate buffer pH 5. The suspension was shaken for a few minutes and then the cellulose was centrifuged. The excess [14 C] biotin in the supernatant was counted and the avidin content of the egg was calculated from the amount of biotin removed. It is still possible that some avidin is not adsorbed onto the CMC (e.g. because of complexing with DNA) and this would not be included in the assay.

TABLE II. AVIDIN CONTENT OF SINGLE EGGS

(1)

<u>Replicate Analysis on Single Eggs</u> <u>(mg avidin/egg)</u>			
	<u>Egg 1</u>	<u>Egg 2</u>	<u>Egg 3</u>
Result 1	1.60	1.78	2.20
Result 2	1.75	1.40	1.98
Result 3	<u>1.64</u>	<u>1.62</u>	<u>2.25</u>
Average	1.66	1.60	2.14
S.D.	4.3%	11.8%	6.75%

Average S.D. = 7.6%

(ii) Avidin Content of Different Eggs from the Same Breeder
(mg/egg)

<u>Breeder</u>	<u>Day</u>	<u>Ellis</u>	<u>Smith</u>	<u>Singleton</u>	<u>Campbell</u>
Egg 1	1.05	1.42	0.95	0.45	0.925
2	1.1	1.31	1.44	0.28	0.39
3	1.7	0.55	1.18	0.74	0.65
4	1.4	0.875	1.18	0.45	0.50
5	1.25	0.75	1.2	0.39	0.77
6		1.04		0.38	0.125
Average	1.3	0.99	1.2	0.45	0.56
S.D.	20%	34%	14.5%	34%	47%

Average = 0.9
Average S.D. = 30%

(iii) Average Avidin Content/Egg, Determined on Batches of Six
Eggs from Different Breeders (mg/egg)

1.84	0.78	1.30	0.99	0.70	1.20
0.45	0.56	0.63	1.66	1.25	0.95
					0.75

Average = 1.08 ($\pm$ 0.44) mg/egg. S.D. = 41% Range = 0.45 to 1.84 mg/egg

(c) Results

The results of these determinations are given in Table II. The estimates of avidin content of individual eggs are reasonably reliable, the standard deviation of replicate determinations on the same egg being of the order of 8%. However, there is considerable variation in the avidin contents of different eggs, even if obtained from the same breeder. In some cases the scatter was much greater than in others. It is not clear from the results to what extent the apparent differences in the average content of avidin in eggs from one breeder or another are significant. Nevertheless the senders which appeared to supply eggs with the most avidin were selected.

4. Large Scale Preparation of Avidin

(a) Materials Eggs

Eggs The eggs used were classified as "large" and were delivered to the laboratory within a few days (2-5) of laying. If processing did not begin immediately they were stored in the cold room (4-6°) but never for more than a further 2-3 days.

CM cellulose Whatman CM 70 was used throughout. It was of considerable importance that it was thoroughly washed before use. CMC, when new, contains a sparingly soluble component which has a very strong affinity for proteins and gives soluble complexes (Green 1963^a). This "soluble CMC" could be gradually leached out of the material by prolonged washing. In addition, as metal ions had been shown to contribute to the instability of avidin, the CMC was given a wash with EDTA and ion-free water was used for the final wash.

New CMC was washed several times by suspension in a large volume of distilled water, allowing it to settle and decanting to remove fines. After degassing by evacuation on the water pump, the washing was repeated as more fines appeared to be liberated, and the cellulose was finally degassed again and packed into a column. The column was irrigated with the following solutions in the sequence indicated, the quantities given being the amount used per 100 g of dry CMC starting material; 0.5 N NaOH containing 0.5 M NaCl, 600 ml; distilled water, 10 l; 1 mM EDTA, 5 l; and ion-free water, 10 l. The NaOH solution often resulted in the appearance of some bright yellow substance as it percolated through the column. This material washed off quite readily and did not appear to result in any impairment of the adsorptive power of the CMC. After the washing the CMC was unpacked from the column and sucked dry on a Büchner funnel. If the CMC was poured onto the Büchner funnel in a thick suspension there was no need for any filter paper and a rapid and efficient removal of excess water was achieved. The cellulose was pressed out as dry as possible. This product is referred to as CMC filter-cake. It was stored in well-stoppered containers at 4°. On drying samples overnight at 110° different batches of filter-cake were found to contain 80-83% water.

Ammonium carbonate Analar "ammonium carbonate" solutions were prepared in ion-free water and their concentration recorded in percentages of weight/vol.

(b) Method

Egg whites were separated manually with care to ensure minimum contamination by yolk. Lots of 500 ml of pooled whites were diluted with equal volumes of ion-free water and homogenised to ensure thorough dispersion of the thick parts. The suspension was centrifuged at about 3000 g for 15 min or 1,500 g for 30 min and the small amount of heavy material which sedimented to the bottom (mostly ovomucin plus chalazae) was discarded. The resulting solution comprised about 5% protein, the avidin concentration being of the order of 0.002%, and the pH was 8.5-9.

Two large scale preparations of avidin were conducted and they are sufficiently different to warrant a description of both.

(c) Preparation D26

To 44 l of egg white solution obtained from 720 "Sykes hybrid" eggs was added 800 g CMC filter-cake and the pH adjusted to 7.0 with 140 ml 3 N H₂SO₄ with constant stirring. The mixture was well stirred for a further few minutes and allowed to settle for 30 min. The bulk of the supernatant was siphoned off and the remainder recovered by a brief centrifugation in 1 l lots. The CMC was then washed by resuspension in a few litres 0.05 M ammonium acetate buffer pH 9, allowed to settle and decanted. A thick slurry of the CMC was then poured carefully onto a Büchner funnel without paper and allowed to settle. When a sufficient bed of cellulose had formed on the bottom of the Büchner funnel gentle suction was applied. The CMC was then washed on the funnel with a few litres of the pH 9 buffer and finally well pressed out. The filter-cake so obtained was weighed (batch I) and a small sample (about 1 g) was taken for total avidin assay.

TABLE III LARGE SCALE AVIDIN PREPARATIONS

Experiment No.	D 26			D 31		
Eggs	Sykes hybrid			Rhode Island x Light Sussex hybrid		
Number	720			900		
Vol. of diluted egg white	45 L			54 L		
Batch No.	I	II	III	I	II	III
CMC wt. added (g)	800	500	500	1200	800	800
CMC wt. recovered (g)	710	728	635	935	748	650
Avidin content (mg)	320	317	150	320	200	230
Total avidin (mg)	787			750		
CMC column	65 x 9 cm			70 x 9 cm		
Hold-up volume	3.8 L			4.0 L		
Final yield	336 mg			450 mg		

To the egg white supernatant 500 g CMC filter-cake was added and the isolation and washing process repeated as above (batch II). A third batch adsorption was similarly performed (batch III). The supernatant at this stage was suitable for the isolation of ovalbumin and ovomucoid. This was done and the details will be given in a later section. Each batch of CMC filter-cake with adsorbed basic proteins was then resuspended in the pH 9 buffer and thoroughly degassed on the water pump. The outlet of the column (9 x 100 cm fitted with a sintered glass disc) was closed off and the column one-third filled with the pH 9 buffer. Fresh CMC filter-cake (500 g) was suspended in a few litres pH 9 buffer, degassed and poured onto the column. After allowing the CMC to settle to the bottom of the column, the flow of buffer was permitted from the outlet and the CMC packed down with the buffer flowing. The batch III CMC suspension was then poured onto the column as a slurry. Entrapped air bubbles were released by stirring and the CMC packed down with the buffer flowing. Batch III was layered next in the same manner, followed by batch I. The final column of CMC was packed under slight pressure (1-2 lbs/sq.in) and was now ready for elution. The relevant data on this preparation are given in Table III.

(d) Preparation D₃₁

The 54 l of diluted egg white resulting from 900 Rhode Island and Light Sussex hybrid eggs supplied by Mr. Richers were extracted as before with three batches of CMC. In this case an attempt was made to minimize possible avidin losses by washing the CMC with 0.025 M ammonium acetate, pH 7, but when the CMC was finally packed onto the column the eluting buffer did not flow through it. The

cellulose was resuspended in 0.025 M ammonium acetate pH 9 buffer and washed by decantation, but after repacking the column blocked up again. The column was unpacked once more and the CMC washed by resuspension in 50 l of 0.05 M ammonium acetate pH 9 and washed on the Büchner funnel. After resuspension and degassing the material was repacked into the column and the flow rate was found to be adequate. However, by this time all the batches of CMC had become mixed by the repeated unpacking of the column and a considerable amount of the material had been lost by the repeated decantation. The details of this preparation are also given in Table III. The washing and eluting of the two preparations were carried out in the same way.

The column was washed overnight with one to two hold-up volumes of 0.25% ammonium carbonate at a flow rate of about 5 ml per min, which brought the E_{280} of the effluent down to less than 0.05. Elution was thereafter performed by a stepwise increase in ammonium carbonate concentration. The elution schedule and the effluent pattern for these columns are given in figs. 2 and 3. Fractions of 250 ml were collected by hand and the column flow rate was maintained at about 18 ml/min. The fractions were then assayed and those containing the avidin (fig. 2, tubes 9-16 and fig. 3 tubes 8-14 inclusive) were pooled. For D_{26} the solution was concentrated on a short column of CMC and dialysed. The solution was acidified with acetic acid to a pH between 5 and 6 and then well degassed at the water pump. The total protein content of the solution was estimated from the E_{280} using a factor of $E_{1\text{ cm}, 280}^{1\%} = 15.6$, which was the value obtained experimentally with crude avidin. For concentration purposes a loading of about 5 mg protein/ml of column

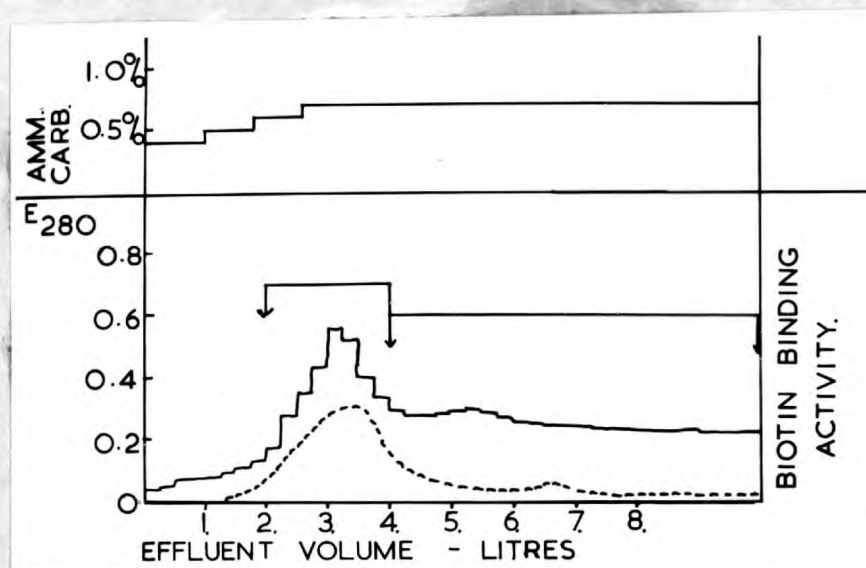


Fig. 2. Ammonium Carbonate Elution after Batch Adsorption of Basic Egg White Proteins (Preparation D 26)

Column, CM-cellulose (9 x 65 cm); flow rate, 18 ml/hr; fractions, 250 ml. — E_{280} ; ---- biotin binding activity in arbitrary units. The scale for the biotin binding activity ordinate is selected in such a way that for pure avidin the two curves would coincide.

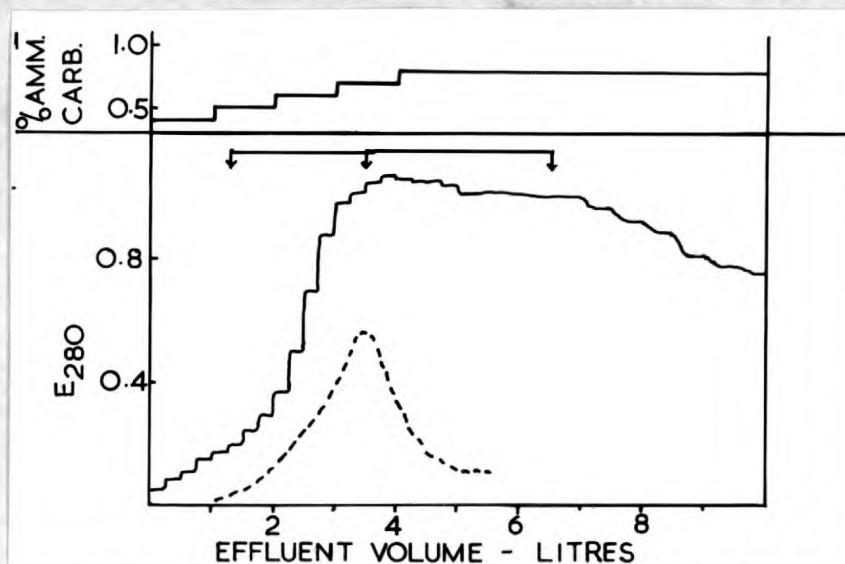


Fig 3. Ammonium carbonate elution after batch adsorption of basic egg white proteins (Preparation D 31)

As for fig. 2.

volume was used. The column was equilibrated with 0.01 M ammonium acetate buffer pH 5.5, and the protein solution then run on at a flow rate of not more than 0.5 cm/min. With an influent E_{280} of 0.8 the effluent E_{280} was less than 0.04. Elution was performed with 2% ammonium carbonate solution but it was important to wash the column first with ammonium acetate pH 9 buffer (0.05 M) until the pH of the effluent was 9 to prevent the formation of CO_2 on addition of the carbonate solution. The material emerged as a sharp peak with the solvent front. Fractions containing protein were pooled and dialysed for 48 hours at 4° in Visking bags on a rocking dialyser against ion-free water.

5. Selection and Development of Further Purification Procedures

Large scale preparations of avidin yielded material with an $E \frac{1}{1} \frac{\%}{cm} 280_{m\mu}$ of 15.6 and a biotin binding capacity of 6 γ biotin/ml $E_{280} = 1$ or 9.4 γ biotin/mg, compared with 8 γ biotin/mg for the most active preparations of Fraenkel-Conrat et al. (1952a). Small scale re-chromatography on CMC with a linear concentration gradient of ammonium acetate buffer pH 9 yielded some avidin with a biotin binding capacity of 7.6 γ biotin/ml $E_{280} = 1$ or 11.8 γ biotin/mg avidin (N.M. Green, unpublished results). This clearly showed that the large scale preparations of avidin, although producing a product with a higher specific activity against biotin than any previously obtained did not in fact yield pure avidin. Comparison of the E_{280} and activity curves of the elution diagram (figs. 2 and 3) in any case indicated a fairly high proportion of inactive protein in the avidin preparation. A series of small scale column chromatographic experiments were therefore conducted

to find conditions which gave the highest biotin binding activity and the best coincidence of activity and E_{280} plots on the elution curves. In all cases linear gradients of ionic strength were employed for elution and each chromatograph was performed without any change in the pH of the buffer.

(a) CG50 Chromatography

(1) Methods Of the polyanionic resins commercially available the only one besides CMC which had been found generally useful for the chromatography of basic proteins is Amberlite IRC 50 or the special chromatographic grade, CG 50. Like CMC this is also a polycarboxylic acid but the backbone is a synthetic cross-linked polymethacrylic acid. This resin has been used extensively in the Rockefeller Institute Laboratories and the general conditions described by Moore and Stein (1956) were followed. Unlike CMC, the CG 50 is very slow to come to ionic equilibrium and its capacity for adsorbed protein is much lower. However the affinity or strength of binding of avidin was found to be far greater for CG 50 than for CMC.

Amberlite CG 50 (Type II) 200-400 mesh which is a special grade of Amberlite IRC 50 (also known as XE 64) was obtained from Rohm and Haas Company. Fines were removed by vigorously stirring the resin in a large volume of water and pouring off the supernatant after settling for 15 min. After repeating the decantation four or five times the resin settled fully in 15 min leaving a clear supernatant. The resin was cycled twice between the Na^+ and H^+ forms by washing with NaOH and HCl respectively as described by Hirs, Moore and Stein (1953). The second NaOH wash also contained 10^{-3}M EDTA to remove trace metals from the column.

Mechanically the technique of setting up, filling and running of the columns was the same as for CMC except that much slower flow rates were used and much longer equilibrium times were required to obtain identical pH values for influent and effluent. It was noticed that the CG 50 parts with sodium ions more readily than with protons. The columns were therefore packed with the resin in the sodium form and then washed with a buffer of the desired pH until the equilibrium was attained (usually 2-3 days). Maximum flow rate for washing, adsorption or elution from this resin was 2 cm/hr; but 1 cm/hr was used when maximum resolution was required.

Because of the slow attainment of equilibrium the CG 50 resin was not subjected to changes in pH and protein adsorption was performed at the same pHs that used for elution but at a lower ionic strength. Before use the column was washed until equilibrium was attained with the buffer which represented the starting point of the elution gradient. Prolonged washing of the column was necessary, not only because of the slow rate of equilibration but also because a small amount of material with a significant extinction at 280 m μ leached out of the resin. After several days of continuous washing the optical density of the column effluent at 280 m μ had dropped to less than 0.02 where it remained as long as the flow was not interrupted. At this stage the column was given a brief wash (less than one hold-up volume) with a buffer at the same pH as that used for equilibration but at about one-tenth the ionic strength. The protein was adsorbed onto the column in the latter buffer and elution was resumed with the starting buffer. In most cases a gradient of ionic strength was then applied

to effect elution of the protein. Up to 1 mg protein could be chromatographed/ml of total column volume; but for best resolution 0.2 - 0.4 mg/ml of column was used.

Whenever a gradient of constantly changing concentration was employed for column elution a two-chamber series-coupled arrangement of open vessels was employed. This device (due to Parr, 1954) is described in detail by Svensson (1960) and, in all the work in this study (on CMC or CG 50), was used with two identical vessels resulting in a linear gradient. For all column chromatography experiments, except the very large scale preliminary avidin purification (100 cm x 9 cm column), the effluent was collected in discrete fractions in automatic fraction collectors. Drop counting was the preferred method of measuring fractions but slight variations in the fraction size were observed when peaks of high protein concentration were eluted. This was due to the lowered surface tension which caused a decrease in the size of the drops. Fractions collected from different columns varied from about one quarter of a hold-up volume for very small columns to one-fiftieth for large ones.

When the effluent from a column had been collected all tubes were first assayed for total protein by measuring E_{280} . Those tubes which were likely to contain avidin were then assayed for biotin binding activity by the radioactive biotin method. The effluent diagram relating these two properties was then plotted. From a consideration of these diagrams the effectiveness of the system in purifying avidin and/or in resolving the different avidins could be evaluated.

(11) Results All the experiments described in this section were performed on avidin D₂₆. The study of the behaviour of lysozyme on CG 50 (Tallan and Stein, 1953) was taken as a starting point for the present investigation. The lysozyme (egg white) was eluted at pH 7.12 in a buffer 0.2 M in respect to phosphate in about 6 hold-up volumes. However under similar conditions avidin was not eluted even after 30 hold-up volumes. Ethanolamine ($pK_a = 9.47$) was used to obtain a higher pH. By bringing 0.25 M HCl to pH 9.5 with this base a buffer was produced with ionic strength 0.25. This buffer soon brought off the bulk of the applied material in a single peak. A gradient of ionic strength from 0.1 to 0.25 in ethanolamine hydrochloride buffer at pH 9.5 produced a wider spread of the active material. It also seemed that the material consisted of two components running almost together. They were eluted at a region in the gradient where the ionic strength was approximately 0.2. On this column the concentration gradient was checked in the effluent by determining the chloride content of various fractions along the run. The results fitted approximately to the expected straight line but did not give as close a correspondence as that found when a similar check was made on the effluent of a CMC column.

Ethanolamine hydrochloride buffers of pH 9.5 and at a constant ionic strength of 0.17 or 0.20 did not effect satisfactory elution of the avidin in terms either of biotin-binding activities on an optical density basis or in terms of fractionation of the avidin into more than one component. Nor did a buffer of constant ethanolamine hydrochloride concentration ($I = 0.05$, $pH = 9.5$) and a gradient

of increasing NaCl concentration give good results in either respect. For the next chromatogram the pH of the buffer was raised to 10 and an ethanolamine gradient was reintroduced. This eluted the avidin at an ionic strength of 0.18 and again suggested a split into two components. Owing to the strong tendency of ethanolamine to absorb CO_2 from the air these buffers may in fact have been mixtures of ethanolamine hydrochloride and ethanolamine carbonate or carbamate. As the elution reservoir or gradient device was exposed to the air for some time during elution, there might have been much more carbonate in the buffers at the end than at the beginning of a run. If both carbonate and carbamate were having specific effects on the elution of the avidin this may have further complicated the effects of the various buffers.

Ethanolamine carbonate buffers were therefore prepared by bubbling CO_2 into ethanolamine solutions of known concentration until the required pH was obtained. A gradient elution from 0.05 M to 1.0 M ethanolamine adjusted to pH 9.5 with CO_2 resulted in a symmetrical peak at a point on the gradient corresponding to 0.5 M ethanolamine. As the ethanolamine is very nearly half ionised at this pH, this represents an ionic strength maximum of 0.25, but probably in fact considerably less due to carbamate formation (see Jensen et al, 1954). The material of highest activity over the peak bound 6.8 γ of biotin/ml $\text{E}_{280} = 1$, compared with a value of 6 for the starting material and 7.6 for the best results on CMC columns. This system therefore was not giving any advantage over CMC in terms of activity against biotin, nor was there a useful resolution of the avidin into the multiple components.

Boric acid (pK_a 9.2) is a suitable alternative buffer in this range. In addition its ability to form charged complexes with carbohydrates may affect the elution of glycoproteins specifically in a mixture with proteins and bring about a separation not produced by other buffers. As experience with ethanolamine buffers had indicated that pH 10 brought about slightly better resolution of the different components of avidin than pH 9.5, the former pH was chosen for the borate experiments. As sodium borate (sodium tetraborate, borax) in solution gives rise to equivalent amounts of NaH_2BO_3 and boric acid, the ionic strength of the solution is numerically equal to the normality of sodium ions. To prepare a borate buffer of given ionic strength sodium borate and sodium hydroxide both of a normality (as determined by titration with standard HCl) equal to the desired ionic strength were mixed until the required pH was attained. Using a gradient from $I = 0.02$ to $I = 0.4$ in borate buffer, pH 10, the avidin was eluted in a symmetrical peak at an ionic strength of 0.22. Repeating the experiment with a much more shallow gradient over the range $I = 0.18$ to $I = 0.22$ the avidin emerged as a main component followed and slightly overlapped by a second active component. The maximum biotin-binding activity of both components was $8.3 \mu\text{g/ml}$. $E_{280} = 1$ ($13 \text{ } \gamma/\text{mg}$). In both respects, activity and fractionation, therefore, this result represented a considerable advance over any previous column.

If the various avidins differ from one another in net charge this difference should be at a maximum near the isoelectric point of the protein (about pH 10.5). As avidin appeared to be highly

stable in alkali, chromatograms were run at pH values around 10.5. Sodium carbonate-bicarbonate buffers were prepared by mixing solutions of equal molarity of Na_2CO_3 and NaHCO_3 to the desired pH. A series of chromatograms was performed over the range pH 10.4 - 10.6 with concentration gradients from 0.02 - 0.1 M. Elution usually occurred when the concentration of eluent was somewhere between 0.04 M and 0.07 M, the actual position being very sensitive to pH. In some cases the avidin emerged as two separate components but conditions were difficult to standardize. Elution patterns were sensitive to changes in pH of 0.01 and were affected by column length in an unusual way, resolution improving with shorter columns. Borate-carbonate buffers at pH 10.5 brought about no improvement, so for further development onto a preparative scale sodium borate-hydroxide buffer at pH 10 was chosen.

A single eluting buffer which gives a finite distribution of the protein between eluent and resin can often be used with CG 50 instead of a gradient (Moore and Stein, 1956). With pH 10 borate buffers the main peak of the avidin emerged at an ionic strength of about 0.2. Chromatograms were therefore run using this buffer at constant ionic strengths of 0.210, 0.200 and 0.195. These columns did resolve the avidin into more than one active component but the peaks were very spread out with much tailing. As a result very shallow concentration gradients were re-introduced in the method finally adopted.

(iii) Detailed Description of Preparative CG 50 Chromatogram A column of CG 50, conditioned as described above, was washed with 0.5 N NaOH plus 0.5 N NaCl containing 10^{-3} M EDTA, ion-free water, and finally boric acid (0.2 M) until the effluent pH was approximately 10. Borate

buffer, pH 10.0 and ionic strength 0.195, was then run through the column for 48 hours by which time the pH of the effluent was 10.0. Crude avidin from a large CMC column (250 mg) was made up in 5 ml borate buffer pH 10 of 0.02 ionic strength and applied to the top of the column. Elution was continued with the 0.195 ionic strength buffer.

The elution diagram for this chromatogram is given in fig. 4. Fractions 48-69 inclusive were pooled (solution A), fractions 73-130 inclusive yielded solution B and 144-169 inclusive, solution C. The three solutions were dialysed against ion-free water and concentrated by adsorption onto separate CMC columns at pH 5. They were eluted with 2% ammonium carbonate. The fractions containing the concentrated proteins from each column were then dialysed in a rocking dialyser overnight in the cold room and centrifuged at 90,000 g for 15 minutes to remove a slight turbidity. The clear supernatants were finally freeze-dried, yielding the three preparations of avidin, A, B and C. The total recovery of 280 mμ adsorbing material from this column was 81% and was calculated by cutting out the area under the elution curve and weighing the paper. However the three pooled fractions after concentrating, dialysing, centrifuging and freeze-drying yielded 60 mg. or 24%.

(b) CMC Chromatography

For further purification, CG 50 chromatography was very tedious and recoveries were rather low, partly because some avidin became denatured (as indicated by its precipitating on dialysis). Further investigations were thus carried out to find conditions which would give the most active avidin by CMC chromatography. With this supporting medium the yields were much better and ~~where~~ there was no

evidence of the avidin becoming insoluble during the later stages of the purification. The large scale preparation of avidin (D₂₆, p22 fig. 2) afforded the opportunity to reinvestigate the conditions for CMC chromatography of avidin. Tubes 9-16 inclusive were pooled (2,130 ml with O.D.₂₈₀ = 0.4) concentrated on a CMC column and yielded 725 E₂₈₀ units of protein solution which was dialysed and stored in the frozen state (-20°). The activity of this solution against biotin was 5.7 γ biotin/ml. E₂₈₀ = 1.

During the large scale avidin preparation on CMC nearly the whole column was occupied by adsorbed proteins and most of the column was virtually saturated with protein. The degree of separation obtained under these conditions cannot be expected to be very high. A much lower column loading was therefore used when the long trail of very low specific activity material (fractions 17-35) from the D₂₆ column was rechromatographed. From the elution diagram (fig. 5) it can be seen that at this loading a much better resolution is obtained, than from the large scale column although the elution conditions were the same. The first peak, emerging at 0.7% ammonium carbonate, was the avidin with an activity over the peak of 7.5-8.0 γ biotin/ml E₂₈₀ = 1 but it was partly overlapped on the tailing edge by an inactive impurity. Tubes 27-49 inclusive were preserved.

Two preparations were now on hand from the D₂₆ column, the main peak material (tubes 9-16, fig. 2) and the rechromatographed tail end material (tubes 27-49, fig. 5). A small scale column was then run on the main peak material and eluted with a concentration gradient of ammonium acetate buffer pH 9.0. A small inactive peak was eluted first (at 0.15 M) partly overlapped by the avidin (at 0.2

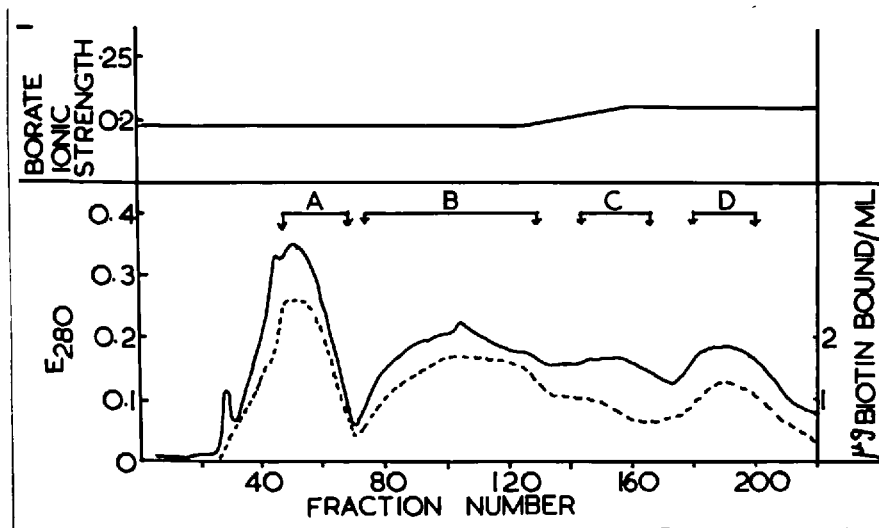


Fig. 4. Preparative Amberlite CG 50 Column Column, 4 x 48.5 cm; Fractions, 9.15 ml; flow rate, 30 ml/hr; holdup volume, 206 ml; 250 mg crude avidin (similar to the pooled active material from fig. 2 or 3). Elution by sodium borate buffer (pH 10.0), ionic strength as indicated.

———— E₂₈₀ - - - - - biotin binding activity

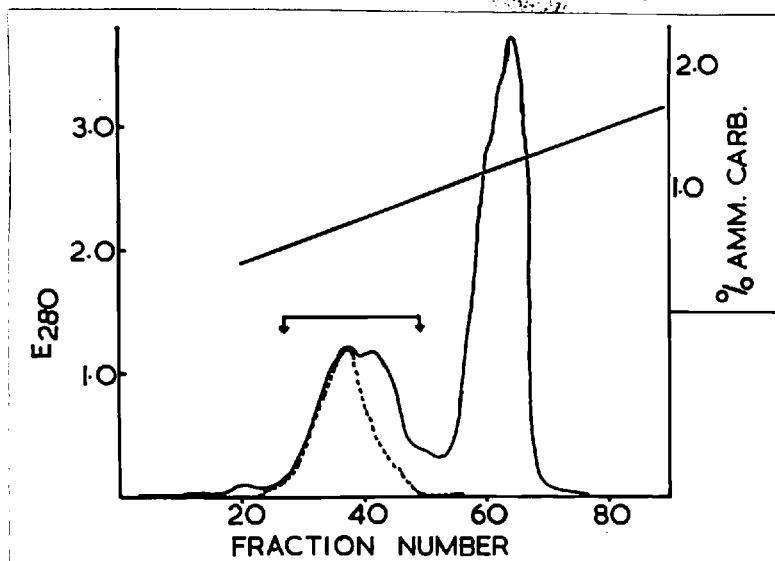


Fig. 5. Rechromatography of Tail Ends from Fig. 2. Elution by ammonium carbonate concentration gradient as indicated. Column, CM-cellulose (4 x 54 cm, 580 ml holdup volume); fractions, 25 ml; flow rate, 0.2 cm/min; total load, 1.5 - 2.0 g protein.

———— E₂₈₀ - - - - - biotin binding activity (see Fig. 2).

M), which had an activity of 7.5 $\mu\text{g/ml}$ $E_{280} = 1$ over the peak. In this case the tailing edge of the avidin peak did not appear to be contaminated. The difference between this result and that of fig. 5 may have been due to the main peak material and the tail end material of D_{26} containing different impurities or to the acetate and carbonate anions giving different types of separation. A sample of material from the ammonium carbonate column (fig. 5) tube 46) was therefore run on a small scale column and eluted with ammonium acetate buffer pH 9. An inactive peak emerged early in the run, clearly separated from the active material which followed it. It was now clear that the ammonium acetate pH 9 buffer was giving a different type of separation from the ammonium carbonate elution. The material from the main peak of D_{26} was therefore rerun with ammonium acetate pH 9 elution. The avidin emerged as a symmetrical peak (fig. 6) showing a very close correspondence between O.D. and activity curves. The peak material (tubes 102-150) was concentrated on a CMC column, eluted with 3% ammonium carbonate, desalted on Sephadex G 25 and freeze-dried, yielding 175 mg of material designated avidin D 26.1

The rechromatographed tail end material (fig. 5) was then pooled (tubes 27-49) and chromatographed with ammonium acetate gradient as eluent. The first peak to emerge at 0.14 M on the gradient was completely devoid of anti-biotin activity. The active material which followed it had a peak at 0.20 M but was not quite symmetrical (fig. 7) although, as on the previous column (fig. 6), the specific activity on an optical density basis was very constant over the whole peak. It was divided into two lots (A, 120-168 and B, 169-220) which were separately

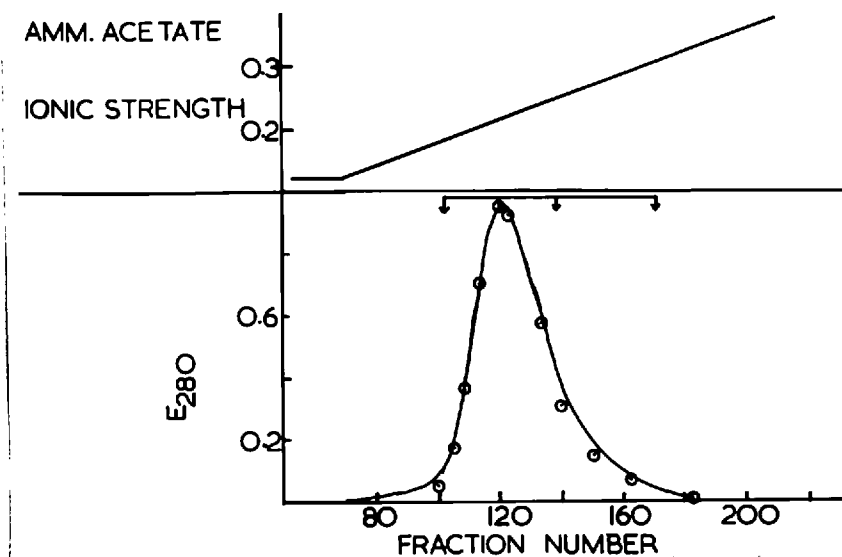


Fig. 6 Rechromatography of Main Peak from Fig. 2. Elution by ammonium acetate buffer (pH 9.0) concentration gradient as indicated. Column, CM-cellulose (4 x 50 cm); fractions, 18 ml; flow rate, 0.2 cm/min; total load, 750 mg protein.

—— E_{280} $\circ$ biotin binding activity (see fig.2)

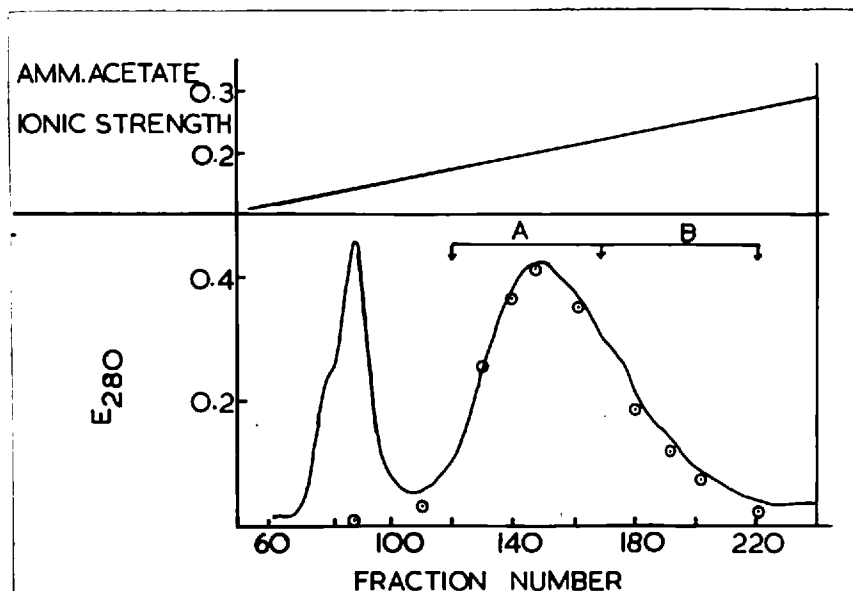


Fig. 7. Rechromatography of Active Peak from Fig. 5. Elution by ammonium acetate buffer (pH 9.0), concentration gradient. Column, CM-cellulose (4 x 31 cm); fractions 12.5 ml.

—— E_{280} $\circ$ biotin binding activity (see fig.2)

concentrated on CMC columns, eluted with ammonium carbonate and dialysed. In order to reduce the time needed for freeze-drying the samples were then concentrated ^{By} pervaporation. The solutions in the bags (Visking tubing tied off at both ends) in which they had been dialysed were hung up in front of a fan in a cool, well darkened room. After a few hours the volume had been reduced to almost a quarter and they were then freeze-dried to yield the preparation D 26.3 A (110 mg) and D 26.3 B (51 mg).

6. Description of the Final Method

From the experiments described above a final method was adopted for the purification of avidin which involved three CMC chromatograms: (i) a large scale column eluted with ammonium carbonate resulted in a crude avidin preparation which represented nevertheless a purification of about 1000 times from the original egg white, and contained about 50% avidin; (ii) rechromatography with the same solvent under conditions of lower loading which separates the avidin from all but a small fraction of the contaminants and finally (iii) chromatography under the same conditions as (ii) but with acetate instead of carbonate as the anion, in which case the remaining impurity is eluted before the avidin. The results for a full preparation of avidin by this method are presented below.

From the large scale column described above (D₃₁, page 24 fig. 3) fractions 8-26 were pooled and chromatographed with ammonium carbonate elution. From the effluent diagram (fig. 8) it will be seen that the avidin was well separated from most of the inactive material but there was partial overlapping by a minor contaminant. Tube 60 from

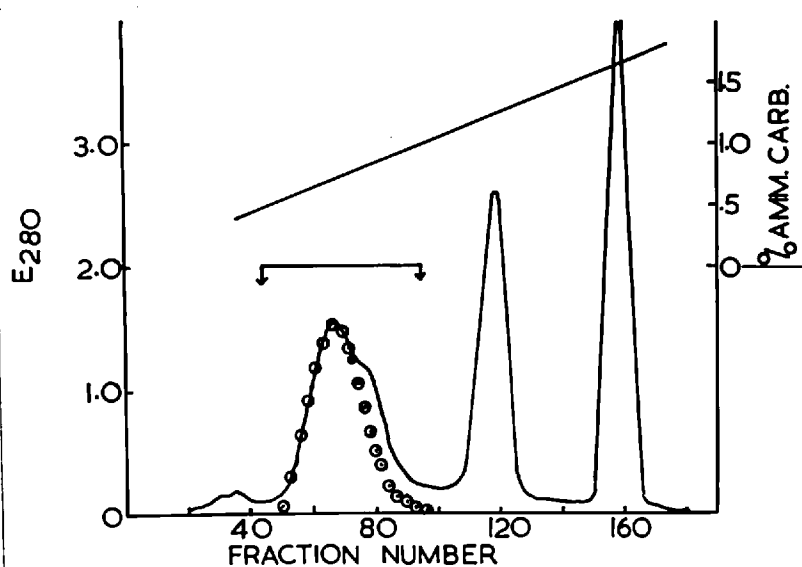


Fig. 8. Rechromatography of Active Material from Fig. 3
 Ammonium carbonate concentration gradient elution. Column, CM-cellulose (6 x 56 cm); flow rate, 0.12 cm/min; fractions, 36 ml; total load approx 3 g. — E₂₈₀ ○ biotin binding activity (see fig. 2)

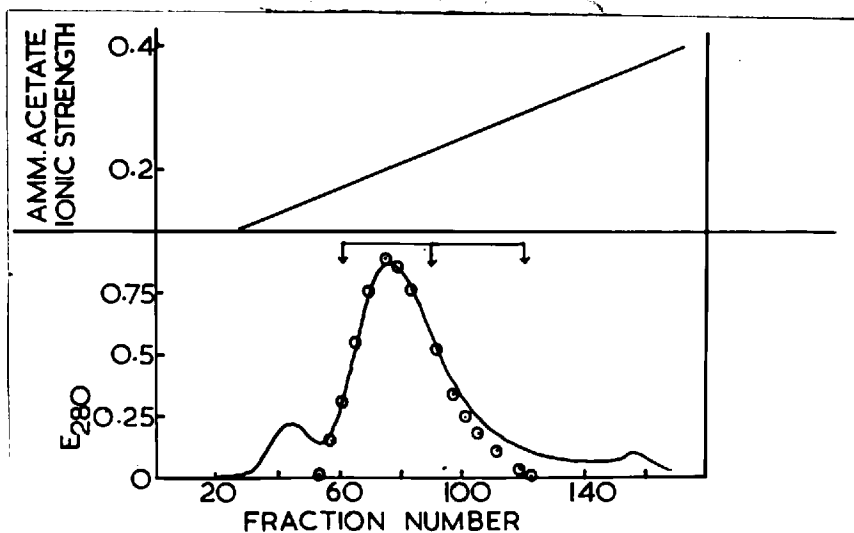


Fig. 9. Rechromatography of Active Material from Fig. 8.
 Ammonium acetate buffer (pH 9.0) concentration gradient elution. Column, CM-cellulose (6 x 40 cm); fractions, 36 ml; total load approximately 1 g. — E₂₈₀ ○ biotin binding activity (see fig. 2)

this run, on spectrophotometric titration with biotin (page 13) gave as high an activity as was ever obtained with any avidin (9 γ biotin/ml $E_{280} = 1$). Tubes 44-94 inclusive, containing 32% of the starting material, were pooled, acidified and rechromatographed with a gradient of ammonium acetate buffer pH 9 as eluent. In this case (fig. 9) an impurity emerged before the avidin. In this case, too, a tube on the leading edge of the curve (fraction 63) was titrated with biotin in the spectrophotometer and bound 9 γ biotin/ml $E_{280} = 1$. By radiobiotin assay the specific activity was constant over the peak and tubes 61-90 were pooled, concentrated, dialysed and freeze-dried to yield 450 mg of avidin D 31. The tail end of this run together with a miscellaneous collection of avidin residues which had been collected in the laboratory over three years were subjected to chromatography under the conditions of the second and third columns described above, and yielded 134 mg of reclaimed avidin which was fully as active against biotin as the best fresh preparations.

7. Discussion on Isolation of Avidin

As discussed above (page 10) it was essential to isolate the avidin by a method which would preclude the formation of complexes with naturally occurring acidic macromolecules in the egg white. Isolation of avidin free of nucleic acid by CMC chromatography (Rhodes et al, 1958) indicated that this ion-exchange material may be particularly well suited for the purpose. In the present isolation procedure the CMC served three purposes: (1) being a highly acidic polymer it was able to compete successfully with the naturally occurring acidic macromolecules and prevent them complexing with the

avidin; (ii) conditions were so arranged so that only the most basic proteins were adsorbed. As avidin is one of the most basic proteins in egg white - the most basic 5% of the protein contains all the avidin - this was a fairly selective adsorption of avidin (lysozyme being the major contaminant); (iii) the adsorbed proteins were fairly selectively eluted from CMC by the use of buffers with gradually increasing salt concentration.

Adsorption of material from solution onto an insoluble supporting medium is most efficiently done on a column. In this case, however, a batch process was preferred for two reasons:- (i) it proved to be necessary to lower the pH of the native egg white in order to get most of the avidin to adsorb onto the CMC. It was desirable to have the CMC present in suspension while the pH was being lowered in order to compete with the naturally occurring acidic macromolecules as the avidin became more positively charged; (ii) as only a small fraction of the egg white proteins were being adsorbed a large volume of egg white was used relative to the amount of CMC. It was found that owing to its high viscosity (even when diluted with an equal volume of water) and the presence of sticky, mucinous materials in the egg white, the flow soon became impractically slow if large volumes of white were run onto CMC columns.

Preliminary experiments to find conditions for most efficient batch adsorption in terms of ratio of CMC to diluted egg white, number of batches required and optimum pH were performed by N.M. Green (unpublished work). Three batch adsorptions using 0.5 g dry weight of CMC/100 ml of diluted egg white at pH 7.0 adsorbed approximately 80% of

the avidin. Not much material less basic than avidin was adsorbed under these conditions. In summary, batch adsorption onto CMC directly from diluted egg white proved to be a highly efficient means of isolating the basic proteins. By elution with ammonium carbonate, an overall purification of 1200 to 1600 times was obtained in virtually one step.

It is of some interest to compare the chromatographic behaviour of avidin with that of the other proteins of similar basicity which were also adsorbed onto CMC. Ammonium carbonate was originally chosen for elution because of its volatility. (This greatly facilitated the assay procedure because the excess radioactive biotin could be determined on evaporation of the supernatant, without the need for self-adsorption corrections.) The successive use of CMC columns eluted with carbonate and acetate proved to be particularly effective as the two anions eluted avidin and its closest contaminants in a different order, and this greatly facilitated the purification.

Before making these comparisons on a quantitative basis, it is first necessary to ascertain whether the position reached in the gradient, when the peak is eluted, or the elution volume (R_F^*) is the relevant parameter for the evaluation of the positions of the peaks. In the first type, resolution is largely independent of column length and dependent rather on the slope of the gradient. In the second case, which requires a true, reversible distribution coefficient yielding a reasonable R_F (between 0.1 and 0.7), the entire length of the column

* The R_F value in column chromatography has been defined by Boardman and Partridge (1955) as the hold-up volume of the column/total volume of effluent which flows from the moment of application of the solute until the top of the peak is eluted. It is exactly equivalent to R_F as defined by Consden, Gordon and Martin (1944).

is involved in the fractionation and the dimensions of the column greatly affect the degree of separation obtainable. The CMC chromatography described above corresponds closely to the first type as the peaks emerged at very nearly the same position on the gradient over a very wide range of conditions which differed in respect to column dimensions, slope of the gradient or loading of the column. It has therefore been accepted that the point on the concentration gradient at which a particular peak emerges characterises the elution of that substance. The points of elution (represented by the position on the gradient) of avidin and the two closest contaminants is set out in Table IV.

TABLE IV ELUTION OF PROTEIN FROM CMC AT pH 9.0

Protein	Point at which eluted	
	Ammonium Acetate Ionic strength	Ammonium Carbonate % w/v
Contaminant A	0.14	0.85
Avidin	0.20	0.70
Contaminant B	0.20	1.20

Unfortunately ammonium carbonate, in solution, gives rise to a complex equilibrium of ammonium ions, bicarbonate ions and carbonate ions, the ionic strength of which cannot be derived without a knowledge of the equilibrium constants for the various reactions. As these constants are not well established a direct comparison of the behaviour of the the three proteins in the presence of the two eluting buffers in terms of ionic strength of elution cannot be made. However it is of interest to note that the ratio of the concentrations at which the two contaminants

are eluted are virtually identical in the two systems. It appears thus that it may be the avidin which is behaving essentially differently in the two cases. If this is so, then the avidin is somewhat less basic in the ammonium carbonate solution than in the acetate. It is possible to speculate that ion binding of bicarbonate or carbamate to the avidin is responsible for this, but it is not possible at this stage to say why this should be so.

The behaviour of avidin on CG 50 contrasts sharply with that of lysozyme. On the CMC columns described above, lysozyme was eluted well after avidin in agreement with the results of Rhodes et al (1958). However, on CG 50, lysozyme is eluted with 0.2 M phosphate buffer pH 7.18 (Tallan and Stein, 1953) whereas the avidin required a pH of about 9 to 10 before it could be eluted at similar salt concentrations. As no direct comparisons have been made between the behaviour of proteins on CMC and CG 50 it is not known whether lysozyme or avidin exhibits the more usual type of behaviour.

It is clear from the results that a better resolution of the components of avidin is obtained on CG 50 than on CMC. This can be seen, for example, by a comparison of figs. 9 and 10 which show chromatograms of the same material on CMC and on CG 50, in both cases under optimum conditions. Where a single symmetrical peak occurs on CMC, a partial resolution into components is observed on CG 50. On the other hand, with bovine pancreatic ribonuclease the opposite is the case. Material which gives rise to a single peak on CG 50 is resolved into two fractions on CMC (Taborski, 1959).

C CHARACTERIZATION AND ANALYSIS OF AVIDIN

The values obtained for specific biotin binding activity for avidins A, B and D 31 were significantly higher than any previously described. Stored in a dessicator over CaCl_2 in the cold room as freeze-dried powders, these materials did not show any measurable loss of activity over many months and remained readily and completely soluble in water and in moderately strong salt solutions. These preparations were characterised in a number of respects and analyses were carried out on their overall composition.

(1) Methods

(a) Homogeneity

It is clear from section B that CG 50 chromatography represents a very sensitive system for assessing the degree of contamination by inactive components, if any, and for investigating the chromatographic homogeneity of the active component(s) of avidin. As the amount of material available was limited, it was not examined by other methods used for the assessment of protein homogeneity, e.g. ultracentrifugation or electrophoresis.

For avidin obtained from eggs from a single breeder and isolated solely by CMC chromatography, a CG 50 column was used on an analytical scale. The column was equilibrated with sodium borate-sodium hydroxide buffer, pH 10 and ionic strength 0.2, and then washed overnight with the same buffer but with $I = 0.02$. Protein (6mg) was applied in about 0.5 ml of the latter buffer and the column then eluted with a gradient of borate buffer pH 10 under the conditions of fig. 10, p 61.

(b) Dry Weights

Samples of protein were dried at 110° in an all glass pistol. A weighing bottle of Pregl pig containing the protein was placed in the stem together with its ground glass lid and the pistol heated for 15-20 min. The pistol was then evacuated on an oil pump to less than 10^{-5} cm of Hg and maintained at 110° with the pump running for about a further hour. With the outlet tap closed, the pistol was removed from the jacket and allowed to cool. Dry air was admitted slowly into the pistol and the weighing vessel was quickly removed, stoppered and weighed on a Stanton microbalance which was found to give a reading reproducible to 0.005 mg.

(c) Ultraviolet Spectrum and Extinction Coefficient

The extinction coefficient of avidin and the spectrum as a whole were determined on a Unicam SP 700 double beam recording spectrophotometer. The usual precautions were observed, such as ensuring good matching of the cuvettes before use, ensuring complete freedom from turbidity (by high speed centrifugation if necessary), absence of significant amounts of stray light and the use of the instrument in its most sensitive optical density region.

(d) Total Nitrogen

(1) Development of the Method. To ascertain whether the amino acids and amino sugars in avidin, both of which could be determined with a precision of about 2%, accounted for all the nitrogen present, and also to determine the amount of avidin present in a given solution (e.g. for estimation of $E_{1\text{ cm}}^{1\%}$, 282 $m\mu$) an accurate nitrogen determination was required. At the same time the small amount of avidin

available demanded the use of a micromethod. A method by which one could estimate between 10 and 50 $\mu\text{g N}$ with an accuracy of about 2% would be required. The Kjeldahl procedure described by Hiller, Plazin and van Slyke (1948) which originally required about 1 mg N per estimation was first used but was adapted for application on a considerably smaller scale. Samples of NH_4Cl containing 10, 20 or 50 $\mu\text{g N}$ were distilled from NaOH solution in a microdistillation apparatus, collected under nitrogen in dilute boric acid and titrated manually or automatically on a Radiometer titrator. The variation in results between replicates was equivalent to 5 $\mu\text{g N}$ or more. As the titration was adequately reproduceable the variation arose from the distillation. It was independent of the amount of ammonia distilled and even blanks varied to an extent equivalent to about 4 $\mu\text{g N}$. The following measures were taken to reduce this variation: addition of KMnO_4 and H_2SO_4 , or of NaOH to the steam generator; scrubbing of the nitrogen with soda lime, H_2SO_4 and NaOH solutions before use; a constant temperature cell for the collection and titration of ammonia; omission of the boric acid for greater sensitivity; and the provision of extra splash heads to ensure that no small drops of liquid were carried over with the steam; but these all had no effect.

This method was therefore considered unsuitable for use on this scale and a procedure which does not require distillation was investigated. Jacobs (1960) had shown that in a Kjeldahl digest neutralised to pH 5 with NaOH and suitably diluted, ammonia could be estimated directly by a ninhydrin procedure. Later a digestion method on a very small scale in sealed glass vials was described (Jacobs, 1962). This method was adopted, but with a number of modifications. In place

of the 10-20 μ l of sulphuric acid recommended for digestion, 50 μ l of a digestion mixture was used, as metal catalysts were found to be necessary for complete digestion. In the original method the digest after neutralization is diluted so that 2 ml plus 2 ml ninhydrin reagent, when reacted and diluted to 10 ml gave a suitable optical density at 570 m μ . In order to reduce the blank values, in the modified method 1 ml of diluted, neutralised digest plus 1 ml of ninhydrin reagent were allowed to react and diluted to 20 ml. This required four times the concentration of ammonia in the reaction mixture to give the same final optical density. It was found that these conditions still gave a linear calibration curve which showed no sign of levelling off over the range employed. Finally the ninhydrin reagent described by Moore and Stein (1954), which employs hydrindantin, was found more stable and reproducible than the reagent containing stannous chloride as used by Jacobs (1962).

(11) Final Method Digestion mixture was prepared by adding 0.2% anhydrous copper sulphate (dried at 250 $^{\circ}$) and 0.04% finely powdered "Analar" selenium to "Micro Analytical Reagent" sulphuric acid from a newly opened bottle. The mixture was heated to boiling in a conical flask and allowed to cool with an inverted beaker resting over the neck of the flask. If stored for more than a few days in a glass stoppered bottle some metal salts crystallised out but this did not appear to have any effect on the digestion. Because of the difficulty in excluding atmospheric contamination by ammonia and moisture this solution was not kept for more than a week. Sodium hydroxide for neutralization of the digest was a 50% w/v solution which had been allowed to stand until any carbonate

present had settled to the bottom. Approximately 0.08 ml was needed for each titration and it was dispensed from a 1 ml horizontal micro-burette controlled by a threaded plunger. The tubes for digestion were prepared from borosilicate glass tubing 8 mm outside diameter with 2 mm walls cut into 16 cm lengths and well washed with hot sulphuric-nitric acid mixture and distilled water. Each length was then heated in the centre and divided into two 8 cm tubes with one end sealed off. Care was taken not to contaminate the open end of the tube by touching, and the tubes were finally cleaned and annealed by heating in a muffle furnace to 600-650° and allowed to cool slowly in the furnace.

Hydrindantin was made by reducing "Analar" ninhydrin with ascorbic acid (Moore and Stein, 1954). Ethylene glycol monomethyl ether ("methyl cellosolve") was treated with 5 g/l stannous chloride dihydrate and allowed to stand for 24 hours to remove peroxides. It was distilled immediately before use. The ninhydrin reagent contained 2% ninhydrin, 0.3% hydrindantin and M sodium acetate buffer pH 5.5 in 75% aqueous "methyl cellosolve" as described by Moore and Stein (1954). It was made up on the day it was required and never stored. Sodium acetate, acetic acid, citric acid and NaOH were all of "Analar" grade. At first the ninhydrin reaction mixture was heated in ground glass stoppered test tubes, but it was later found more convenient to use standard 12 x 150 mm Pyrex test tubes and rubber stoppers.

In the final method 10 μ g nitrogen in a final volume of 15 ml gave an optical density of 1.0 at 563 $m\mu$, which is equivalent to an ϵ of 2.1×10^4 . A sample of material containing 50 μ g nitrogen was

used in each digestion tube as this was sufficient to allow several replicate determinations on the neutralized digest. The standard solution consisted of 2.681 g glycine/l, protein solutions of 3 mg/ml were used and distilled water served as blank. Solution (0.1 ml, or on some occasions 0.5 ml of 0.6 mg. protein/ml) was pipetted into a weighed digestion tube and, after re-weighing, the tubes were dried down in a dessicator over P_2O_5 and NaOH. Digestion mixture (0.05 ml) was added with a Carlsberg constriction pipette and the tubes were sealed. They were warmed slightly to reduce the viscosity of the sulphuric acid which could then be spread over the entire inner surface. The tubes, in a pyrex beaker, were placed for 30 min. in a muffle furnace maintained at 460-480° (as measured by the thermocouple see p. 71).

When cool, each tube was cut into two by scratching with a diamond and applying a hot glass rod. The contents of the lower half were transferred with a Pasteur pipette to a calibrated 5 ml flask. Both halves were thoroughly rinsed with not more than 2 ml of water, all washings being added to the flask with the same Pasteur pipette. Citrate buffer pH 5.0 (2 M) was added (0.5 ml) and the contents of the flask were titrated with concentrated NaOH to pH 5. Samples (about 0.5 μ l) were removed with a fine Pasteur pipette, as the end point was approached, and the pH was checked on indicator paper. The flask was finally made to the mark with water and well mixed. Samples (1 ml) were transferred to 12 x 150 mm test tubes, ninhydrin reagent (1 ml) was added and the tubes were stoppered and heated in a boiling water bath for 15 min. Each tube was diluted with 50% (v/v) aqueous ethanol

to give an optical density reading between 0.5 and 1.0. With an initial sample of 50 μg nitrogen, each tube contained 10 μg and a final volume of 15-20 ml was suitable. The diluted solutions were thoroughly shaken to reoxidise excess hydrindantin (Moore and Stein, 1954) and finally read in 1 cm cells at 563 m μ , which was found to be the extinction peak on the spectrophotometer in use at the time.

(e) Total Phosphorus In view of the presence of different numbers of phosphate groups in three fractions of hen's egg albumin, the phosphate content of the different fractions of avidin obtained on CG 50 chromatography was of particular interest. A micro modification of the Fiske and Subba Row method was used which results in a final volume of 1 ml (Bartlett, 1959). 1-Amino-2-naphthol-4-sulphonic acid was recrystallised from hot aqueous sodium bisulphite-sodium sulphite solution and the ammonium molybdate ("Analar") solution was stored in a polythene bottle in the dark. Samples were digested in tapered 12 ml. centrifuge tubes with H_2SO_4 and H_2O_2 at 160°. It was found essential to use tubes washed with hot sulphuric-nitric mixture to obtain low blanks and good reproducibility.

(f) Amino Acids Except for cystine, tryptophan, asparagine and glutamine, the amino acids of avidin were determined, after acid digestion of the protein, by continuous ninhydrin analysis basically as described by Spackman et al (1958) but with a few modifications (Crumpton and Wilkinson, 1963). The work was performed in the Department of Immunology, St. Mary's Hospital Medical School on the Spino Model 120 Amino Acid Analyser with the kind collaboration of Dr. Crumpton, Mr. Diamond and Mr. Wilkinson. The analyser was calibrated with the Spino Standard Amino Acid Mixture to which nor-leucine was added in the same concentration

as the other amino acids. Samples of this mixture (2 ml) containing 0.5 μ mole of each amino acid were used for each determination.

To weighed amounts of solutions (containing about 4 mg protein) in 16 mm x 150 mm thick walled rimless Pyrex test tubes 2 μ moles of norleucine were added and the mixtures were dried in vacuo over P_2O_5 . The protein was hydrolysed in 3 ml constant boiling HCl in well evacuated (0.05 mm Hg) sealed tubes in a boiling toluene bath (110°). Hydrolysates were dried at 40° in vacuo in a rotary evaporator.

Tryptophan was determined from the extinction at 282 $m\mu$ and 291 $m\mu$, the wavelengths of the avidin maximum and fine structure peak respectively. The extinctions were corrected for background absorption, by a linear extrapolation from the 340-360 $m\mu$ region, and for tyrosine absorption by using the tyrosine content obtained from the chromatographic analysis. These corrections amounted to about 6% at 282 $m\mu$ and 3% at 291 $m\mu$. The tryptophan extinction coefficients were assumed to be the same as for free tryptophan at its maximum (279 $m\mu$, ϵ = 5,500) and fine structure peak (288 $m\mu$, ϵ = 4,570). In this way the shift in the tryptophan spectrum due to combination in the protein is automatically corrected for. The results from the two wavelengths agreed to within 4% and an average of the two values was taken.

Cysine was determined after oxidation to cysteic acid by a modification (Moore, 1963) of the method of Schram, Moore and Bigwood (1954). Protein samples (about 4 mg) in a thick walled Pyrex test tube were treated with 2 ml of preformed performic acid at 0° overnight. Excess performic acid was destroyed with HBr and the mixture evaporated to dryness. Constant-boiling HCl was then added and the oxidised protein

hydrolysed and analysed as described above. The cysteic acid content relative to the amount of protein oxidised was calculated by reference to stable amino acids such as alanine and leucine determined in the same analysis. In this case the Technicon Autoanalyser was used and chromatography of the amino acids was performed by the method of Piez and Morris (1960) using only one column and a gradient of increasing pH and salt concentration for elution. This column was calibrated with 0.2 - 0.3 μ moles of the same amino acid mixture as before to which cysteic acid had been added to the same concentration as the other amino acids. The cysteic acid emerged as a sharp peak with the gradient front.

(g) Amide Nitrogen As the amide groups of asparagine and glutamine are removed during hydrolysis a separate analysis of those groups is needed if the total nitrogen in the protein is to be accounted for. The analysis also permits the total number of free carboxyl groups in the protein to be calculated. The usual method of mild acid hydrolysis followed by distillation of the liberated ammonia under slightly alkaline conditions produces only very little non-specific deamination in proteins. With glycoproteins, N-acetyl glucosamine provides further alkali labile nitrogen and the above method is unsuitable. A step for freeing the ammonia liberated by mild acid hydrolysis from as much of the other alkali labile nitrogen as possible has therefore to be introduced into the method.

Protein (5 mg) was hydrolysed in 2 N-HCl (1 ml) at 100° in a sealed tube for 3 hours. Ammonia and highly basic peptides were precipitated by the addition of 1 ml of 30% (w/v) phosphotungstic acid ("Analar" grade stored in a polythene bottle in the dark) and the mixture was left

in the cold overnight (Johansen et al, 1960). The precipitate was spun down, washed with 1 ml of 30% phosphotungstic acid and finally made up in 10 ml, 0.2 M sodium acetate buffer pH 6. This results in a slightly turbid solution which should be free of the hexosamine liberated by hydrolysis. Samples were incubated in Conway microdiffusion units (type 2A, Conway, 1950) overnight at 5° after addition of an equal volume of saturated K_2CO_3 (Tracey, 1952). The centre well contained 0.2 ml sodium citrate buffer (0.2 N) pH 5. After incubation the contents of the centre well were transferred to a 12 x 150 mm test tube and the well rinsed out with 0.8 ml of the sodium citrate buffer, the washings being added to the contents of the test tube. Ninhydrin reagent (1 ml; Moore and Stein (1954)) was added and the colour developed and read as described for the total nitrogen analyses above.

(h) Hexose Total hexose content of avidin was determined directly on the whole glycoprotein by the orcinol-sulphuric acid procedure of Winkler⁽¹⁹⁵⁵⁾ as described by Francois, Marshall and Neuberger (1962) but with slight modifications. Orcinol monohydrate (B.D.H., 25 g) was placed in an 800 ml round bottom flask and dried at 80° in vacuo on a rotary evaporator, to remove the water of crystallization. The dried residue was recrystallized from 500 ml. 1:1 toluene: petroleum ether (80-100°). The crystals were filtered off and dried in air protected from the light. When the orcinol-sulphuric acid reagent was prepared by mixing one volume of 1.6% aqueous orcinol and 7.5 volumes 60% v/v sulphuric acid the heat generated caused some colouration of the solution and increased the blank value. Preparation of the reagent by dissolution of orcinol (0.188% w/v) in cold, 53% (v/v) sulphuric acid

resulted in lower blanks. The reagent was not kept for more than a day. The procedure of Francois et al (1962) was followed and for small samples was scaled down ten times. Mannose, recrystallized by the method of Isbell et al (1955), was used as standard and, with the micromodification, 25 μ g gave an $E_{1\text{ cm}}$ at 505 $m\mu$ of 0.6 to 0.7 on different occasions (ϵ approximately 4.7×10^3).

(i) Hexosamine. The method of Elson and Morgan (1933) as modified by Kraan and Muir (1957) was followed for the determination of hexosamine after its liberation by hydrolysis with 4 N HCl at 100° . The p-dimethylaminobenzaldehyde was recrystallized from HCl according to Adams and Coleman (1948) and from ethanol by rapidly dissolving it in a minimum volume at $30-40^\circ$ and cooling it to -20° . Acetylacetone was redistilled and the $138-148^\circ$ fraction stored at -10° . Small samples were redistilled in vacuo immediately before use. Glucosamine hydrochloride was recrystallized by dissolving in water with stirring and slight warming (not above 50°) until saturated and then filtering into a large volume of 95% ethanol. The solution was allowed to stand in the cold overnight and the crystals were filtered off and dried. The Kraan and Muir (1957) method was scaled down slightly to a final volume of 5 ml instead of 10, and 30 μ g of glucosamine yielded a final $E_{1\text{ cm}}$ at 530 $m\mu$ of 0.6 ($\epsilon = 1.8 \times 10^4$).

(j) Adsorbed Ammonium Salts Since the protein was finally eluted with ammonium buffers the preparation was tested for bound volatile ammonium salts. Protein (about 1 mg) in 0.2 ml 0.2 M acetate buffer, pH 6, was placed in the outer rim of a Conway microdiffusion unit (type 2A). The centre well contained 0.2 ml of 0.2 M citrate buffer, pH 5. Saturated K_2CO_3 (0.2 ml) was placed in the outer rim

separately from the protein solution and, after sealing the diffusion unit, the two solutions were mixed and incubated in the cold room (5°) overnight. The inner well was then washed out and assayed for ammonia as described for amide determination.

(k) Biotin Content of Avidin Preparations It was not ascertained whether the method of isolation of avidin would separate it from avidin-biotin complex. As the binding of biotin would not change the size or net charge of avidin appreciably it is possible that any biotin present in the egg white would be found in the final avidin preparation. The biotin content of avidin preparations was therefore determined. The method involved the following steps:

- (i) Denaturation of the avidin to release the biotin (by heat);
- (ii) Addition of a known excess of native avidin to complex with the released biotin; (iii) Addition of a known excess of radioactive biotin, more than enough to back titrate any avidin not already in combination with "cold" biotin; (iv) Removal of all avidin-biotin complex (by CMC) and counting the excess radioactive biotin.

Preliminary experiments with avidin, to which about 1% of its capacity of radioactive biotin had been added, demonstrated that after heating at 120° in a sealed tube for 60-80 minutes more than 80% of the added radioactivity could be recovered.

Protein solution, (1 ml) containing 0.6-0.7 mg, was heated in a sealed tube at 120° for 80 minutes. Fresh avidin solution (0.02 ml containing about 12 µg) was added, followed after a few minutes by 0.02 ml solution containing 2,000 cpm biotin, then 0.1 ml M pyridine-acetate buffer pH 5 and finally 100 mg CMC filter cake. The mixture

was shaken gently, centrifuged briefly and 0.5 ml of the supernatant removed and counted. The control consisted of 1 ml of water.

2. Results

As the various avidin preparations differed in a number of respects a brief summary of their origin and preparation will be given here. Avidin A and avidin B are the first and second fractions respectively from the large scale CG 50 column (page 34). These preparations were derived from a pooled batch of eggs. Relatively small quantities of these materials were prepared and they were not analysed as fully as avidin D 31. This preparation was derived from eggs from a single breeder entirely by CMC chromatography (page 40). The preparation known as D 26.3 is of special interest and will be discussed separately. D.26 is essentially identical with D 31 except for the breed of chicken (see page 22).

(a) Purity This term is used to indicate the degree of contamination of the avidin preparations by non-avidin protein, as opposed to the term "heterogeneity" which is used to indicate the number of different molecular forms of the avidin. The final preparative chromatogram for each preparation furnishes the chief criterion used to assess its purity, by the degree of coincidence of the E_{280} and biotin binding activity curves in the effluent diagram. As the avidin peaks were collected in 20 to 50 fractions a fairly good assessment could be made of the activity over the peak.

Avidins A and B emerged from the preparative CG 50 column with a constant specific activity (13.5 γ /mg) over almost the whole of both peaks. The small regions of slightly lower activity were not

collected. The activity of these preparations is about 2% lower than that of D 31. If this difference is significant it may be due to the avidins A and B containing a small proportion of material which had become denatured on the column. This is suggested because these preparations gave rise to a slight precipitate after being concentrated and dialysed, while the avidin prepared on CMC did not. The third component of this column (avidin C) either possessed slightly lower intrinsic activity or was associated with some impurity. It was obtained in very small amount and was not further investigated.

The preparations derived solely by CMC chromatography, avidin D 26 and D 31, also showed a very close correspondence between activity and E_{280} in the final chromatograms, but in these cases a slightly lower specific activity was noticeable on the trailing edge of the peaks. This trail was not included in the final pooled samples which only contained the most active fractions. Nevertheless, analytical scale chromatograms of these preparations on CG 50 eluted with borate buffers still showed a slight contamination of inactive protein, which appeared on the trailing edge of the second peak (fig. 10). The specific activity of these materials over the peak on CMC and on CG 50 had a constant value of $8.8 \text{ } \mu\text{Ci/ml } E_{280} = 1$ (equivalent to $13.8 \text{ } \mu\text{Ci/mg}$). The purity of the avidin preparations in this study was thus probably of the order of 95%.

(b) Biotin Binding Capacity In view of the uncertainties of the radioactive biotin assay at both extremes of the range (page 11) a full titration was carried out. To a series of tubes containing known amounts of biotin increasing amounts of avidin were added and the excess

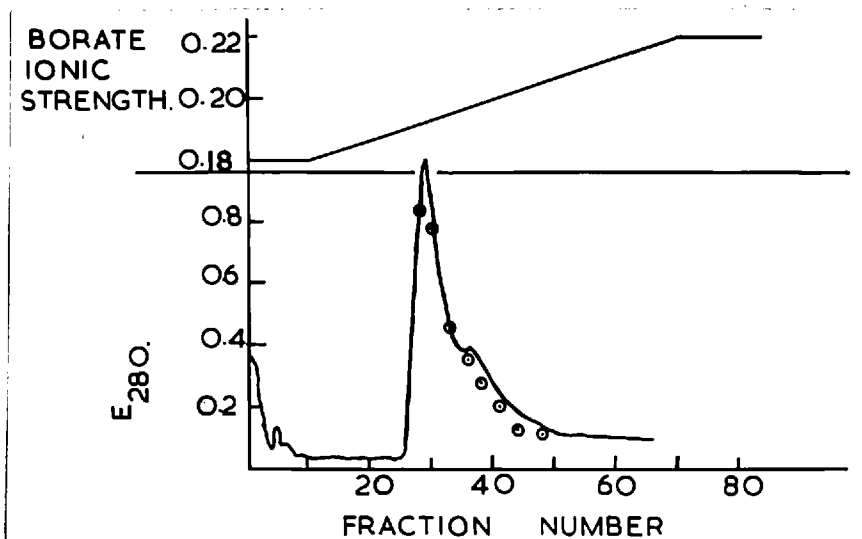


Fig. 10 Rechromatography of Avidin D 31 from Fig. 9 on CG 50

Column, Amberlite CG 50 (1 x 20 cm); flow rate 1 - 1.5 ml/hr; fractions, 0.5 ml — E_{280} ○ Biotin binding activity (see fig.2)

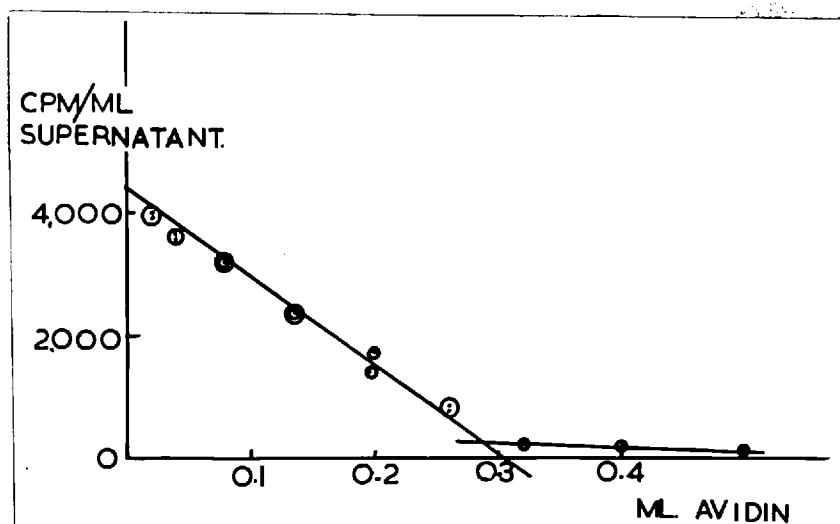


Fig. 11 Titration of Avidin with Radioactive Biotin

Avidin solution ($E_{280} = 0.11$) added to 0.5 ml biotin solution (4,400 cpm/ml) in 0.1 M pyridine-acetate buffer, pH 5.0. Complex removed with CM-cellulose and residual activity in supernatant counted.

biotin ^{14}C assayed after removing avidin-biotin complex with CMC.

The results are given in fig. 11. The intercept of the two straight line regions of the plot or, more accurately, the extrapolated intercept on the abscissa (see Discussion),^{p71} gives the biotin equivalent of the sample. The result for avidin A was $125 (\pm 5) \times 10^3$ cpm/ml $E_{280} = 1$ or 13.2 μg biotin/mg avidin. Similarly the result for avidin B was $135 (\pm 5) \times 10^3$ cpm/ml $E_{280} = 1$ or 13.05 γ biotin/mg avidin.

In the case of avidin D 31 the larger amount available made it possible to use the more accurate spectrophotometric method. The results are given in fig. 1^{p47} and give a figure of 8.8 γ biotin/ml $E_{282} = 1$ or 13.8 γ biotin/mg avidin. This figure represents an average of several determinations which agreed to within about 2%.

(c) Moisture Content The repeatability of the balance was such that about 3 mg protein could be used for estimation of moisture content. Taring of the weighing vessels and weighing of the dried protein was repeated until three successive weighings agreed to within 0.01 mg. The moisture content of avidin D 31 when stored in a dessicator over CaCl_2 in the cold room was $8.6 \pm 0.3\%$. On samples of avidin stored in the bench water contents of 9.0% and 10.0% were obtained.

(d) Spectrum and $E_{1\text{ cm}}^{1\%}$ 282 m μ The ultraviolet spectrum of avidin D 31 is reproduced in fig. 12. The concentration of protein in the solution was calculated from determinations of total nitrogen content. A solution containing 100 μg N/ml had $E_{282\text{ m}\mu}^{1\text{ cm}}$ of 1.00. As avidin D 31 contained 15.7% nitrogen on a dry weight basis the $E_{1\text{ cm}}^{1\%}$ 282 m μ was 15.7. For avidin A and B extinction coefficients were determined on an air dry basis and later corrected for moisture content. The values were, for avidin A, $E_{1\text{ cm}}^{1\%}$ 282 m μ = 15.6 and for avidin B, 14.3

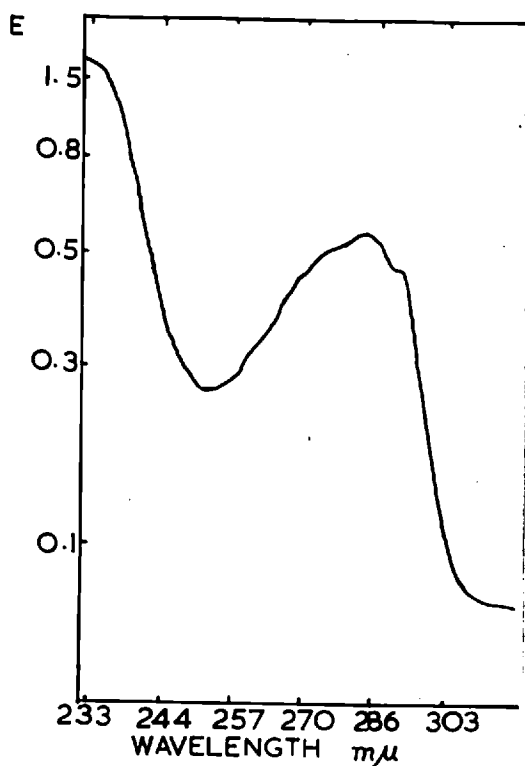


Fig. 12 Ultraviolet absorption spectrum of Avidin D 31 at 0.367 mg/ml. Scale linear in wavenumber.

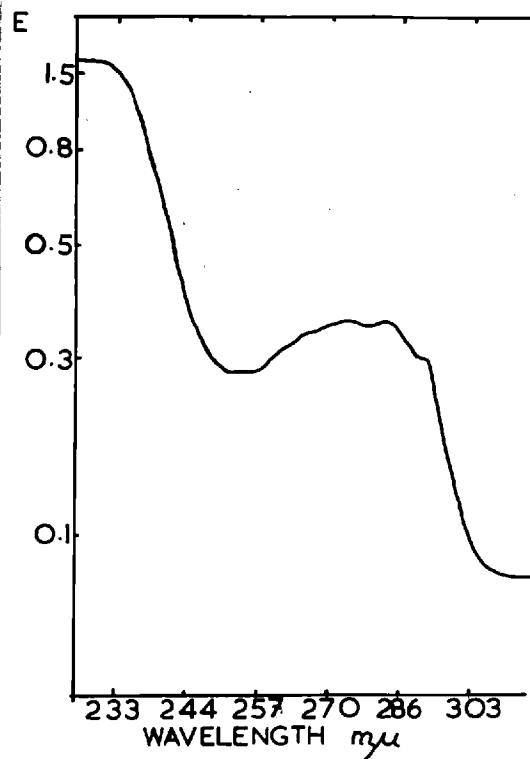


Fig. 17 Ultraviolet absorption spectrum of Avidin D 26.3A (per-vaporated avidin) at 0.306 mg/ml. Scale linear in wavenumber.

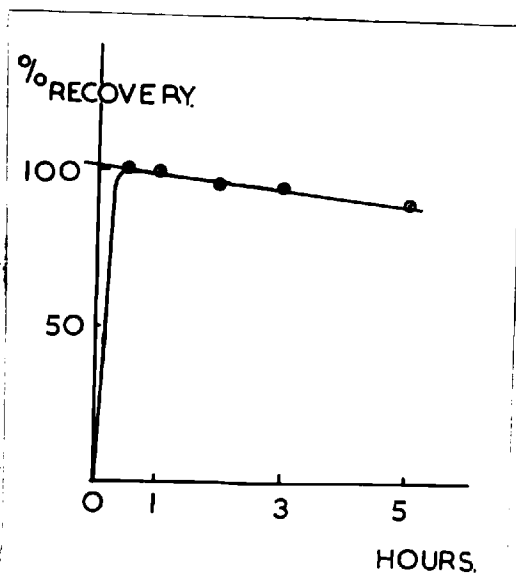


Fig. 13 Total nitrogen determination on samples of Avidin D 31 digested in a sealed tube at 480° for various periods of time. The 100% value is equivalent to 15.7% N

Extinction coefficients were not determined directly on the dried protein as the heat treatment may have affected the extinction of the tryptophan residues which are particularly sensitive to chemical change in avidin.

(e) Total Nitrogen To illustrate the reproducibility of the method, the values obtained for replicate determinations will be quoted. Total nitrogen was determined on solutions of known concentration made up from protein dried at 110° in vacuo, and also on solutions of known extinction at 282 m μ . The results are given in table V. After these results had been obtained some doubt arose as to whether they did in fact represent total recovery of the nitrogen from the samples (see Discussion, page 71). As a result a further series of analyses were performed on samples digested for various periods of time and these results are given in fig. 13.

(f) Total Phosphorus A standard containing 0.5 μ g phosphorus gave an $E_{230}^{1\text{ cm}}$ after subtracting the blank, of 0.45 ($\epsilon = 2.8 \times 10^4$). To check the effect of the presence of protein on the determination, 0.5 μ g phosphorus (as $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$) was assayed in the presence or absence of 1 mg lysozyme. There was no detectable difference in the final result.

Avidins A and B were found to contain 0.07% and 0.046% phosphorus respectively. This represents 1.5 and 1.0 phosphorus atoms per mole, which probably indicates that avidin may contain one or two phosphorus atoms and that Avidin A is a mixture of the two types.

(g) Amino Acids Replicate analyses on the standard amino acid mixture showed a maximum variation of $\pm 3\%$ with a single batch of

reagents. Avidin A and B were hydrolysed for one period of time only (24 hours) due to the scarcity of material, and corrections for destruction of threonine and serine were made using the rates determined on the D 31 preparation. A standard amino acid sample was run on the analyser followed by two runs of the avidin A hydrolysate, a second standard amino acid sample, two runs of the avidin B hydrolysate and finally a third standard. The results from the first and second standard runs were averaged and this was taken as the colour yield from which the results for avidin A were derived. Similarly the average of the results for the second and third standard runs served as the basis for the calculations for avidin B. As the duplicate results agreed closely they were averaged. In the case of avidin D 31 hydrolysis was performed for 24 and 72 hours and duplicate samples from the 24 hour hydrolysate and one from the 72 hour one were analysed. Once again standards were run periodically and the colour yields for the appropriate standards used for the calculations. However, by the time these analyses were done Walsh and Brown (1962) had introduced the use of a known quantity of norleucine as internal standard whereby an allowance is made for any day to day variation in the absolute colour yield of a given ninhydrin solution. The results are set out in table VI.

Cystine of avidin D 31 was fully oxidised to cysteic acid. A standard mixture of amino acids, which this time included cysteic acid, was run before and after samples of a hydrolysate of the oxidised avidin. The results were calculated per 32 alanine residues. Alanine was chosen as standard as it is stable to oxidation and present in avidin in an amount similar to cysteic acid. Tyrosine was completely destroyed by

Table V Results of Total Nitrogen Determination

Solution	Samples taken for digestion (g)	Replicate samples (1 ml) taken for ninhydrin E_{570}	Results corrected for volume of sample $\% \text{ N/ml}$ (averages)	Final Result	
				N/ml E_{280}^{-1}	$\% \text{ N}$
Avidin D 31 $E_{282} = 0.442$	0.099	0.938	44.4	100	
		0.938			
		0.940			
	0.099	0.936	44.2		
		0.938			
		0.938			
Avidin A $E_{282} = 0.859$	0.5013	0.850	79.1	92.8	
		0.853			
	0.5008	0.860	80.1		
		0.862			
	0.5026	0.865	79.9		
		0.859			
Avidin D 31 dried at 110° in vacuo 0.287 mg/ml	0.0996	0.960	435.5	15.7%	
		0.962			
		0.965			
	0.0990	0.938	444.5		
		0.938			
		0.942			
	0.0986	0.942	448.0		
		0.942			

Table VI Composition of Avidin

<u>Avidin Preparation</u>	<u>D 31</u>		<u>A</u>		<u>B</u>	
Total N %	15.7		15.3			
E ₂₈₂ /mg/ml	1.57		1.56			
Biotin/ml E ₂₈₂ - 1	8.8		8.7			
Biotin µg/mg Avidin	13.8		13.5		13.3	
	g/100g	Res/70,800g	g/100g	Res/71,500g	g/100g	Res/71500
Lysine	8.03	39	8.25	40	9.0	44
Histidine	0.97	4.4	0.94	4.4	1.02	4.7
Arginine	8.40	35	8.6	36	9.6	40
Aspartic acid	11.74	63	11.7	63	11.6	63
Threonine	14.53	87	15.1	91	14.8	89
Serine	5.74	39	5.68	39	5.55	37
Glutamic acid	9.76	47	9.05	44	9.0	44
Proline	1.58	9.7	1.52	9.5	1.33	8.3
Glycine	5.13	48	5.04	48	5.06	48
Alanine	2.73	23	2.80	23	2.89	23
$\frac{1}{2}$ cystine	4.75	28				
Valine	5.20	32	4.96	31	5.0	31
Methionine	1.87	8.9	1.89	9.1	1.67	8.0
Isoleucine	6.35	35	6.20	33	6.22	35
Leucine	5.75	31	5.70	31	5.73	31
Tyrosine	1.15	4.5			1.30	5.1
Phenylalanine	7.00	31	6.96	31	6.52	28
Tryptophan	5.50	19	5.4	19	5.4	19
N Ac glucosamine	3.83	12	4.0	13	4.0	13
Mannose	5.80	23				
Amides	1.14%	58				
Phosphorus				1.5		1.0
Biotins bound		4.0		4.0		4.0
Total residues	593		595		597	
Mean res. weight	119		120			
Nitrogen recovered	103.4%					
Weight recovered	100.4%					

the oxidation.

(h) Amide Nitrogen The method used to determine amide nitrogen was first tried out on ovalbumin before being applied to the avidin. In both cases samples containing different amounts of protein were assayed and the final content determined from the slope of the resulting curve (fig. 14). The scatter of the points on the curve gives an idea of the reliability of the method. Ovalbumin by this method gave 0.88% amide nitrogen (28 residues per mole) which is about 10% less than the value of 0.96% (31 residues per mole) obtained by Johansen et al (1960) after hydrolysis in 10 N-HCl at 37° for 250 hours. The avidin sample (D 31) gave a value of 1.14% amide nitrogen, equivalent to 58 residues per mole.

(i) Volatile Ammonium Salts The method was first tested with a sample of ovalbumin. The reagent blank, as well as an incubation containing only water, gave a final E_{570} of 0.05 ($\pm$ 0.005). Egg albumin (1 mg) after incubation with 50% saturated K_2CO_3 gave the same reading. A standard containing 10 μ g nitrogen as NH_4Cl gave a reading of 0.83 ($\pm$ 0.01) in the presence or absence of 1 mg of ovalbumin. Avidins A and D 31 gave a final result indistinguishable from the blank. Ammonium salts equivalent to about 0.5 μ g nitrogen, if present in the protein preparations, would have given a final reading almost double the value of the blank and would have been readily detectable. The preparations tested thus contained less than 0.05% of their weight as volatile ammonium salt nitrogen.

(j) Hexose As the orcinol method was being employed on a tenth of the scale of that previously described, it was checked on oval-

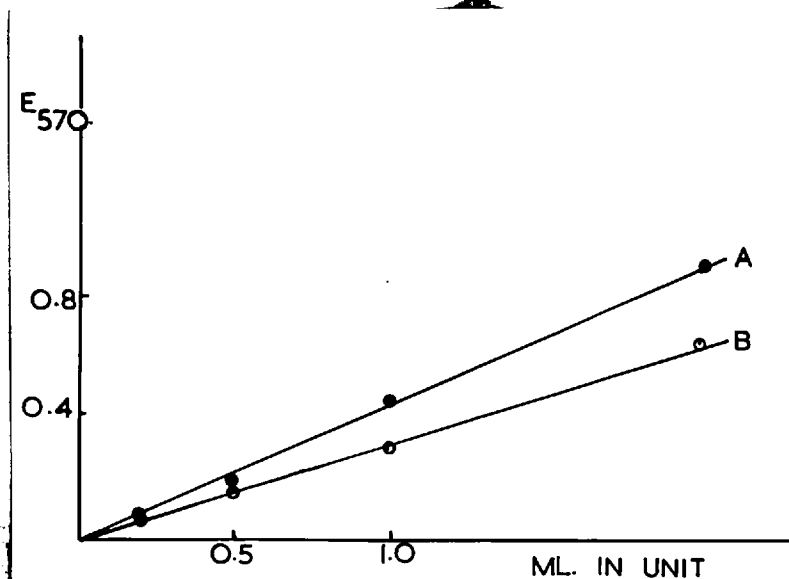


Fig. 14 Determination of Amide Nitrogen as Ammonia Various volumes of a solution (10 ml) of the phosphotungstate precipitate from acid hydrolyzed protein (5 mg) incubated in Conway microdiffusion units and ammonia determined in the centre well with ninkhydrin reagent.

A - Avidin

B- Ovalbumin

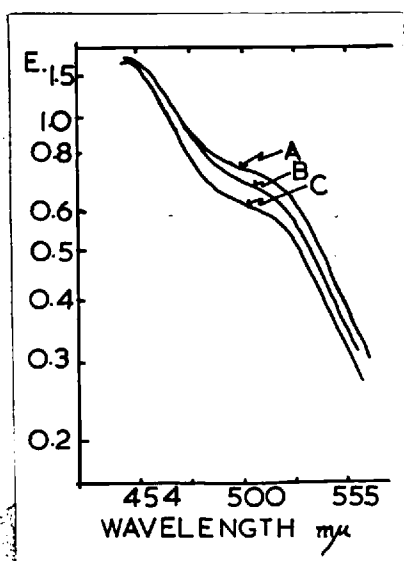


Fig. 15 Absorption spectra of the chromophores produced with the orcinol reagent. A - avidin; B - mannose; C - ovalbumin.

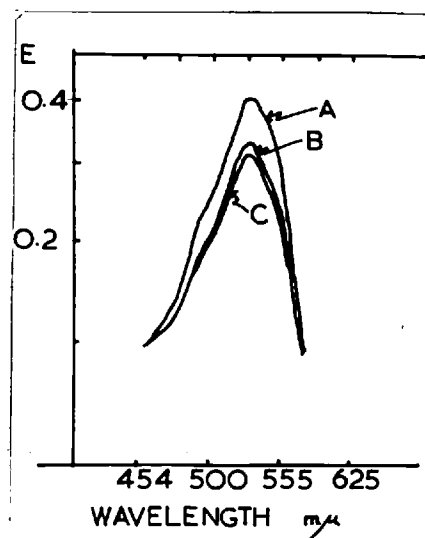


Fig. 16 Absorption spectra of the chromophore produced by the Elson and Morgan reaction. A - glucosamine; B - avidin; C - ovalbumin.

bumin as well as calibrated with the usual mannose standards. The result for ovalbumin (1.96% mannose) was in agreement with the results of Francois et al. (1962). Avidin D 31 gave a value of 5.8% mannose, equivalent to 22.6 residues per mole or 5.7 per biotin bound. The absorption spectra of the chromophores produced by orcinol with mannose, ovalbumin and avidin are virtually identical (fig. 15).

(k) Hexosamines Due to the difficulty in reconciling maximum liberation of amino sugars with minimum destruction of the free residues, analyses were performed on samples of glycoprotein which had been hydrolysed for different periods in 4 N HCl at 100°. With ovalbumin and with avidin there was little significant change after the first two hours up to 6.5 hours when the experiment ended. The results from the plateau of the plots gave 1.25% glucosamine (3.1 residues/mole) for ovalbumin and 3.1% (12.1 residues/mole) for avidin D 31. Avidin A and B were similarly hydrolysed but only one period of time was employed (4 hours). The results were 3.3% glucosamine for avidin A and 3.15% for avidin B. The figures recalculated as N-acetyl glucosamine give for avidin D 31, A and B respectively 3.83%, 4.06% and 3.90%. The absorption spectra of the chromophores produced in the assay by glucosamine, ovalbumin and avidin D 31 are reproduced in fig. 16.

(l) Biotin Content The results were calculated as a percentage of the total biotin binding capacity of the preparations. Avidin D 26 was found to contain 1.0% and 0.9% on duplicate determinations and D 31 0.8% and 0.96%. As the total capacity for biotin is about 13 µg/mg this represents approximately 0.01% biotin by weight in the preparations. This value was not taken into account in the final

calculations of recoveries or of equivalent weight of avidin per mole of biotin bound.

(m) Pervaporated Avidin (D 26.3) The two products D 26.3A and D 26.3B (see page 40) were virtually identical but differed significantly from the rest of the avidin D 26 preparation. The ultraviolet absorption spectra (D 26.3A, reproduced in fig. 17) ^{p 63} were considerably different from that of native avidin, but the same as that of avidin which had been treated with periodate (Green, 1963^b). It may thus be concluded that the avidin had been oxidised during pervaporation. The preparation was further analysed in a number of respects and the results are listed below together with the figures for avidin D 31 in brackets as well as the D 26.3 value as a percentage of the D 31 value.

$\mu\text{g Nitrogen/ml } E_{280} = 1$	122	(100)	122%
% Nitrogen (dry weight)	15.2	(15.7)	97%
$E_{1\text{ cm}}^{1\%} 282 \text{ m}\mu$	12.8	(15.7)	82%
Biotin bound	10.0 γ biotin/mg avidin	(13.8)	73%
	or 7.8 γ biotin/ml $E_{280} = 1$	(8.8)	89%

Discussion on Analysis of Avidin

(a) Methods Direct determination of specific radioactivity of the biotin by weighing proved to be very difficult to perform on the extremely limited quantities of material available. "Cold" biotin, was recrystallised a number of times, dried to constant weight, and then used to determine the specific biotin binding activity of an avidin preparation, on an E_{280} basis, with considerable accuracy ($\pm 1\%$) by the

spectrophotometric method. A sample of this standardized avidin preparation was then combined with radioactive biotin and the excess biotin removed by dialysis. The specific radioactivity of the complex on an E₂₈₀ basis furnished an accurate measure of the specific radioactivity of the radioactive biotin. This method is due to N.M. Green who used it to standardize the radioactive biotin used in this study.

When increasing amounts of avidin were added to samples of radioactive biotin and the complex removed with CMC a small residual radioactivity was still found after the equivalence point had been passed (page 62 fig 12). This could be due either (i) to the biotin preparation containing a radioactive impurity not bound by the avidin or (ii) to some avidin-biotin complex being bound by the "soluble CMC". If the first explanation was true the point of intersection would be the equivalence point; but if the second possibility accounted for the residual activity, the extrapolated intercept on the abscissa would give the most accurate result. As the biotin had been purified chromatographically and as CMC was known to give rise to a small fraction of soluble material (Green, 1963^a) the second approach was adopted for calculation of the results.

For the digestion of protein in sealed tubes for the total nitrogen determinations the temperature (460-480°C) was registered on the thermocouple of the muffle furnace. Subsequently, from a consideration of the results of Grunbaum et al (1952) it was realized that a thermocouple under these conditions can register 40° to 50° below the true temperature, and furthermore that above 470° losses of ammonia may occur (presumably by oxidation to nitrogen). If the thermocouple used for the digestions in this study was in error, then only about

80% of the ammonia in the tubes may have been recovered. Although standards (glycine) were always digested simultaneously with the samples, incomplete recovery could give rise to errors if the rates of destruction of ammonia were different for sample and standard. However, as the overall colour yield obtained for the standards ($\epsilon = 2.1 \times 10^4$) is very close to theoretical (2.16×10^4 ; Troll and Cannan, 1953) there does not appear to have been much loss of ammonia. Nevertheless samples of avidin were digested in the usual way, but for varying periods of time to determine the rate of destruction of the ammonia. It was clear from the results (fig. 12) ^{p 63} that the rate was too slow to cause any significant error in the results.

The method used for determination of tryptophan in avidin differed from the standard procedure (Beavan and Holiday, 1952) as the tyrosine content was known from the amino acid analysis. It was not necessary, therefore, to determine both amino acids by ultra-violet methods - using the spectral shift in 0.1 N NaOH to get an estimate of the tyrosine content - and the simpler method described above was used instead.

The amino acid analyses of avidin were complicated by the presence of glucosamine in the hydrolysate. Its position in the column effluent is sensitive to very small changes in composition of the eluting buffer or of the temperature of the column. In the method of Spackman et al (1958) it is usually eluted after phenylalanine on the long column, and before lysine on the short column. In some chromatograms of hydrolysates of avidin by this method glucosamine was eluted before, or sometimes simultaneously with, tyrosine. On the

single column procedure employed with the Technicon autoanalyser glucosamine is eluted in the region alanine, valine, cystine. With some of the elution procedures used with this column glucosamine overlapped alanine or cystine in the effluent diagrams making the particular amino acid inestimable. With the Technicon "Chromohead" micro-amino acid procedure finally adopted, under carefully controlled conditions glucosamine is eluted between alanine and valine where there is a sufficient gap to allow for any small variations in the position of glucosamine.

(b) Results The biotin binding activity of avidin D 31 (13.8 μg biotin/mg avidin) gives an equivalent weight of 17,700 g per biotin bound. Molecular weight values obtained with this material are 66,000 by sedimentation and diffusion (not extrapolated to zero protein concentration) and between 67,000 and 70,000 by equilibrium sedimentation (Green 1964). Earlier molecular weight results on less pure material are similarly between 60,000 and 70,000 (Fraenkel-Conrat et al, 1952a). It is thus most likely that avidin binds four moles of biotin per mole, which gives a molecular weight on a biotin binding basis of 70,800.

Comparison of the amino acid analyses of the 24 and 72 hour hydrolysates of avidin D 31 show the usual destruction of serine, threonine and cystine. The slight reduction in tyrosine in the 72 hour hydrolysate and the increase in histidine are possibly also significant. For the most labile amino acids, serine, threonine and cystine, the rate of destruction was derived by comparison of the values at 24 hours and 72 hours and the content of these amino acids at zero hydrolysis time was derived by linear extrapolation.

Amino acid values of the best previous avidin preparation, Avidin A (Fraenkel-Conrat et al, 1959 a,b) agree fairly closely on the whole, to those presented in table VI. The early results are consistently 10 to 20% lower (average value 13.5%) on a g/100 g protein basis but 16% of this preparation was not accounted for as amino acid or carbohydrate. Overall biotin-binding capacity of this material was 8 to 9 $\mu\text{g}/\text{mg}$, but taking into account that it contained about 10% avidin-biotin complex (Fraenkel-Conrat et al, 1952b), and about 16% non-protein contamination, the avidin itself bound about 11.3 μg biotin/mg. This is 82% of the value for avidin D 31 and indicates a relatively low contamination of non-avidin protein.

The cystine values in table VI require some comment. Fraenkel-Conrat et al (1952b) found very little cystine after hydrolysis of avidin with a mixture of concentrated hydrochloric acid and 95% formic acid (1 ml and 0.8 ml respectively for 10 to 40 mg of protein - Lewis et al, 1959). As their material was slightly contaminated by non-avidin protein they concluded that avidin itself may well have no cystine at all. On the other hand, only 40% of the total sulphur of their preparation (0.95%) could be accounted for. In the present study direct determination from the chromatograms of 6 N HCl digests yielded approximately five half-cystine residues per mole of protein, after allowing for destruction by linear extrapolation to zero hydrolysis time. The cysteic acid content from separate determinations on digests of performic acid oxidized protein, however, was 28 to 29 residues per mole. It is likely that the rapid initial rate of destruction of cystine is related to the high tryptophan content of the material and perhaps also to the presence of carbohydrate.

There are no free sulphydryl groups in avidin whether determined by nitroprusside tests or by the p-chloromercuribenzoate method of Boyer (1954), and whether the tests are performed on the native protein or after denaturation in guanidine hydrochloride (Lewis et al, 1950 and N.M. Green, personal communication).

The hexose present in avidin was characterized by Fraenkel-Conrat et al (1952b). Mannose was the only neutral sugar found in the purest avidin samples. Consequently in the present analyses mannose was taken to be the only hexose in avidin. The identity of the absorption spectra of the orcinol chromophore of mannose, ovalbumin and avidin is compatible with this, but many hexoses give the same chromophore.

The hexosamine of avidin has not been identified unequivocally. After hydrolysis in 4 N HCl for various times avidin gave a chromophore with the Ronile and Morgan (1935) reaction (under the conditions of Kraan and Muir, 1957) identical with that produced by treating glucosamine or ovalbumin in the same way (see page 68), but a large number of amino sugars give the same spectrum (see e.g. Crumpton 1959). The colour yield given by avidin hydrolysates in this reaction was approximately constant over a range of preliminary hydrolysis times extending from 3 to 8 hours as reported for glucosamine (Johansen et al, 1960, Marshall and Neuberger, 1960). However, other amino sugars, e.g. galactosamine, have a similar order of acid stability to that of glucosamine. The position of the hexosamine peak on the amino acid chromatograms is potentially a most useful method for its positive identification, but due to the erratic results obtained, no positive conclusions can be drawn from the present analyses. In the absence of

an exact characterization of the hexosamine it was calculated as N-acetyl glucosamine for the estimation of total recovery of avidin in terms of known constituents.

D 31 is the avidin preparation most exhaustively analysed for amino acid and carbohydrate constituents. By summation of the residue weights in g/100 g protein on a dry weight basis, 100.4% of the weight of avidin is accounted for. The nitrogen content of avidin by summation of known constituents is 16.25% but the nitrogen value obtained is 15.7% or 96.6% of the expected value. It is difficult to see where the source of the discrepancy lies. It may be due to the summation of a number of small errors which all happen to be in the same direction, e.g. overestimation of arginine or lysine, overestimation of amide nitrogen, underestimation of total nitrogen. The total sulphur content from the cystine and methionine values is 1.3%. Fraenkel-Conrat et al (1952b) reported a total S content of 0.95%, which, if corrected for the presence of about 15% of non-protein contaminant in their preparation (see page 74) gives a final value of 1.1%. It is thus apparent that all the weight, nitrogen and sulphur of avidin have been accounted for in terms of amino acids and carbohydrates. The extremely stable binding of biotin by the molecule will thus have to be explained in terms of a binding site made up only of amino acids and/or carbohydrate. It is unlikely that the carbohydrate plays an important role in the biotin binding as Streptavidin, a very similar protein recently found in Streptomyces, which has a similar affinity for biotin, has no carbohydrate (Tausig and Wolf, 1964 and N.M. Green, personal communication). (See also Chalet and Wolf, 1964).

PART III OVOMUCOID

A Introduction

1. Historical

In 1873 Bechamp reported a material in hen's egg white not precipitated by ethanol, and in 1890 Neumeister described from the same source a protein resistant to coagulation by heat. These are probably references to the substance which Mörner (1894) isolated, showed was a glycoprotein and termed ovomucoid. The early literature has been reviewed by Meyer (1945), Lineweaver and Murray (1947), Fevold (1951), Laskowski and Laskowski (1954) and Warner (1954). Interest in ovomucoid was given great impetus in 1947 when Lineweaver and Murray showed unequivocally that it was the component in egg white responsible for inhibition of trypsin. They used a method of isolation which did not involve heating and their paper will be taken as the starting point for this review of the literature.

Ovomucoid, which comprises about 12% of egg white protein, is not the only antitryptic substance in hen's egg. "Ovoinhibitor", an egg white protein which inhibits bacterial and fungal proteinases (Matsushima, 1958), is also active against trypsin and chymotrypsin (Rhodes et al, 1960; Feeney et al, 1963). A livetin fraction, carefully prepared from yolk with a minimum of contamination by egg white, had approximately 6% of the antitryptic activity (on a weight basis) of ovomucoid (Lineweaver and Murray, 1947).

2. Methods of Preparation

The study of the nature and composition of ovomucoid has been hindered by the lack of any generally applicable method for its isolation in a pure state. Most investigators since 1947 have used material obtained by acetone or alcohol precipitation at pH 3.5 as described by Lineweaver and Murray (1947) and Fredericq and Deutsch (1949) respectively. Both products are mixtures with about the same specific activity against trypsin although they contain different impurities (Noble et al, 1957).

Egg white proteins have been fractionated by precipitation with varying ethanol concentration, ionic strength (I), pH and temperature in a manner analogous to the Cohn method for plasma (Forsythe and Foster, 1950). The final supernatant (pH 4.6, -15° , $I = 0.04$ and an ethanol mole fraction of 0.11) containing the ovomucoid was concentrated, dialysed and freeze-dried. This fraction was not characterized by the authors and has subsequently been shown to contain a contaminating glycoprotein (13.6% hexose, 13.8% hexosamine and 3% sialic acid) with no inhibitory activity towards trypsin (Ketterer, 1962 and 1965).

A new method for preparation of ovomucoid arose out of the complete fractionation of egg white proteins on CM cellulose, where the antitryptic activity emerged in more than one fraction (Rhodes et al, 1958). By a combined use of CM- and DEAE-cellulose columns, Rhodes et al (1960) isolated three ovomucoid fractions by procedures which did not necessitate the use of protein precipitants. However, the material was subjected to fairly high acidities (pH 3.5-4.0) and to adsorption onto the surface of a carboxylic acid resin where the effective acidity may be even higher.

The antitryptic activity of the preparations obtained by the methods of Lineweaver and Murray (1947) and Fredericq and Deutsch (1949) has been enhanced by further purification. On triethylaminoethyl (TEAE) cellulose chromatography both products gave two peaks without antitryptic activity as well as one active peak which was somewhat asymmetrical (Jevons, 1960). The active material was later fractionated into a number of peaks (Beeley and Jevons, 1965). Electrophoresis of either ovomucoid preparation on cellulose columns under optimum conditions gave a few inactive peaks on elution as well as one active peak which was quite symmetrical (Jutisz et al, 1957; Noble et al, 1957). After repeating this purification twice the active peak had 1.6 times the specific activity of the starting material on an E_{280} basis. But after dialysing and freeze-drying, the final product had a specific activity on a weight basis not very different from that of the starting material, which the authors interpreted as a loss of activity in the final stages of preparation.

CM-cellulose column chromatography of the Lineweaver and Murray ovomucoid results in an increase in specific activity of 55% on an E_{280} basis (Feeney et al, 1963). However, material eluted from CM-cellulose does not rechromatograph as a single peak under the same conditions (Chatterjee and Montgomery, 1962). This suggests that this treatment may cause some degradation, possibly due to the acid conditions required (pH 3.7 - 4.3). On DEAE-cellulose chromatography at pH 7 the Lineweaver and Murray (1947) preparation gave an active peak which rechromatographed as expected and contained 67% of the original hexose and 82% of the 280 m μ absorption of the starting material.

It has been known since the classical work of Longworth et al (1940) that at low ionic strength and in the vicinity of its isoelectric point ovomucoid is electrophoretically heterogeneous. The presence of separate components in ovomucoid was clearly demonstrated by Fredericq and Deutsch (1949). Bier et al (1953) distinguished five components present both in the Lineweaver and Murray (1947) and the Fredericq and Deutsch (1949) materials with respective isoelectric points and relative proportions as follows: 4.41, 30%; 4.28, 45%; 4.17, 17%; 4.01, 6%; 3.83, 2%. Fractions enriched in each of the components were isolated by electrophoresis convection and showed no marked differences in antitryptic activity, hexose content, hexosamine content or sedimentation behaviour. Even the material purified by DEAE-cellulose chromatography (Chatterjee and Montgomery, 1962) split into four migrating species on electrophoresis at pH 4.6 and $I = 0.01$. Isolation of ovomucoid with the aid of an ion-exchange cellulose (Rhodes et al, 1960) yielded one fraction which did not adsorb onto CM-cellulose at pH 3.5. This material accounted for 1% of the ovomucoid and contained 4% sialic acid or four residues per mole (Warren (1959), thiobarbituric acid method). The remainder of the ovomucoid adsorbed onto the cellulose at that pH and was eluted in two roughly equal fractions at pH 4.1 and pH 4.4. These contained 0.6% and 0.4% sialic acid respectively. The active peaks obtained on TBAE cellulose chromatography did not show any differences in the hexose to hexosamine ratios, but in total carbohydrate and in sialic acid content significant differences were found (Beeley and Jevons, 1965).

Ovomucoid preparations usually give a single symmetrical peak on ultracentrifugation (Fredericq and Deutsch 1949; Rhodes et al, 1960; Chatterjee and Montgomery, 1962). Immunological studies on the Fredericq and Deutsch (1949) product indicated heterogeneity (Wetler and Deutsch 1950), but material purified by column electrophoresis was homogeneous by immunoelectrophoresis (Jutisz et al, 1957). The slight antichymotryptic activity often associated with ovomucoid (Weil and Timasheff 1960) is due to contamination by another inhibitor (Feeney et al, 1963) and thus the absence of such activity can be used as additional evidence for purity. Ovomucoid has not been crystallised.

5. Composition

(a) Amino Acids Analytical values have been obtained by two groups of workers for ovomucoid prepared by the method of Lineweaver and Murray (1947). Lewis et al (1950) hydrolysed the material in 6 N HCl for 18 hours under reflux and assayed the amino acids by microbiological methods. Taking 24% as the content of carbohydrate (see later) their figures give an overall recovery of 90% of the weight. Stevens and Feeney (1963) analysed by the Moore and Stein method the hydrolysate obtained after 22 hours at 110° in 1,000 volumes of constant boiling HCl. In this case inclusion of the carbohydrate as determined by the same authors (18.7%) gives 96% of the weight as the overall recovery.

Both results indicate approximately one half-cystine residue per eleven amino acids. The same proportion was found by Deutsch and Morton (1961), whether the cystine was determined directly or as the more stable cysteic acid or S-carboxymethylcysteine derivatives. There are no free sulphydryl groups (Lineweaver and Murray 1947). Cystine and methionine account for virtually all the sulphur of the protein (2.2%, Lineweaver and

Murray, 1947). Tyrosine content by spectrophotometric analysis of different fractions of ovomucoid varies from 4.5 to 5.9 residues per mole (Rhodes et al, 1960).

Amide content was determined on ovomucoid prepared by the Lineweaver and Murray (1947) procedure, using a method which reduced concomitant deamination of hexosamine to a minimum (Marshall and Neuberger, 1960), and was found to be 0.45% or 9.3 moles per 28,800 g of ovomucoid. From amino acid values of Lewis et al (1950), the molecule thus has 32 acidic and 22 basic groups.

Ovomucoid has only one amino acid chain as the molecular weight after cleavage of all disulphide bridges is essentially unchanged (see Table VIII). The carboxyl end is a phenylalanine residue (Penasse et al, 1952). Ovomucoid is however not attacked by carboxypeptidase nor is dinitrophenylated ovomucoid, although phenylalanine is a substrate for the enzyme and the enzyme is not inhibited by ovomucoid (Noble et al, 1957). This unreactivity cannot be due to the free carboxyl group being esterified as ovomucoid does not contain any alkaline hydroxylamine labile bonds (Harbon et al, 1963). Dinitrophenylation yielded 0.8 moles DNP-alanine per mole of ovomucoid indicating that alanine is the free amino end of the chain (Fraenkel-Conrat and Porter, 1952 and Noble et al, 1957).

(b) Carbohydrate Content Glucosamine from ovomucoid has been identified as the crystalline hydrochloride, chromatographically (paper and Dowex-50 columns), and as arabinose after treatment with ninhydrin. Similarly mannose has been characterised as the phenyl-hydrazone and galactose as the 1-methyl-1-phenyl-hydrazone and the

tetramethyl ether. Both hexoses have been identified by paper chromatography in a number of solvents (Stacey and Woolley 1940; Dixon, 1955; Gottschalk and Ada, 1956; Bragg and Hough, 1961; Chatterjee and Montgomery, 1962). The presence of sialic acid in ovomucoid, reported by Odin (1958), has been subsequently confirmed by Rhodes et al (1960), Jevons (1960) and Chatterjee and Montgomery (1962).

A study of the conditions required for release of glucosamine for quantitative analysis indicated 4 N HCl at 100° for about 8 hr to be optimum (Marshall and Neuberger, 1960). The value so obtained (15.5%) is in good agreement with the value expected from the acetyl content of the material. It is of interest that when determining acetyl groups, Marshall and Neuberger (1960) also found three equivalents of formic acid per mole of ovomucoid liberated after acid hydrolysis. The origin of this acid is still obscure. Sialic acid content of ovomucoid has been determined by the thiobarbituric acid assay of Warren after liberation by $0.1\text{ N H}_2\text{SO}_4$ at 80° for 60 min (Rhodes et al, 1960). Material released in the same way was also examined by the Svennerholm column separation procedure and reported as N-acetylneuraminic acid (Chatterjee and Montgomery, 1962).

Estimation of total neutral sugar in ovomucoid by treating the glycoprotein with orcinol or anthrone in the presence of a high concentration of mineral acid usually indicates a value of about 8 to 10%. However free hexose recovered even from mild acid hydrolysis is considerably less than this (e.g. 7% (Dixon, 1955); 5.7% (Gottschalk and Ada, 1956); 5.7% (Chatterjee and Montgomery, 1962)). Such losses can

TABLE VII CARBOHYDRATE COMPOSITION OF OVOMUCOID

Author Reference	Method of preparation	% Nitrogen in Preparation	Constituent in percentage (g/100g)				Sialic Acid	Acetyl Residue	Hydrolysis for release of hexosamine (a)
			Glucosamine free base	Total neutral sugar	Mannose	Galactose			
1.	(e)	13.2	17	9.7					2 <u>N</u> - 8 hours
2.	(f) + electrophoresis		13.0-14.4	8.2 - 9.9					2 <u>N</u>
4.	(f)	13.5	9.2	10	5	2		4.3	2 <u>N</u> - 1 - 32 hours
5.	(g)		14.1	8.3	4.3	1.4			2 <u>N</u> - 6 hours
7.	(f)	13.2	15.2					3.7 ^(d)	4 <u>N</u> - 8 hours
8.	(e)	13.4	12.5		6.3 ^(b)	1.7 ^(b)		3.8	2 <u>N</u> ^(c) - 48 hours
9.	(f) + chromatography	12.7	14.6		4.7	1.0	0.5		4 <u>N</u> - 8 hours
10.	chromatography	11.8		8.4					
11.	(f)	13.3	13.6	5.1			0.4		4 <u>N</u> - 8 hours
12.	(e)		13.27	8.46			0.93		4 <u>N</u> - 4 hours
12.	(f)		15.36	8.74			1.14		4 <u>N</u> - 4 hours
12.	phenol precipitation	14.1	14.78	8.9			1.20		4 <u>N</u> - 4 hours

(a) HCl at 100° was used in all cases, but the concentrations and times varied as indicated. (b) In the case of ovalbumin it has been found that the method used by Bragg and Hough (1961) gives values about 35% too high (Clamp & Hough, 1963). (c) H₂SO₄ (d) These authors also found 0.3% formyl residue. (e) Alcohol + trichloroacetate pH 3.5 (Reference 1) (f) Acetone + trichloroacetate (reference 3). (g) Heat coagulation (reference 6).

References for Table VII

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|-----------------------------------|--------------------------------|--------------------------------|-------------------------|
| 1. Fredericq & Deutsch (1949) | 2. Bier et al. (1953) | 3. Lineweaver & Murray (1947) | 4. Dixon (1955) |
| 5. Gottschalk & Ada (1956) | 6. Mörner (1894) | 7. Marshall & Neuberger (1960) | 8. Bragg & Hough (1961) |
| 9. Chatterjee & Montgomery (1962) | 10. Neuberger & Papkoff (1963) | 11. Stevens & Feeney (1963) | 12. Chosson (1964) |

result from destruction of the liberated sugar and also from the trapping of neutral sugars onto deacetylated hexosamines (Gottschalk and Ada, 1956). All the individual values for mannose and galactose given in table VII were derived by mild acid hydrolysis (e.g. 0.25 N or 1 N H_2SO_4) followed by quantitative paper chromatographic separation and estimation either by direct densitometry or after elution. Due to the difficulty in getting good recoveries of mannose and galactose a number of workers record the results as a ratio of mannose to galactose only, e.g. 6:2 (Stacey and Woolley, 1940, 1942); 7:2 or 5:2 by different methods (Dixon, 1955); 4.8:1, 5.1:1 or 5.0:1 on different ovomucoid preparations (Chosson, 1964).

(c) Nature of the Carbohydrate The use of 10% $\text{Ba}(\text{OH})_2$ at 100° to obtain a preparation rich in carbohydrate (used for ovomucoid by Stacey and Woolley 1940, Bragg and Hough, 1961, Tanaka 1961a) is today considered undesirable. Proteases are a far less drastic and more specific method for removing peptide material and have been used for this purpose by Tanaka (1961 b,c), Hartley and Jevons (1962), Montgomery and Wu (1963), Neuberger and Papkoff (1963) and Montreuil et al (1963).

Several glycopeptides with different hexose to hexosamine ratios were isolated from ovomucoid hydrolysates obtained by the action of $\text{Ba}(\text{OH})_2$, pancreatin or trypsin (Tanaka, 1961 a,b,c). Glycopeptide mixtures were obtained from ovomucoid after digestion by Pronase P (Neuberger and Papkoff 1963) or by papain (Montgomery and Wu 1963) and were essentially similar in composition. After removal of amino acids by gel filtration the glycopeptide mixture was separated into a number

of fractions either by chromatography or by electrophoresis. These showed differences in carbohydrates or amino acids or both. Molecular weights of the glycopeptides were determined by a number of methods and all lie in the region of 2,000 to 3,000. As the carbohydrate of ovomucoid totals 6,000 to 7,000 g/mole it must be present in the form of a few separate prosthetic groups, most probably three. Most of the glycopeptide material contained 8 moles of glucosamine and about 4 to 5 moles of mannose per end group (DNP or phenylthiohydantoin). Smaller amounts of galactose were found in most glycopeptides and N-acetylneuraminic acid in one fraction. The tentative composition for the heterosaccharide of ovomucoid indicated by these results viz. three carbohydrate moieties each containing 8 N acetyl glucosamines and 4 to 5 neutral sugars would give overall sugar contents of 15.4% glucosamine and 8-10% neutral sugar in good agreement with the reported values on the whole protein.

The bond linking at least a part of the carbohydrate to the protein was shown to be between glucosamine and aspartic acid by the isolation of compounds containing only these residues from partial acid hydrolysates of ovomucoid glycopeptides (Tanaka, 1961d). At least one glycopeptide yielded approximately one mole of ammonia on hydrolysis in constant boiling HCl for 22 hours in a sealed tube which suggested the linkage was via an asparagine residue (Montgomery and Wu, 1963). As the phenylthiocarbonyl derivative of this glycopeptide cyclized in acid to the phenylthiohydantoin without detaching the carbohydrate, it was concluded that at least some of the carbohydrate is linked via a glycosylamine type of linkage involving the amide group of asparagine and most probably the reducing group of N acetyl glucosamine as in hen's egg

albumin (page 3). Threonine and cystine have also been found associated with ovomucoid glycopeptides and in the case of threonine there is some evidence that the hydroxyl function is not free (Chosson, 1964).

The arrangement of the monosaccharide units within the heterosaccharide of ovomucoid was first studied by Stacey and Wooley (1940, 1942), using material precipitated by ethanol from the supernatant of heat coagulated egg white. Hydrolysis of the methylated carbohydrate prepared directly from this material yielded very different results from the hydrolysis of material prepared by the method of Fredericq and Deutsch (1949) and methylated by almost the same procedure (Bragg and Hough, 1961). Periodate studies have yielded equally contradictory information (Bragg and Hough (1961) and Chatterjee and Montgomery (1962)) and it is thus not possible to draw conclusions about the nature of the heterosaccharide at this stage.

By isolating and identifying the products of Dowex 50 hydrolysis of ovomucoid, Montreuil and Chosson (1962) and Chosson, Montreuil and Scheppler (1962) have shown that N-acetylactosamine is a constituent of the heterosaccharide. Recently N-acetylglucosamine has been released from ovomucoid (presumably from the non-reducing end of the heterosaccharide chain) by a ram testes enzyme extract to the extent of 2 - 3 moles/mole of protein (Caygill and Jevons, 1964).

6. Physical Characteristics

The physical characteristics which have been determined for ovomucoid are shown in table VIII. The sedimentation pattern is independent of pH within the range 1.4 to 11.6 and independent of concentration between 0.5 and 1.5% (Fredericq and Deutsch, 1949). No detailed

TABLE VIII PHYSICAL CONSTANTS OF OVOMUCOID

Author	Lineweaver & Murray (1947)		Fredericq and Deutsch (1949)	Rhodes et al. (1960)	Deutsch & Morton (1961)			Chatterjee and Montgomery (1962)
Method of preparation	T.C.A. and acetone at pH 3.5		T.C.A. and alcohol at pH 3.5	Chromatography at pH 3.5-4.4	Fredericq and Deutsch (1949) further purified			Lineweaver and Murray (1947) further purified
Further treatment	-	heated	-	-	-	heated	reduced & alkylated	-
Molecular weight	28,800 (1)	27,900 (1)	27,000(2)	27,000(2)	31,500(2)	41,200(2)	32,600(2)	
S_{20}^0 , w (Svedberg)			2.8	- 2.49	2.62	2.62	1.92	2.8
D_{20} , (10^{-7} cm ² sec ⁻¹)			8.0	7.7	6.01	4.59	4.56	
$\bar{V}$ (ml.g.)			0.685	0.71				
Axial ratio			6.3		12-14	10	25-30	
$[\alpha]_D^{20}$	-56°							-78°
$E_{1\text{ cm}}^{1\%}$ 280 mμ				6.1(3)	4.8(4)	7.3(4)	4.0(4)	4.1

(1) By osmotic pressure

(3) From Rhodes et al. (1958)

(2) By sedimentation and diffusion

(4) Derived from the published spectra.

studies have been published on the shape of the molecules but the frictional ratio of 1.35 indicates a globular protein of approximately spherical shape, which is consistent with the low α -helix content found by optical rotatory dispersion (see below).

In a few respects the behaviour of ovomucoid is not as expected for a simple globular protein of molecular weight 28,800, but the presence of 25% carbohydrate complicates any comparison with pure proteins. Thus, with respect to diffusion constant, behaviour on gel filtration on Sephadex G 100 (Whitaker, 1963) and escape time on dialysis (Craig et al, 1958) ovomucoid appears to occupy a somewhat larger volume than a typical protein of the same molecular weight. These facts, together with the marked resistance of ovomucoid to precipitation - even the fully dinitrophenylated derivative is water soluble (Fraenkel-Conrat and Porter, 1952; Noble et al, 1957) - can be taken as suggestive of a highly hydrated molecule.

The unusual stability of ovomucoid to precipitating agents and the fact that activity against trypsin is not irreversibly lost in 9 M urea, even at elevated temperatures (Lineweaver and Murray 1947) ^{also} may be due ^{in part} to the extensive disulphide crosslinking of the molecule. Most of the antitryptic action is retained after exposure to acidic conditions even at relatively high temperatures. In fact at low pH the specific optical rotation of the protein becomes slightly less negative than it is in neutral solutions, which tends to suggest a folding up of the molecule into a more ordered configuration (Jirgensons et al, 1960; Herskovitz and Laskowski, 1962). This conclusion is confirmed by the finding that whereas above pH 5 most of the tyrosyls of ovomucoid are

accessible to solvent perturbation (as shown by the ability of the solvent to shift the ultraviolet absorption spectrum of the tyrosyl residues), below pH 4 a large proportion of these residues are inaccessible to the solvent and thus presumably buried within the molecule (Herskovitz and Laskowski, 1962). Exposure of ovomucoid to alkaline conditions on the other hand brings about irreversible loss of antitryptic activity at a rate dependant upon pH and temperature. At room temperature, for example, at pH 11 or 12 complete loss of activity occurs and this, as in the case of the low pH changes, is accompanied by an increase in dextrorotation of the solution (Jirgensons et al, 1960). This alkali-induced change in specific rotation is difficult to explain on the basis of conformational changes only, particularly in view of the fact that the intrinsic kinematic viscosity of ovomucoid solutions increases on the addition of alkali, suggesting unfolding of the molecule. This anomolous rotation change is not due merely to the presence of a large proportion of carbohydrate, as orosomucoid (40% carbohydrate) behaves normally, the rotation becoming more negative in solutions of very high or low pH compared with neutral conditions (Jirgensons 1957), nor is it due to any marked changes in the general shape of the rotatory dispersion curve but it may be related to the high cystine content of the protein. The rotatory dispersion curve of ovomucoid fits a one term Drude equation with a λ_0 of 237 m μ , which is typical of a globular protein with low α -helix content (Jirgensons et al, 1960).

7 Biological Activity

Ovomucoid, in common with a number of other proteins, inhibits the proteolytic and esterase activities of trypsin (see reviews by Laskowski and Laskowski, 1954, and Green and Neurath, 1954). The existence of a complex between ovomucoid and trypsin has been inferred because: (a) on electrophoresis a mixture of ovomucoid and trypsin runs as one component with a mobility intermediate between that of the two starting materials and (b) ultracentrifugation of the mixture produces one sedimenting boundary with a sedimentation coefficient of 3.8 compared with a value of 2.6 when either are run alone (Sri-Ram et al, 1954a; Rhodes et al, 1960). However a one to one molar relationship is only a first approximation as there is good evidence for the occurrence of complexes involving more than one trypsin per ovomucoid, (but not more than one ovomucoid per trypsin, Sri-Ram et al, 1954a).

Interaction between trypsin and ovomucoid in the absence of substrates occurs too rapidly to be measured directly, but the dissociation constant of the complex has been determined to be about $5 \times 10^{-9} M$. If carboxyl groups on the ovomucoid are blocked by esterification or if amino groups on the trypsin are acetylated, complex formation is severely retarded (Frankel-Conrat et al, 1949; Frankel-Conrat et al, 1952; Sri-Ram et al, 1954b). Dissociation of the complex into its components at pH values below 3 (Green, 1953; Sri-Ram et al, 1954a) would suggest that ionization of the carboxyl groups of the enzyme or the inhibitor or of both is essential for interaction. However, complex formation cannot be explained only in terms of electrostatic attraction between molecules of opposite net charge because: (a) at least one trypsin inhibitor (pano-

creatic inhibitor, Green and Work, 1953) has an isoelectric point similar to that of trypsin; (b) a short exposure to pH 12 irreversibly destroys the antitryptic activity of ovomucoid determined at pH 8 but presumably does not drastically alter its net charge; (c) if the active site of the enzyme is occupied with substrate the affinity of the enzyme for protein inhibitors is greatly reduced or abolished (Green 1953); (d) if the trypsin is inactivated by the presence of diisopropylfluorophosphate or HgCl_2 formation of a complex with ovomucoid does not take place (Green 1953). On the basis of studies on protein-protein association as followed by the release of protons it has been proposed that the trypsin-inhibitor reaction consists of a cleavage of one especially sensitive bond in the inhibitor, followed by formation of a covalent bond between trypsin and inhibitor (probably an ester bond between the active seryl of trypsin and the newly formed COOH-terminal of the inhibitor Finkenzstadt and Laskowski, 1965). The complex may then dissociate to form either the native inhibitor or a modified inhibitor with the bond cleaved. At equilibrium at pH 3.75 the modified form predominates; however kinetic control might well produce predominantly the native form. According to this explanation of the inhibition, the only difference between these inhibitors and substrates lies in the rates of decomposition of the complexes and on the dependence of these rates on pH. At neutral pH the enzyme-substrate complexes decompose rapidly to yield products; enzyme-inhibitor complexes decompose slowly. Furthermore, the rates of decomposition of normal enzyme-substrate complexes (for trypsin and chymotrypsin) rise with pH in the range 4 to 8, while those of the trypsin-inhibitor complexes fall (Lebowitz and Laskowski, 1962).

Gradual addition of ovomucoid to a given amount of trypsin at first brings about a reduction in activity which is directly proportional to the amount of ovomucoid added. But as the equivalence point is approached, additional ovomucoid has less and less effect and even in large excess does not entirely abolish the enzymatic activity (Gorini and Audrain, 1953). This has been interpreted in a number of different ways and may be of significance if the antitryptic action of ovomucoid is a part of its natural role in the developing embryo.

One aspect of the inhibition which was difficult to establish with certainty is the extent to which it is competitive with substrate. The difficulty is due to the dissociation constants of the inhibitors (in the region of 10^{-9}M) being very much smaller than the Michaelis constant of the enzyme when acting on the usual protein substrates (about 10^{-2}M). As a result competitive inhibition could not be demonstrated by the usual methods. With benzoyl-arginine ethyl ester (where the trypsin has a Michaelis constant of about 10^{-5}M) on the other hand it was shown that the protein inhibitors could displace substrate and vice versa, which clearly demonstrated a competitive type of inhibition (Green 1953).

In view of the deviation from linearity it is not possible to determine the exact equivalence point in the titration of trypsin with ovomucoid. Activity of ovomucoid is thus best expressed as twice the amount required to produce 50% inhibition of a given activity of trypsin, and the substrate must also be specified as this will affect the result (Castanada-Agullo, 1950; Fraenkel-Conrat et al, 1952). It

is well established that even thoroughly recrystallized trypsin can contain a variable proportion of inactive and non-trypsin material (Liener, 1960; Cole and Kincade, 1961; Buck et al, 1962; Maroux et al, 1962). It is thus not possible to compare accurately different ovomucoid preparations where the activity is expressed in terms of weight (or E_{280}) of "trypsin". Such results are only useful for direct comparison of activities determined side by side.

The striking effect of the injection of fresh egg white into rats (0.5 to 1.5 cc/adult rat) first noted by Parker and Parker (1924) was described in detail by Selye (1937). The ovomucoid fraction of Hektoen and Cole (1928) gave a full reaction at 15 mg/adult rat, whereas the rest of the egg white was inactive (Leger and Masson, 1948). As material prepared by the method of Lineweaver and Murray (1947) is also fully active (Ankier and West, 1964) it is most probable that it is in fact the ovomucoid which is responsible for these pharmacological effects. The syndrome (which is also produced by 100,000 molecular weight dextran) is characterized by a general pruritus followed by conspicuous oedema of the extremities, an increase in the haematocrit and a lowering of the body temperature. The action is much greater in adrenalectomised rats or following the administration of thyroxin, the egg white in these cases producing severe shock with vascular collapse (Leger and Masson, 1948; Halpern and Briot, 1950). The effects in normal rats are due largely to an increased capillary permeability which can be demonstrated directly by very sensitive dermal tests using Evans blue. In this case 0.05 ml of protein solution containing 1 μ g nitrogen is sufficient to elicit a response (Halpern, 1956).

Due to its similarity to anaphylaxis the syndrome was classified as anaphylactoid (Leger, Masson and Prado, 1947) and is considerably attenuated by synthetic antihistaminic drugs (Leger and Masson, 1947). Corticosteroids give no protection in intact nor in adrenalectomised animals but adrenaline is highly effective in both cases (Halpern and Briot, 1950). The action of ovomucoid on the rat is often attributed to its ability to release histamine (Schoachter and Talesnick, 1952; Halpern, 1956) but it is not identical with an anaphylactic reaction as it can be distinguished in dermal tests from the results of a classical antigen-antibody reaction (Roche e Silva, 1956). In addition, prior administration of reserpine, which depletes the animal of 5-hydroxytryptamine, or bromo-lysergic acid diethylamide (BOL 148) a specific antagonist to 5-hydroxytryptamine, both prevent the anaphylactoid response to ovomucoid or to dextran (Parratt and West, 1957). It is thus likely that the effects are due more to released serotonin than to released histamine. It has recently been shown that in some strains of rat a recessive mutation in an autosomal gene has the effect of preventing the anaphylactoid reaction. Pure strains of non-reacting rats have been propagated (Harris, Kalmus and West, 1963).

8. Comparative Biochemistry of Avian Egg White Ovomucoids

Ovomucoids prepared from egg whites from eleven different species by adsorption and elution from CM and DEAE cellulose columns under approximately the same conditions as for chicken ovomucoid (see page 78) had similar total nitrogen contents (11-13%) and were very low (less than 1%) in tryptophan (Rhodes et al, 1960). Their inhibitory activities towards trypsin and chymotrypsin, however, showed some

striking differences and resulted in the classification of the different species into classes which inhibited respectively per mole of ovomucoid (a) one mole of trypsin but not chymotrypsin, (b) one mole of chymotrypsin but not trypsin, (c) one mole of trypsin and one mole of chymotrypsin, either separately or simultaneously and (d) two moles of trypsin and one of chymotrypsin, either simultaneously or separately. For duck ovomucoid (class d) the inhibition of either trypsin or chymotrypsin is quite independent of the presence of the other protease, indicating that the combining sites are specific and separate. Acetylation or carbamylation of turkey ovomucoid (class c) does not affect its antichymotryptic activity, as is the case for the anti-tryptic activity of chicken ovomucoid (class a) and the anti-chymotryptic activity of pheasant ovomucoid (class b). However, anti-tryptic activity of turkey ovomucoid is rapidly lost on blocking of the free amino groups, providing further evidence for the independence of the different protease-specific sites on the ovomucoid (Stevens and Feeney, 1963).

Egg whites of the various species investigated (Feeney et al, 1960) differed in ovomucoid content from 11% (chicken) to 30% (cassowary). Egg whites of both members of the order Casuariiformes, emu and cassowary, were exceptionally rich in ovomucoid and in total antitryptic activity (not all of which was accounted for as ovomucoid). The sialic acid content of the ovomucoid from these two species was very high (emu 10.5%, cassowary 5.4%), and ovomucoid from the sialic acid poor egg whites of two members of the order Anseriformes, duck and goose, on the other hand had undetectable or no sialic acid.

B. Gel Filtration Studies on Ovomucoid

1. Introduction

On passage through a column of cross-linked dextran (Sephadex) a mixture of substances of different molecular sizes will emerge in descending order of size, i.e. the larger the molecule the less will be its elution volume. A molecule which is totally excluded from the gel will emerge in the void volume (V_0) of the column (the void volume is the volume of interstitial fluid which fills the space between the gel grains). On the other hand a molecule whose diffusion is not at all hindered by the gel matrix will emerge with an effluent volume equal to the total volume of water in the column (i.e. the void volume plus the water imbibed by the gel grains).

Consideration of the results of gel filtration in other applications (for example Lindner et al, 1959) suggested a method of isolating ovomucoid as the trypsin complex in a highly purified form. If the crude ovomucoid preparation were fractionated and the fraction with molecular weight 25,000 to 30,000 retained (and similarly with crude trypsin), then on gel filtration of a mixture of ovomucoid and trypsin so purified the fraction with molecular weight 50,000 to 55,000 should represent pure complex. If the crude ovomucoid (or the trypsin) contains any impurities with a molecular weight of about 25,000 these must be separated from the complex (as must any excess of either component of the complex) if the method is to succeed. Thus the gel filtration conditions must be such that a protein of M.W. 25,000 is well resolved from one of 50,000.

Even when complexed with ovomucoid, trypsin is not completely devoid of activity, but it is virtually inactive - and possesses maximum stability - in slightly acid solution. Gel filtration was therefore performed at pH 5. Ammonium acetate buffer at an ionic strength of 0.1 was used throughout the series of experiments unless otherwise indicated.

Two preparations of ovomucoid were used. One was prepared by the method of Lineweaver and Murray (1947), and the other by CM- and DEAE-chromatography according to Rhodes et al (1960; see page 78). A few other well-characterised proteins were used as markers. Ovalbumin was crystallized at its isoelectric point from egg white 50% saturated with ammonium sulphate, and recrystallized twice (Warner, 1954); chymotrypsinogen was obtained from L. Light & Co Ltd., γ -globulin (7 S from horse) was kindly donated by Mr. R. Weir; trypsin was obtained from Novo Industries, Denmark. The Sephadex (G 75, pulverized, and G 100, bead form, both of medium particle size) was obtained from Pharmacia Ltd. The G 100 was further fractionated by dry sieving and the fraction between 50 μ and 100 μ (100 to 300 mesh, which accounted for 75% of the material) was used. The Sephadex was conditioned for use by soaking in buffer for at least two days, and degassed on the water pump before being poured into columns. For packing purposes columns were fitted with extension tubes and filled with buffer. The gel particles were permitted to settle through the buffer with the outlet closed until a considerable layer had packed down at the bottom. With the outlet open the packing was continued until the column was virtually full. Closing the outlet and removing the extension tube rendered the column

ready for use. To improve resolution with the G 100 three columns were used in series. The columns (150 cm x 5 mm) were all filled as described above. The outlet of the first was then connected to the bottom of the second so that the material travelled up this column, the top of which was connected to the top of the third column, the effluent being finally collected in the usual way. The column was treated with a silicon anti-wetting agent before use. The thoroughly cleaned tubes were filled with a solution (5% v/v) of M.S. 1170 in methyl ethyl ketone. After a brief soaking the solution was run out and the tubes cured at approximately 100° for 30 minutes.

3. Results

A column (150 cm x 1 cm, $V_t = 120$ ml) was filled with G 75 Sephadex and protein samples (10 mg) were applied in 0.5 ml. By comparing elution volumes from separate runs it was seen that trypsin and chymotrypsin emerged at about the same point and the ovalbumin slightly ahead. Application of a mixture of ovalbumin and trypsin yielded a slight separation, the ovalbumin emerging as a shoulder on the leading edge, and the trypsin at the peak, of the eluted material. To improve resolution further, by reducing vertical diffusion of material, a column of small cross-sectional area (and increased length) was required.

The 450 cm x 0.5 cm column ($V_t = 105$ ml) was thus devised and samples were applied in volumes of 0.25 ml. With this column packed with G 75 good resolution of chymotrypsinogen and ovalbumin was obtained. When ovomucoid was passed through this column it emerged slightly earlier than the ovalbumin did. The 450 cm column clearly showed that despite its lower molecular weight ovomucoid was excluded

from Sephadex G 75 to a greater extent than ovalbumin, and was in fact totally excluded from this gel. Both ovomucoid preparations emerged as a single symmetrical peak and thus neither appeared to contain significant amounts of substantially smaller molecular weight contaminants. This unexpected exclusion of ovomucoid meant that the original idea of isolating pure complex would probably not be possible, but it was now of interest to see how far ovomucoid would be excluded from gels of larger pore size and whether the material would still give a symmetrical peak on a gel where the ovomucoid did not penetrate the beads.

The 450 cm column was thus repacked with Sephadex G 100. Application of a mixture of chymotrypsinogen and ovalbumin showed that G 100 gave a better resolution of these two proteins than did G 75. Ovomucoid emerged from this column as an almost symmetrical peak at a position a little ahead of that occupied by ovalbumin. The differences in elution volume for these two glycoproteins was too small to give a separation when the two were run together. Their exact relative location was determined by running each one together with a marker of chymotrypsinogen or 7S γ -globulin. Besides the first run of ovalbumin and chymotrypsinogen, mixtures of ovalbumin and 7S γ -globulin, ovomucoid and chymotrypsinogen, and ovomucoid and 7S γ -globulin were also applied to the column. These experiments also demonstrated that the elution volume for a given protein was highly reproducible and not affected by the presence of other proteins. The position of trypsin in this system was also determined. A mixture of ovomucoid and 7S γ -globulin was also run in 0.05 M Tris buffer, pH 8,

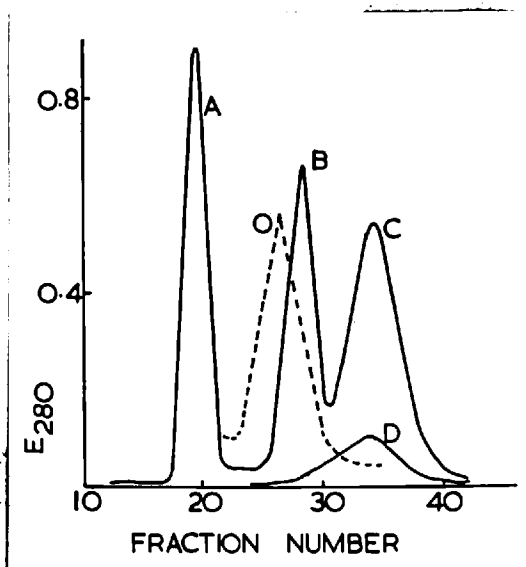


Fig 18. Gel Filtration of Ovomucoid and Other Proteins on Sephadex G 100

O - ovomucoid; A- γ -globulin
B- ovalbumin; C - chymotrypsinogen
D - trypsin.

The fig. is compiled from 4 runs as follows: O+A; O+C; B+A; B+C.
Column - 450 x 0.5 cm. Sample vol. 0.25 ml. Fractions - 2 ml.

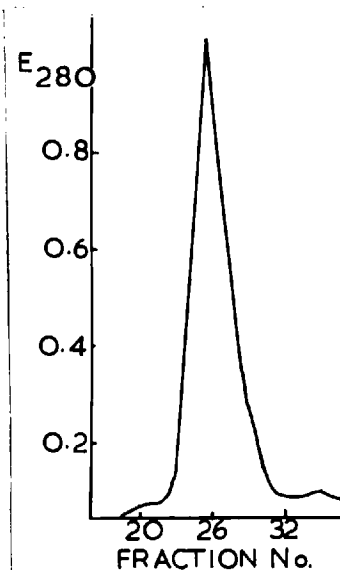


Fig. 19. Gel Filtration of Ovomucoid plus Trypsin on G 100

Column etc. as for fig. 18.

10 mg ovomucoid + 5 mg trypsin
Applied in 0.25 ml.

TABLE IX. Properties of Proteins Used in Gel Filtration Study

Protein	Peak Vol. ml.	Molecular Wt $\times 10^{-5}$	Diffusion Coefficient $\times 10^7$	Sedimentation Constant $\times 10^{15}$
γ -globulin	39	140	4	7
ovomucoid	53	28	6.1-7.7	2.6
ovalbumin	56	44	7.5	3.5
chymotrypsinogen	68	25	10	2.6

plus M NaCl and it was found that over this range their elution volumes were quite independent of pH.

The results for the gel filtration of ovomucoid and the marker proteins on Sephadex G 100 are superimposed in fig. 18. The figure is compiled from four separate runs. In addition the elution diagram of trypsin is also superimposed. In table IX the elution volumes of the proteins are tabulated together with their known physical characteristics.

The final experiment in this series was to run a mixture of 10 mg ovomucoid and 5 mg trypsin in 0.25 ml on the ^{cm} 450 column to ascertain where the complex ran relative to the excess ovomucoid. A single symmetrical peak resulted (fig. 19) with almost no material eluting in the trypsin position (a useful criterion of purity for active trypsin, as only trypsin complexes with ovomucoid) but the complex did not separate from the excess ovomucoid. Ovomucoid contains almost no tryptophan and the ratio E_{280}/E_{295} in the ultraviolet extinction spectrum is 6.6. With trypsin, on the other hand, due to the presence of tryptophan this ratio is 2.14. For fraction 24 from this column the ratio E_{280}/E_{295} was 3.52 and for fraction 28 it was 4.2. The trypsin (i.e. the complex) was thus present largely in the leading part of the peak and the excess ovomucoid predominantly in the trailing part, but the resolution was too poor to be of any preparative value.

4. Discussion

In general terms the elution volume of a substance on gel filtration is dependent on its molecular size and, most probably, to some extent on its shape. For a homologous series of substances - with

the same general shape, density (or partial specific volume) and degree of hydration - the size, or gyration radius, correlates fairly accurately with the molecular weight. Homologous series of this nature whose behaviour on gel filtration have been studied are dextrans of increasing chain length and globular proteins of roughly spherical shape. In these cases elution volumes (V_e) have been found to bear a strict relationship to molecular weight, a plot of V_e/V_0 against $\log$ molecular weight gives a good linear relationship (Granath and Flodin, 1961; Whitaker, 1963; Roubal and Tappel, 1964; Andrews 1964)*.

* For Sephadex G 75 and G 100 all theoretical treatments are consistent with a simple all-or-none exclusion effect operating in the gels. The gel matrix can be considered as consisting of two phases, one where the macromolecule is free to diffuse, and one where it is prohibited. The relative proportion of the two phases in the gel for a given solute is the factor which determines its elution position and is dependent upon the molecular size of the solute. In the available regions the solute is considered to behave approximately in the same manner as under conditions of free diffusion in a liquid, and the transition to prohibited area is very abrupt with negligible gel present of an intermediate type. However in Sephadex G 200 a second effect of steric and frictional interaction of the solute molecules with the gel matrix plays an important role (Ackers, 1964). In this case in large parts of the available regions of the gel the macromolecules have a restricted diffusion, which can be considered by reference to a model in which these regions are represented by uniform cylindrical channels with a radius of the same order of magnitude as that of the macromolecule.

Restricted diffusion of a similar nature to that in G 200 is also probably the operative factor in the behaviour of macromolecules in gelatin and agar gels as studied by Allison and Humphrey (1960). These authors pointed out that one important difference between free diffusion and the diffusion they were observing was that the effect of shape (or axial ratio) on the two processes differed. They postulated that the use of their gels gave information other than the simple diffusion coefficient. If molecules with similar diffusion coefficients (i.e. in free solution) have similar end points by their method, it is likely that they have similar sizes and shapes. The effect of shape on G 100 chromatography, where diffusion is either free or prohibited, may therefore be significantly different from the effect of shape on the, predominantly, restricted diffusion process of G 200 chromatography. This may account for the apparently anomalous behaviour of lysozyme and cytochrome c when their behaviour on the two types of gel is compared. On G 100 cytochrome c behaves normally but lysozyme does not (Whitaker, 1963), whereas on

A theoretical formula has been deduced relating the degree of exclusion of a molecule to its molecular weight (Porath, 1963).

Application of this treatment to the results Granath and Flodin (1961) obtained on gel filtration of a series of dextrans, showed a very close agreement between the predicted and the observed results. The central assumption in this treatment that the radius of gyration of the macromolecules is proportional to the square root of their molecular weights should theoretically only be true for randomly coiled macromolecules and not for globular proteins (Laurant and Killander, 1964). However, Andrews (1964) showed that globular proteins give as good a fit to the theoretical expectation as dextrans do.

Applying the empirical relationship between elution volume and log. molecular weight (Whitaker, 1963 and Andrews, 1964) to the above results gives the line shown in fig. 20. The results of other authors are also drawn on the graph for comparison. The differences in slope and height of the lines are probably due to slight differences in water regain values of the different batches of gels used and to differences in particle size as both factors affect the elution volume. On the basis of these results, ovomucoid behaves as a globular protein of molecular weight 48,500. Whitaker (1963) reported an apparent molecular weight from gel filtration on G 100 for ovomucoid of 45,000.

Previously reported cases of unexpected exclusion of macromolecules from Sephadex (e.g. tectonic acids, Critchley et al, 1962)

G 200 the reverse situation pertains (Leach and O'Shea, 1965). The above considerations may also lead to an extension of Allison and Humphrey's conclusion, to the effect that if molecules behave similarly on G 100 and G 200 they have similar shapes and sizes.

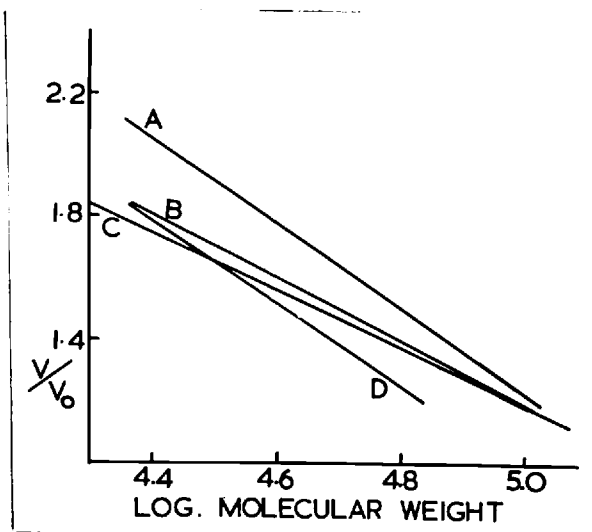


Fig. 20 Elution Volume (V) of Globular Proteins per Unit Column Volume on Sephadex G 100 vs. log Molecular Weight

For reasons of clarity the points have been omitted.

A - Andrew (1964); B - Whitaker (1963); C - Roubal and Tappel (1964); D - present study.

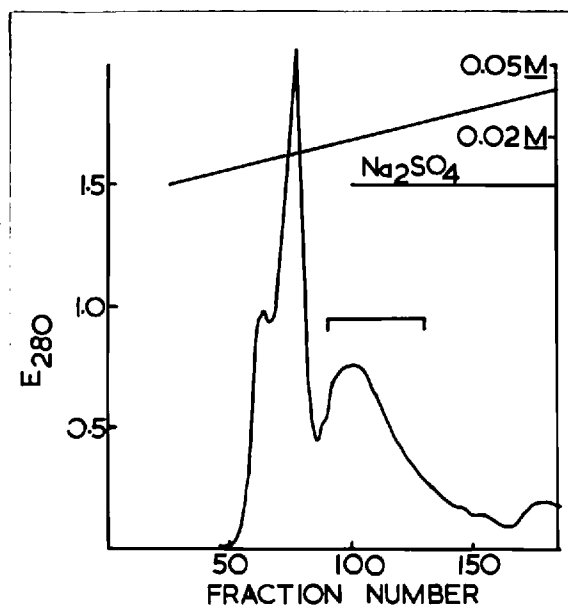


Fig. 21 Chromatography of Crude Ovomucoid on DEAE-cellulose

Sodium sulphate concentration gradient elution in pH 7.0 phosphate buffer (0.002 M). Column, 2.6 x 49.5 cm; fractions, 10 ml, 2 g ovomucoid (Preparation I) applied. Antitryptic activity all in peak under bracket.

have been explained partly by the fact that the dextran chains of Sephadex have a small content of acidic groups which may repel strongly negatively charged molecules. The apparently anomalous exclusion of ovomucoid, however, cannot be explained as a charge effect, for, on gel filtration at pH 3.5, where the protein is virtually uncharged or slightly cationic, ovomucoid behaves exactly as it does at pH 5 or pH 8. This exclusion relative to globular proteins is more likely to be accounted for by the relatively large proportion of carbohydrate in the molecule. Dextrans are far more excluded from Sephadex than proteins of the same molecular weight. Sephadex G 75 when calibrated with dextrans has a lower molecular weight limit for complete exclusion of about 40,000 (manufacturers information and Granath and Flodin, 1961) whereas with proteins the corresponding figure is about 150,000 (Andrews, 1964). Similarly with Sephadex G 100 the corresponding figures for carbohydrates and proteins respectively are approximately 100,000 and 300,000. These figures however are obtained at the limit of the resolution of Sephadex and are subject to considerable variation. A more accurate comparison can be obtained from the behaviour of ribonuclease (M.W. 13,700) and the heaviest of Granath and Flodin's (1961) dextran fractions which are both eluted in virtually the same position near the middle of the range of Sephadex G 75. The number average molecular weight of the dextran fraction is 3,180 or 4.3 times smaller than the molecular weight of ribonuclease*.

* This would explain the very good separation on gel filtration between glycopeptides and carbohydrate-free peptides (Marks et al, 1962)

The most likely explanation for the greatly expanded structure, of carbohydrates in solution when compared with proteins is that they are highly hydrated and carry a layer of water around each polysaccharide chain. The absence of helical structures and hydrophobic regions in polysaccharide molecules also tends to result in more expanded overall structures than that usually found with the more highly organised protein molecule. That carbohydrates bind considerable amounts of solvent water has been shown by equilibrium dialysis (Ogston and Phelps, 1961) by osmotic pressure measurements (Laurent and Ogston, 1963) and by solubility studies (Laurent, 1963), whether in solution or set in a gel (Laurent and Killander, 1964). The low partial specific volume of carbohydrate (0.61; Granath, 1961) relative to proteins (0.73) may also be related to this difference in affinity for water. Probably the interior of most protein molecules is not accessible to solvent and they thus displace more volume than carbohydrates where the whole macromolecule is hydrophilic and does not possess a hydrophobic or inaccessible central region.

Ovomucoid has a partial specific volume which lies between the usual value for carbohydrate and protein (0.68-0.7) and would thus appear to have an affinity for water intermediate between the two extreme cases. This assumption would account for other exceptional physico-chemical properties of ovomucoid such as high resistance to precipitation by protein precipitants or by heat. The unexpected behaviour of ovomucoid towards denaturing agents has previously been noted in connection with optical rotatory dispersion studies and led Jirgenson et al (1960) to postulate a central core of protein surrounded by a shell of carbohydrate.

As the relation between molecular volume (or elution position on gel filtration) and molecular weight is different for carbohydrate and protein, calibration of gel filtration columns with neither type of macromolecule will allow predictions to be made for the behaviour of glycoproteins. From experiments on the behaviour of proteins on columns of granulated agar gel, Polson (1961) concluded that the degree of separation of proteins on gel filtration is largely dependent upon the differences in diffusion coefficient and to a lesser extent on differences in molecular weight. Laurant and Killander (1964) provided a novel theoretical approach to gel filtration based on the assumption that the molecular radius derived from the diffusion constant by Stokes Law is the appropriate size parameter which determines the elution volume of a substance. As the results for dextrans and proteins fit their behaviour as predicted by this theory equally well it is worth a detailed consideration here.

The rate that a substance moves down a gel column is dependent upon the fraction (K_{av}) of the gel phase into which the substance can readily diffuse. For the experimental determination of K_{av} the elution volume (V_e) of the substance, the total volume (V_t) of the gel bed and the void volume (V_o) of the column must be determined. K_{av} can then be derived from the relationship

$$K_{av} = \frac{V_e - V_o}{V_t - V_o} \quad (1)$$

For a gel of dextran chains in the form of straight rigid rods of infinite length randomly distributed, Ogston (1958) has calculated theoretically the fraction available for spherical particles of

known radius (r_s). Applying this formula Laurant and Killander (1964) arrived at the following theoretical expression for K_{av}

$$K_{av} = e^{-Lr} (r_s + r_r)^2 \quad (2)$$

where L is the concentration of rods in the gel (cm rod/cc) and r_r is the radius of the rod. This radius (r_r) was derived as 7.10^{-8} cm, and the value of L was calculated from the water regain of the gels used. It was found to be related to the concentration (C) in g/ml of dextran in the gel by the simple expression $L = 4.C \times 10^{11}$ cm/cc. It is thus possible to calculate the K_{av} of a substance on Sephadex assuming its radius (r_s) is the radius of an equivalent sphere derived from the diffusion constant by Stokes formula ($D = RT/6 \eta rN$). The authors compared K_{av} values thus calculated with the values obtained experimentally and found a very good fit both for proteins and for carbohydrates.

This suggests that the property of a molecule measured by its diffusion constant gives a better guide to its behaviour on gel filtration than does its molecular weight. Conversely, gel filtration would give more reliable information about the diffusion constant of a substance than of its molecular weight.

If equations (1) and (2) are combined and the relevant constants substituted, an equation can be derived relating the elution volume (V_e) of a substance on a gel column directly to its diffusion constant. For the 450 cm column of Sephadex G 100 described above, $V_t = 105$ ml, $V_0 = 38$ ml, $L = 2.9 \times 10^{12}$ cm/sec, $r_r = 7 \cdot 10^{-8}$ cm and the equation becomes

$$V_e = 67(e^{-9.1 \times 10^{-4}(7+213/D)^2}) + 38 \text{ mls,}$$

where D is the diffusion constant $\times 10^7$. For chymotrypsinogen which has a diffusion constant of 9.5×10^{-7} , the predicted V_e is 68 ml and for ovalbumin ($D = 7.5 \times 10^{-7}$) 57 ml, both in very good agreement with the values obtained experimentally (see Table IX).^{p101} Using the formula the other way round ovomucoid (V_e 53 ml) gives a diffusion constant of 6.35×10^{-7} . This is approximately in accord with one recently reported value (6.01, Deutsch and Morton, 1961) but differs considerably from another (7.7, Rhodes et al, 1960); which makes it difficult to draw firm conclusions until this discrepancy in the published diffusion constants is resolved.

Addendum In a further study of the behaviour of proteins and glycoproteins on gel filtration (Andrews, 1965) it was found that most glycoproteins (ovalbumin being an exception) are excluded to a greater extent than would be expected on a basis of molecular weight. However, on the basis of diffusion coefficients they do conform moderately well.

C. Isolation and Characterization of Ovomucoid

1. Introduction

All previous methods for isolation of ovomucoid from egg white have included either precipitation of contaminant proteins at pH 3.5 or chromatography on an acid resin which involved similarly low pH solutions. As one of the outstanding points of interest about this glycoprotein is its heterogeneity, in which connection the rather acid labile sialic acid may be of importance, it seemed worth while to seek a method of isolation which involved a minimum exposure of the material to acidic conditions. In addition, it had not been

shown that any of the well established methods of isolation of ovomucoid yielded pure products (except perhaps for the method of Feeney et al (1963) which appeared after this work was started).

2. Preparation of Crude Ovomucoid

(a) Preparation I. A large batch of crude ovomucoid (Preparation I) was isolated from egg white from which avidin and lysozyme had been isolated as described in Part I. The egg white (from Light Sussex-Rhode Island hybrid hens) was originally diluted with an equal volume of ion-free water and centrifuged. After three batch treatments with CM cellulose, ammonium sulphate was added to the final supernatant to half-saturation. The mixture was left in the cold overnight and then acidified to pH 4.6 with dilute sulphuric acid. The well-stirred mixture was left in the cold for a further 24 hours and then filtered on a Büchner funnel. To 25 l. of filtrate was added 7.2 kg ammonium sulphate which was sufficient to saturate it at that temperature (5°). The mixture was again well stirred and allowed to settle over two days in the cold. The precipitate was filtered off, redissolved by the addition of the minimum quantity of distilled water and desalted on Sephadex G 25. The fractions containing protein were pooled and freeze-dried. This preparation contained nearly all the proteins of egg white except for avidin, lysozyme and most of the ovalbumin. By antitryptic assay it was found to consist of about 30% ovomucoid, and was used as a starting material for most of the chromatographic investigations on ovomucoid described below.

(b) Sample prepared by the Lineweaver and Murray Method

Pooled white from 36 large, fresh eggs was mechanically stirred and the acetone-trichloroacetate reagent (1 part 0.5 M trichloroacetic

acetone plus 2 parts acetone) added until the pH was 3.5 (pH meter). After a further 30 minutes stirring the mixture was left overnight at 5°. It was centrifuged in the cold and the ovomucoid was precipitated from the supernatant with 2½ volumes of acetone. The precipitate was collected by centrifugation and redissolved in a minimum quantity of water. On dialysis some precipitation occurred and the material was cleared by centrifugation before freeze-drying. The final yield was 5 g.

3. Antitryptic assay

Activity of trypsin was assayed by the method of Erlanger et al (1961) with DL-benzoylarginine p-nitroanilide (BAPA) as substrate. The BAPA was prepared by method (b) of Erlanger et al (1961) by coupling 2 g of benzoylarginine with p-nitroaniline in the presence of concentrated phosphoric acid and phosphorus pentoxide in diethyl phosphite. The racemised phosphorylated intermediate was converted directly to the crystalline hydrochloride which was isolated. Recrystallization from 20% v/v acetic acid in the presence of charcoal yielded 2 g (60% yield) of BAPA (m.p. 258 - 260°) and faint yellow in colour.

Trypsin hydrolyses L-BAPA to benzoyl arginine and p-nitroaniline. As the nitroanilide has an absorption maximum at 315 mμ, the release of nitroaniline (λ_{max} = 380 mμ) can be readily followed. This is done at 410 mμ, as there is still some absorption by anilide at 380 mμ. To illustrate the inhibitory effect of increasing amounts of ovomucoid on the activity of trypsin by this method a substrate was prepared by diluting 1 ml of a solution (40 mg/ml) of BAPA in dimethylsulphoxide to 50 ml with 0.05 M tris buffer (pH 8.2) containing

0.02 M CaCl_2 ; the BAPA is first dissolved in the organic solvent and then diluted with water as it is only sparingly soluble in water. To a series of tubes each containing 1 ml substrate, increasing amounts of solution containing 0 to 10 μg of a crude ovomucoid preparation was added. Trypsin solution (0.1 ml, containing 5 μg) was then added to each tube and after 25 min at 24° the reaction was stopped by the addition of 0.2 ml 30% (v/v) acetic acid and the O.D. at 410 $\text{m}\mu$ was recorded in 2 cm cuvettes. The activity was found to decline linearly with increasing concentration of ovomucoid over the range of the test.

For routine assay of antitryptic activity, e.g. of column effluents, the substrate was made up freshly by mixing 10 ml 0.1 M tris buffer pH 8.2; 0.4 ml M CaCl_2 ; 0.2 ml of a solution of 40 mg/ml BAPA in dimethylsulphoxide; and 10 ml of water. This substrate was always maintained at 37° as the BAPA has a tendency to precipitate out even at room temperature. To 1 ml of substrate the desired amount of unknown or standard ovomucoid was added followed by trypsin, usually 5 or 10 μg . After incubation at 37° , usually for about 10 mins, reaction was stopped with 0.2 ml of 30% (v/v) acetic acid and the extinction at 410 $\text{m}\mu$ was recorded. The amount of the sample which would be required to inhibit a given amount of trypsin could thus be ascertained.

4. Methods for Further Purification of Ovomuroid and/or Fractionation into Multiple Components

(a) Use of DEAE Sephadex

(1) Methods and Materials The cross-linked dextran, "Sephadex" is available with ionic substituents attached to the chains, such as the diethylaminoethylether "DEAE-Sephadex". This material differs from DEAE-cellulose in that: (i) its total ion-exchange capacity is four to five times greater, and (ii) the possibility exists of obtaining a separation by the processes of ion-exchange chromatography and gel filtration acting simultaneously, thus giving a better fractionation than by either method alone. Two forms of DEAE-Sephadex are available. The more highly cross-linked (A-25) tends to exclude molecules larger than about M.W. 10,000, and thus the less cross-linked (A-50), where the ionic groups inside the gel are accessible to much larger molecules, was used in the present investigation.

The DEAE-Sephadex was prepared for use by first allowing it to swell for two days in distilled water, and then decanting several times to remove the fines. The material was cycled through the hydrochloride form with 0.5 N HCl (treatment with HCl being as short as possible to prevent hydrolysis of glycosidic bonds of the dextran); and then through the free base form, with 0.5 N NaOH, before equilibrating with the starting buffer. A pH of 5.0 was originally chosen as the ovomucoid should be anionic at this pH but most other proteins in the mixture (e.g. conalbumin) would pass directly through the resin. In the course of the investigation, it

became apparent that very small variations in pH and ionic strength of the ammonium acetate buffer had a marked effect on the behaviour of the proteins on the column. The following method was thus used to prepare accurate ammonium acetate buffers. A stock solution of acetic acid was prepared and standardized against sodium hydroxide. To prepare a buffer the required amount of this solution was titrated with ammonia to pH 7.0. This solution was then brought to the required pH by the addition of acetic acid. This yielded a buffer where concentration of ammonium ion was exactly known and hence the ionic strength.

(11) Results and Discussion At pH 5.0 in 0.10 ionic strength buffer it was found that much of the protein of the crude ovomucoid preparation (Preparation I) passed through the column unadsorbed and that this fraction did not contain ovomucoid. On continued elution with the same buffer an active ovomucoid peak emerged followed by a second inactive peak. Application of a gradient of NaCl at this stage eluted two to three more peaks, all of which had no antitryptic activity. It thus appeared that, as far as the ovomucoid was concerned, a reasonable distribution coefficient existed between the resin and the eluting buffer, fulfilling the requirement of true partition chromatography and no gradient was required in this region of the elution. There was no suggestion of a resolution of different components with this system nor was the ovomucoid very well resolved from inactive components. The ionic strength of the eluting buffer was thus reduced in subsequent columns to obtain a greater degree of adsorption of the ovomucoid. An ionic strength of 0.09 instead of

0.100 resulted in a better spread of the first three peaks. A further reduction of the ionic strength to 0.08 brought about still more separation of the peaks.

The material from the active peaks of these chromatograms was not extensively investigated, but on an E₂₈₀ basis its antitryptic activity was considerably higher than that of the standard ovomucoid preparations of Lineweaver and Murray (1947) or Fredericq and Deutsch (1949). The procedure appeared therefore to offer a simple method for obtaining ovomucoid in a relatively pure state. To prepare this ovomucoid in a more convenient amount the whole procedure was repeated on a larger scale. The above experiments were performed on 0.9 x 33 cm columns and the larger scale column was 2 x 40 cm. This column was used with the same starting material (Preparation I) and buffer (ammonium acetate, I = 0.085, pH = 5.0) but did not give comparable results. The proportion of the second (active) peak was much less than previously and the third peak had become virtually a trail of the second. In experiments intended to elucidate this anomaly the volume of solution in which the sample was applied was found to have some effect on the results, but this could not account for the failure to reproduce the original results on a larger scale. The system was critically affected by small changes in conditions and it appeared as if there was some variable factor (perhaps concerned with the pretreatment of the resin or the packing of the column) which could not be traced. As a result of this method was not pursued further.

(b) Use of DEAE-Cellulose

(1) Methods and Materials Chatterjee and Montgomery (1962) described the chromatography of crude ovomucoid (Lineweaver and Murray,

1947) on DEAE-cellulose. In the present study, in view of the erratic results with DEAE-Sephadex, chromatography of ovomucoid on DEAE-cellulose was attempted along the lines of the above method.

DEAE-cellulose (Whatman DE 50, total exchange capacity approximately 1.0 meq/3) was conditioned for use by soaking in 1 N NaOH and then filtered on a Büchner funnel (no paper required). The material was washed with additional 1 N NaOH until no more coloured material was removed, and then resuspended in 1 N HCl. It was filtered immediately, washed with water on the Büchner funnel and then resuspended in 1 N NaOH. It was filtered again and washed with water until free from alkali. The cellulose was then resuspended in water and the suspension adjusted to pH 7.0 with NaH_2PO_4 solution (0.2 M) on the pH meter. The suspension was degassed, allowed to settle and decanted to remove fines. Settling and decanting was repeated a few times using 0.002 M phosphate buffer, pH 7.0, as the suspending medium and the material was finally packed into a column and the 0.002 M phosphate buffer (pH 7.0) run through overnight.

(11) Results and Discussion Chatterjee and Montgomery (1962)

equilibrated the DEAE-cellulose and adsorbed the ovomucoid onto the column in phosphate buffer ($I = 0.005$, pH = 7.0). Elution was effected by a linear gradient (the slope of the gradient was not reported) of sodium sulphate from zero to 1.0 M in 0.05 M phosphate buffer (pH 7.0). In the present investigation 0.002 M phosphate was used as the equilibrating and adsorbing buffer, and crude ovomucoid (Preparation I) as the starting material. After adsorption of the protein the column was washed with about one hold-up volume of the starting buffer of the gradient (0.05 M buffer pH 7.0) which eluted all the ovomucoid activity with the front. This indicated (1) that in the experiments of

Chatterjee and Montgomery (1962) the gradient was superfluous and the starting buffer was sufficient to bring off the ovomucoid; or (ii) that the DEAE cellulose used by the above authors (Brown Company, Type 40, exchange capacity 0.88 m eq/g) bound protein considerably more strongly than the Whatman material (see however, page 120); or (iii) that there is a misprint in the publication.

The peak eluted from DEAE-cellulose with the front of the 0.05 M phosphate buffer (pH 7.0) was very sharp and contained about 70% of the E_{280} absorbing material applied to the column. It could be seen by eye that it contained iron-conalbumin (pink colour, $\lambda_{\max}$ 470 m μ) and its antitryptic activity indicated a content of about 40% by weight of ovomucoid. It was thus clear that very little resolution of the ovomucoid was taking place, but merely its elution with the buffer front. As a result the experiment was repeated with the salt gradient applied in the dilute phosphate buffer (0.002 M) instead of in the more concentrated one (0.05 M) which is itself capable of eluting ovomucoid and its associated proteins from the resin.

The first peak of the above column was therefore pooled, dialysed against 0.002 M phosphate buffer (pH 7.0) and applied to a column equilibrated with the same buffer. Washing with the 0.002 M phosphate buffer did not bring off any material. The gradient of sodium sulphate was then applied in this buffer at a slope of 1.25 mmoles/cm (up to 0.2 M). This brought off a single sharp peak almost immediately, which comprised virtually all the E_{280} absorbing material applied to the column. The front part of the peak contained the iron-conalbumin but it was not at all separated from the ovomucoid. The

peak was again pooled, dialysed against 0.002 M phosphate buffer (pH 7.0) and reappplied to the column. Elution was performed as for the previous column except that the slope of the gradient of sodium sulphate was reduced to 0.3 mMoles/cm and only taken as far as 0.05 M. The iron-conalbumin was now partially resolved as a shoulder on the leading edge of the peak and the antitryptic activity emerged as the trailing edge of the same peak. This represented the best resolution of the ovomucoid on DEAE-cellulose at this stage but it was still only about 50% pure on a weight basis. Another chromatogram was run with Preparation I as starting material and eluted in the same way as the one just described. The results were very similar and the region of the peak containing the ovomucoid was pooled and used later for chromatography on CM cellulose (page 121).

In the next column a still shallower slope was used and a modified procedure was adopted for packing the column (Porath and Hjerten, 1962). The column (2.6 cm diameter, 55 cm long) was extended with a 50 cm length of tubing (joined by binding with tape) and fitted with a large filter funnel similarly joined to the top. The column was filled with 0.002 M phosphate buffer, pH 7.0, and the outlet closed. A dilute suspension of the DEAE-cellulose (pretreated and equilibrated as described above) was poured into the funnel. The contents of the funnel were mechanically stirred by means of a rod which projected several cm into the extension tube. When the cellulose had percolated through the buffer and a layer had settled on the bottom of the column the outlet was opened and the packing was continued with the buffer flowing. Stirring was maintained continuously

and the level of buffer in the funnel replenished if necessary.

When the cellulose had packed down in this way into a column about one and a half times the length of that finally required the stirrer and funnel were removed and the column finally packed down under approximately 10 p.s.i. pressure.

Crude ovomucoid (2 g of Preparation I) was applied to the column in 0.002 M phosphate buffer (pH 7.0). Elution was effected by a gradient of sodium sulphate (0.13 mMoles/cm) in this buffer at a flow rate of approximately 0.2 cm/min; 10 ml fractions were collected. This resulted first in the elution of a sharp but inactive peak followed by the ovomucoid (fig. 21^{p.105}). In this column the ovomucoid was clearly resolved from the inactive material as a second peak, but there was no suggestion of a resolution of the ovomucoid into its several components.

In the following chromatograms of ovomucoid on DEAE-cellulose NaCl was used as the eluting salt and ovomucoid partially purified by the Lineweaver and Murray (1947) method was used as starting material. Using Whatman's DEAE-cellulose and a NaCl concentration gradient of 0.5 mMole/cm to 0.1 M in phosphate buffer (pH = 7, I = 0.02) one peak was eluted which contained a shoulder of inactive material on the leading edge and two shoulders on the trailing edge. The peak and trail were antitryptic. A second gradient up to 0.5 M NaCl did not elute any further material. Two other commercially available DEAE-celluloses (Cellex-D, fine grade supplied by Bio-Rad, kindly given by Miss E.M. Press; and Sigma Chemicals' fine grade) which were both much finer in particle size than the Whatman's were tried out under the above conditions. However, both adsorbents gave essentially the same results as

the Whatman product, the ovomucoid being eluted as an irregular, assymetric peak only partially resolved from inactive impurity and without any resolution of the different components of ovomucoid.

(c) The Use of CM-cellulose

During the fractionation of total egg white protein (Rhodes et al 1958) as well as in the course of subsequent work in the same laboratory (Rhodes et al, 1960 and Feeney et al, 1963) ovomucoid was chromatographed on CM-cellulose. The consideration that absorption onto the acidic surface of a carboxyl resin may catalyse the hydrolysis of sialic acid residues from ovomucoid resulted in a hesitation to use it in the present study (cf. Chatterjee and Montgomery, 1963). However the unrewarding results obtained with basic ion exchange resins led to a reinvestigation of its use, despite this possibility. The method described by Feeney et al (1963) appeared to yield ovomucoid least contaminated by other proteins and was used as the starting point for the following study.

Ovomucoid from a DEAE-cellulose column (page 119) was dialysed against 0.1 M ammonium acetate buffer pH 4.0 and then applied to a CM-cellulose column (1.5 x 34 cm) equilibrated with the same buffer. All the material was adsorbed under these conditions, washing with 100 ml of the starting buffer bringing off no 280 mμ adsorbing material. A gradient of 0.1 M ammonium acetate from pH 4.0 to pH 4.6 over 300 ml brought off a peak with a slight suggestion of consisting in fact of two components running very close together. The whole peak showed high antitryptic activity. A more highly purified ovomucoid preparation (the pooled active peak from fig. 21) was employed for the next chromatogram on the same column. The pH gradient from pH 4.0 to

pH 4.6 was this time applied in a total of 450 ml. The material eluted from this column emerged as two partly overlapping peaks which both had high antitryptic activity. The ovomucoid was this time clearly resolved into two components. When this procedure was applied to samples of ovomucoid obtained by methods other than chromatography on DEAE-cellulose, however, the same result was not obtained. A sample prepared by the method of Lineweaver and Murray (1947) yielded only one peak of active material with a slight shoulder on the leading edge. On some occasions only a single symmetrical peak was obtained. Even the crude preparation of ovomucoid which had been used as starting material for the DEAE-cellulose chromatography did not result in two active peaks under the above conditions.

(d) Aminoethylated Ovomucoid

(1) Introduction The study of the S- β aminoethylcysteinyl derivative of ovomucoid was undertaken for two reasons. Chromatography of ovomucoid on ion exchange celluloses had proved to be particularly unrewarding and it was hoped that the fully unfolded protein would evince a more satisfactory chromatographic behaviour. Secondly the unfolded, reduced and alkylated derivative could be used for structural studies, employing enzymic or chemical rupture of bonds. With a highly crosslinked protein, rupture of a few bonds (e.g. the two methionine residues in the protein) may not produce separate fragments if they are still held together by disulphide bridges.

The sulphydrylblocking agent, ethyleneimine, was introduced by Raftery and Cole (1963) as a more efficient method of producing the S- β -aminoethylcysteine (AEC) derivative than the bromoethylamine reagent

previously employed (Cavallini et al, 1955; Lindley, 1956). After treating a reduced preparation of insulin B chain with ethyleneimine, amino acid analysis of a hydrolysate yielded 91% of the cystine as AEC (Raftery and Cole, 1963). No other amino acid was modified by the reagent, with the possible exception of phenylalanine (88% recovery). Recently Hofmann (1964) treated chymotrypsinogen with bromoethylamine under milder conditions than those used by previous workers. Amino acid analysis showed complete conversion of the cystine to AEC. Recoveries of other amino acids were quantitative except for phenylalanine (88%) and histidine (84%). The cationic nature of the new side chain raises the isoelectric point of the protein. In the case of ovomucoid this facilitates chromatography of the protein at a pH near its isoelectric point which is desirable for separation of closely related species.

(ii) Preparation β -Mercaptoethanol (Eastman Kodak) was redistilled immediately before use and collected under nitrogen. Guanidine hydrochloride was prepared by neutralizing the "Analar" carbonate salt with HCl, evaporating to dryness, and recrystallizing from methanol containing 10% v/v concentrated HCl. The buffer used was 2-amino-2-methyl propane 1:3 diol-HCl (Raftery and Cole, 1963; abbreviated hereafter to AMFD). The buffer solution (5 M) was prepared by adding solid amine to a solution of the acid to prevent the formation of carbonates. It was stored in a flask with a ground glass neck, fitted with a soda lime tube. Ethyleneimine was obtained as a colourless liquid from Light & Co. in sealed ampoules and was used without further purification. For reasons of safety it was

dispensed with an all glass syringe and never by pipette. Ovomucoid prepared by the method of Lineweaver and Murray (1947) from fresh large eggs (yield from 48 eggs, 7 g) was used as starting material.

The method adopted for reduction of ovomucoid in quantities up to 100 mg. was derived by slight modification of that of Crestfield, Moore and Stein (1963) which is in turn very similar to that of Anfinsen and Haber (1961). Guanidine hydrochloride was used in place of urea as it is a more efficient denaturant and free from undesirable side reactions. A smaller volume of reaction mixture was used (4.25 ml instead of 12 ml), AMFD buffer was used in place of "tris" and reduction was allowed to proceed for 24 hours instead of 4. To 100 mg ovomucoid in a screw-topped glass bottle (capacity 4.25 ml) the following additions were made in the order stated: guanidine hydrochloride solution (8 M , 3.2 ml), EDTA solution (50 mg/ml Na_2 salt, 0.2 ml), AMFD buffer (5 M , pH 8.6, 0.3 ml). The solution was degassed in a vacuum desiccator to remove dissolved oxygen and mixed. Freshly distilled β -mercaptoethanol (0.2 ml, a 50 times molar excess over the disulphide on the protein) was then added. The bottle was filled to the brim with distilled water, a disc of "polythene" slid over the top to exclude air and the lid screwed securely down to seal the bottle. The solution was mixed by repeated inversion and then allowed to stand at room temperature for 24 hours. The contents were then transferred by means of an all glass syringe to a flask containing 14 ml AMFD buffer (5 M , pH 8.6, representing 25 moles buffer per mole mercaptoethanol) and 1.6 ml ethylenimine (13 moles per mole mercaptoethanol) and allowed to stand at room temperature for 30 min. The solution was finally dialysed overnight in the cold room on a rocking dialyser

against distilled water. The dialysed solution was freeze-dried to yield the aminoethylated ovomucoid.

For preparation of the ovomucoid derivative, on a larger scale, guanidine hydrochloride solution (8 M , 3 ml) was added to 2 g ovomucoid in a specimen bottle, and the mixture allowed to stand overnight, for complete dissolution to take place. The clear solution was transferred to a dialysis bag of 4/32 Visking tubing with a total capacity of about 5 ml. The specimen bottle was washed out twice with 0.25 ml EDTA solution (50 mg/ml Na_2 salt) the washings being added to the contents of the bag. The bag was tied off, enclosed in a nylon mesh "sock" and placed in a screw cap bottle of 30 ml capacity. Mercaptoethanol solution (100 ml) was prepared by adding 10 ml AMFD buffer (5 M , pH 8.6) and 5 ml EDTA solution (50 mg/ml, Na_2 salt) to 57.3 g guanidinium chloride. Water was added to a volume of 93 ml and the solution was transferred to a screw-capped bottle. Nitrogen was bubbled through for an hour, freshly distilled mercaptoethanol (7 ml) was added and the bottle securely closed. The bottle containing the dialysis bag of protein solution was then filled with mercaptoethanol solution, securely closed and left at 37° overnight. The outside solution in this bottle was changed three times at 24 hour intervals and the bottle stored at all times at 37° . The dialysis bag was finally removed and its contents added to 25 ml AMFD buffer (5 M , pH 8.6) containing 2.5 ml ethylenimine. The mixture was allowed to stand for 30 min at room temperature and then dialysed and freeze dried as for the small scale preparation.

(iii) Chromatography of Aminoethylated Ovomucoid The

approximate isoelectric point of the modified ovomucoid was first determined by electrophoresis on cellulose acetate strips. A sample of serum was run concurrently on an adjacent strip to serve as a set of markers. In borate buffer at pH 8.9 the protein moved towards the anode with a mobility about the same as that of serum β -globulin. In maleate buffer at pH 6 the protein was distinctly cationic with a greater mobility than that of γ -globulin. By intrapolation the isoelectric point of the protein was assessed to be about 8.5.

Chromatography was performed exclusively on CM-cellulose. The material was adsorbed at pH 6.0 in phosphate buffer ($I = 0.02$). Elution with a concentration gradient of this buffer to $I = 0.2$ produced some resolution of the material but no clear fractionation into discrete peaks. Elution of material adsorbed in the same way with an increasing concentration of "tris" buffer at pH 8.0 did not effect any improvement in resolution. When the material was applied to the column in 0.1 M "tris" buffer pH 8.0 one peak emerged unadsorbed followed by two small peaks. Subsequent application of 1% ammonium carbonate solution eluted the fourth and largest peak. On application of the starting material in 0.1% ammonium bicarbonate solution most of it was again unadsorbed. Subsequent elution with a concentration gradient of ammonium bicarbonate from 0.1% to 0.5% eluted a few small, poorly resolved peaks. A very similar result was obtained by application of the protein in 0.1% ammonium bicarbonate (pH = 8.3) and elution with a gradient of increasing pH to 0.5% ammonium carbonate solution, pH = 9.0. Similar experiments at pH 8.5

and pH 9.0 only served to confirm that the ovomucoid derivative required a pH well below 8.0 in order to adsorb onto the CM cellulose.

For the next column the starting material was therefore applied in sodium phosphate buffer pH 6.0, $I = 0.02$. Even here some material passed directly through the column. The column was then washed with 0.1% ammonium bicarbonate solution (pH = 8.3) until the pH of the effluent was 8.3. It was found that the pH of the effluent rose rapidly to about 9.0 where it stayed for some time before reaching equilibrium at pH 8.3. Two small peaks were eluted by this operation. Application of a concentration gradient of ammonium bicarbonate from 0.1% to 1.0% eluted a number of poorly resolved peaks. On testing the fractions with orcinol reagent the first of the peaks eluted with the gradient, as well as the unadsorbed material, were found to contain hexose. Glycylglycine ($pK = 3.2$) was also tried as eluting buffer for the aminoethylated ovomucoid. Protein was adsorbed in phosphate buffer pH 6.0, $I = 0.02$ and the column then washed with glycylglycine buffer 0.01 M, pH 8.0 until the effluent pH was 8.0. Elution was continued with a concentration gradient in glycylglycine buffer from 0.01 to 0.1 M. However a very poor resolution resulted, the material merely leaching out continuously.

Consideration of all the above results led to the conclusion that adsorption of the modified protein onto CM cellulose at pH 6.0 in phosphate buffer followed by elution with an ammonium bicarbonate concentration gradient appeared to be the most promising system. The next series of chromatograms were thus devoted to ascertaining the optimum conditions for this system in respect to the loading of the column, the

pH and slope of the eluting gradient etc. The pH of a freshly prepared solution of ammonium bicarbonate was found to be very close to 8.3 at any concentration between 0.1% and 1%. However, it is an unreliable buffer and on standing the pH gradually increases to about 8.7 in a few days. As a result 0.02 M "Tris" at pH 8.3 was introduced as buffer and elution was effected with an increasing concentration of ammonium bicarbonate in this buffer. Ammonium bicarbonate solutions were always freshly prepared on the day they were used. The pH overshoot in the effluent when ammonium bicarbonate (pH 8.3) was applied to a column equilibrated with sodium phosphate (pH 6.0), was probably due to the phosphate being washed out rapidly but the sodium being only slowly displaced with ammonium ions. As a result, ammonium phosphate buffer at pH 6.0 was employed in place of the sodium phosphate, and this did in fact eliminate the pH overshoot on equilibration with the higher pH buffer. The ammonium phosphate buffer was prepared from diammonium phosphate solution adjusted to pH 6.0 with phosphoric acid. The concentration used for preliminary equilibration of the column and application of the protein was 0.02 M in respect of ammonium ion.

The method finally evolved will be described on the scale used for chromatography of 200 mg of aminoethylated ovomucoid. The column (2 cm x 50 cm) was equilibrated with ammonium phosphate buffer pH 6.0, 0.02 N in ammonium ion. Starting material (200 mg) was applied in this buffer, and a further 300 ml of the same buffer passed through the column. "Tris" buffer (0.02 M, pH 8.3) was then applied to the column and after the passage of 250 ml a gradient of ammonium bicarbonate from 0.2% to 1.0% in 960 ml of the tris buffer was applied. The column was finally eluted with the tris buffer containing 1% ammonium carbonate

plus M Na_2SO_4 to bring off the last peak. The fractions collected (5 ml) were assayed for E_{280} and hexose by the orcinol reaction. Selected tubes at the protein peaks were also used for sialic acid determination. The results for this column are represented in fig. 22. The fractions indicated in the figure were pooled, dialysed and freeze dried.

Analysis of Aminoethylated Ovomucoid. Fraction III (7.5 mg) and fraction IV (17.4 mg) were analysed for their amino acid composition. Hydrolysis (in constant boiling HCl) was performed in well evacuated sealed tubes. The evaporated hydrolysate was analysed on the Technicon amino acid column using the micro bead column (page 55). Aminoethylcysteine was eluted between ammonia and lysine. The colour yield for pure AEC was taken from Hofmann (1964). The results are set out in table XII (page 160). On all amino acid charts cystine was totally absent. Oxidation by performic acid resulted in a very small peak eluting with the solvent front but as repeated reduction and alkylation did not diminish the size of this peak it was taken to be some oxidation product other than cysteic acid.

From the results (page 160) it appears most likely that peak IV does in fact represent ovomucoid. The high values for isoleucine and methionine, however are difficult to understand. In the case of peak III on one of the three analyses carried out a small additional peak appeared near the AEC, and the size of the AEC was about 65% of that in the other two charts. If the small peak represents some impurity which was not always separating from the AEC the value in the table may be too high. In this case it should be about 6 residues of AEC per

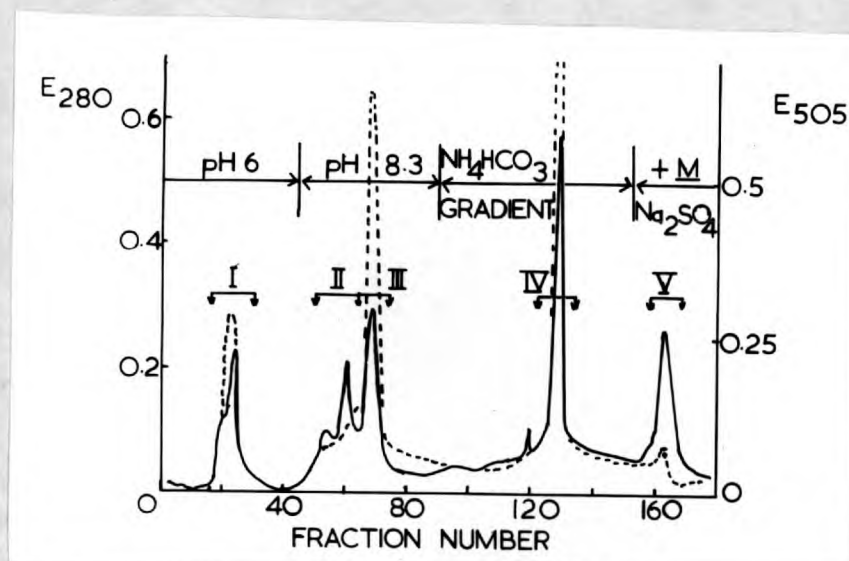


Fig 22 Chromatography of AEC-ovomucoid on CM-cellulose

200 mg applied in pH 6.0 phosphate buffer (0.02 M), pH raised with tris buffer (0.02 M), NH_4HCO_3 gradient and M Na_2SO_4 elution in the tris buffer
 — E_{280} ---- Hexose (orcinol reaction)

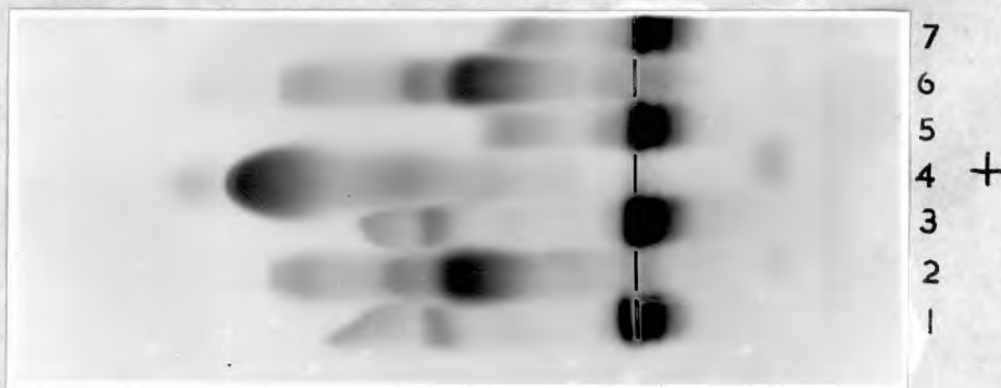


Fig. 23 Starch Gel Electrophoresis of Ovomucoid and the Trypsin Complex

Na acetate buffer pH 4.6; $I = 0.01$; 25 ma; 16 hours

1,3 ovomucoid - Lineweaver and Murray (1947; 2LM); 2 1M ovomucoid plus trypsin

5,7 ovomucoid - Neuburger and Papkoff (1963; NP) 4 Trypsin

6 NP ovomucoid plus trypsin

28,800 g. Insufficient material was available for reliable quantitative carbohydrate analyses on these materials. However, hexose, hexosamine and sialic acid were all found to be present in both materials.

(e) Analytical Starch Gel Electrophoresis of Ovomucoid

(1) Introduction Starch gel electrophoresis (Smithies, 1955) possesses a greater ability to separate complex protein mixtures than any other method, with the possible exception of electrophoresis in the recently introduced polyacrylamide gels. The degree of separation obtainable is particularly striking with groups of proteins which are basically identical but differ either in the degree of polymerisation (e.g. haptoglobins, Smithies, 1959b) or in the number of charges carried (e.g. orosomucoid, Schmidt, 1962). These two cases illustrated the two factors which principally govern the behaviour of protein in the gels, viz. size and net electrophoretic charge (cf. Smithies, 1962).

The major application of starch gel electrophoresis has been in the field of serum proteins. The technique has been used for separation of egg white proteins (Lush, 1961) but under these conditions ovomucoid was not resolved from ovalbumin and could not be identified on the gels. In addition, difficulty with staining the ovomucoid by the usual Naphthalene Black dye complicated the study by this method. Similar difficulties in location of ovomucoid on starch gels by the usual staining procedure have been reported by other workers (Feeney et al, 1963).

(11) Methods and Materials The apparatus for starch gel electrophoresis consisted of trays, slot-former lids and buffer reservoirs made to the specifications of Smithies (1959a) for vertical electrophoresis. Electrodes were of platinum wire and the power supply was a full wave rectifier of variable power output (without provisions for constant current or constant voltage operation). Partially hydrolysed starch suitable for gel electrophoresis was obtained from Connaught Medical Laboratories, Toronto. Two preparations of ovomucoid were employed. One was prepared chromatographically according to the method of Rhodes et al (1960) and was similar to the material described by Neuberger and Papkoff (1963). The other was a commercial preparation obtained from Light & Co. and was prepared by the method of Lineweaver and Murray (1947). Agarose was obtained from Seravac Laboratories Ltd. and had been prepared according to Russell et al. (1964). The preparation (batch 21) contained a small amount of denatured protein-chlorophyll complex. It was found that this could be removed by preparing a solution of the agarose (0.8%) by heating in a boiling water bath in 0.01 M sodium acetate buffer (pH 4.5). The solution was then placed in a 45° bath and after about an hour the contaminating material came out of suspension as a flocculant precipitate. This was removed by centrifuging at 45° and a clear colourless supernatant remained. This was allowed to cool and reheated in a boiling water bath for 10 minutes when required. Trypsin was obtained from Novo Industries, Copenhagen, and the other egg white proteins used as markers were prepared when needed. Ovalbumin was kindly donated by Dr. R.D. Marshall and was preparation P (Johansen et al., 1961). Lysozyme was a by-product of the avidin preparation and was prepared

by CM cellulose chromatography and recrystallized according to Fevold and Alderton (1949). Conalbumin (iron complex) was isolated and recrystallized according to Warner and Weber (1951). The dye Eriochrome Black T was obtained from E. Gurr Ltd. Ethylene glycol was purified by shaking with "Biodemimrolit" mixed bed resin (5% w/v) and charcoal, filtered and redistilled. Sodium acetate buffers at pH values between 4 and 5 were used throughout. To prepare buffers of accurately known ionic strength a stock solution of 5 N acetic acid was prepared and standardized. For the preparation of a buffer the requisite quantity of acetic acid was pipetted out, diluted to nearly the required volume and then titrated with sodium hydroxide to the theoretical end point on the pH meter using the relation $\text{pH} = \frac{1}{2}(\text{pK}_w + \text{pK}_a + \log c)$. The pH of the solution was then brought down to the required value with acetic acid and the buffer finally diluted to volume.

For the preparation of the gels, starch (60 g) was added to 500 mls of buffer in a 2 l round bottomed flask and heated on a naked flame with constant swirling, past the point of maximum viscosity and until the suspension became fluid again (Smithies, 1959b). The temperature was kept below 80° and the suspension was thoroughly degassed by boiling for a minute or two on the water pump before pouring. The slot-former lid was laid down on the hot gel (which completely filled the tray) and kept down by weights. The gel was left to cool at room temperature and was used 6 to 16 hours after pouring. The usual method of applying the sample is to place a solution in the slots and seal them with molten petroleum jelly. This method suffers from the disadvantages (a) that when the current

is first switched on, the protein concentrates at the face of the gel and, unless the slot is very accurately levelled, the dense layer of protein at the top of the slot decants to the bottom producing spreading of the band; and (b) protein solution is liable to leak out between the gel and the tray or past the petroleum jelly onto the surface of the gel. For preparative purposes where a slot the full width of the gel was used these drawbacks became serious and another procedure was devised for application of the sample into the gel.

A gel of agarose (0.8%) was prepared in 0.01 M acetate buffer pH 4.5 and rendered fluid by heating in a boiling water bath for at least 10 minutes. The sol was then placed in a 45° bath, at which temperature it remained fluid for several hours. To the protein solution at 45° an equal volume of agarose sol was added and, after mixing in the 45° bath, the solution was pipetted into the slot, where it solidified almost immediately. As the agarose gel at this concentration (0.4%) provides very much less resistance to migration of protein than does the starch, the band-sharpening effect observed when the protein is applied in solution is not diminished by this procedure. On the other hand, electrodecantation and leaking are completely prevented, and this method of applying samples was used routinely.

The exposed surface of the gel was sealed with a thin sheet of "polythene" and the gel subjected to electrophoresis in a vertical position by immersing the bottom end directly into the lower reservoir and connecting the top end by a filter paper wick to

the upper buffer reservoir (Smithies, 1959a). Electrophoresis was performed for about 12 to 20 hours taking care that the total power supplied to the gel was not too high, so as to cause heating of the gel above about 30°. It was found that about 3.5 watts was the maximum that the gel could dissipate without exceeding this temperature. After the run the gel was removed from the tray, sliced with a thin taut wire and the two halves separated. A report that Eriochrome Black T was a useful protein dye (Clark, 1962) led to its trial on starch gels. It was employed in the same manner as had been developed for Naphthalene Black (Smithies, 1955). The gel was soaked in a saturated solution of the dye in a methanol:water:acetic acid mixture (5:5:1) for 30 min and then washed with the methanol:water:acetic acid mixture until the background was clear (2 to 4 hours minimum but preferably overnight). This resulted in very satisfactory and permanent staining of all proteins used in these studies, and gave very low background staining. Gels could be preserved in this fluid in sealed containers or, preferably, photographic records could be made. An approximately 4% protein solution diluted with an equal volume of agarose solution produced optimum loading of the gels and each slot in the analytical gels held 0.07 to 0.08 ml of solution. For a better demonstration of the minor components of the ovomucoid preparation this loading was doubled. The single full-width slot used in the preparative gels had a capacity of 0.8 ml and 30 mg of ovomucoid was applied in this volume of the usual 0.4% agarose solution.

(iii) Results and Discussion For the first series of experiments the conditions of Bier et al (1953) for free boundary electrophoresis were applied to the starch gels. Sodium acetate buffer was used for preparing the gels and for filling the electrode vessels. The same pH was used throughout but a higher buffer concentration was employed in the electrode vessels to reduce pH changes resulting from electrolysis. Electrophoresis was performed at about 150 volts across the whole system for about 16 hours. It was found that, in general, good resolution of the different components of ovomucoid by starch gel electrophoresis required the same conditions as did free boundary electrophoresis; viz. low ionic strength and approximately isoelectric pH. The best results were obtained at pH 4.65 and ionic strength 0.01. The requirements of the system were found to be fairly critical, good resolution only occurring over a pH range of ± 0.1 of a unit and the ionic strength had to be controlled to within approximately ± 0.002 . The apparatus contains two buffer reservoirs at each end of the gel, connected by suitable wicks. The one reservoir contains the electrode and the other is connected to the gel. At first all chambers were filled with 0.1 M sodium acetate buffer. In later experiments better results were obtained by using 0.3 M buffer in the electrode vessels and 0.05 M buffer in the vessels directly in contact with the gel.

Under optimum conditions both ovomucoid preparations gave two major, and one or two minor bands moving towards the anode and fairly evenly spaced. The intensity of staining (and hence presumably the proportion in the starting material) was highest for the

slowest moving band and decreased rapidly towards the anode, the fastest moving band (the fourth one) only being visible in heavily loaded applications. This pattern was virtually identical for the two ovomucoid preparations but the cathodically migrating contaminants differed considerably. To ascertain whether the group of evenly spaced anodic bands were all ovomucoid, samples of the starting material were mixed with a slight excess of trypsin and then electrophoresed. All the negatively charged material in both ovomucoid preparations now disappeared and a diffuse band appeared at a position intermediate between that of the trypsin and that of the original ovomucoid bands. ^{P130} (fig. 23). Thus all anodic bands in the original gels were ovomucoid and the complex with trypsin is stable even at pH 4.6 (cf. Sri-Ram et al, 1954^a). A second new band, smaller and more cathodic than the main new band also appeared. This material probably corresponded^a with the (trypsin)₂-ovomucoid complex reported by Sri-Ram et al (1954). Two of the cathodic contaminants of the Lineweaver and Murray preparation of ovomucoid were identified by the use of known markers as lysozyme and conalbumin, but a few other minor bands remained unidentified. Under the conditions used, ovalbumin was found to be well resolved from ovomucoid and not coincident with it as in the work of Lush (1961).

Although the multiple forms of ovomucoid were demonstrated by the appearance of banding, resolution was rather poor and attempts were made to improve this by the use of gels containing urea, which has been shown in other cases to improve resolution of materials on starch gels (Smithies, 1962 and Cohn and Porter, 1964). Electro-

phoresis of ovomucoid in the presence of high concentrations of urea posed a special problem, however, due to the very narrow range of conditions of pH and ionic strength over which good resolution was attainable. The small amount of ammonium cyanate formed from the urea in solution, particularly on heating, and the ammonium carbonate produced by hydrolysis of the cyanate, as well as the ammonia resulting from the biuret reaction, all result in increase in pH and ionic strength of the solution (see Marier and Rose, 1964). All these reactions produce ionic species which reduce the electrical resistance of the solution. To measure the total sum of these processes a Wheatstone bridge type conductivity meter was used with a standard conductivity cell. The cell was not calibrated to give absolute conductivities. All measurements were taken in the same cell, and as they were only for comparative purposes, the results were recorded directly as resistance (in ohms, Ω) of the solution in the standard cell. As a standard of reference, the resistance of sodium acetate buffer (pH 4.6) was measured at different concentrations.

A solution of urea was prepared (9.5 M) without heating. The pH was found to be 10 and the resistance 8,000 Ω . The pH was then brought down to 4.5 with acetic acid (resistance was now 4,000 Ω) and the solution immersed in an 80° bath. Changes in temperature, pH and resistance with and without the presence of Zeocarb 225 H⁺ resin (2% w/v) were followed with time (see fig. 24). Without the resin present the final resistance was equivalent to a 50 mM sodium acetate buffer (about five times that used in the gels); but in the presence of the resin the rise in pH and drop in resistance

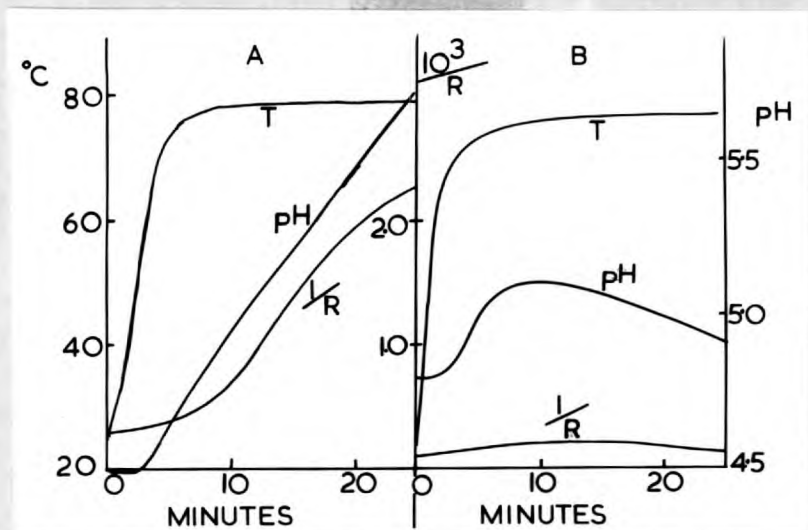


Fig. 24 Effect of Heating 9.5 M Urea in an 80° Bath
T, temperature; R, electrical resistance in the
standard cell ($1/R$ is a measure of conductivity).
A - no further additions; B - in the presence of 2%
w/v Zeocarb 225 H⁺ resin.

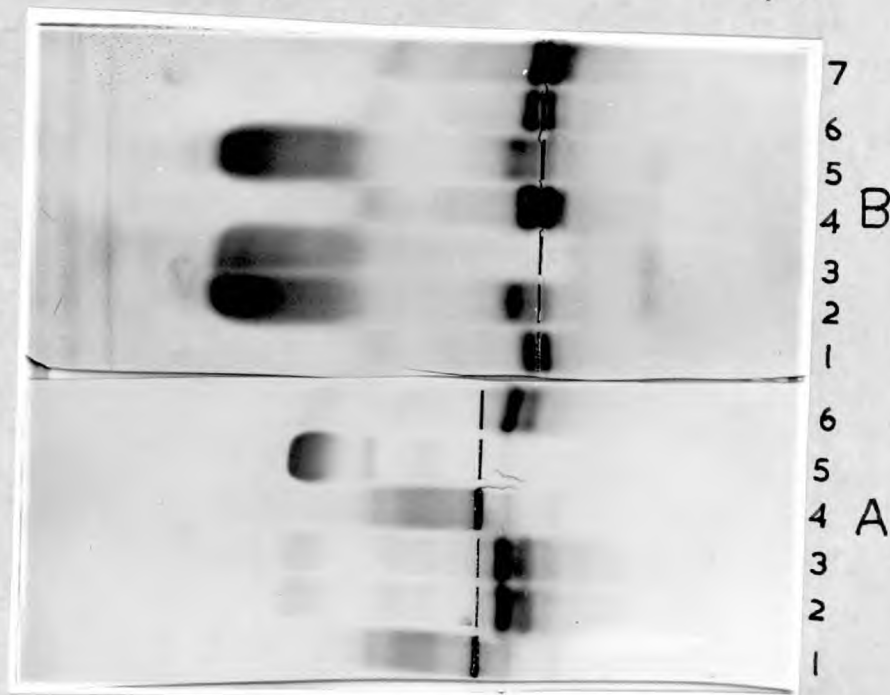


Fig 25. Starch Gel Electrophoresis of Ovomucoid and the
Trypsin Complex in the Presence of Urea. A - 8 M urea;
I = 0.008; pH = 5.0; 23 ma; 150 v; 20 hr.; 1, 4 -
ovalbumin; 5, lysozyme; 2,3,6 ovomucoid. B - 6 M urea;
pH = 4.5; I = 0.01; 1,4,6,7 ovomucoid; 3 trypsin
2,5 ovomucoid plus trypsin.

were reduced to negligible proportions. A modified procedure was thus adopted for preparations of gels containing 8 M urea, whereby resin is present in the mixture as long as any heating is required, but is removed before addition of the buffer. To prepare 500 ml (sufficient for one tray) of gel containing 8 M urea and 12% starch, urea (288 g) was stirred with 150 ml water containing 2 g of Zeocarb 225 H⁺ resin (16-50 mesh) and heated until completely in solution (approximately 60°). Starch (72 g) was suspended in 146 mls of water in a 2 l round bottom flask and the warm urea solution, containing the suspended resin, was added with vigorous swirling. The mixture was then heated in an 80° water bath with constant swirling for 10 minutes, and cooled in a water bath at room temperature for 20 to 30 minutes. In the presence of urea the gel is slow to set and remained fluid at this point even though the temperature had dropped to 40°. The resin was now filtered off through a muslin cloth and, as a check, the resistance was always measured at this stage and was usually 4,000 to 6,000 Ω in the standard cell. To 450 ml of this solution (five-sixths of the total volume) 50 ml of sodium acetate buffer (I = 0.10) was added, the solution well mixed, degassed and finally poured into a tray and allowed to set overnight. The final pH of the solution was always found to read approximately 0.8 of a unit higher than that of the buffer solution added. A buffer of pH 4.0 was thus added to obtain gels with a pH of 4.8, which was found to give the best results. Gels containing 6 M urea were prepared similarly.

The final resistance of the urea gel after addition of the buffer was approximately 2,8000 Ω , more than twice as high as that of

aqueous gels containing the same concentration of buffer. This meant that a higher voltage could be applied across the urea gel for the same total power. The loading of the slots was done as described previously, i.e. in 0.4% agarose and without any urea. A potential of about 200 volts was applied to the gels for 16 to 20 Hours. The buffer boxes were filled with sodium acetate buffer ($I = 0.1$) at the same pH as the gel but without urea. Subsequent slicing, staining and photography of the gels was as for those without urea.

As in the case with the aqueous gels, in the presence of urea, ovomucoid only showed clear banding over a narrow range of conditions. The ionic strength again had to be about 0.01 and the best results were obtained between pH 4.5 and 5.0 in 6 or 8 M urea. To obtain bands with the same apparent electrophoretic mobilities the gels containing urea required a pH 0.2 of a unit higher than in the absence of urea. The pattern of the bands and their relative staining intensity was not affected by the presence of urea; but the bands were much sharper.

It has long been known that there is a marked increase recorded on the pH meter when urea is added to a buffer. In the course of the present study when acetic acid was titrated in 8 M urea an apparent pK of 5.4 was obtained (compared with 4.76 in water) and with borate a similar increase in the recorded pH at half neutralisation was observed from 9.2 in water to 10.1 in 8 M urea. In this connection a recent study of the pH of urea solutions is of great interest (Bull et al, 1964). With half-neutralized solutions of p-nitrophenol and

acetic acid and with very dilute KOH solutions, an increase of about 0.8 of a pH unit was consistently observed in 8 to 9 M urea. With very dilute HCl solutions this increase was 1.63 of a pH unit and with half-neutralized ammonium hydroxide 0.3 of a unit. However, when determined by conductance methods the pK_a s of acetic acid, p-nitrophenol and ammonia were found to be 0.9, 1.0 and 0.6 of a pH unit lower respectively in the presence of 9.44 M urea than in water. The authors concluded that urea increases the dissociation of weak acids and increases the ionization of water. They postulated that the urea drastically reduced the activity of hydrogen ions, leaving the activities of other ions more or less unchanged. As a result it is not possible to predict what the isoelectric point of a protein really is in 8 M urea, but it is probably significantly lower than in water. The fact that in the presence of urea a slightly higher pH (0.2) was required to obtain the same apparent electrophoretic mobility is probably due to the urea-containing gel presenting a considerably higher resistance to the movement of the protein band.

Trypsin is not enzymatically active in the presence of 6 M urea (Harris, 1956) and as the active site appears to be necessary for complex formation with ovomucoid (see introduction) it was of interest to investigate the effect of urea on the complex. The gels containing urea afforded an opportunity of doing so. Preformed complex (containing excess trypsin and in the absence of urea) was placed in the slots of a 6 M urea gel (in the usual 0.4% agarose gel) and electrophoresed in the usual manner. Comparing these results (fig. 25) with a similar experiment without urea (fig. 23) the complete absence of

the ovomucoid-trypsin complex in the presence of 6 M urea is apparent. This confirms the general conclusion that only enzymatically active trypsin is capable of forming complexes with naturally occurring trypsin inhibitors. It further demonstrates that 6 M urea will bring about dissociation of the enzyme-inhibitor complex. The odd appearance of the ovomucoid may be due either (i) to the complex first migrating cathodically into the gel and then dissociating, leaving the ovomucoid significantly displaced although the displacement of the trypsin is not observable; or (ii) to the ovomucoid being modified by the trypsin during the process of association and dissociation (see page 92).

For electrophoresis of ovomucoid, the chief advantage of the gels containing urea was the greater compactness and clarity of the bands, ie the reduction in diffusion relative to electrophoretic mobility. This may have been due merely to the presence of a high concentration of solute in the aqueous phase of the gel and not to any specific effect of the urea on the protein. Other relatively inert solutes such as glycerol, sucrose and ethylene glycol were tried and found to be free of undesirable side reactions and much simpler to use than urea. Gels were prepared in the usual way with the addition of the required amount of the particular solute. A gel containing 12% starch, 30% (v/v) glycerol and 0.008 M sodium acetate buffer at pH 4.7 gave a final resistance of 4,200 Ω in the standard cell and was electrophoresed with the buffer boxes containing sodium acetate buffer pH 4.7, $I = 0.3$. An overall voltage of 240 v. produced a current of 13.5 ma (3.24 watts) and gave a good resolution

With discrete bands in 12 hours. Sucrose (50% w/v) and 8% starch in 0.008 M sodium acetate buffer pH 4.7 gave a good gel with a final resistance in the conductivity cell of 5,500 Ω . An overall potential of 360 v at a current of 10 ma (3 watts) was applied for 16 hours, but there was very little movement of the protein bands. This retardation of electrophoretic mobility appeared to be a function of the molecular size of the added solute and ethylene glycol was thus tried in an attempt to combine the beneficial effect of a large non-aqueous phase in the gel with the minimum retardation effect.

Ethylene glycol was employed at a concentration of 40% v/v of the aqueous phase together with 12% starch and sodium acetate buffer, I = 0.008, at pH values between 4.6 and 4.9. The optimum pH value was found to be 4.85. The two reservoirs in direct contact with the gel contained 0.05 M sodium acetate buffer as well as 40% v/v ethylene glycol and the electrode compartments contained 0.3 M aqueous sodium acetate buffer, all at the same pH as the gel. These gels had a resistance under standard conditions of about 5,000 Ω and an overall potential of 230 v (resulting in a current of 13 ma, or 3 watts) was applied to the gel for 20 hours. As with all the above gels the slicing, staining and washing of the gels was performed in the usual way. As can be seen from fig. 26 the presence of ethylene glycol in the gels resulted in a considerable sharpening of the bands but permitted much less movement of the bands from the slots. The general pattern of the bands and their relative staining was again the same with or without the ethylene glycol, as was also the case for glycerol and sucrose. The various conditions employed for starch gel electrophoresis of ovomucoid are summarized in table X.

Table X Conditions Employed for Starch Gel Electrophoresis

Starch Conc'n	Additional Solute	Resistance in standard cell (ohms)	Solution in Electrode vessels	Solution in buffer boxes	Potential (volts)	Current (m.amps)	Ionic strength of gel	pH of - gel
12%	- (Analytical)	1,200	0.1 M	0.1 M	150	20	0.01	4.6
12%	- (Preparative)	1,000	0.3 M	0.05 M	150	25	0.015	4.6
12%	8 M urea	2,800	0.1 M	0.1 M	200	15	0.01	4.85
12%	30% v/v glycerol	4,200	0.3 M	0.3 M *	240	13.5	0.008	4.7
8%	50% w/v sucrose	5,500	0.3 M	0.1 M	300	10	0.008	4.7
12%	40% v/v ethylene glycol	5,000	0.3 M	0.05 M [†]	230	13	0.008	4.85

* plus 30% w/v sucrose † plus 40% v/v glycol

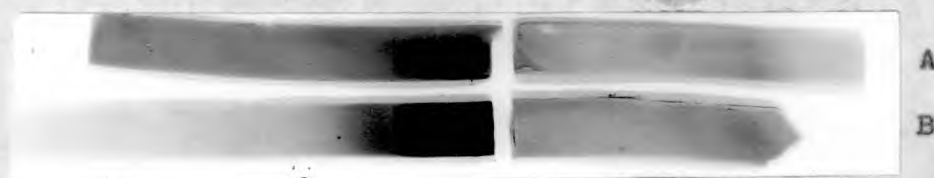


Fig. 26 Effect of Ethylene Glycol on starch gel electrophoresis of ovomucoid. A - pH, 4.55; I, 0.012; + 40% v/v ethylene glycol, 20 hrs, 13 ma, 230 v. B - Typical guide strip for standard aqueous preparative starch gel (see text)

(f) Preparative Starch Gel Electrophoresis

(1) Methods and Materials With added experience, and as a result of small technical modifications, complete separation of the different bands could be obtained with the standard aqueous starch gels without the addition of a large non-aqueous phase. Although the aqueous gels resulted in far greater spreading of the bands they were easier to use and resolution was sufficient to suggest its development into a preparative method. Of the two methods for mechanical disruption of the gel, maceration and freezing and thawing, Smithies (1959b) preferred the second, but even here the recovery was very poor and the isolated material was heavily contaminated by soluble starch. Moretti et al (1958) introduced the use of a second electrophoresis for eluting material from isolated segments of starch gel. The system was chiefly applicable to proteins with a fairly high electrophoretic mobility as the protein had to traverse a considerable distance before it could be recovered. In addition the apparatus was fairly elaborate and only one protein could be eluted at a time. A simplified apparatus based on the same principle (Gordon, 1960) was also only capable of eluting one band at a time. The method was considerably improved (Bocci, 1961) by the use of a tray in which up to four segments of gel could be eluted simultaneously. However, location of the eluted protein is the difficult feature of this method. Tsuyuki (1963) described a tray for the simultaneous elution of a large number of isolated gel segments. Each segment was placed in a compartment made partly out of a bent plastic microscope slide, but with two opposite sides of dialysis membrane. In effect, the compartments are adjacent to each other in the tray but separated by

the membranes. The current flows through all the chambers, elution taking place in each one. Each protein is trapped by the membranes. The apparatus is however rather elaborate and the making of each chamber a fairly delicate operation. At about the same time an apparatus was described for barrier electrophoresis (Vande Woude and Davis, 1963) where dialysis membranes were used to separate different compartments of the apparatus. The membranes were held in place between segments of the tray, the whole structure being held together with screws. By a combination of these ideas, a tray was devised and constructed for the simultaneous elution of starch gel segments by means of a second electrophoresis.

The tray was made from "Perspex" and was composed of two end pieces and eight individual segments which could be assembled together to form the tray and held in place by brass rods furnished with washers and nuts at each end. During assembly a section of dialysis membrane was inserted between each segment so that the final apparatus consisted of a series of compartments separated by the membranes. The use of silicone grease between each face and membrane ensured a water tight fit. The membranes were soaked and the holes punched before assembly and, once set up, the apparatus could be used repeatedly as long as it was stored filled with water to prevent the membranes from drying out (see fig. 27).

The starch gel (12%) was prepared in sodium acetate buffer, pH 4.6 to 4.7, $I = 0.015$ and poured in the usual way but only one slot of 12 cm was made which occupied almost the full width of the gel. When fully set the gel was loaded with 30 mg of crude ovomucoid in 0.8 ml of 0.4% agarose gel at 45° and covered with a sheet of "polythene". The

gel was subjected to electrophoresis with 0.3 M sodium acetate in the electrode chambers and 0.05 M sodium acetate buffer in the ~~ones~~ adjacent to the gel (all at the same pH as the gel) at 150 volts and 25 ma for 20 to 24 hours. After electrophoresis a narrow locating strip was cut from each edge of the gel and a third one from the middle of the gel. These were sliced, stained and washed in the usual way and then returned to their places, alongside the remaining sections of gel in the tray. The regions containing protein as indicated by reference to these strips were cut out yielding two segments of gel for each band of protein (one from each side of central locating strip). As two protein bands were isolated from each gel and two gels were run concurrently eight slices of gel were obtained from each run. Each of these was then placed in a separate compartment of the elution apparatus. The gel segment occupied approximately half the space in the compartment. The remainder of the space in the compartment was filled with 0.01 M acetate buffer pH 5.0 to the level of the gel slice. A block of rubber was placed on the slice to keep it in place and to prevent the passage of electric current through any buffer which may otherwise have covered the slice. When the whole elution apparatus was set up any unused compartments were filled with the 0.01 M acetate buffer to the same level as the others. The two end compartments were connected with filter paper wicks to the electrode vessels (capacity 5 l) filled with 0.05 M sodium acetate buffer pH 5.0. Electrophoretic elution was performed for 24 hours at a current of 20 ma. In later runs the pool of buffer in each compartment was covered with molten 45° paraffin wax, to prevent evaporation, which

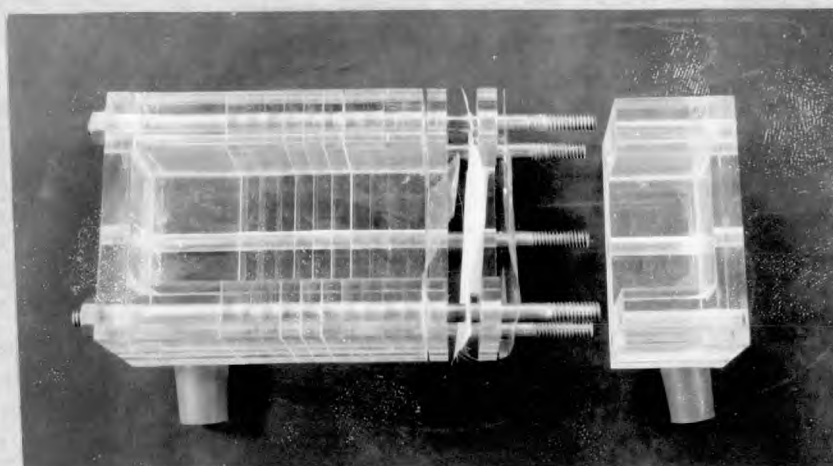


Fig 27 Apparatus for Electrophoretic Elution of Proteins from Starch Gel

In this tray the nylon mesh membranes used by Lloyd and Mears (1964) to keep the starch gel segment in place have been incorporated. Dialysis membranes and nylon mesh membranes are placed alternately between the segments and the starch gel piece in every alternate segment such that the protein migrates through the nylon mesh and is trapped by the dialysis membrane.

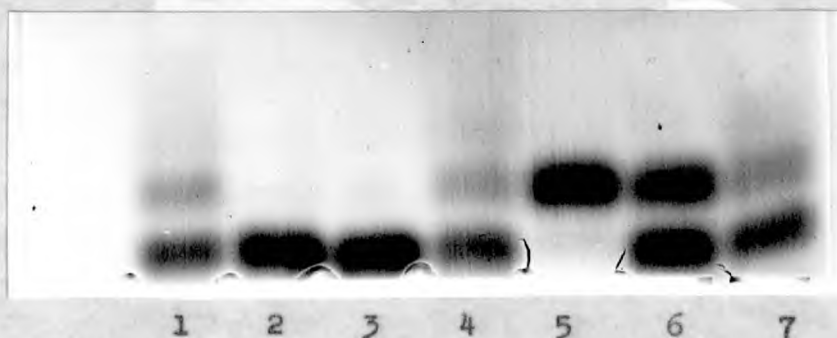


Fig 28 Effect of Neuraminidase on Re-electrophoresis of ovomucoid fractions isolated from starch gel

1,4,7 - starting material; 2 - slow fraction plus neuraminidase; 3 - slow fraction; 5 - fast fraction; 6 - fast fraction plus neuraminidase.

otherwise resulted in increased buffer concentration in the chambers with subsequent fall in electrical resistance accompanied by an excessive amount of heating. At the end of the run the gels were removed, each compartment washed out and the appropriate lots pooled. The dialysed freeze-dried residues from these buffers were found to be considerably contaminated with soluble starch and the preparations were therefore further purified by column chromatography on DEAE cellulose columns.

Materials eluted from the two major bands from two gels (total starting material 60 mg) were pooled into one "fast" fraction and one "slow" fraction (the terms "fast" and "slow" refer to their relative rates of migration towards the anode). The material was dialysed against phosphate buffer I - 0.005, pH = 7.0 and adsorbed onto a DEAE cellulose column (1 cm x 40 cm) equilibrated with the same buffer. Elution was effected by a linear increase in Na_2SO_4 concentration from 0 to 0.25 M over 200 ml of the phosphate buffer. The fractions containing protein were pooled, extensively dialysed and freeze-dried. The final yield was 14 mg of slow fraction and 8.5 mg of fast fraction. Correcting for the material removed in the guide strips this represents a recovery of 50% of the remainder. The proportions of fast and slow material isolated are roughly in accord with their relative staining intensity as judged by eye.

These two fractions represent a complete separation of two electrophoretically distinct species of ovomucoid and also probably a higher degree of purity than obtained by any other method. As the procedure was very laborious and time consuming only relatively small

amounts of the two fractions were finally collected, but it was possible to carry out a reasonable analytical programme on the materials using exclusively micro-analytical methods. All analyses described below were performed on about 10 mg of each fraction.

(11) Analysis of Eluted Material

Methods Amino acid analyses were performed with the kind collaboration of Mr. Wilkinson on the Spinco 120 amino acid analyser according to the method of Spakman, Moore and Stein (1958) as outlined previously (see p. 53). A sample of each fraction (3 mg) was hydrolysed in the presence of 2 μ moles norleucine in 1 ml 6 N-HCl at 110° for 24 hours. Cystine was determined as cysteic acid after performic acid oxidation of 1 mg of material (page 54). Moisture content was determined by drying in vacuo at 110°. Stock solutions were prepared of both fractions and of the crude ovomucoid used as starting material by adding water (0.1 ml, by weight) to 3 mg of each fraction.

N-acetyl neuraminic acid was liberated by mild acid hydrolysis or with neuraminidase^(L. Light and Co.). For acid hydrolysis 10 μ l of stock solution was mixed with an equal volume of 0.2 N H₂SO₄ in a 3 ml conical centrifuge tube, which was sealed with a rubber stopper and heated in an 80° water bath for 60 minutes. Evaporation of the solution was found to be considerable, condensation taking place on the upper part of the tube and on the stopper. A second method was consequently used in which hydrolysis was performed in sealed melting point capillary tubes. This resulted in less destruction of liberated sialic acid as ascertained by the use, in both cases, of control solutions of free sialic acid. In the first method 30 μ l of water was then added to the tube as the determination of sialic acid was

always performed on a 50 μ l sample. When the capillary tube was used, the hydrolyzed solution was gently blown out into a suitable tube and the capillary rinsed with 20 and then with 10 μ l of water, the washings being added to the tube. For enzymic liberation of sialic acid, 10 μ l of stock solution was incubated with 40 μ l of neuraminidase solution and 2 μ l of toluene in a rubber stoppered 3 ml conical centrifuge tube at 37° overnight. Subsequent experiments with only 10 μ l of neuraminidase solution gave equally good recoveries. As a check on the activity of the enzyme and on the absence of aldolases in the preparation, a sample of sialyl-lactose (kindly provided by Mr. E.R.B. Graham) was included in all analyses. The sialyl lactose preparation contained no free sialic acid but did not appear to be pure as its bound sialic acid content (by acid hydrolysis) was only 19% (theoretical, 41%). However it did serve as a comparative control to ensure complete recovery of sialic acid by the enzymic method. In all cases liberated sialic acid was determined by the method of Aminoff (1961) on a reduced scale except that the chromophore was extracted with cyclohexanone as in the Warren (1961) procedure instead of by the butanol-HCl mixture. This modification (suggested by Mr. E.R.B. Graham) results in a more complete extraction of the chromophore.

Sample or standard N-acetyl-neuraminic acid (50 μ l) was incubated with periodic acid (0.025 M NaIO_4 in 0.150 N H_2SO_4 , 25 μ l) for 30 minutes at 37°. Sodium arsenite (2% w/v solution in 0.5 N HCl, 20 μ l) was then added and the solution shaken until colourless (1 - 2 minutes). After addition of 200 μ l 0.1 M thiobarbiturate (thiobarbituric acid, recrystallized from water, adjusted to pH 9.0 with NaOH) the solution was heated at 100° for 75 minutes and then cooled in an

ice bath. Concentrated HCl (10 μ l) and cyclohexanone (500 μ l) were finally added and after vigorous shaking the mixture was cooled in ice-water and centrifuged at 15° . The organic layer was pipetted off and its absorption spectrum recorded in a 1 cm micro-cell.

Both fractions of ovomucoid were subjected to starch gel electrophoresis. A sample of each fraction was also electrophoresed after treatment with neuraminidase. Seven slots in a single gel were used and for each one 30 μ l of sample was mixed with 30 μ l of 0.8% agarose at 45° and the total mixture applied (except slot 6, where the sample was 40 μ l). For the first, fourth and seventh slots the sample comprised 20 μ l of the stock solution of starting material plus 10 μ l of 0.01 M sodium acetate buffer pH 4.6. Slot two contained 20 μ l of the stock solution of the slow fraction which had been incubated with 10 μ l of neuraminidase solution and 2 μ l toluene at 37° for 24 hours. Slot three was the same as two except that buffer replaced the neuraminidase. Slot five was similar to three, but with the fast fraction and slot six contained 30 μ l of fast fraction which had been incubated with neuraminidase (10 μ l), as for slot two. Electrophoresis was performed as described for the preparative starch gels above.

To determine whether, and to what extent, the preparations were contaminated with soluble starch they were subjected to mild acid hydrolysis and glucose was specifically determined with hexokinase and glucose-6-phosphate (NADP) being measured spectrophotometrically (Slein 1963). To stock solutions of fast and slow fractions (20 μ l) and to a sample of starting material (1 mg), 0.5 ml N-HCl was added, the tubes

sealed and heated at 100° for 3 hours. The tubes were opened and titrated to neutrality with 2 N NaOH using an external indicator. For each glucose determination two tubes were set up, each containing (a) 0.7 ml ATP solution [tris buffer pH 8.0 (0.1 M, 10 parts); CaCl_2 (0.1 M, 2 parts); serum albumin (4 mg/ml, 1 part) adenosine triphosphate (0.01 M ATP, Na_2H_2 , 1 part]; (b) 0.05 ml hexokinase solution (300 units/ml, where 1 unit catalyses phosphorylation of 10 μg glucose/min at 30° and pH 8.0, Berger et al, 1946); (c) 0.05 ml glucose-6-phosphate dehydrogenase solution (1 unit/ml, where 1 unit catalyses the oxidation of 1 μmole glucose-6-phosphate/min at 25° , Glazer and Brown, 1955); and (d) 0.6 ml of water, giving a total volume of 1.4 ml. Standard glucose or hydrolysate (50 μl) was added to one tube and 50 μl water to the other. The solutions were transferred to 2 cm silica microcuvettes (capacity 0.8 ml) which were placed in the double beam recording spectrophotometer. The instrument was set at 340 $\text{m}\mu$ and adjusted so that the difference in optical density between the two cuvettes ($\Delta E_{340\text{m}\mu}$) was zero. The solutions were returned to the tubes, the last addition, NADP solution (3×10^{-3} M NADP. NaH_2 , 0.1 ml) was made, and after restoring the solutions to the cuvettes, $\Delta E_{340\text{m}\mu}$ was recorded with time. The maximum was reached after about 5 minutes and was found to be linearly related to the amount of glucose in the sample over the range tested (2-10 μg). No correction was made for possible destruction of glucose during hydrolysis.

For assay of total hexose (by orcinol), glucosamine and anti-tryptic activity, solutions of each fraction and of the starting material were prepared by dissolving 5 mg in 150 μl of water. Two 50 μl samples were taken for the orcinol assay and the residue diluted by

the addition of 100 μ l water. Two 50 μ l aliquots were taken from this solution for glucosamine assay, the remainder being once again diluted with 100 μ l of water. The final solution served for measurement of antitryptic activity using again two 50 μ l samples. The methods used for orcinol, glucosamine and antitryptic assays have been described above (page 56, 57, and 112). They were scaled down for present purposes as follows. For total hexose determination 0.425 ml orcinol solution (1.88% in 53% v/v H_2SO_4) was added to the sample (50 μ l, containing approximately 50 μ g ovomucoid), heated at 80° for 15 minutes, cooled and read at 505 m μ in a 1 cm microcuvette. For estimation of glucosamine the sample (50 μ l, containing approximately 15 μ g ovomucoid) was hydrolysed with an equal volume of 8 N HCl for 8 hours at 100° in a sealed tube, and then evaporated to dryness. The residue was redissolved in 50 μ l water, mixed with 100 μ l acetyl-acetone solution and heated at 100° for 20 minutes. After addition of ethanol (300 μ l) and Ehrlich's reagent (50 μ l) the solution was heated at 65° for 10 minutes, cooled and read at 530 m μ in a 1 cm micro-cuvette. Determination of antitryptic activity was performed by adding 1 ml of substrate and 100 μ l of solution containing 10 μ g trypsin to the samples (50 μ l, containing about 5 μ g ovomucoid). After incubation at 37° for 10 minutes reaction was stopped by the addition of 0.2 ml 30% v/v acetic acid and the solution read at 410 m μ in 1 cm microcuvettes. Control tubes contained no ovomucoid and 5 μ g trypsin.

Analytical Results Moisture content of both fractions of ovomucoid, which were stored in stoppered tubes in the cold, was 15% and all analytical values are given on a dry weight basis. In addition, samples were found to be contaminated by soluble starch (see later) and this has also been subtracted from the weight of the samples before calculation of the results presented below. Amino acid analyses of the fast and slow fractions are summarized in table XII^{P160}. The starting material used for preparing the two fractions is the same as that for which Stevens and Feeney (1963) reported amino acid analyses and these values have been reproduced in the table for comparison. The phenylalanine peak was severely overlapped by glucosamine. The glucosamine contribution, as derived from the clear peak obtained on the short basic amino acid column, was subtracted to give a result for phenylalanine.

Determination of standard N-acetyl neuraminic acid (NANA) over the range 0.2 to 1.5 μg in 0.05 ml by the modified micro-Aminoff method resulted in a good linear calibration with a molar extinction coefficient of 91.3×10^3 and blank readings of about 0.02. Reproducibility was of the order of $\pm 1\%$. When standard NANA was subjected to the acid treatment used for liberation of sialic acid in the 3 ml conical tubes (total sample volume 20 μl) the recovery was 78%. Under these conditions the slow fraction yielded less than 0.05% of sialic acid and the fast fraction 0.23%. When acid hydrolysis was performed in sealed capillary tubes the recovery of standard samples was 90% and the sialic acid results were 0.056% for the slow fraction and 0.56% for the fast fraction. By this method, too, the starting material

contained 0.5% sialic acid, the sialyl-lactose 19% and the chromatographic sample of crude ovomucoid 0.87%

The rate of enzymatic liberation of sialic acid from ovomucoid was determined by incubating a sample of the starting material (7 mg) with 0.5 ml enzyme solution at 37° and removing aliquots (0.05 ml) at various times for analysis. The ratio of enzyme to substrate was 7 units per mg ovomucoid or 1.2 units per µg of sialic acid. One unit is the amount of enzyme required to liberate 1 µg NANA from a human serum glycoprotein substrate (Fraction I - Dische) in 15 minutes at 37°. The results obtained, after different time of incubation, as a percentage of sialic acid in the sample and as a percentage of the final value were: 1 hour 0.43% (76%); 3 hours 0.53% (92.5%); 6 hours 0.57% (100%) and 16 hours 0.57% (100%). A second sample incubated with 0.45 units (nominal) per µg sialic acid for 16 hours also gave a value of 0.57% sialic acid. For determination of sialic acid in the two isolated ovomucoid fractions by enzymatic liberation ^{and for the re-electrophoresis.} a series of incubations were performed including standards and controls with enzyme substrate ratios of 2 to 3 units/µg NANA. Recovery of the NANA control in this case was 100% and the sialyl-lactose preparation yielded 17% sialic acid (90% of the acid liberation value). The slow fraction contained 0.07% sialic acid and the fast fraction 0.61%. The sialic acid content of the chromatographically prepared ovomucoid by this method was 0.71%. All the sialic acid results are summarized in table XI.

Table XISialic Acid Content of Fractions of Ovomuroid Isolated Electrophoretically

	Residues Sialic Acid Per Mole of Ovomuroid	
	Released by Acid	Released by Enzyme
Starting material	0.81	0.66
Slow fraction	0.05	0.06
Fast fraction	0.35	0.37

The value for total hexose for both fractions was 27.4% on a dry weight basis. This includes the glucose resulting from the starch contamination, which was then determined specifically by the enzymatic method. For the glucose determination the mixture containing both enzymes, ATP and glucose standard or hydrolysate (but no NADP) was checked on the spectrophotometer. No E_{340} change occurred over about 5 minutes, after which time the NADP was added. The maximum ΔE_{340} obtained with a 5 μ g glucose standard in a 2 cm cuvette was 0.21 in a total volume of 1.55 ml, which gives a molar extinction coefficient for the NADPH of 6.1×10^3 . This is very close to the value of 6.22×10^3 quoted in the method followed. When a tube was set up containing 120 μ g of hydrolysed starting material addition of NADP brought about no change in E_{340} , but introduction of 5 μ g of glucose to this mixture gave the expected optical density change. Ovomuroid therefore contains no glucose and also does not interfere with the test. When their hydrolysates were assayed, the isolated materials were found to contain 17.5% glucose in the slow fraction and 15.7% in the fast fraction. If these values are subtracted from the total hexose figures and also from

the total weight of the materials, mannose contents of 13.9% for the fast and 12% for the slow fractions are obtained. These values are probably too high as the values for glucose are most likely too low. The low values for glucose are due to the fact that no correction was made for destruction of glucose during acid hydrolysis.

Glucosamine analyses were straightforward and yielded 14.8% and 13.8% in the slow and fast fractions respectively. For the activity assays the trypsin solution was prepared from a freeze-dried preparation stored in the cold. To correct for moisture content and any other non-protein contaminants the concentration of the solution was derived from the E_{280} using the value $E_{1\text{ cm}, 280\text{ m}\mu}^{1\%} = 15.9$ (Seravac Laboratories Ltd., descriptive booklet on enzymes). One mg of trypsin was found to be equivalent to 1.1 mg of slow and 1.15 mg of fast fraction. These figures are derived by doubling the amount of ovomucoid required to produce a 50% inhibition of the trypsin as discussed above (p. 94). Carbohydrate and activity values are summarized together with the amino acid results in table XII.

The results of the re-electrophoresis experiment on the two fractions before and after neuraminidase treatment are shown in fig. 28. ^{p149} The fractions are electrophoretically homogeneous with negligible cross-contamination. The multiple bands in the original electrophoretograms of ovomucoid are, therefore, clearly not artefacts produced by the conditions employed, but do represent different species, which when isolated, behave predictably on electrophoresis. The slow fraction is virtually sialic acid free and, as expected, is not altered by neuraminidase treatment. The original fast fraction (which contained approximately

TABLE XIIAmino Acid Composition of Ovomucoid Fractions (Res/28,000 g)

Preparation	Starch Gel		Aminoethylated		Stevens & Feeney (1963)
	Slow	Fast	Peak IV	Peak III	
Alanine	10.5	11.0	9.0	11.1	10.3
Aminoethylcysteine			10.8	8.4	
Arginine	5.2	5.8	4.26	4.7	5.53
Aspartic Acid	28.2	28.0	28.3	22.5	27.4
$\frac{1}{2}$ cystine					16.8
Cysteic Acid	14.6	15.4			
Glutamic Acid	12.7	13.9	15.9	17.3	13.6
Glycine	14.1	14.4	15.0	14.5	14.2
Histidine	3.9	3.9	3.15	3.8	4.5
Isoleucine	2.7	2.9	4.6	6.4	3.08
Leucine	10.1	10.8	8.68	6.2	10.2
Lysine	12.1	12.1	10.8	9.8	11.8
Methionine	1.73	1.92	3.8	3.4	2.0
Phenylalanine	4.7	3.6	4.28	6.1	4.35
Proline	7.3	7.2	5.5	6.18	5.85
Serine	9.5	9.9	12.1	15.6	10.2
Threonine	12.6	12.6	14.6	15.9	13.1
Tyrosine	5.1	5.0	5.28	4.5	5.48
Valine	13.7	12.8	10.9	6.62	14.4
Hexose	16.6	22.0			
Hexosamine	22.4	22.4			

half a mole of sialic acid per mole of protein) ran as two fractions after neuraminidase incubation. One, which we may call the F_f fraction, in the same position as the original fast fraction and another, the F_s fraction, in the same position as the original slow fraction. This is consistent with the explanation that the original fast fraction is a mixture of two species of ovomucoid, in about equal amounts, which both have the same net charge at the pH of the experiment. One of these appears to be free of sialic acid (the F_f fraction) and the other probably contains one mole of sialic acid per mole of protein (the F_s fraction). If this is so, the difference between F_f and F_s , after neuraminidase treatment, is one charged group per mole as the sialic acid carboxyl is fully charged at pH 4.6. The sialic acid-free F_f component must therefore also differ from the original slow fraction by one charge per mole. This could be due to a single group such as a difference in a basic amino acid, or to a number of groups which are only partly ionised under the conditions of electrophoresis such as two side chain carboxyl groups of pK 4.6.

(g) Discussion

The solubility of ovomucoid in the presence of protein denaturants like acetone and trichloroacetate led to the development of methods for its isolation from egg white in a highly concentrated state. However two related properties of these ovomucoid preparations soon became apparent. The ovomucoid was not entirely free from other proteins; and the ovomucoid itself consisted of a number of slightly different molecular forms. With the advent of powerful new tools for protein purification in the decade that followed, these problems were

investigated in a number of laboratories (see p. 179). When this project was undertaken no definitive chromatographic method had been published either for obtaining pure ovomucoid or for separating the different components which had been demonstrated by electrophoresis.

The methods which had been developed for purification of ovomucoid - chromatography on TEAE cellulose, or DEAE cellulose, or column electrophoresis - all used as starting material preparations obtained by selective precipitation at pH 3.5. The method of Rhodes et al (1958) employing CM cellulose chromatography, also involved the use of similarly acidic solutions. The first consideration when the present study was initiated was to investigate methods of isolation which avoided the use of such low pH conditions. In addition none of the four methods mentioned above had been shown to yield pure ovomucoid. For the first reason the crude ovomucoid referred to as preparation I, which had not been subjected to conditions below pH 4.6, was used as starting material for the chromatography on DEAE-Sephadex and DEAE-cellulose.

The results obtained on DEAE-Sephadex are rather difficult to understand. With a combination of gel filtration and ion-exchange chromatography operating, it is possible that the system is susceptible to very small changes in conditions. In a descriptive booklet on Sephadex ion exchange materials recently produced by the manufacturers (1965) it is pointed out that the rate at which equilibrium is attained on DEAE-Sephadex is rather slow. As a result it is necessary to employ slow flow rates with these columns; and that if the flow rate is too fast considerable loss of resolution occurs. This fact was possibly not sufficiently appreciated at the time. From the results obtained

on chromatography of preparation I on DEAE-cellulose it appears that the ovomucoid was being eluted more or less simultaneously with some other protein or proteins. In spite of the low isoelectric point of ovomucoid it was very readily eluted from the basic cellulose at pH 7.0. It emerged at an ionic strength of about 0.04 (in sodium sulphate and in sodium chloride). This is only fractionally higher than that needed for elution of conalbumin which has an isoelectric point about 2 to 2.5 units higher. A factor which is possibly worth considering when proteins are being eluted at such low ionic strengths is the effect of one protein on the elution of another. If a contaminant protein is eluted before the ovomucoid and emerges with an optical density at 280 mμ of about 0.5 its concentration is of the order of 1 mg/ml. If we assume, for the sake of argument, that the molecular weight of the protein is 50,000 this gives a molar concentration of about $4 \times 10^{-5}M$. For computation of ionic strength of salt solutions the concentration is multiplied by the square of the number of charges on the particle. This is probably not applicable to macromolecules, but we can possibly use it to obtain an approximate order of magnitude. A protein of molecular weight 50,000 would have about 450 residues, and if, say 50 of these carry a charge then if, for purposes of elution, the sign of the charge is immaterial, the ionic strength resulting from the protein in solution would be about 0.04. This calculation may well be widely incorrect, but does indicate at least the possibility of protein-protein interaction on a column at very low ionic strength.

Whether the elution of contaminants was actually bringing off the ovomucoid as well, or whether their elution was merely coincidental it was clear that the chromatography was not removing all

contaminants from the ovomucoid. Some other procedure, such as selective precipitation, was required. The most common method for preliminary purification of ovomucoid is to remove most of the inactive material by precipitation in the presence of acetone and trichloroacetate in the cold at pH 3.5. Although this procedure was at first considered undesirable, it does not appear to alter the molecule significantly as it is claimed that the chromatographic behaviour of ovomucoid (including its heterogeneity) is not altered by this treatment (Maeda and Peeney - private communication). Consequently ovomucoid partially purified by the Lineweaver and Murray (1947) method was used for the later series of chromatograms on DEAE-cellulose. However, it was still not found possible to obtain discrete peaks which would have indicated resolution into chromatographically pure proteins.

The results of the chromatography of ovomucoid on the basic ion exchange materials may be compared with those of Jevons (1960) and Beeley and Jevons (1965). Starting with ovomucoid partially purified by precipitation of inactive material at pH 3.5 and employing TEAE-cellulose, chromatography was performed at pH 6.8 and elution effected by a NaCl concentration gradient. Here too, as indicated by the anti-trypsin activity of the final preparations, the ovomucoid was not freed from all inactive protein. In the only other study of the chromatography of ovomucoid on basic ion exchange resins published (Chatterjee and Montgomery 1962), the elution diagram is not given.

Chromatography of ovomucoid on CM-cellulose in some respects produced more useful results than chromatography on the basic

exchange resins. From antitryptic assays the products appeared to be far less contaminated by inactive material than those derived from DEAE-cellulose. From the point of view of purification of ovomucoid by chromatography therefore, this would be the method of choice. This is fully in accord with the results of Feeney et al (1963).

In this study attention was always focused on the problems of the heterogeneity or multiple forms of ovomucoid and their resolution. In this connection, as with the problem of ovomucoid purification, the basic exchange materials did not appear to yield useful results. This is in contrast to the results of Beeley and Jevons (1965) who derived a number of peaks (though poorly resolved) on TEAE-cellulose chromatography. Although these do not appear to represent pure ovomucoid a resolution into different molecular forms may have been obtained. The resolution of the different ovomucoids by chromatography on CM-cellulose is somewhat puzzling. From the results of the starch gel electrophoresis and also according to Feeney (personal communication from Maeda) prior exposure of ovomucoid to mildly acidic conditions does not affect the heterogeneity. However on CM-cellulose chromatography the history of the materials before application to the column did appear to affect the result. Only the preparation obtained from DEAE-cellulose chromatography could be resolved clearly into more than one active peak and the material obtained after acid precipitation of contaminants could not.

In spite of the low isoelectric point of ovomucoid and its apparent large excess of negative charges it was only very weakly bound to DEAE cellulose and eluted at very low ionic strength. It appears

likely that the very hydrophilic carbohydrate groups of the molecule are situated on the outside of a highly crosslinked globular protein core, and that only very few charges are sterically sufficiently accessible to form salt bridges with the resin. This may explain the very weak binding and unsatisfactory chromatographic behaviour of ovomucoid on ion exchange columns. The degree of accessibility of the charged groups to the resin is likely to be conformation dependent (as is the case for the accessibility of the tyrosine side chains to solvent perturbation) and this may create an additional difficulty in obtaining reproducible chromatograms.

The results of the chromatography of the aminoethylated materials indicated that these fully unfolded proteins do in fact give much more discrete peaks and better resolution of the different proteins present. Amino acid analysis of the aminoethylated materials from the two main peaks yielded no cystine at all. Even if considerable destruction of cystine takes place on acid hydrolysis the total absence of cystine from the amino acid charts most probably indicates its total absence from the protein. From a comparison of amino acid values it is clear that the major peak represents ovomucoid, as a well-resolved, sharp and symmetrical peak. However, the significant discrepancy in the values for one or two of the amino acids cannot be accounted for at this stage.

The main differences between electrophoresis in gels and the classical free boundary method are:

(a) the protein sample in gel electrophoresis is applied in a thin band which enables the different species present to move clear of each other; as opposed to the deep layer of solution where only the front and

back boundaries separate,

(b) that in the gel matrix the water is bound to the polysaccharide chains thus making convection, electrodecantation and other flow phenomena impossible as well as reducing diffusion of the protein bands. The very high viscosity of the gels provides a far more stable medium for electrophoresis than the fluid conditions prevailing in free boundary conditions, and finally

(c) proteins separate not only by charge but also by virtue of their size.

Consideration (b) above also probably accounts for the fact that, whereas on starch gel electrophoresis fractionation occurred, attempts by Jutisz et al (1956) and Noble et al (1957) to obtain fractionation by column electrophoresis according to the method of Porath, did not meet with success.

In an independent study on the fractionation of ovomucoid by starch gel electrophoresis Wise et al (1964) arrived at very similar optimum conditions and obtained a very similar resolution of the ovomucoid to that obtained in the present study. In this work, too, the most basic component appeared by eye to be present in the highest concentration. In the present study this conclusion was confirmed by the actual isolation of the material as described above. However, by free boundary electrophoresis (using ovomucoid of the same origin as that of the above studies) the second most basic component is present in largest amount as judged by the areas under the peaks (see p.⁸⁰). It is most probable that the two main bands in the starch gel correspond to O₁ and O₂ of Bier et al (1953) but for some unknown reason their relative concentrations are reversed.

In the present study a far better resolution of the different components of ovomucoid was obtained on starch gel electrophoresis than on ion-exchange chromatography. This was also found in a study on the α_1 acid glycoprotein of serum (Schmid et al, 1962). Although five bands could be demonstrated by starch gel electrophoresis only a single peak was obtained on DEAE-cellulose chromatography. Fractions from different regions of the peak when subjected to electrophoresis in starch gels clearly showed that only a very low degree of resolution was taking place on the column. The greater resolution of mixtures of proteins which differ in net charge obtained on gel electrophoresis compared with that obtained on ion-exchange chromatography is possibly due to the fact that in the case of chromatography only surface charges which can form salt bridges with charged groups on the resin (and not charges in sterically inaccessible regions of the protein - such as those covered by carbohydrate) are operative; whereas in electrophoresis the location of the charged group within the tertiary structure of the glycoprotein is immaterial.

It is clearly desirable to exploit the high resolving power of electrophoresis in a gel phase for the isolation of pure protein species. The method described above, although producing the desired results is too time consuming to be of general applicability. Since this work was completed a very similar apparatus, although on a slightly larger scale, has been described (Lloyd and Mears, 1964). It is of interest to note that recently a method of preparative electrophoresis on a column of polyacrylamide gel has been developed which may prove to be the method of choice for carrying out this type of fractionation in the future (Jevans et al, 1964).

From the results of the re-electrophoresis of the isolated ovomucoid fractions after they had been treated with neuraminidase, a few useful conclusions and speculations can be derived. If there is one charge difference between F_s and F_f , the original slow and fast fractions also differ by a unit charge per mole. As the slow fraction has about half the electrophoretic mobility of the fast fraction this would indicate that their respective absolute net charges are about one and two. The difference in isoelectric point between the two main fractions of ovomucoid (Bier et al, 1953) is 0.12 of a pH unit. The only groups whose ionization is affected in this region are the side chain carboxyl groups, whose average pK is about 4.5; and the degree of ionization of a group in the vicinity of its pK is related to the pH of the medium in an approximately linear fashion. The pH difference between the experiment and the isoelectric point of the slow fraction (which results in a net charge of one on the slow fraction) is about the same as the pH difference in isoelectric points between the two fractions (which also results in a unit charge difference). This would give an isoelectric point for the slow fraction of 4.48, compared with the value of 4.41 of Bier et al (1953) but it must be borne in mind that no account has been taken of possible endosmotic effects in the present study. Another conclusion is possible from the above considerations. To get an overall change of one full charge per mole (the charge difference between the two ovomucoids) with a pH shift of 0.1 of a unit (the difference in isoelectric point between the species) would require 20 to 25 groups with pK 's in the region of 4.5, or more groups if their pK 's are significantly out of this range. This

value compares reasonably with the calculated side chain carboxyl content of 32 per mole.

The conclusion from the electrophoresis experiment to the effect that the sialic acid alone does not account for all the electrophoretic variation in ovomucoid is in accord with the results of Beeley and Jevons (1965). After neuraminidase treatment of ovomucoid they found two major components on free boundary electrophoresis corresponding to O₁ and O₂ of Bier et al. A result very similar to one obtained on starch gel electrophoresis of ovomucoid after enzyme treatment, where it was noted that the two major bands were still observable. (This experiment is not described in detail above as it yielded no information which was not already derived from the re-electrophoresis of the isolated fast fraction after enzyme treatment.) The significance of the distribution of sialic acid in the fractions these workers derived by chromatography is less clear. All fractions contained sialic acid, the content increasing with the resistance of the material to elution. As the antitryptic activity of the fractions indicates the presence of contaminating material these results are difficult to evaluate.

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