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THE UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

STUDIES OF PARTIAL STRUCTURE AND SYNTHESIS OF HEN'S OVALBUMIN

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the

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M. REBECCA ENOCHS

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STUDIES OF PARTIAL STRUCTURE AND SYNTHESIS OF HEN'S OVALBUMIN

APPROVED BY

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DISSERTATION COMMITTEE

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STUDIES OF PARTIAL STRUCTURE AND SYNTHESIS OF HEN'S OVALBUMIN

CHAPTER I

INTRODUCTION

Hen ovalbumin was one of the first proteins to be crystallized, having been first crystallized by Hofmeister in 1890 (1). It is present in relatively large proportion, comprising about 65% of the total protein present in egg white. Due to its ready availability and its ease of crystallization using ammonium sulfate precipitation (1), the protein has been widely studied for its protein structure and composition. Several reviews of such studies exist in the literature (1, 2).

In this laboratory, interest in the study of this particular protein was initiated for two main reasons. First, the existing structural studies were inconclusive in several respects, since they had not been done with modern techniques. Uncertainty about the structure, and the basis of the apparent heterogeneity of the protein, suggested that these questions might be resolved in studies using modern methodology, not available for the earlier studies. Secondly, egg ovalbumin was of interest, as, in the mature hen oviduct it constitutes 64% (3) of the total protein synthesized. It would, therefore, provide a good system for the identification of specific protein biosynthesis. Many studies have been done with this system, primarily from the aspect of its hormonal

regulation.

To study all of the structural and biosynthetic properties of ovalbumin would normally involve a large number of approaches with different technical facilities, which would not be feasible for a dissertational study. However, it was felt that all these problems could be explored using modern peptide mapping techniques. Peptide mapping has proven to be a powerful tool in modern molecular biology. For example, this technique was used to show the structural difference between normal and sickle cell anemia hemoglobins (48). Mapping techniques have been used extensively in the comparison of structurally related proteins. More recently, mapping by diagonal electrophoresis (32) has provided a new approach to finding overlaps during protein sequencing. For these reasons this methodology was applied to studies of the egg ovalbumin molecule. The specific experimental designs which were used are discussed later.

A review of the literature pertaining to the studies undertaken in this work will be given. First, the literature covering the past structural studies will be presented, and then studies related to biosynthesis of this protein.

Ovalbumin has a molecular weight of approximately 45,000 (1), and is an acidic protein, having approximately 75 acidic amino acid residues and 39 basic residues (4). Its amino-terminal has been found to be acetylated (5) and, as shown by Narita (6), ^{14}C-acetate is incorporated into the amino-terminal peptides synthesized in mature oviduct minces.

It has been reported by Lush (2) that there were two genetically

distinct forms of ovalbumin. These genetic variants were shown to display different electrophoretic mobilities. The slower moving type was termed A, and the faster termed B. These two strains of ovalbumins were found to have similar, but not identical reactivity to rabbit antisera (7), and a single peptide difference was observed. Although strain A is most predominant, in some flocks of chickens, Leghorns included, both A and B occur (8). It was also demonstrated that quantitative differences occur in eggs laid by the same hen on different occasions, indicating a physiological regulation of heterogeneity (8).

Another source of heterogeneity of ovalbumins, other than the genetic one, is in the carbohydrate moiety. Ovalbumin consists of approximately 3.2% carbohydrate (1). Some heterogeneity in this component has been found by Cunningham (1), using Dowex-50 chromatographic separation of glycopeptides obtained from digestion with various proteases. Glucosamine residues have been reported to range in number from 2-5, and mannose from 5 or 6 to 12 (1, 4).

The third and most pronounced source of heterogeneity lies in the phosphorus content. Longsworth (1) found, in 1941, that ovalbumin gave three components in free boundary electrophoresis between pH 5-10. He identified the major component as A_1, and the minor components as A_2 and A_3. The phosphorus content of ovalbumin had been determined by Osborne and Campbell, in 1900, (1) to be an uneven number, always less than two moles per mole of protein, and varying slightly with each preparation. Perlmann (12), in 1952, determined a value of 1.8 phosphorus for ovalbumin. She was the first to determine that the different electrophoretic components contained different numbers of phosphate groups

esterified to the protein. Since there were no means to fractionate the three components at that time, she used enzymatic dephosphorylation to separate and isolate the three different electrophoretic forms. Prostatic phosphatase released 46% of the phosphorus from A_1, yielding an electrophoretic component identical to A_2. Intestinal phosphatase removed all phosphorus from A_1 and A_2 to give a product identical to A_3. She found the relative amounts of these components in unfractionated ovalbumin to be 85% of A_1, 14% of A_2, and a trace of A_3. The phosphorus content of total protein was 1.8; of A_2 it was 0.9; and of A_3 it was 0.0.

Although the evidence presented by Perlmann (12) was strongly in favor of a difference in phosphate content being the major source of heterogeneity, it could not be implicated as the only one. Lush, in his study of genetically different ovalbumin fractions, found electrophoretic heterogeneity even in pure A_3. This would suggest that differences also exist in carbohydrate and other structural features of ovalbumin.

Although the distribution of cysteine and half cystine residues has been extensively studied (1, 2), the results have been varied and inconclusive. To determine the total of the two, Akabori (2) developed a procedure in which he specifically converted both disulfide and sulfhydryl sulfurs to hydrogen sulfide, by heating the protein with hydrazine. He observed seven residues of hydrogen sulfide per mole of protein. This was in agreement with the number of cysteic residues determined by taking the difference between total sulfur content and the number of methionine residues. Amino acid analyses have revealed values of seven residues (1), and eight residues (4), for the total number of half cystines.

The number of sulfhydryl groups has been variously reported to be three, four, and five (2). It was found that denaturation of the molecule exposed more sulfhydryl groups to reactive agents than were apparent in the native protein. Diez et al. (9) used four different methods of assaying for sulfhydryls, with both native, and denatured protein. He consistently found a value of four sulfhydryls when using denatured protein. This value is in agreement with that of Winzor and Creeth (1, 2), and of Fothergill and Fothergill (10). Winzor and Creeth (1, 2), determined that there were two disulfide bonds by estimating the amount of mercuric chloride taken up by ovalbumin before and after reaction with sulfite. In contrast, Fothergill and Fothergill (10), using differential labeling with iodo(^{14}C)acetate before reduction and with iodo(^{3}H)acetate after reduction, found four sulfhydryls and one disulfide. These authors further indicated that the disulfide bond was found in a peptide near the carboxy-terminal of the protein. Thus the number of sulfhydryls is still in doubt, being probably four or five, and the number of disulfides being probably one or two. Flavin and Anfinson (11) found twelve different dipeptides containing cysteic acid. This could account for a minimum of six, or a maximum of thirty cysteine or half cystine residues. Further studies to help elucidate this problem will be reported in the present work.

It has been well established that there are three forms of ovalbumin, containing two, one, and no phosphorus moieties (9). However, the points of attachment of these phosphates were still uncertain when this study was begun. It was also not clear whether or not the sites of phosphorylation are specific or random. Perlmann (12), cites evidence that

at least one phosphate is esterified to the alcoholic hydroxyl group of a serine residue. She isolated a peptide containing one phosphorus moiety, plus a serine residue. Since the removal of the phosphorus from A_2, by intestinal phosphatase is a much slower reaction than removal of one phosphorus from A_1 by prostatic phosphatase, Perlmann hypothesized that this might be due to attachment of the second phosphorus to the quanido nitrogen of an arginine residue.

Flavin and Anfinson, alternatively have presented evidence for both phosphorus atoms to be esterified to serine residues (13). They isolated phospho-peptides from partial acid hydrolysis of ovalbumin, both before and after removal of one phosphate with prostatic phosphatase. Two possible sequences were indicated by the NH_2-terminal analyses of his isolated peptides. These were Asp-Ser(P)-Glu and Glu-Ser(P)-Ala. The first sequence was present both before and after treatment with phosphatase and would thus appear to be the sequence present in A_2. The second sequence disappeared after enzymic digestion, indicating it was the more labile phosphorus peptide in A_1. From the amino acid compositions he obtained, the possible sequence of the first peptide could be Asp-Ser(P)-Glu-Ile-Ala, although sequence studies were not reported. These sequences are similar to those found by Milstein (14), who determined two unique sequences containing phosphoserine from ovalbumin. These sequences have been confirmed in this study.

An additional unsettled question regarding the phosphorylation of ovalbumin, was the specificity of phosphorylation. Since A_2 contains only one phosphate, the first possibility was that this single phosphorylation site is randomly chosen between two sites. The alternative was

that one of the sites must be phosphorylated first. This has been clarified in this work, which shows that the latter is the case.

By use of radioautographs of extracts of hen oviduct incubated for different times with L-^{14}C-Phe, Sanger and Hocguard (15) have shown that ovalbumin A_3 is an intermediate in the biosynthesis of A_1.

Some of the earlier studies of ovalbumin synthesis, using hen oviduct tissue (16-19), attempted to define the cell fractions in which this protein and its precursors were being synthesized. There is evidence that ovalbumin is synthesized in a fraction sedimenting with some mitochondria plus microsomes. There is also some disagreement over whether or not incorporation occurs in a lipoprotein precursor. In these earlier studies the specific synthesis of ovalbumin was checked by fractionation on a CM-cellulose column, as described by Rhodes et al. (20). Using this criteria, Carey (19) found incorporation into ovalbumin A_3 and A_2, but not into A_1. In this work incorporation was also observed to be primarily into A_3 and A_2, with some incorporation also into A_1.

Many studies have related the synthesis of proteins in oviduct tissue to the effect of hormones. Dingman et al. (21) reported in 1969, that estrogen and progesterone injections into immature chicks caused an increase in incorporation of labeled RNA precursors into nuclear high molecular weight RNA in oviducts incubated in vitro after such injections. They also observed an increased amount of nuclear t-RNA in oviduct tissue removed from hormone injected chicks. Further studies indicated an increase in m-RNA synthesis and increased numbers of polysomes (22,23); although another study indicated the effect of progesterone might be on decreasing catabolism (24). The induction of ovalbumin m-RNA by estrogen

has been demonstrated by extracting RNA of polysome fractions from oviducts of chicks previously injected with estrogen, and incubating these RNA fractions in a cell-free system (25). The synthesis of ovalbumin was demonstrated in this system by measuring incorporation of radioactivity into a protein fraction which formed a precipitate with antiovalbumin. A single peak was also seen after co-electrophoresis with cold ovalbumin on sodium dodecyl sulfate (SDS) polyacrylamide gels. This group has also, recently isolated some protein initiating factors from washed polysomes (26, 27). According to these papers, ovalbumin peptides, synthesized in a cell-free system, will bind to antiovalbumin when passed through an affinity chromatographic column containing bound antiovalbumin. Further, Palmiters' group (3) has demonstrated binding of ovalbumin synthesizing polysome fractions to antiovalbumin. The use of immuno-precipitation of specific protein, polypeptides, or of actively synthesizing polysome fractions, is thus established as a very powerful tool, when used in conjunction with cell-free systems for the determination of specific m-RNA activity. An additional criterion of synthesis of a specific protein has also been reported by Rhodes et al. (28). He chromatographed tryptic peptides of oxidized protein from the incorporating system, on Dowex ion exchange columns. The correspondence of peaks to those obtained using pure ovalbumin was used as a criteria of ovalbumin synthesis.

The author's interest in the biosynthesis of ovalbumin was in establishing a different assay of the synthesis of specific ovalbumin peptides, which would be independent of the completion of the polypeptide chain, or of its correct three-dimensional structure. Many assays for protein synthesis are described in the literature. In addition to the

ones already described for oviduct systems, there are similar reports for the biosynthesis of myosin (29, 30). A unique system in which a m-RNA from one mammalian species is placed in a mammalian cell-free system of another species to direct synthesis of a protein specific to tissue of the first species, was described by Lockard and Lingrel (31). In this system separation of the inserted messenger directed protein was accomplished on CM cellulose columns and co-chromatographed with pure cold protein.

The assay which I have attempted to develop in this work is based on the fingerprint mapping of specific protein peptides. These peptides can be identified by their electrophoretic mobility at various pH's. To eliminate the need to determine the yield of peptide obtained from biosynthetic systems, peptides were co-electrophoresed with tritiated ovalbumin peptides as a reference. Losses due to paper absorption or other causes, are thus less important, as it is the ratio of carbon-14 to that of tritium in each peptide spot which is measured. This ratio should give a linear curve if the amount of tritium is constant, but the yield of carbon-14 peptide varies. Therefore it is proposed that a peptide synthesized in an <u>in vitro</u> system can be identified as belonging to a specific protein, if it maps in the same place as the reference peptide, and if its $^{14}C/^{3}H$ ratio falls along the linear correlation line established using pure ovalbumin peptides with each label.

The assay will be discussed in relation to other assays, as to its reliability and usefulness as an assay for specific protein biosynthesis. Its usefulness in further demonstrating peptide synthesis in a uni-directional manner from the amino to the carboxyl-terminals will also be discussed.

CHAPTER II

MATERIALS AND METHODS

Materials

Carboxypeptidase A (diisopropylphosphofluoridate (DFP) treated, 5X crystallized water suspension) was obtained from Mann Research Laboratories, New York, New York.

Dansyl chloride (DNS), 5-dimethyl-amino-1-naphthalene sulfonyl chloride was obtained from K&K Laboratories, Inc., Plainview, New York.

Ninhydrin was obtained from Pierce Chemical Company, Rockford, Illinois.

Phenylisothiocyanate (PITC) came from Fisher Scientific Co., Fair Lawn, New Jersey, and was distilled before use.

Dye markers used in high voltage electrophoresis were Orange G and Xylene Cyanol Blue FF, obtained from K&K Laboratories, Inc., Plainview, New York.

Egg albumin, 5X crystallized, came from Pentex, Kankakee, Illinois.

Alpha chymotrypsin, 3X crystallized, was purchased from Worthington Biochemical Corp., Freehold, New Jersey.

Trypsin, 2X crystallized, L-(1-Tosylamido-2-phenyl)ethyl chloromethyl ketone (TPCK) inhibited, came from Sigma Chemical Co., St. Louis, Missouri.

Thermolysin, 3X crystallized, was obtained from Calbiochem., San Diego, California.

Papain suspension was obtained from Worthington Biochemical Corp., Freehold, New Jersey.

Dioxane, napthalene, ethlene glycol, and methanol, all scintillation grade, were obtained from Eastman Co., Rochester, New York.

PPO (2,5 diphenyloxazole), POPOP (((p-bis((2-(5-phenloxazoly)))-benzene))), scintillation vials, standard ^{14}C-toluene and ^{3}H-toluene solutions, ^{14}C and ^{3}H algal amino acid mixtures, and Omnispray, all were purchased from New England Nuclear, Boston, Massachusetts.

Kodak Royal Blue X-ray film came from Greb X-Ray Co., Oklahoma City. X-ray film holders, Kodak K-d19 developer, Rapidfix, and trays came from Hale's Photography Supplies, Oklahoma City, Oklahoma.

DL-O-Phosphoserine (Ser-P), came from the California Foundation for Bio-Medical Research, Los Angeles, California.

Gas mixture of 95% O_2 - 5% CO_2 was obtained from Anesthesia Services Inc., Oklahoma City, Oklahoma.

Canalco Disc Gel Electrophoresis equipment and accessories were from Canalco Industrial Corp., Bethesda, Maryland.

Other laboratory chemicals were purchased from Fisher Scientific Company, Houston, Texas.

Methods

Carboxymethylation

Carboxymethylation of ovalbumin was done in a 6 M guanidine solution, made in 0.2 M N-ethyl morpholine (NEMAC) buffer, pH 8.0, con-

taining 0.2% EDTA. A 50X molar excess of iodoacetic acid over sulphydryls was added and the pH readjusted to 9.5. Ovalbumin was then added to a 2.5% concentration and reacted at room temperature approximately 30 minutes. The reaction mixture was then dialyzed against 0.02 M acetic acid to stop the reaction and to remove excess guanidine (43).

Carboxyl-Terminal Determination

Carboxyl-terminal determination by hydrazinolysis (35), was performed as follows. A solution containing 0.1 - 1.0 μmole of peptide was dried over NaOH in a vacuum dessicator. Following constriction at a suitable position, the tube containing the peptide was placed in dry ice and 0.2 ml of 95% hydrazine added through the constriction. The tube was sealed and the contents allowed to react for 24 hours at 80°C. The tube was opened, the contents dried over H_2SO_4 and P_2O_2, dissolved in citrate buffer pH 2.2 and applied to ion exchange chromatography on the column of the amino acid analyzer.

Determination with carboxypeptidase A of the carboxyl-terminal amino acid was done as follows (44). A 25 μl aliquot of (DFP)-treated carboxypeptidase A suspension was added to one ml ice cold distilled water and centrifuged for 5 minutes at 2000 g to remove contaminating amino acids. One hundred μl of 1% $NaHCO_3$ were added to the precipitate. Three drops of freshly prepared 0.1 N NaOH were added to dissolve the enzyme. The pH was brought to 8-9 with 0.1 N HCl, and the solution was diluted to 1.25 ml with 0.2 M NEMAC buffer, pH 8.0, giving an approximate enzyme concentration of 1.0 mg/ml. Thirty μl of enzyme solution, containing approximately 0.02 μmoles of peptide, were added to a 100 μl aliquot of NEMAC. The mixture was allowed to react at 37°C for 5 hours,

dried, and applied to the amino acid analyzer column as above.

Amino-Terminal Determination

Amino-terminals were determined using the dansyl technique of Gray and Hartley (33). DNS-amino acids were identified by thin-layer chromatography on polyamide sheets (34). Sequential analysis was made by the combined dansyl-Edman technique (48).

Cysteic Diagonals

Cysteic diagonal electrophoreses were conducted following the procedure of Brown and Hartley (32). A 1% solution of protein in NEMAC buffer, 0.02 M, pH 8.0, containing 0.002 M $CaCl_2$ was digested with thermolysin at an enzyme to substrate ratio of 1/50. Digestion proceeded for 4.5 hours at 37°C. One drop of acetic acid was added to stop the reaction. The first dimension of electrophoresis was run at pH 3.5 for one hour. The band was cut out, oxidized with performic acid vapors for two hours, then run in the second dimension at pH 3.5.

Papain Digestion

A 20% solution of protein in 0.1 N pyridine acetate buffer, pH 6.5, was digested with papain. About 0.05% mercaptoethanol was added to protect the sulfhydryl groups on the enzyme. An aliquot of a papain suspension was added to make a substrate to enzyme ratio of 50/1. Any precipitate which formed during incubation was centrifuged and discarded. The acidic peptides obtained by digestion with papain were purified by high voltage electrophoresis. The digest solutions were applied as bands of 40 cm length on a full sheet of Whatman 3 MM paper. Sample loads were approximately 1 mg of starting protein per cm of paper. Electrophoresis

was performed at 3 kilovolts, (KV), in pyridine acetate buffer of pH 6.0 or 3.5. The location of peptides was determined using the ninhydrin-cadmium reagent of Heilmen et al. (49). Mobilities were determined relative to aspartic acid at pH 6.0, or serine at pH 2.1.

Fractionation

Commercial ovalbumin was partially fractionated into its differently phosphorylated forms using two alternative columns. The first was a carboxymethyl cellulose column, 2 x 14 cm, eluting with a Na-acetate buffer, with a pH gradient of 4.0 to 6.0. About 50 mg of ovalbumin, dissolved in 10 ml of starting buffer was applied to the column. Alternatively a column of DEAE-Sephadex was employed. The ovalbumins were eluted with 0.05 M Tris buffer, pH 7.5, with a NaCl gradient of 0.05 to 0.5 M. The column was loaded with 100 mg of protein in about 20 ml of starting buffer.

Protein Concentration

Protein concentrations were determined spectrophotometrically at 280 mμ in a Hitachi-Perkin-Elmer Model 139 spectrophotometer. The molar extinction coefficient used, 67,000, was determined for commercially obtained ovalbumin. Protein concentration was measured in some experiments, using the method of Lowry et al. (42).

Oxidation of Protein

Oxidation of protein was done according to a modification of the method of Hirs (45). Performic acid was prepared by adding one part of 50% hydrogen peroxide to twenty parts of 90% formic acid. The solution was allowed to stand for two hours at room temperature in a stop-

pered flask. In another flask, 10 mg of protein were dissolved in 250 µl of 90% formic acid, and 50 µl of anhydrous methanol. The two solutions were cooled at 4° for one half hour and then mixed together. After a reaction time of 2.5 hours, the solution was transferred, with rinsing, to 20 ml excess cold water and lyophilized. Remaining traces of performic acid were removed by redissolving the dry protein in more water and re-lyophilizing.

Amino Acid Analysis

Amino acid analysis was determined by the method of Spackman (50), with a Beckman Spinco Amino Acid Analyzer, Model 120B. This instrument was equipped with a rapid-flow system (flow rate 67 ml/hr) and a high sensitivity range card (5X amplification). Standard amino acid mixtures were chromatographed at frequent intervals to compensate for variability in different preparations of ninhydrin color reagent.

High Voltage Electrophoresis

For the high voltage electrophoretic separation of peptides and amino acids, an apparatus commercially manufactured by Savant was used. The buffers used were: pyridine-acetic acid-water (25:1:225, v/v) pH 6.5; pyridine-acetic acid-water (1:10:90) pH 3.5; and formic acid-acetic acid-water (1:4:45) pH 2.1.

Counting of Radioactivity

Counting of radioactivity was done on a Nuclear Chicago Mark 1 Scintillation Counter. Sample materials were dissolved in about 10 ml of Bray's solution (36), and counted using channel A for ^{3}H and channel B for ^{14}C. Efficiencies were obtained using either a quench curve with the

external standard, or using the internal standard method. The latter method was found to be more reliable since the values obtained from quench curves varied with the age of the voltage regulator, and also with manipulation of the machine when being repaired. Dpm determinations were obtained using the following equations: ^{14}C dpm = cpm (ch B - bkg)/eff ^{14}C (ch B); ^{3}H dpm = cpm A - (^{14}C eff A x ^{14}C dpm B)/^{3}H eff A. The calculation of these figures was simplified by using an Olivetti computer program. The average efficiency for ^{3}H was from 26% to 30%, and for ^{14}C it was 65% to 70%.

Synthesis and Purification of Radioactive Ovalbumin

Radioactive ovalbumin was synthesized using a modification of the method of Palmiter et al. (23). White Leghorn laying hens were killed by decapitation. The oviducts were removed and the magnum portion was cut into small pieces of 50-100 mg. The tissue was placed in an erlenmeyer flask in a 10X volume of a solution of Gibco Medium 199 previously diluted 1:10 with Hank's salts to dilute the amino acid concentration. $NaHCO_3$, (1.2 mg/ml), was added for buffer. The pH was adjusted to 7.4 and the solution passed through a millipore filter. Five hundred units/ml of penicillin and 10 μcuries/ml of ^{3}H-amino acid or 2 μcuries/ml of ^{14}C-amino acid mixtures were added. Since the radioactive amino acids were in solution in 0.1 N in HCl, an equal volume of 0.1 N NaOH was added to neutralize the solution. The minced tissue was added to the medium. The flasks were stoppered with glass wool and placed on a shaker in a 37° warm room, and incubated for three hours. A mixture of 95% O_2 and 5% CO_2 was continuously passed through the medium.

After incubation the tissue was centrifuged at approximately 10,000 x g for 20 minutes. A 10% w/v homogenate was made with 1 mM Na_3PO_4, 15 mM NaCl buffer, pH 7.0. The tissue was homogenized thoroughly with about 20 strokes in a Potter Elvehjem homogenizer. The homogenate was then sonified at a setting of 5, for about three minutes, on a Bronson sonifier.

The homogenate was centrifuged at 30,000 x g for about 5 minutes. The supernate was brought to 50% saturation with $(NH_4)_2SO_4$. After allowing precipitation to occur for about 20 minutes, the precipitation was centrifuged and discarded. A supersaturated solution of $(NH_4)_2SO_4$ was added to the supernate until it became cloudy. The pH was adjusted to the isoelectric point of ovalbumin, pH 4.6, with dilute H_2SO_4. Precipitation was allowed to occur overnight at 4°C. This crude precipitate was collected by centrifugation and dissolved in 0.02 M Tris buffer, pH 8.0. The solution was applied to a Sephadex G-100 column, 5 x 85 cm. The six peaks obtained were examined by polyacrylamide disc gel electrophoresis. The major ovalbumin containing peak was further purified on a column of DEAE-Sephadex (A-50), 4 x 85 cm. Elution was carried out using 0.1 M Tris buffer, pH 8.0, with an increasing NaCl gradient of 0.1-0.5 M. The protein peaks were again analysed by disc gel electrophoresis. Each of the two peaks were then separately fractionated on columns of hydroxyapatite, 3 x 18 cm. Elution was done in a stepwise manner using phosphate buffer, pH 6.8, in the following concentrations: 0.025 M; 0.05 M; 0.075 M; and 0.10 M. Recovery from this column was about 98%. Three peaks were obtained, b-a, b-a', and b-d.

Preparation of Radioactive Fingerprint Maps

Two major methods for preparing fingerprint patterns were used. In the first, hydroxyapatite fraction b-a was used. Quantities of ^{3}H-ovalbumin, ^{14}C-ovalbumin, and commercial nonradioactive ovalbumin were mixed in various ratios, totaling two mgs. The protein mixtures were digested in a 1% solution of NEMAC buffer, pH 8.0. Enzyme to substrate ratio was 1/50, with trypsin and alpha-chymotrypsin. Digestion took place for 24 hours at 37°. The digest was then applied as a 5 cm band on Whatman #1 paper, and subjected to high voltage electrophoresis at pH 3.5, 3 KV, for two hours. The band was then cut out and stitched at right angles on a second sheet of paper. High voltage electrophoresis was repeated at 3 KV, pH 2.1, for 55 minutes. Reference spots were obtained in the second dimension by applying DNS-Asp, DNS-Ala, and DNS-Leu, to one side of the paper at the origin.

The second type of digestion utilized hydroxyapatite fraction b-a', which was oxidized as described above. Protein solutions with mixed isotopes were prepared at a final concentration of 1 mg. Digestion was carried out following the method of Campbell and Sargent (37) for serum albumin. Oxidized protein was suspended in 1 ml of a solution of 1% w/v of $(NH_4)_2CO_3$ containing trypsin. Digestion was allowed to take place at 37°C for 16 hours. The digest was then lyophilized, redissolved 0.1 ml water, and applied as a 5 cm band to Whatman #1 paper. Electrophoresis was carried out at pH 6.0, 3 KV, until the blue dye marker reached a point 15.2 cm from the origin. The band was cut out and stitched to another paper. The second dimension was run at 3 KV, pH 2.1, for one hour.

After staining the fingerprints to identify the peptide spots, it was necessary to remove the color to avoid color quenching of the radioactivity. The peptide spots were traced with pencil and labeled. The mapped papers were then placed in a chamber which contained a beaker of saturated $KMnO_4$:conc. HCl (1:1). The Cl_2 gas evolved effectively bleached the stain.

After bleaching, the papers were allowed to air dry to remove excess Cl_2 gas. The previously marked spots were cut out and eluted with 30% acetic acid. This was determined to be most effective as an eluting agent for these peptides, and did not result in the return of color.

Polyacrylamide Disc Gel Electrophoresis

Polyacrylamide disc gel electrophoresis was carried out as prescribed by the Canalco literature, using a 7½% gel at pH 8.7. The gels were electrophoresed at room temperature, at 3 milliamps per tube.

Preparation of Radioautographs

Radioautographs of fingerprinted peptides were prepared by spraying the papers with Omnispray to intensify the sensitivity. The papers were placed against X-ray films, and stored in cardboard film holders, covered with aluminum foil in a -4°C cold room for 2 months. The films were developed for 3 minutes, placed in a pH 6.8 stop bath for 1 minute, a fixative bath for 10 minutes, and then washed with running water for about 30 minutes.

CHAPTER III

RESULTS

The initial goal of this study was to resolve some of the ambiguity regarding the structure and heterogeneity of ovalbumin. The first objective of this study was to ascertain, using fingerprinting techniques, the number of cysteines and disulfides in ovalbumin. As an additional consequence, some cystine and cysteine sequences were revealed. From the literature, six to eight cysteine or cystine residues were predicted. An analysis of commericial ovalbumin, partially fractionated on a column of DEAE-Sephadex, gave a value of between seven and nine. The diagonal electrophoretic method (32) was used to selectively purify cysteic acid peptides. A thermolysin digestion was done and eight peptides, which migrated out from the diagonal line, were obtained. The amino acid compositions of these peptides are shown in Table 1. The compositions of peptides 3 and 3A, 4 and 4A, and 5 and 5A, are essentially identical. Evidence will be presented showing that these peptides are cysteine peptides, containing free -SH groups. For this reason mixed disulfides are formed upon air oxidation following digestion. These mixed disulfides migrate to different places in the first dimension of electrophoresis. However, they migrate to the same distance in the second dimension after oxidation to cysteic acid. Sequence determination confirmed that these are the same peptides.

TABLE 1

AMINO ACID COMPOSITIONS OF CYSTEIC PEPTIDES

Peptide	Composition
1	Ser, Glu_2, Gly, Ala_2, Lys
2	Cys, Ser, Gly, Phe, Lys, Arg
3	Cys, Ser, Pro, Gly, Tyr
3A	Cys, Ser_2, Pro, Gly, Tyr
4	Cys, Glu_2, Leu
4A	Cys, Glu, Leu
5	Cys, $_t$Ser, Phe
5A	Cys, Ser, Phe
6	Cys, Asp, Ser, Glu_2, Gly, Ala, Ile, Phe
7	Cys, Asp, Thr, Ser_2, Glu_2, Gly, Ala, Phe
8	Cys, Ser, Glu, Gly, Leu

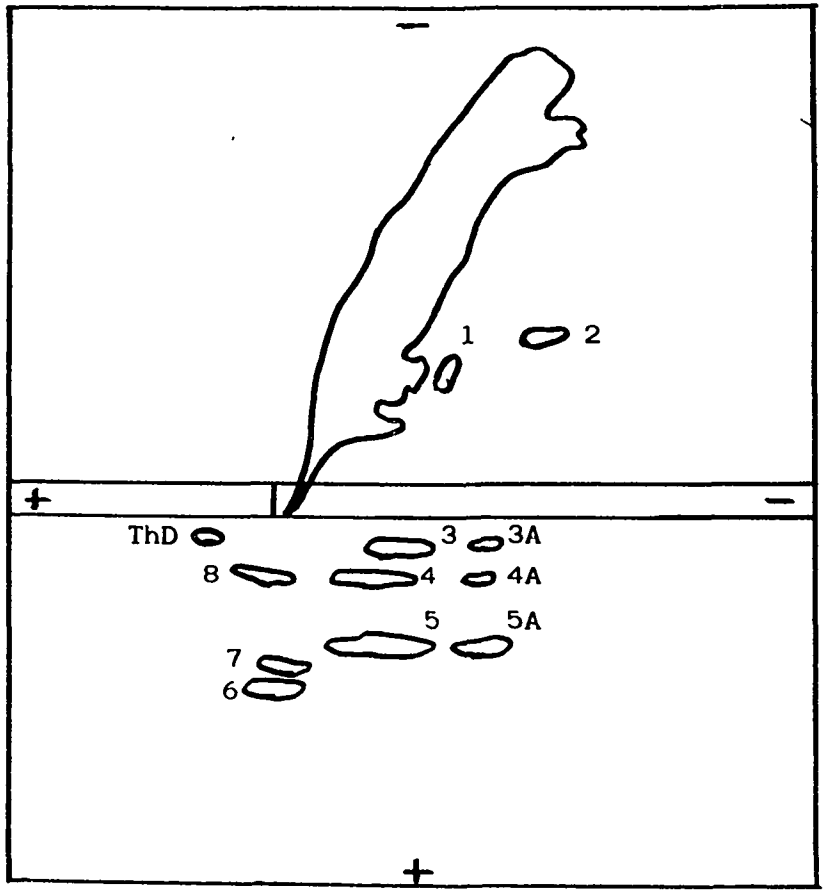
Partial sequences, obtained using the combined procedures of Edman degradation and dansylation (33, 34), are shown in Table 2. Carboxyl-terminal residues were determined by the method of hydrazinolysis (35). As seen in Table 2, peptide 8 is a longer version of peptide 4. Peptide 7 was not obtained in sufficient quantity for sequence determination. From its composition it looks as if it may be from the same sequence as peptide 6. If so, then five unique cys-peptides have been demonstrated in ovalbumin.

As mentioned earlier, there has been some disagreement in the literature over the distribution of disulfide and cysteine residues. To clarify this problem, the technique of carboxymethylation of free sulfhydryl groups (43) was utilized. The protein was denatured in urea or guanidine and reacted immediately with a 50X excess of iodoacetate. This masking of cysteine sulfhydryls prevents the subsequent change in charge due to performic oxidation, and prevents the cysteine peptides from migrating out of the diagonal in the second dimension. Disulfides, however, will not react with iodoacetate unless first reduced. Therefore, the cystine bridged peptides should again migrate from the diagonal after oxidation. Figure 1 shows the peptides obtained before and after carboxymethylation. Peptides 2, 3, 4, and 5 do not appear after blockage of sulfhydryl groups with iodoacetate. This indicates that there are at least 4 cysteine residues in ovalbumin. Using the value of eight cysteic residues determined in this laboratory for ovalbumin, this would leave 2 disulfides. Amino acid analysis of carboxymethylated ovalbumin gave values of three and four for CM-cysteine, and 4 half cystines. If the protein were reduced with mercaptoethanol before carboxy-

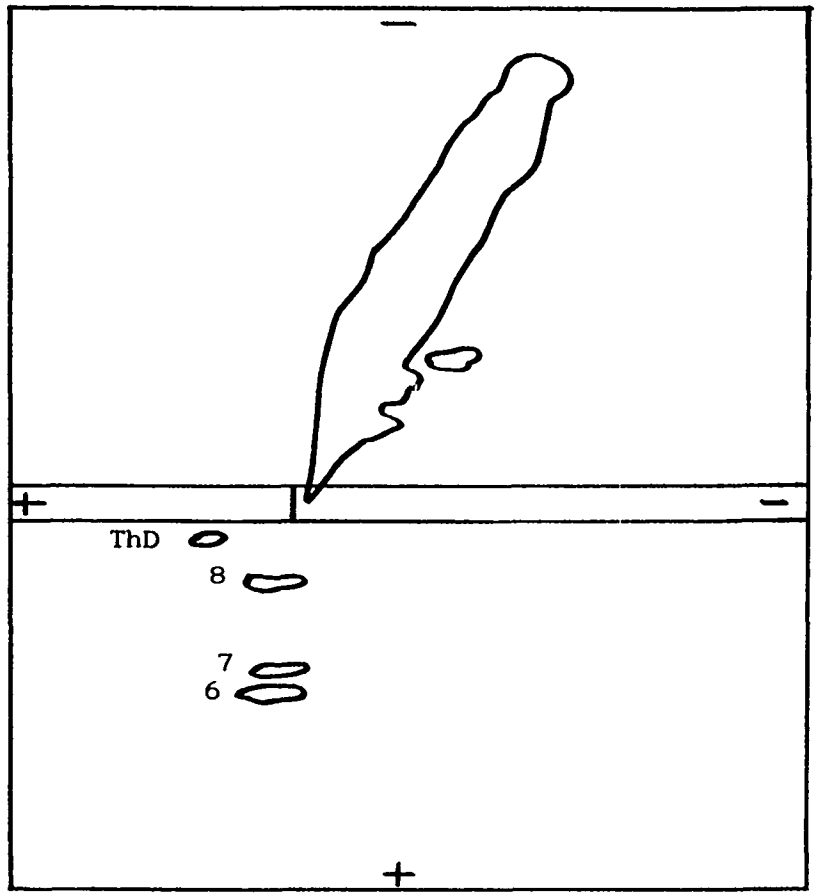
TABLE 2

PARTIAL AMINO ACID SEQUENCES OF CYSTEIC PEPTIDES

Peptide	Partial Sequence
2	Phe-Gly-Cys-(Ser, Lys, Arg)
3 or 3A	Tyr-Cys-Gly-Ser-Pro
4 or 4A	Leu-Glu-Cys
5 or 5A	Phe-Cys-Ser
6	Phe-Gly-Asp-(Ser, Glu_2, Ala, Ile)-Cys
7	Phe-(Gly, Asp, Ser_2, Glu_2, Ala, Cys, Thr)
8	Leu-Glu-Cys-(Ser, Gly)



Diagonal of Thermolysin Digest
of Ovalbumin



Diagonal of Thermolysin Digest of
Carboxymethylated Ovalbumin

Figure 1 - Comparison of cysteic acid peptides from thermolysin digested ovalbumin, before and after carboxymethylation. Peptides numbered from 2-8 contain cysteic acid. Peptide ThD contains phosphoserine.

methylation, to expose S-S linked residues, 5 or 6 CM-cysteines and 2 half cystine residues were observed. This indicates that there are 4 sulfhydryls and 2 cystine groups in ovalbumin. One of the disulfide bridges must be relatively resistant to reduction since it did not become reactive with iodoacetate. Peptides 6 and 7 are likely derived from the same sequence, and from their paired positions in the pattern, it would appear that 6 and 7 may be linked in a disulfide bridge to the sequence in peptide 8 or 4 in the following manner:

```
Phe-Gly-Asp-(Ser, Glu2, Ala, Ile,)-Cys
                                    S
                                    |
                                    S
                         Leu-Glu-Cys-(Ser, Gly)
```

That the other half cystine peptides did not appear in this pattern may be due to several reasons: a) they are contained in large peptides which did not move from the origin, b) they may still be present in the diagonal due to a balance of the negatively charged cysteic acid by a positive charge in the peptide, making it neutral, c) non-specific cleavage may have caused a yield too small to be detected. Other digests were done of ovalbumin to find these peptides, but none of the cysteic peptides from these other digests have been sequenced.

The second goal of this work was to study the phosphoserines in ovalbumin. The object was to use fingerprinting techniques to answer the question of whether the two phosphorylation sites on the molecule were random or had a compulsory order. To determine this, the phosphoserine containing peptides must be first mapped and characterized. Then the ovalbumin must be fractionated to isolate the species containing one and

two phosphoserine residues, (A_2 and A_1, respectively). These fractions were fingerprinted for phosphoserine peptides. If there must be a compulsary order of phosphorylation of the two sites, a single species of phosphoserine sequence would be observed in the molecules containing one phosphate group (A_2). On the other hand, if a random phosphorylation process takes place, then both species of phosphoserine sequence would be observed in ovalbumin A_2. The results are described in the following paragraphs.

While examining the cysteic peptides in the diagonal (Figure 1) it was noticed that one peptide, ThD, was negatively charged in the first dimension. This suggested that ThD might be a phosphoserine containing peptide. The peptide was obtained from preparative electrophoresis and subjected to partial acid hydrolysis with 5.7 N HCl for one hour at 100°. The hydrolysate was applied to a sheet of Whatman #1 filter paper, along with a phosphoserine standard. The paper was electrophoresed at pH 3.5, 3 KV, for about 30 minutes. A strong ninhydrin staining spot was found corresponding to phosphoserine.

To find the remaining phosphopeptides, a papain digest was made and applied as a band on a sheet of Whatman #3 MM paper. It was then electrophoresed at pH 3.5, 3 KV, for about one hour. Seven major peptides were obtained as shown in Figure 2. To determine which were phosphopeptides, partial acid hydrolysis was performed, and the resulting hydrolysates electrophoresed with phosphoserine as standard. The results are shown in Figure 3. There were now a total of five phosphoserine containing peptides: ThD, PD, PE, PF, and PG. The amino acid compositions of these peptides are given in Table 3.

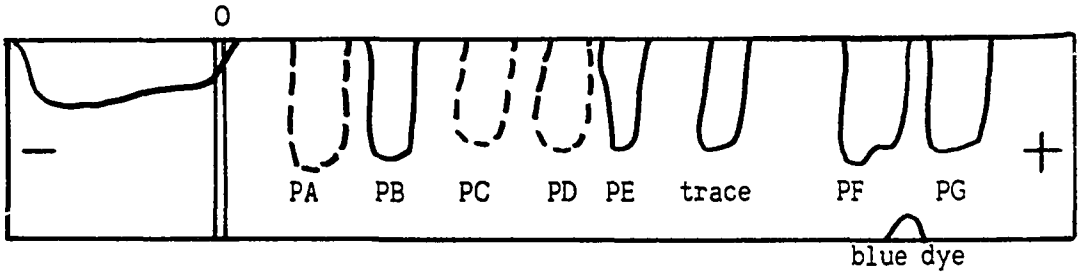


Figure 2 - Acidic peptides obtained from papain digestion of ovalbumin for 16 hours. Peptides were electrophoresed at pH 3.5, 3 KV, for about one hour. PA-PG represent papain peptides. Origin (O), xylene cyanol blue FF (blue dye).

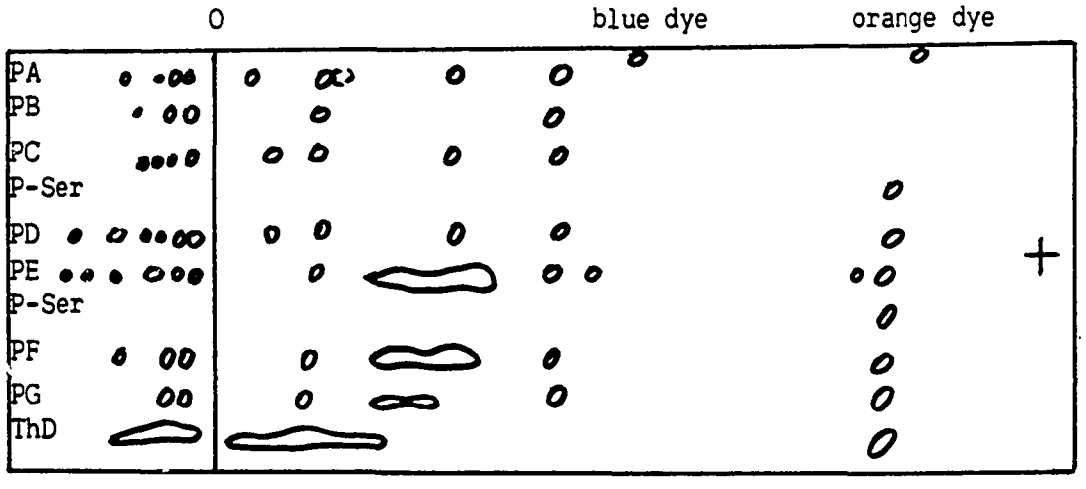


Figure 3 - Electrophoretic pattern of partial acid hydrolysates, of papain peptides, compared to phosphoserine standard. Electrophoresis took place at pH 3.5, 3 KV, for 30 minutes.

TABLE 3

AMINO ACID COMPOSITIONS OF PHOSPHOPEPTIDES

Peptide	Composition
ThD	Ser, Glu, Gly, Ala, $Val_{1.5}$*
PD	Asp_2, Thr, Ser, Glu_3, Gly_2, Ile
PE	Ser, Gly, Glu_2, Ala_2
PF	Asp, Ser, Glu_2, Ala, Ile
PG	Asp, Ser, Glu, Ile

*Val was determined to be 2 residues by 40 hour hydrolysis.

Sequence determination of these peptides was obtained using Edman degradation-dansyl procedures for amino-terminal analysis, and carboxy-peptidase or hydrazinolysis for carboxy-terminal analysis. The aspartyl or glutamyl residues were determined by measuring mobility of the peptide at pH 6.0, before and after removal of the residue by Edman degradation.

Peptide PD: The sequence of PD was found to be: Asp-Glu-Asp-Gln-(Thr, Ser, Glu, Gly_2, Ile). The remainder of this sequence has not been worked out yet.

Peptide PE: PE was found to have the sequence: Ser(P)-Ala-Glu-Ala-Gly-Gln. Glu and Gln were established by mobility at pH 6.0. Also, the decrease in mobility of the peptide after removal of the amino-terminal Ser, indicated that this residue was phosphorylated.

Peptide ThD: ThD was found to be: Val-Val-Gly-Ser(P)-Ala-Glu. It can be seen that this peptide overlaps with PE, giving the phosphoserine containing peptide: Val-Val-Gly-Ser(P)-Ala-Glu-Ala-Gly-Gln.

Peptide PF: Sequence procedures gave the ambiguous sequence: Asp-Ile-(Ser)-Gly-Glu-Ala-Glx. Glx refers to either Glu or Gln.

Peptide PG: PG produced the sequence: Asp-Ser(P)-Ile-Glu.

Further verification of the sequences of PF and PG was made in order to determine whether there were 3 or (if these peptides overlap) 2 unique phosphate containing sequences. Thermolysin digests were made of PG and PF for 5 hours at 37°. The separate digests were then applied to paper as narrow bands and electrophoresed at pH 6.0. These strips were cut out, stitched to another paper, and run at right angles at pH 2.1. The resulting patterns are seen in Figure 4a,b.

The mobility of PG-Tl, and PF-Tl, indicated a negative charge

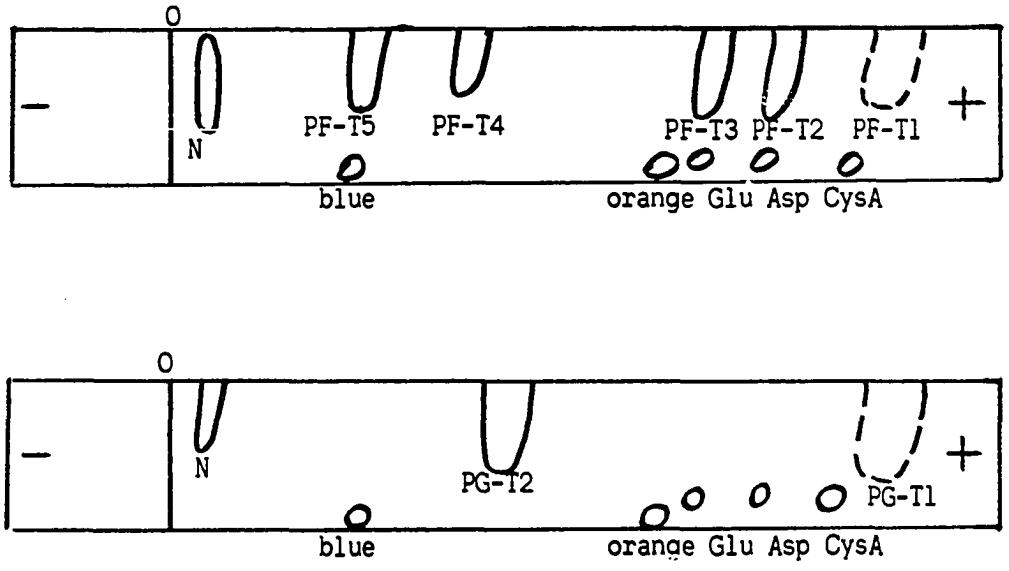


Figure 4a - Thermolysin digests of PF and PG. First dimension of electrophoresis was run at pH 6.0. Neutral (N), PF-T1 to PF-T5 indicate peptides obtained by thermolysin digestion of papain peptide PF; PG-T1 and PG-T2 indicate peptides obtained by thermolysin digestion of papain peptide PG.

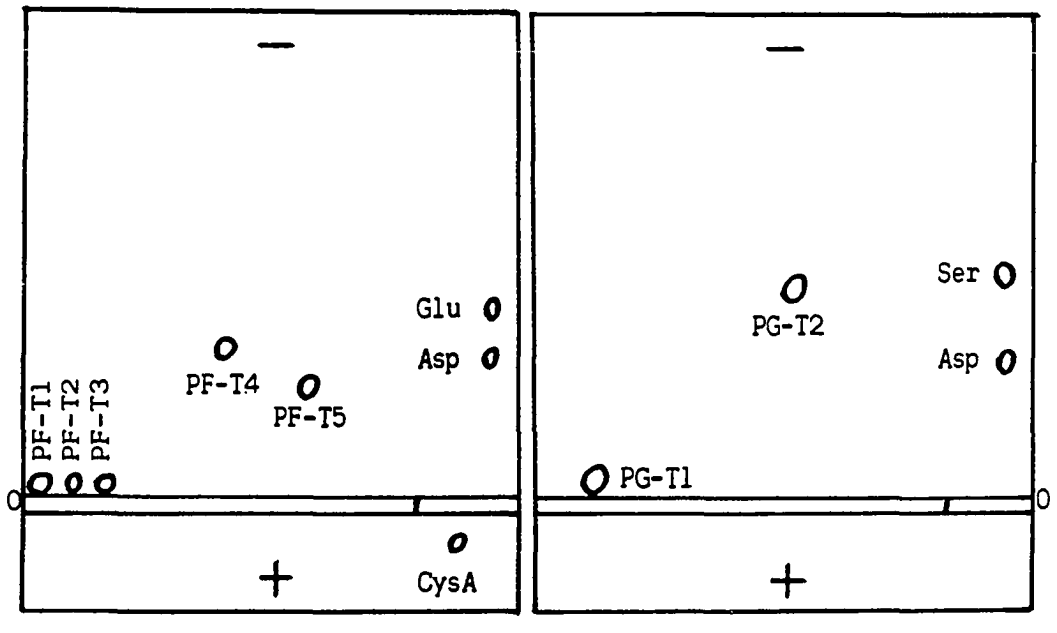


Figure 4b - The second dimensions were run at pH 2.1, 3 KV, for 35 minutes.

of 2. PG-T2, PF-T4, and PF-T5, appeared to have negative charges of 1. The sequence of each of these small peptides was determined as shown in Table 4.

It can be seen then that the sequence of PF and PG, do indeed overlap as follows:

PG - Asp-Ser(P)-Ile-Glu

PF - Asp-Ser(P)-Ile-Glu-Ala-Glu

Two unique phosphoserine peptides, therefore, occur in ovalbumin. Val-Val-Gly-Ser(P)-Ala-Glu-Ala-Gly-Gln, was constructed from peptides ThD and PE, and Asp-Ser(P)-Ile-Glu-Ala-Glu, came from peptides PG and PF.

Having identified the peptides and the sequences to which they belong, it was possible to decide which peptides were specific for the two phosphorylated species of ovalbumin, i.e., A_1 and A_2; or whether the phosphorylation is random. To answer this question it was necessary to obtain purified fractions of the two phosphorylated forms. The first attempt at fractionation was made on a column of carboxymethyl cellulose, according to the procedure of Rhodes, Azari, and Feeney (20). The elution pattern is shown in Figure 5. Peak CM A, which was eluted at pH 4.6, corresponds to ovalbumin A_1 (according to those authors). Peak CM B, eluted at pH 4.9, corresponds to ovalbumin A_2. The only difference between this procedure and that of Rhodes *et al.* was that this procedure used a pH gradient, as described in Methods. Protein from peaks CM A and CM B was digested with papain in the same manner as had been done to obtain peptides PA to PG. The peptides obtained are shown in Figure 6. As can be seen, both phosphoserine sequences are present in each fraction.

TABLE 4

SEQUENCES OF SMALL PEPTIDES OBTAINED FROM THERMOLYSIN DIGESTION OF PF AND PG

Peptide	Sequence
PG-T1	Asp-Ser(P)
PG-T2	Ile-Glu
PF-T1	Asp-Ser(P)
PF-T2	Asp
PF-T3	Glu
PF-T4	Ile-Glu-(Ala)
PF-T5	Ile-Glu-(Ala, Glu_t*)

*t refers to a trace amount.

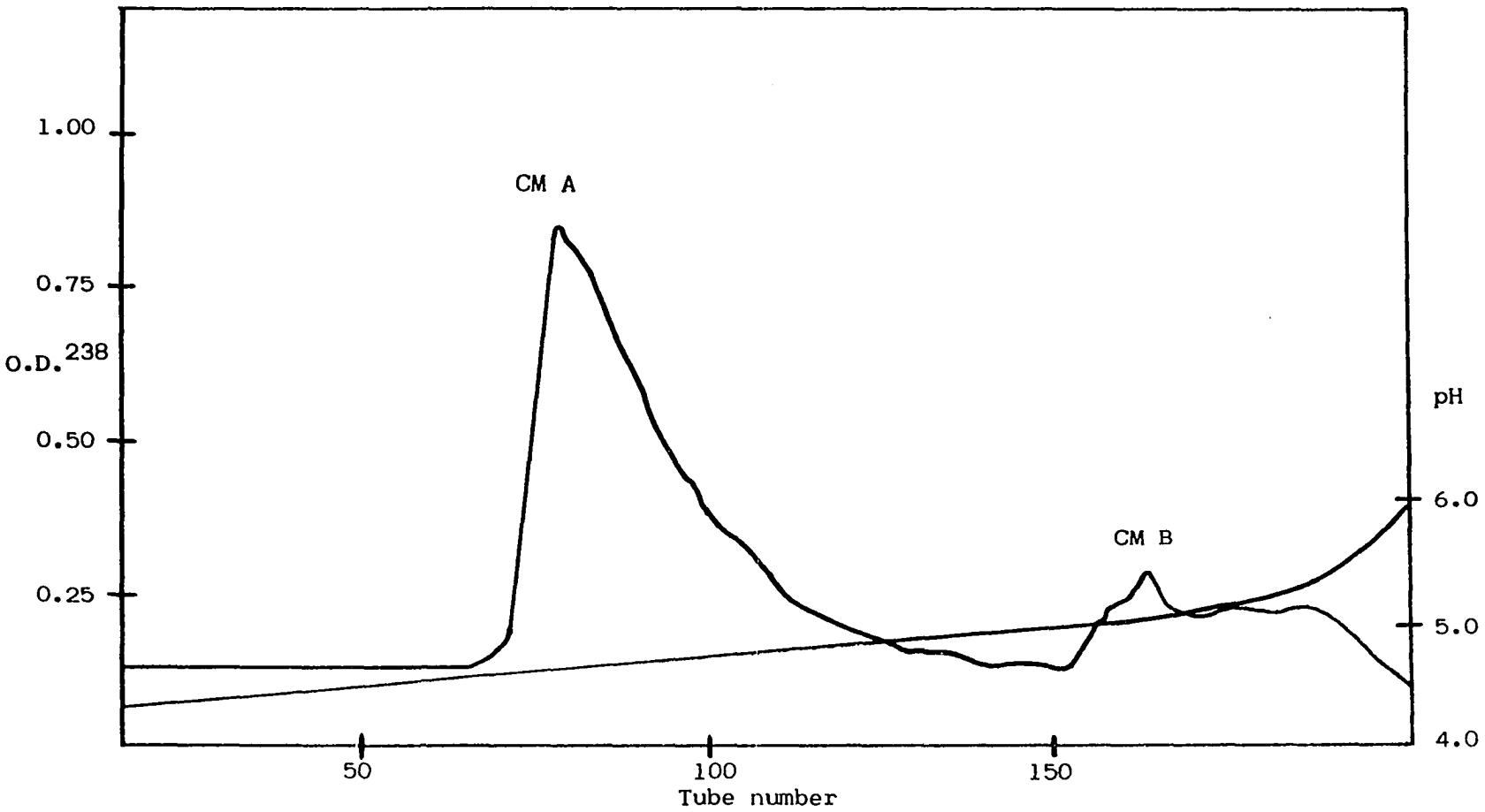


Figure 5 - Fractionation of ovalbumin on CM cellulose.

Elution was performed using a Na-acetate buffer with a pH gradient of 4.0 to 6.0. CMA and CMB represent the two peaks observed. O.D. refers to optical density.

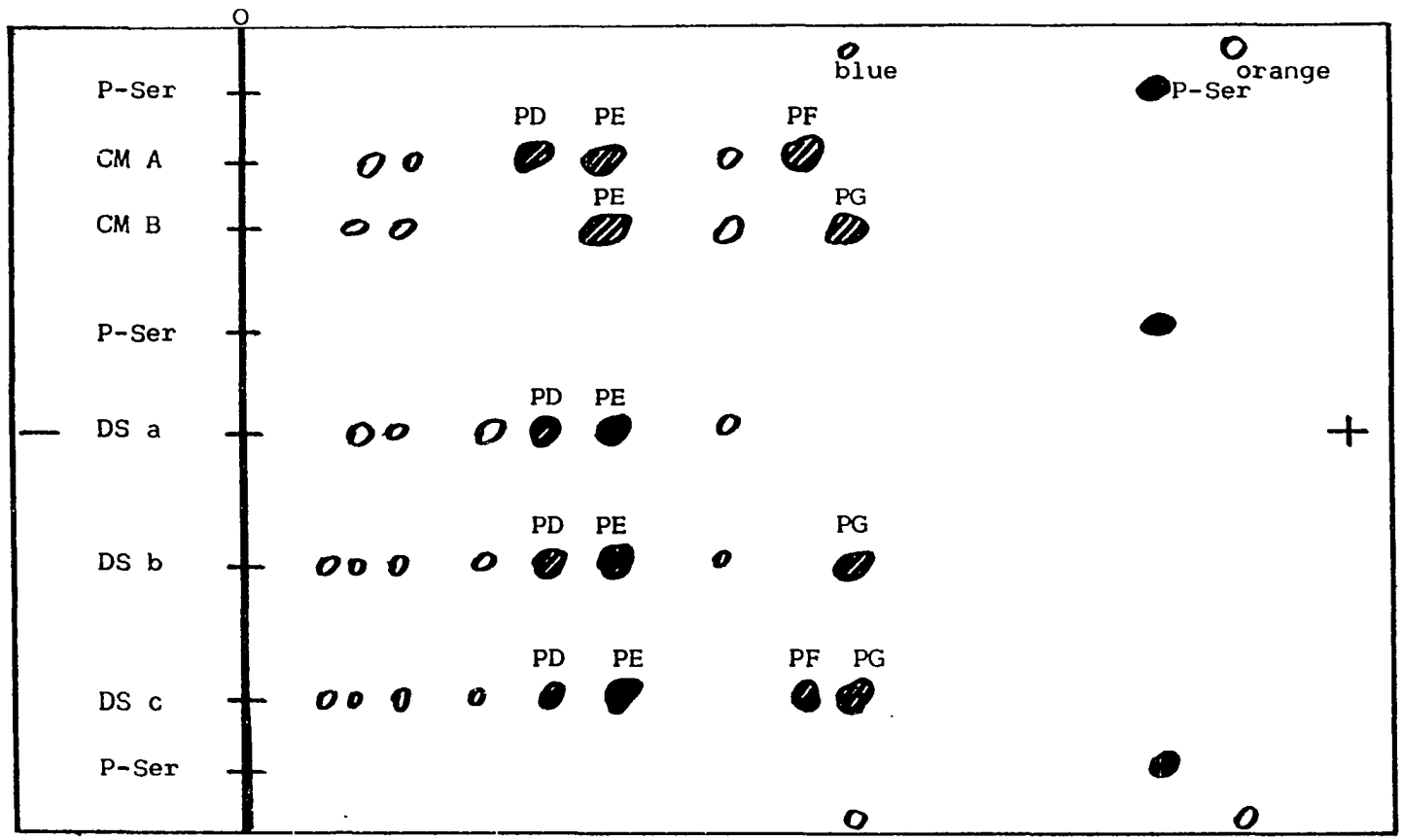


Figure 6 - Phosphoserine peptides of fractionated ovalbumin. Fractions were digested for 16 hours with papain, then electrophoresed at pH 3.5. Shaded spots indicate moderate intensity, ◐; darkened spots indicate highest intensity, ●, of phosphoserine containing peptides.

To further test this apparent random distribution of phosphoserine between the two fractions, ovalbumin was fractionated on a column of DEAE-Sephadex, using an increasing salt gradient. The elution pattern is seen in Figure 7. The purity of these fractions was tested by polyacrylamide gel electrophoresis, Figure 8. Although containing some high molecular weight contaminates, fraction DS a corresponds to the disc gel position of ovalbumin A_2 (4). There is very little A_1 present. DS b is also impure, containing largely A_1, but in addition a substantial amount of A_2 and higher molecular weight contaminants. DS c contains small amounts of A_2 and A_3, but mainly A_1. The papain produced peptides are shown in Figure 6. This peptide pattern distribution indicates that only the phosphoserine sequence found in peptide PE and ThD is present in ovalbumin A_2, whereas, as would be expected, both sequences are present in A_1.

The next phase of this work was the study of the synthesis of ovalbumin. The major purpose of these experiments was to attempt to use protein chemistry mapping techniques to develop an assay for specific ovalbumin peptides. This assay could then be used to identify the synthesis of this protein in an in vitro biosynthesis system.

The initial step was to produce purified, labeled ovalbumin, some of which was labeled with ^3H and some with ^{14}C. The purified proteins would be used to establish the assay. Various ratios of the pure labeled ovalbumins would be mixed and digested together. The position in fingerprint maps and the $^{14}C/^3H$ ratio of these peptides would then be determined for each mixture. A linear relationship should exist between the total isotope ratio of the protein, and the ratio of labels in the

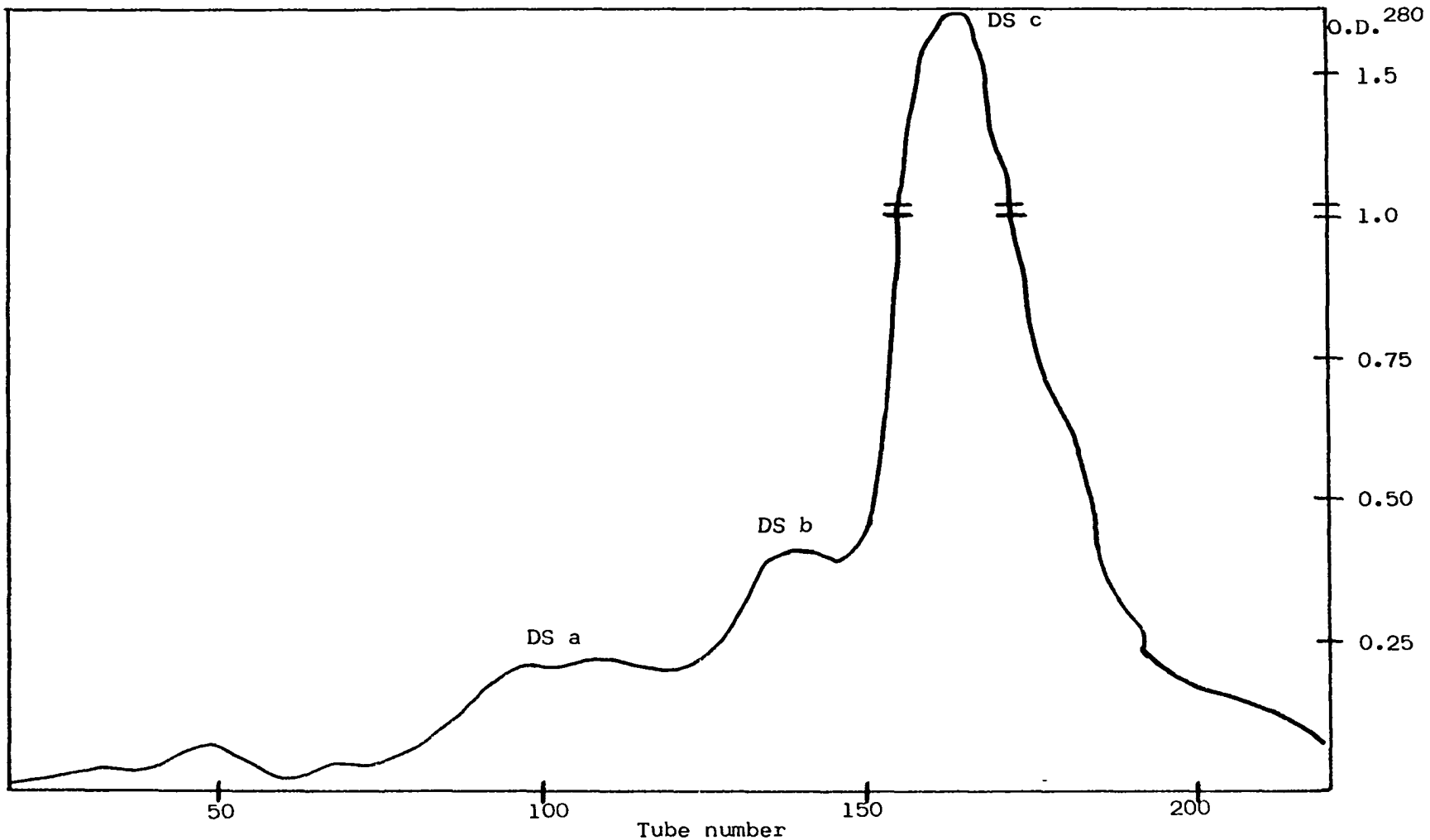


Figure 7 - Fractionation of ovalbumin on DEAE-Sephadex. Eluted with 0.05 M Tris, pH 7.5 with an increasing salt gradient from 0.05 to 0.50 M. DS a - DS c represent the three peaks observed.

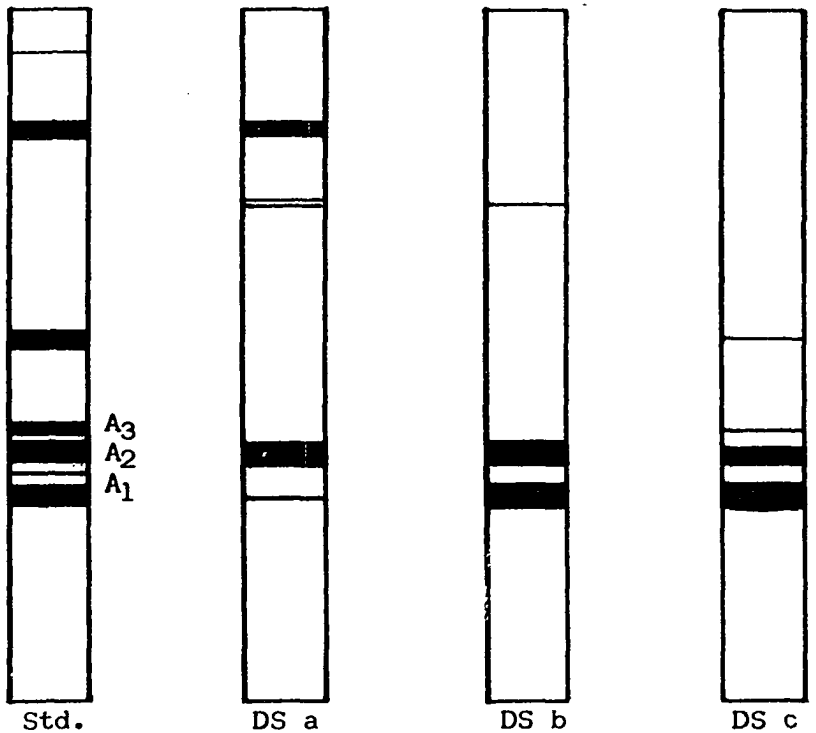


Figure 8 - Polyacrylamide disc gel electrophoresis of DEAE-Sephadex fractionated ovalbumin. A3 represents non-phosphorylated ovalbumin; A_2, singly phosphorylated ovalbumin; and A_1, doubly phosphorylated ovalbumin. Std. represents the electrophoresis of commercially obtained, unfractionated ovalbumin. Gels were stained with Coomassie Blue.

peptides. If this could be established, then the assay could be tested with an impure protein mixture labeled with ^{14}C. This crude protein was digested along with pure ^{3}H-ovalbumin. The position and isotope ratio of ovalbumin peptides should correspond to the position and slope of the linear line established with pure labeled ovalbumins.

Oviducts of actively laying White Leghorn hens were removed and incubated in tissue culture medium, as described in Methods. The purification steps were then performed in the following order. First, precipitation of contaminating proteins were accomplished with 50% saturation of $(NH_4)_2SO_4$. Crude ovalbumin was then precipitated with saturated $(NH_4)_2SO_4$ solution, at its isoelectric pH. This crude ovalbumin was then chromatographed on Sephadex G-100 (Figure 9). The major ovalbumin containing peak was further chromatographed on DEAE-Sephadex (Figure 10). The two peaks from DEAE-Sephadex were each separately chromatographed on hydroxyapatite (Figure 11). The disc gel patterns of these purification steps are shown in Figure 12. The highest yield of ovalbumin A_3 and A_2 was obtained by hydroxyapatite purification of peak b from the DEAE column. This gave quite pure preparations of A_3 (b-a), and A_2 (b-a'). Peak b-d, although it contained ovalbumin A_1, was very impure. These three fractions were further compared by their fingerprint patterns, and the results of partial acid hydrolysis. A good fingerprint separation was obtained using three dimensions of electrophoresis. Each fraction was digested with alpha chymotrypsin and trypsin for 17 hours. They were then applied to paper as a narrow band and electrophoresed at pH 3.5. This strip was then electrophoresed at right angles at pH 6.0. The neutral band from pH 6.0 was then cut out and further electrophoresed at

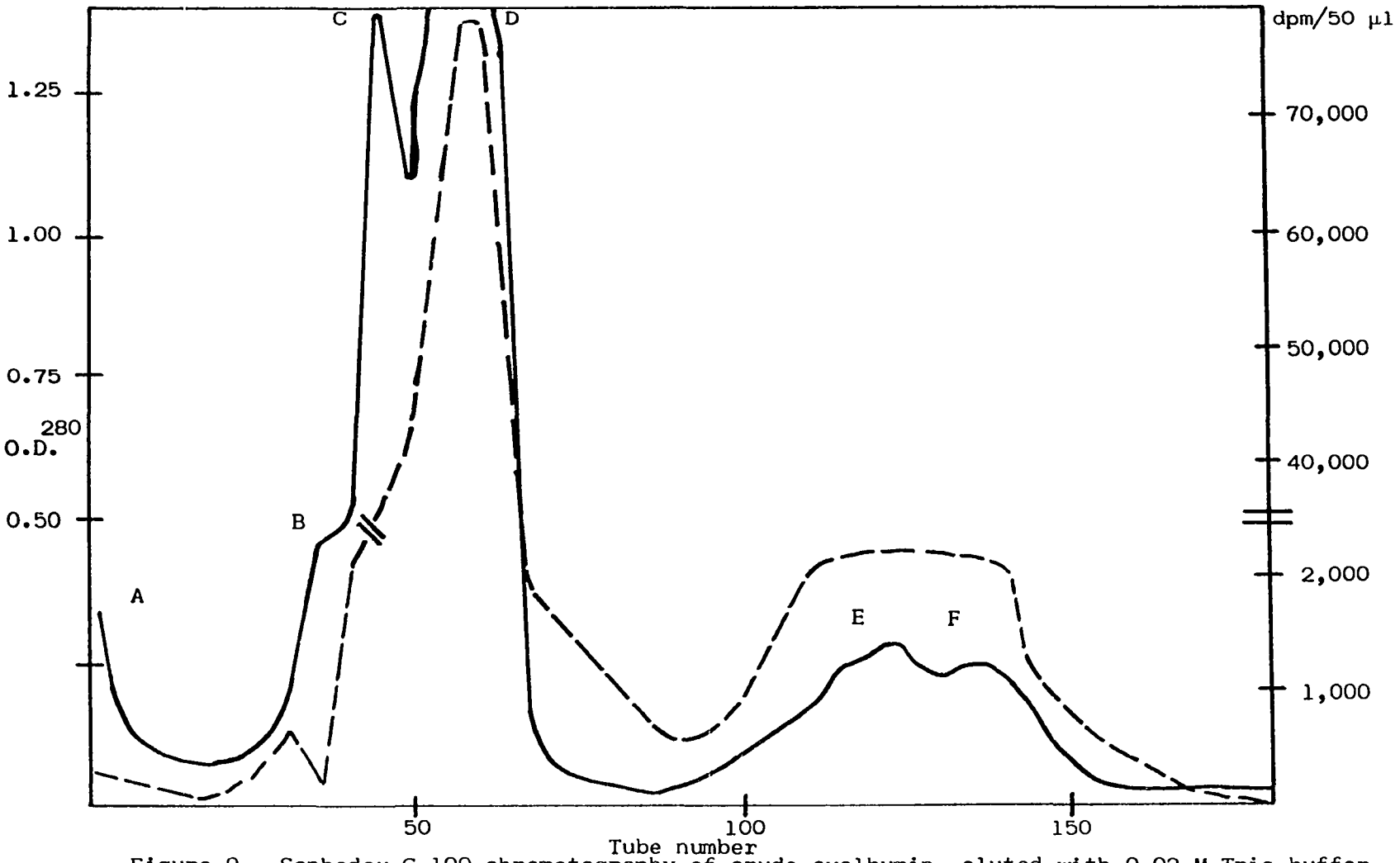


Figure 9 - Sephadex G-100 chromatography of crude ovalbumin, eluted with 0.02 M Tris buffer, pH 8.0. Solid line represents optical density at 280 mµ. Dashed line represents dpm/50 µl.

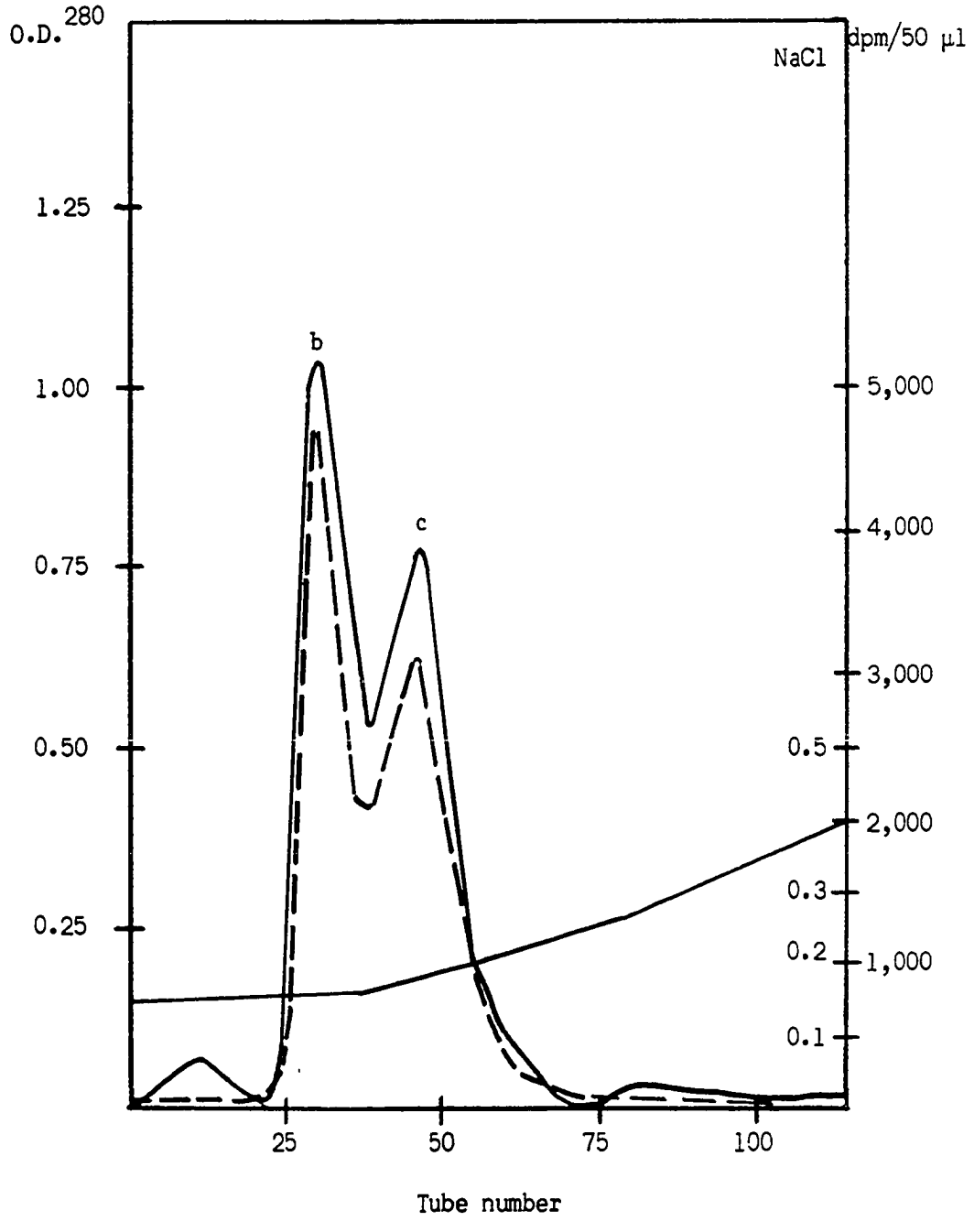
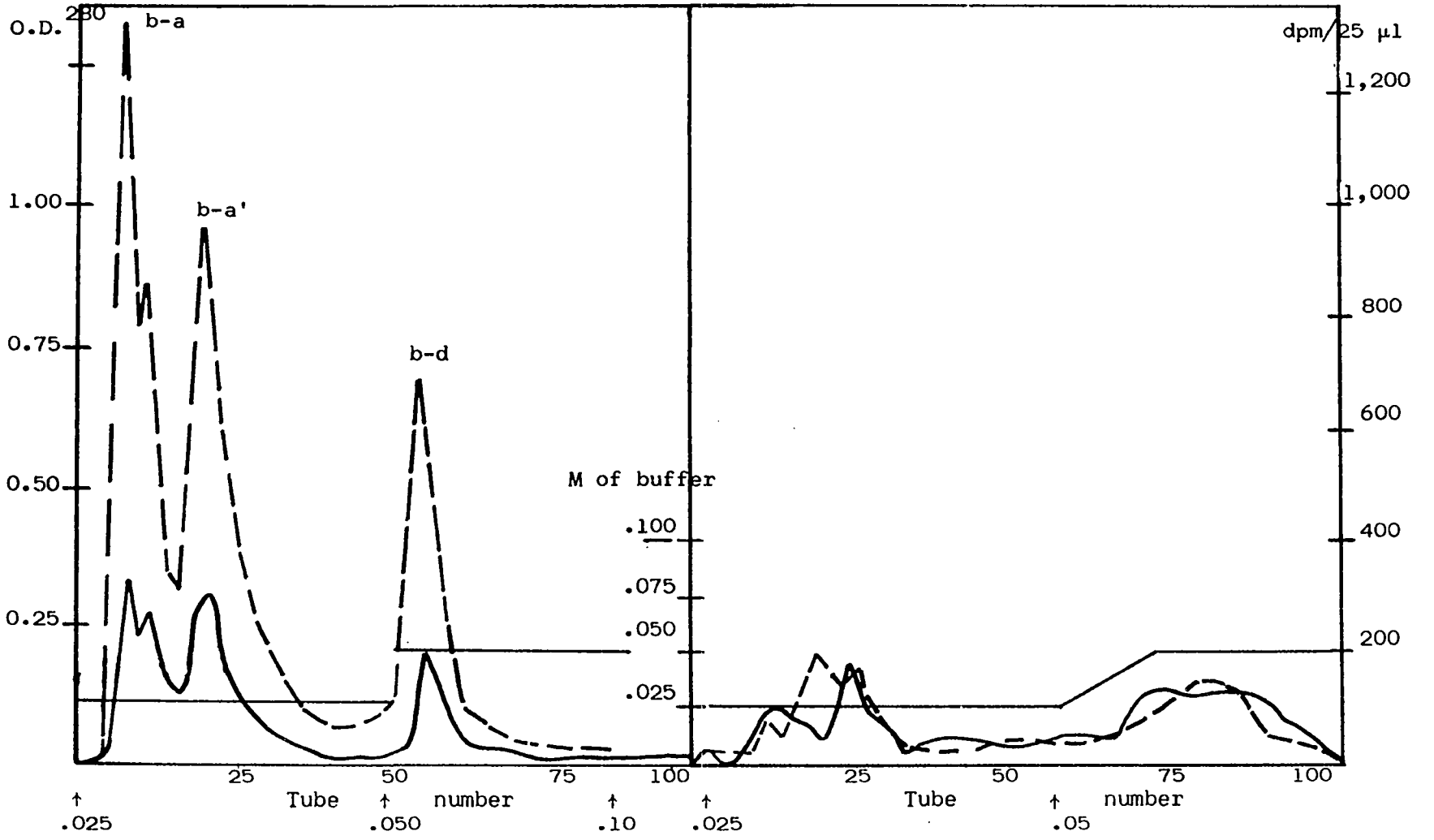


Figure 10 - DEAE-Sephadex chromatography of peak D from Sephadex G-100. Elution was with 0.1 M Tris, pH 8.0, with a 0.1 to 0.5 M NaCl gradient. Solid line represents protein. Dashed line represents dpm/50 µl.

Figure 11 - Hydroxyapatite separation of DEAE peak b on the left, and DEAE peak c on the right. Elution was done in a stepwise manner using increasing concentrations of phosphate buffer, pH 6.8. Solid lines represent optical density of protein. Dashed lines represent dpm/25 µl. Horizontal solid lines indicate concentration of eluting buffer. Arrows along abscissa indicate change of buffer to indicated concentrations.


O.D. 280
b-a
b-a'
b-d
1.00
0.75
0.50
0.25
M of buffer
.100
.075
.050
.025
dpm/25 µl
1,200
1,000
800
600
400
200
25
50
75
100
Tube number
.025
.050
.10
.025
.05

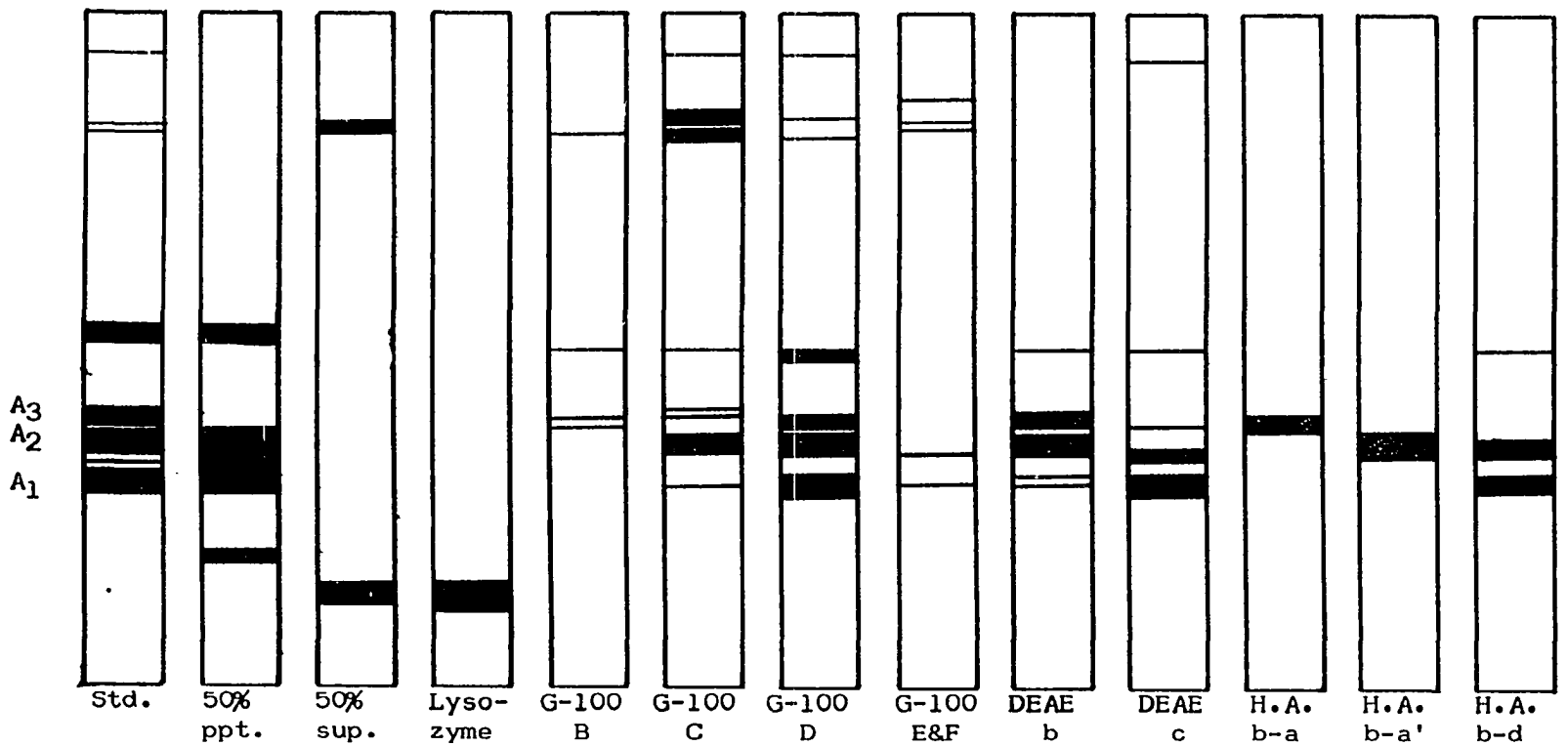


Figure 12 - Polyacrylamide disc gel electrophoresis of the steps in the purification of radioactive ovalbumin fractions. Gels were 7½% and were run at pH 8.9. Fractions A_1, A_2, A_3 stand for ovalbumin fractions containing 2, 1, and 0 phosphates. 50% ppt. represents $(NH_4)_2SO_4$ precipitation; Sup. = supernatant; G-100 = Sephadex G-100; DEAE = DEAE Sephadex (A-50); H.A. = hydroxyapatite. Gels were stained with Coomassie Brilliant Blue.

right angles at pH 2.1. These extensive fingerprint patterns revealed that there are significant differences in the peptide patterns of all three fractions. The fingerprints are shown in Figure 13.

The three fractions were then each subjected to partial acid hydrolysis for one and one-half hours. They were then electrophoresed at pH 2.1 along with standard phosphoserine and phosphothreonine. As can be seen in Figure 14, fractions b-a' and b-d appeared to contain phosphothreonine instead of phosphoserine. Fraction b-a had a spot which ran slightly faster than phosphoserine toward the anode. These spots were cut out and counted in the scintillation counter to detect any radioactivity in these phosphorylated amino acids. Radioactivity was detected in the spot from b-d (235 cpm). To further test whether this was radioactive phosphothreonine, or whether the radioactivity came from some peptide at the origin, partial acid hydrolysates were then electrophoresed at pH 3.5. The results of this experiment confirm the presence of phosphothreonine in fractions b-d and b-a'. Fraction b-a, however, did not show any phosphoserine or phosphothreonine. The spot in pH 2.1 was probably cysteic acid. No radioactivity was detected in any of the phosphothreonine spots. Therefore, b-a is probably ovalbumin A_3, the unphosphorylated form. Fractions b-a' and b-d contain phosphothreonine instead of phosphoserine, however, these are not radioactive. There was definitely a difference in phosphorus content, and, as shown in the fingerprint patterns, there are some peptide structural differences. It was therefore decided that each fraction should be used separately in the assay procedures.

The assay procedure involved two digests. The first one was an

Figure 13 - Fingerprint patterns of fractions from hydroxyapatite purification. Top row was electrophoresed at pH 3.5 as a band, then run at right angles at pH 6.0. Bottom row, shows neutral band from pH 6.0, run at right angles at pH 2.1. bl. - blue reference dye; or. - orange reference dye. Asp., (aspartic acid), and Ser, (serine) were used as references.

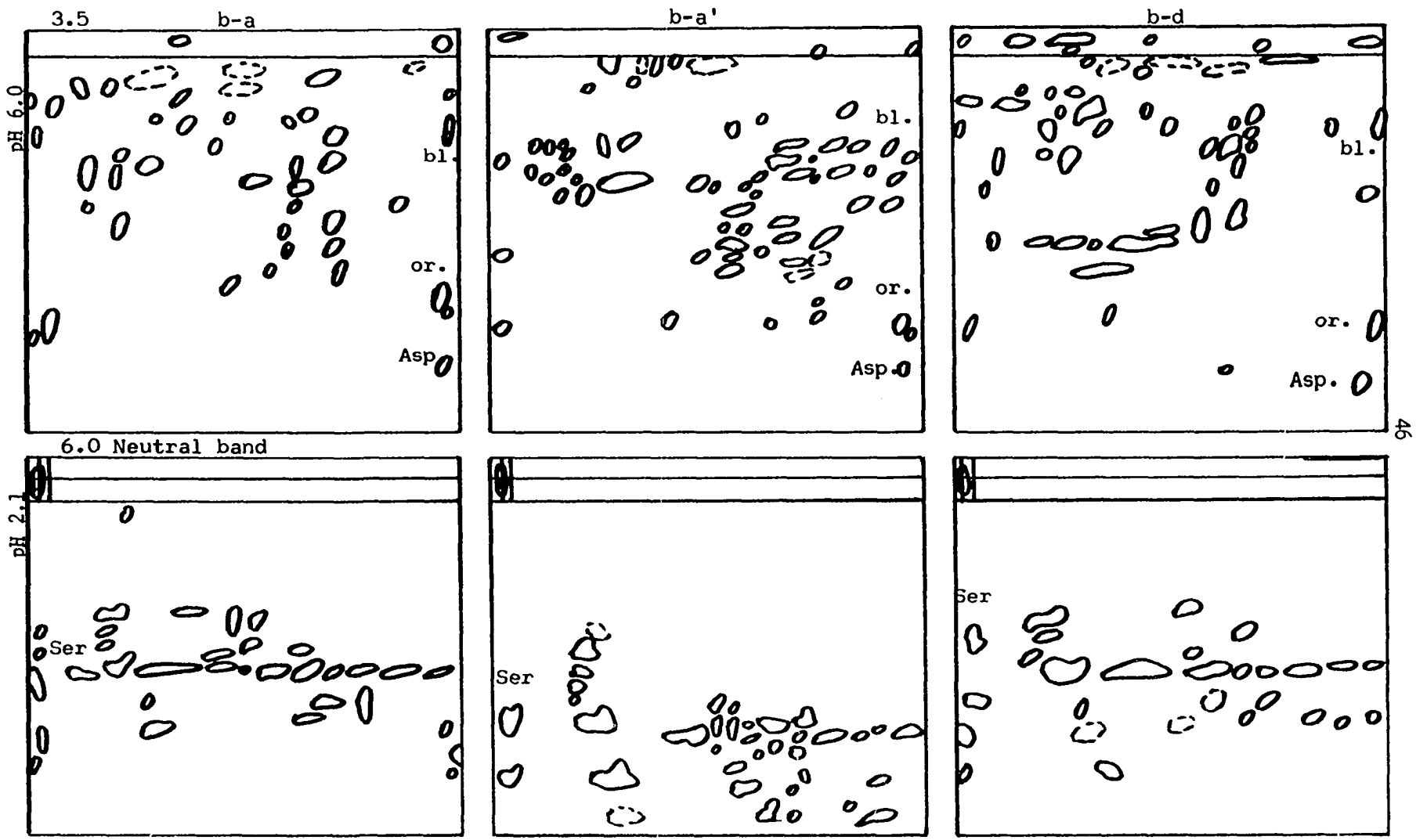
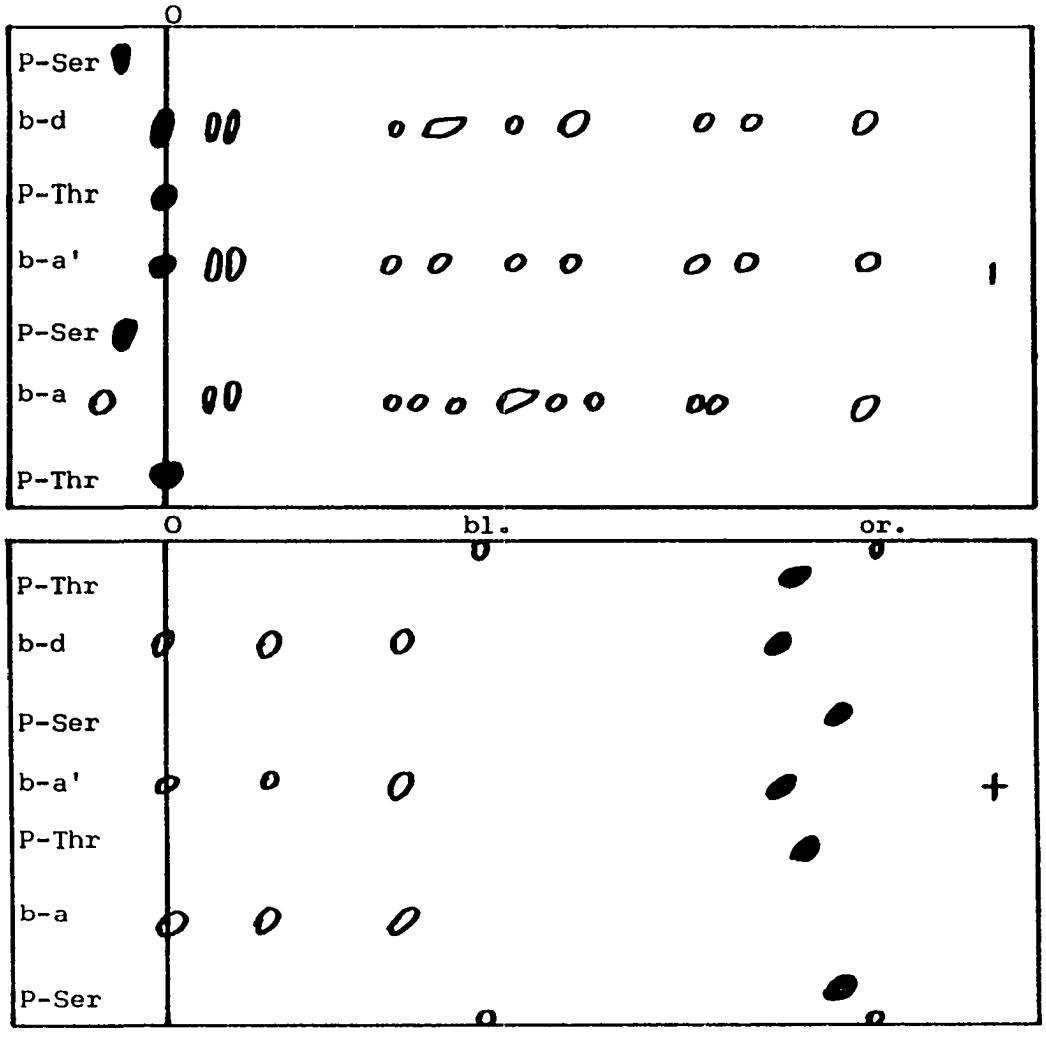

3.5
b-a
b-a'
b-d
pH 6.0
bl.
or.
Asp
bl.
or.
Asp.
bl.
or.
Asp.
6.0 Neutral band
pH 2.1
Ser
Ser
Ser

Figure 14 - Results of partial acid hydrolysis of radioactive ovalbumin fractions. O = origin; bl. = blue dye; or. = orange dye. Darkened spots represent phosphoserine or phosphothreonine.


O
P-Ser
b-d
P-Thr
b-a'
P-Ser
b-a
P-Thr
O
bl.
or.
P-Thr
b-d
P-Ser
b-a'
P-Thr
b-a
P-Ser

alpha-chymotrypsin digestion of fraction b-a. Identical digests were made of ^{3}H-ovalbumin plus ^{14}C-labeled ovalbumin, mixed in various proportions. After digestion for 24 hours, an aliquot was taken from each digest to provide the total radioactivity of each isotope in the digests. This was used to obtain the "theoretical" isotope ratio for the digest. The remaining solution was applied to Whatman #1 paper, and electrophoresed as a band at pH 3.5 for 2 hours. The band was then cut and sewn to another sheet of paper and run at pH 2.1 for 55 minutes. The patterns obtained in this way were often varied, although the main features of the patterns remained similar. This made it difficult to identify closely positioned peptides from one electrophoresis to another. A typical pattern is shown in Figure 15. A radioautograph was made of the fingerprint to determine where the majority of radioactivity was located, and to test for separation of radioactivity along with the ninhydrin sensitive spot.

The assay was dependent on determining the ratio of dpm of ^{14}C to the dpm of ^{3}H in each spot. So it was necessary to determine an efficient means of counting the radioactive spots. Three separate tests were used. The first (Table 5a) was a comparison of a direct count of paper spots in the scintillation vials with that of a water-NH_3 eluate. Spots of ^{3}H and of ^{14}C amino acid mixtures were applied to paper. They were allowed to dry, then eluted, or cut out for counting. Elution gave a consistently higher efficiency of counting. However, when eluting spots from a fingerprint pattern NH_3-water could not be used since it would bring back the strong ninhydrin color, thus producing strong color quenching. Therefore, a different eluting agent was necessary. A peptide solution was spotted on paper in identical amounts. The spots were

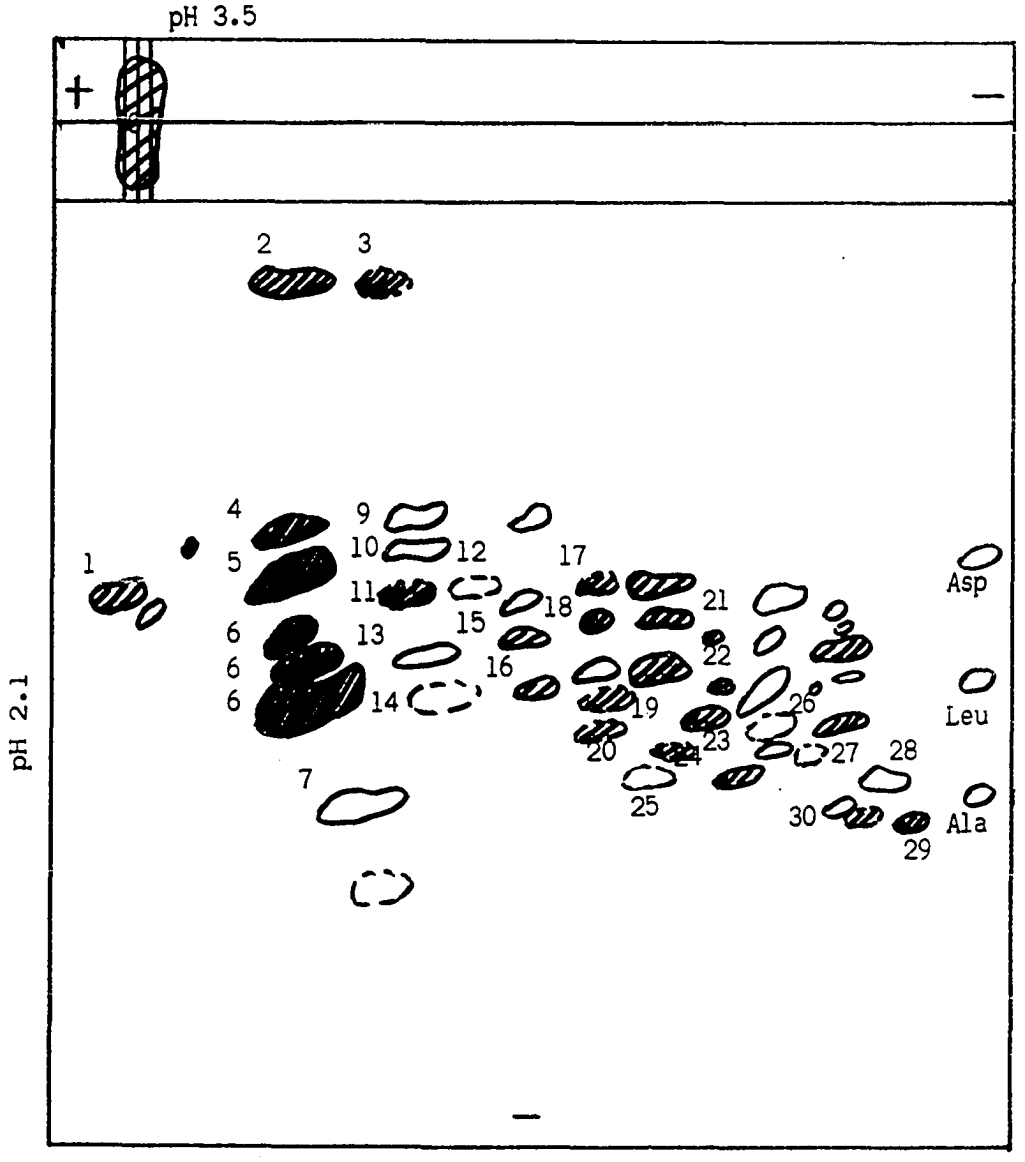


Figure 15 - An example of the fingerprint pattern obtained by digestion of b-a with alpha-chymotrypsin and trypsin. The first dimension was at pH 3.5, and the second at pH 2.1. Numbered peptides are those which occurred most reproducibly in the positions indicated. There are three peptides labeled (6), because these peptides were impossible to separate well. Dashed lines merely indicate peptides which had an orange or yellow color, instead of red, when stained with ninhydrin. Shaded peptides are those which showed up in radioautograph of this fingerprint. Darkness of shading indicates the intensity of the spot on the radioautograph. 0 = origin at pH 3.5.

TABLE 5a

COMPARISON OF EFFICIENCY OF COUNTING PAPER DIRECTLY IN THE VIALS ELUTION WITH NH_3-WATER

Isotope	dpm		% of Total		Total Applied in μCi
	Elution	Paper	Elution	Paper	
^{3}H	127,382	69,306	57%	31%	$1x10^{-1}$
^{3}H	535,029	227,527	60%	26%	$4x10^{-1}$
^{3}H	1,122,843	557,472	51%	25%	1
^{3}H	1,479,923	917,425	44%	28%	1.5
^{14}C	19,730	16,297	89%	73%	$1x10^{-2}$
^{14}C	58,737	46,411	100%	84%	$2.5x10^{-2}$

Duplicate aliquots of labeled amino acid mixtures were spotted on filter paper and dried. One set of spots was then cut out and counted directly in the scintillation vial. The second set of spots were eluted and then dissolved in scintillation fluid before counting.

then eluted with the reagents shown in Table 5b. The eluates were dried, hydrolyzed, and put in the amino acid analyzer. The quantity of each of four amino acids detected was compared. Finally, several spots from a fingerprint pattern were divided in half. Half was eluted with 30% acetic acid, and the other half was placed in the vial without elution. Although all the counts were low, the eluted halves consistently had higher counts (Table 5c).

The peptides given numbers in Figure 15, were those most consistent in position. These peptides were eluted and counted, using double-label settings. The ratio of counts of the two isotopes for each peptide was then plotted versus the ideal ratio for each proportional digest. These graphs (Figures 16, 17) indicate that there appears to be a linear, if not a 1:1 correspondance, of the theoretical to the experimental ratios. The mean value of each point, taken from all the peptides mapped, was computed. The route mean square (rms) was computed to show variance. The regression line obtained using these values was drawn from the slope and y-intercept obtained from the Olivetti computer program for a regression line. The average degree of correspondence of the theoretical to experimental was also obtained. This data is presented in Figure 18.

There is, from these data, a 1 to 1 correspondence between the theoretical and the experimental values. To test whether this slope could be used to identify authentic ovalbumin peptides, a digest was made using 1 mg of pure ^{3}H b-a plus 1 mg of an impure, crude protein mixture obtained by trichloroacetic acid precipitation of total protein from a mince incubated with ^{14}C-amino acids. The peptides obtained from

TABLE 5b

COMPARISON OF ELUTION OF A PEPTIDE BY VARIOUS AGENTS

Eluate	μmoles of Amino Acids			
	Asp	Glu	Gly	Ala
6 N HCl	0.2405	0.5027	—	—
0.1 N NaOH	0.1314	0.2069	—	—
6% Acetic A.*	0.4538	0.6604	0.5409	0.3393
30% Acetic A.	0.3546	0.8501	0.7420	0.4176

*Ref. (39, 40).

Identical aliquots of a peptide solution were spotted on filter paper. The spots were eluted with the indicated agents, dried, hydrolyzed, and then quantitated by amino acid analysis.

TABLE 5c

COMPARISON OF DIRECT COUNT OF PAPER SPOTS VERSUS
THAT OBTAINED AFTER ELUTION
WITH 30% ACETIC ACID

Spot #	^{3}H - bkg. dpm		% Increase	^{14}C - bkg. dpm		% Increase
	Paper	Eluate		Paper	Eluate	
1	12	59	80%	39	84	54%
2	6	16	65%	83	129	36%
3	12	33	63%	90	100	9%

Fingerprint spots were divided in half. One half was eluted with 30% acetic acid and counted. The other half was placed in the vial on the paper. Dpm's are expressed after subtracting the background. The percent increase of the eluate over the paper = % increase.

Figure 16 - Graphs of peptides showing isotope ratios, b-a, chymotryptic digest. Theoretical ratios were obtained from aliquots of the total digests for each mixture. Experimental ratios were obtained from peptide elution and scintillation counting. Small upper lefthand numbers represent individual peptides.

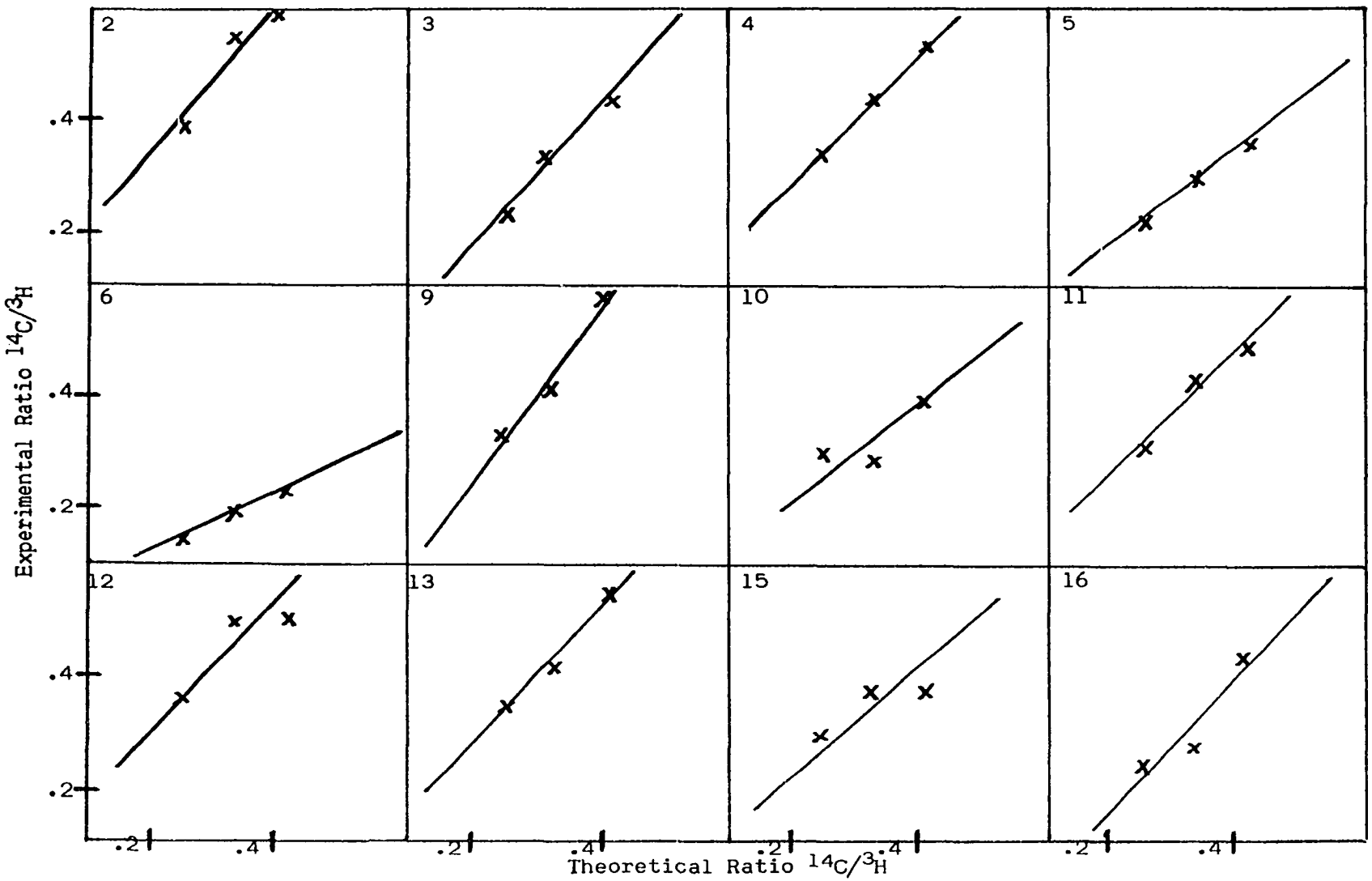
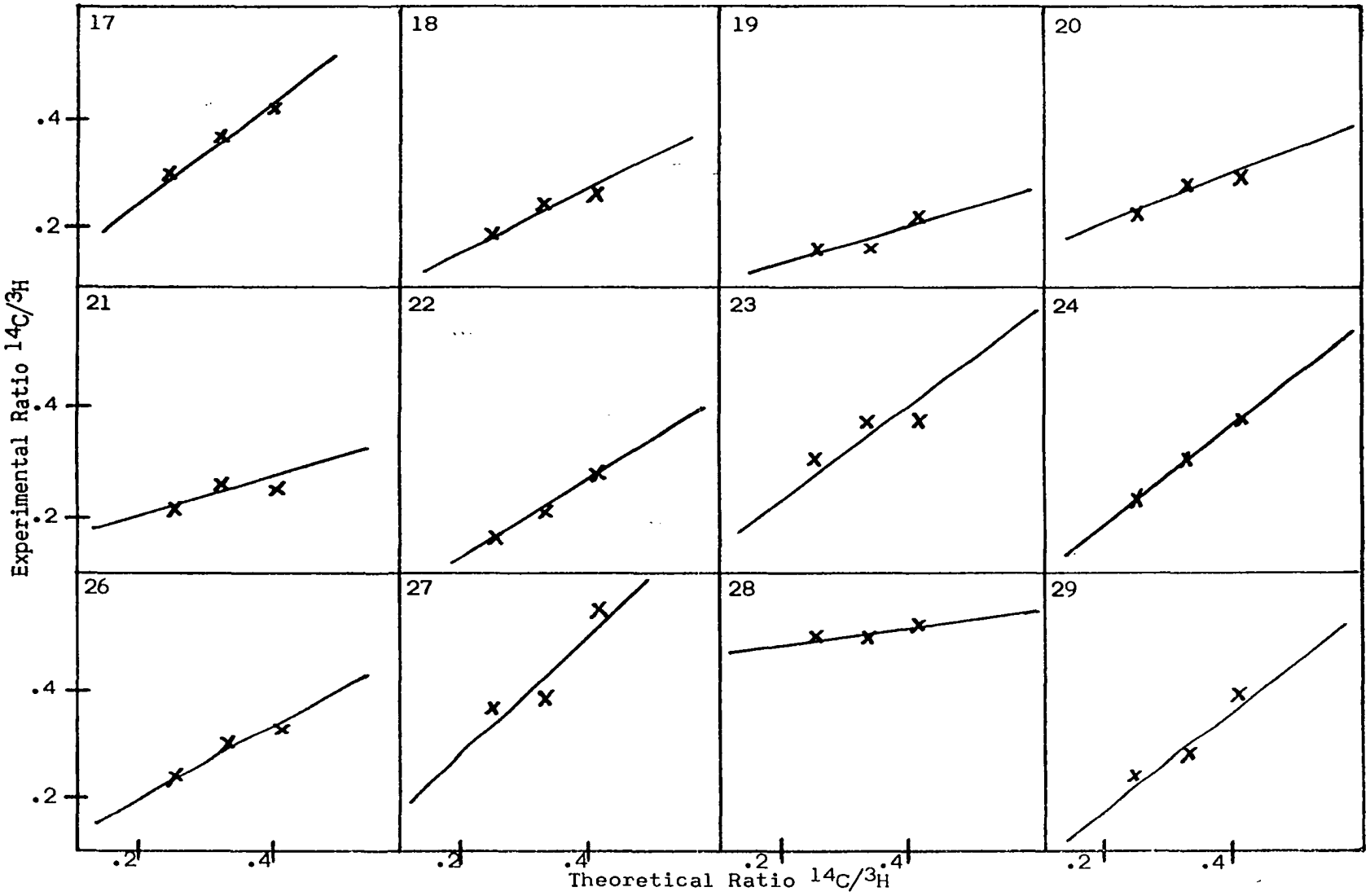

2
3
4
5
6
9
10
11
12
13
15
16
.4
.2
.2
.4
Experimental Ratio $^{14}C/^{3}H$
Theoretical Ratio $^{14}C/^{3}H$

Figure 17 - Graphs of peptides showing isotope ratios, b-a--Continued


17
18
19
20
21
22
23
24
26
27
28
29
.2
.4
Experimental Ratio $^{14}C/^{3}H$
Theoretical Ratio $^{14}C/^{3}H$

a fingerprint map of this digest are seen in Figure 19. The darkened spots are those which correspond in position to peptides seen in the standard map. These peptides, plus several others, were eluted and counted to obtain isotope ratios. These ratios compared to the theoretical ratio were then plotted on the graph of the regression line. Several peptides fall within statistical range of the line, but a large number do not. Several reasons for this will be discussed later.

Due to the difficulty of unambiguously identifying the peptides by location in the chymotryptic map, it was decided to use an enzyme with specific cleavage. Also, since the peptides had rather low counts, with highest counts in the large peptides at the left on the map (see radioautograph, Figure 15), it seemed advantageous to break down the secondary structure of the protein before digestion, so that perhaps more peptides would be formed from these high count areas. Therefore, the protein of ovalbumin fraction b-a' was oxidized, as described in Methods, and digested with trypsin. A very reproducible and clear map was obtained (Figure 20). A radioautograph of this fingerprint indicated clear correspondence of radioactive with ninhydrin sensitive spots. These spots were not as dark as those in Figure 15, but they were more uniformly labeled. Isotope ratios were again obtained for four different proportions of isotope. The graphs of these are found in Figures 21-22. It is evident that the correspondence between theoretical and experimental ratios is much less in these peptides. Several possible reasons for this will be discussed later. Again, a regression line was calculated and peptide ratios from a crude ^{14}C plus pure ^{3}H mixture were plotted on the line. The peptide pattern is seen in Figure 23 and regression line in Figure 24.

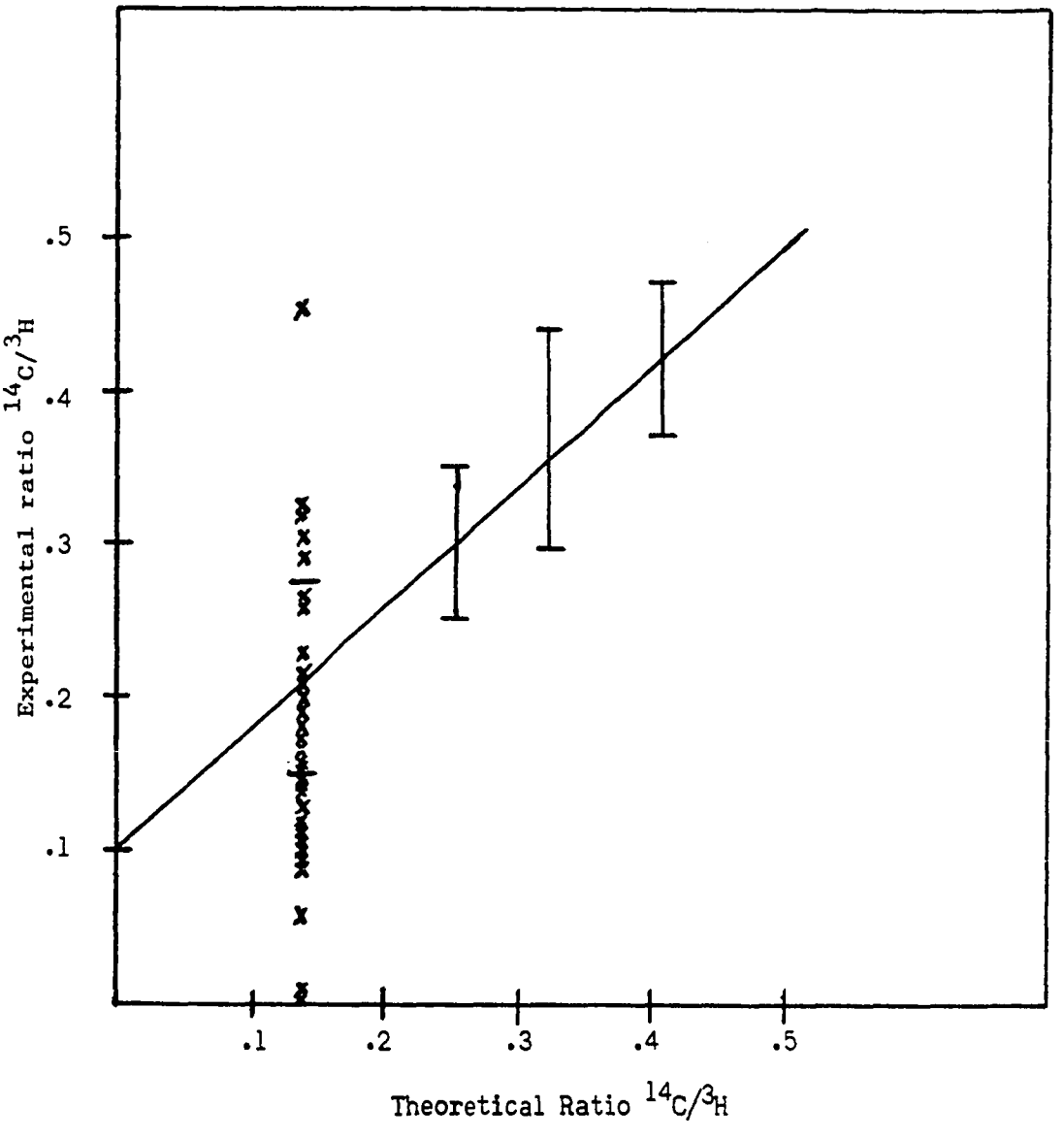


Figure 18 - Regression line of digest of fraction b-a. Slope equals 0.78, degree of correspondence, r, equals 1.0, X equals the unknown peptides from crude digest, plotted against the theoretical ratio of that mixture.

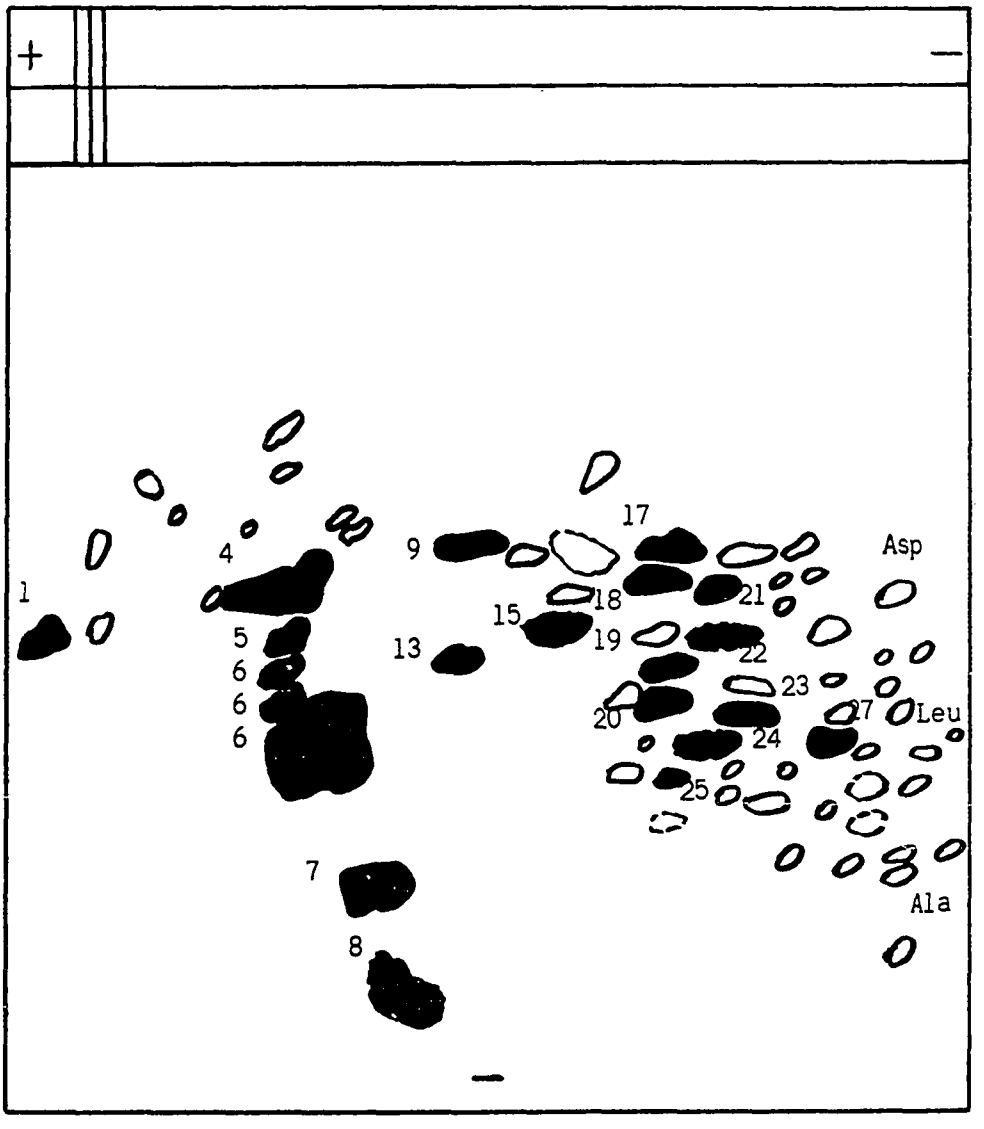


Figure 19 - Fingerprint pattern obtained from 1 mg ^{3}H-b-a plus 1 mg crude ^{14}C protein. Darkened areas correspond to spots in the standard pattern. Digestion was with alpha-chymotrypsin plus trypsin. The first dimension was electrophoresed at pH 3.5, the second at pH 2.1.

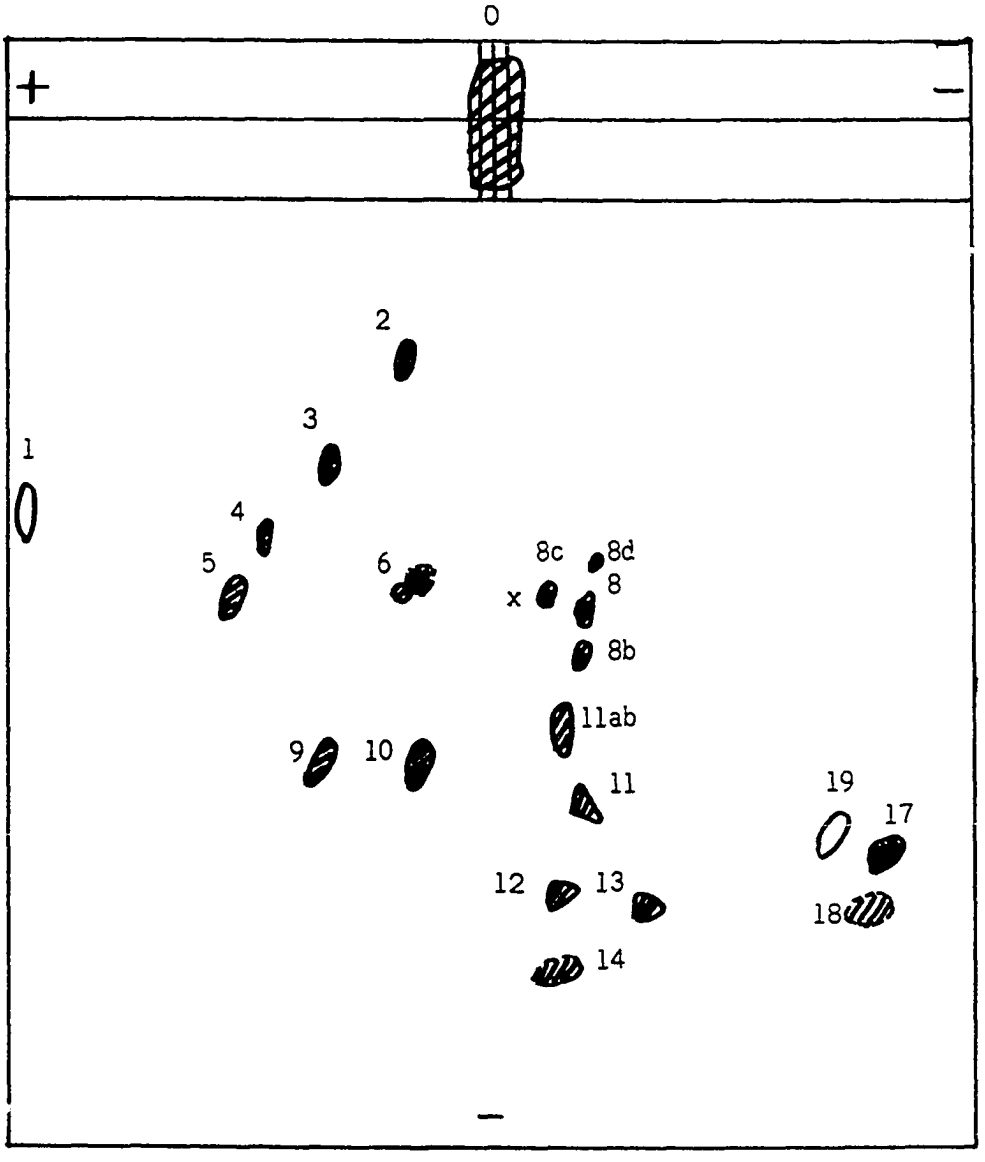
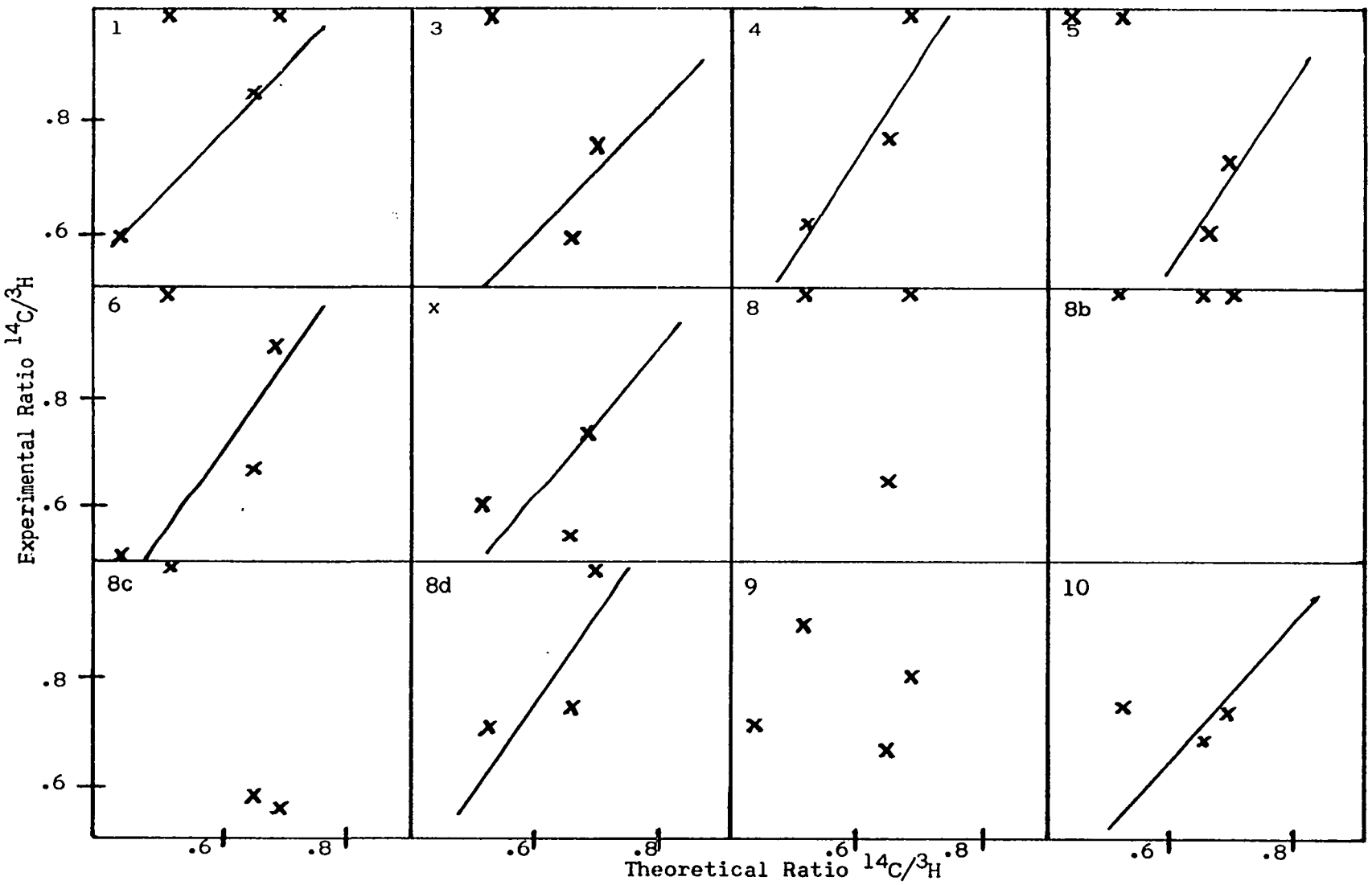


Figure 20 - Fingerprint pattern of oxidized b-a', digested with trypsin. The first dimension was run at pH 6.0, the second at pH 2.1. O = origin at pH 6.0. Shaded peptides are those which showed up in radioautograph of this fingerprint. Darkness of shading indicates the intensity of the spot on the radioautograph.

Figure 21 - Graphs of isotope ratios of tryptic peptides from oxidized b-a'. Theoretical values came from aliquots of digestion mixtures. Experimental values came from eluted peptides. Peptides are numbered in upper lefthand corners.


1
3
4
5
6
x
8
8b
8c
8d
9
10
.8
.6
.6
.8
Experimental Ratio $^{14}C/^{3}H$
Theoretical Ratio $^{14}C/^{3}H$

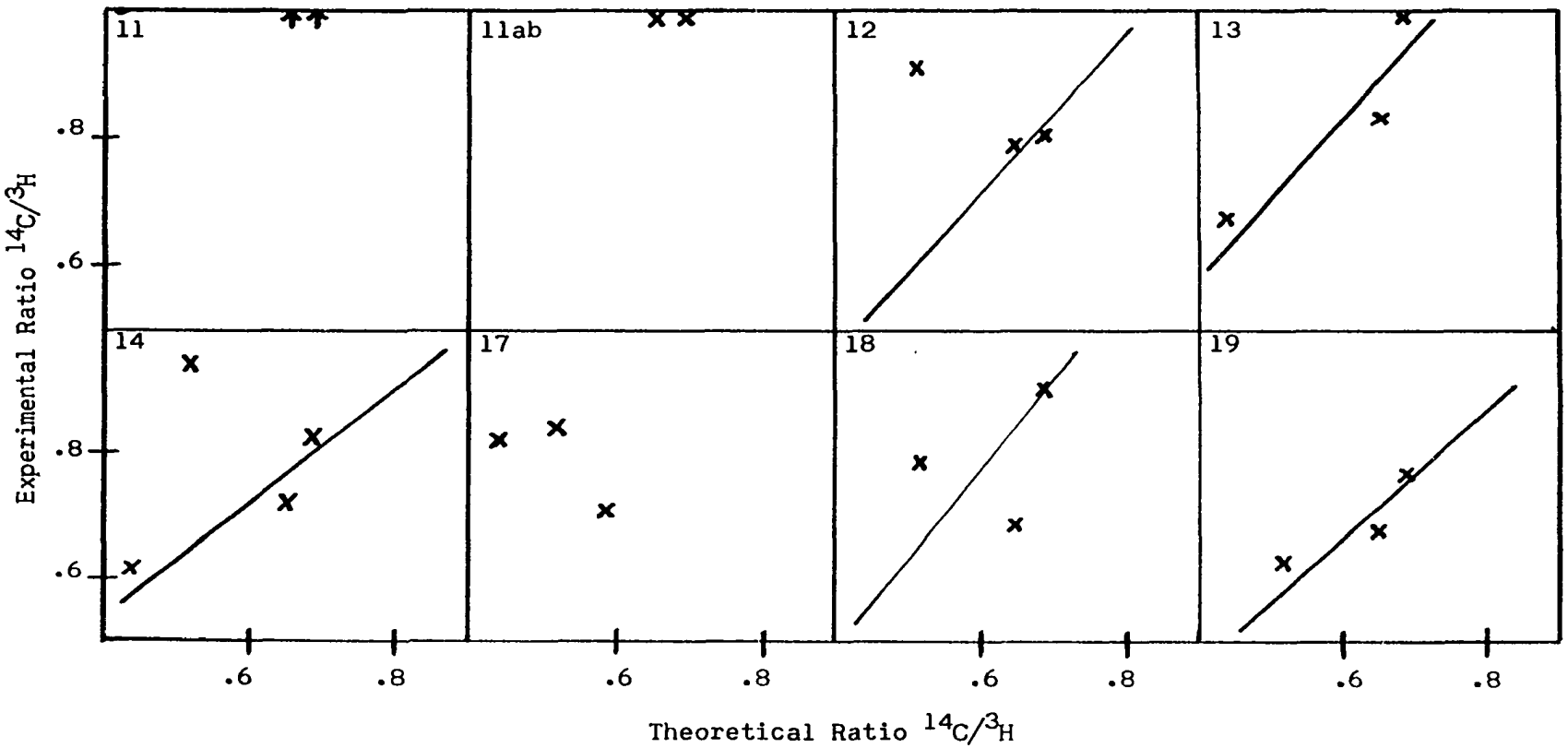


Figure 22 - Graphs of isotope ratios of tryptic peptides from oxidized b-a'.

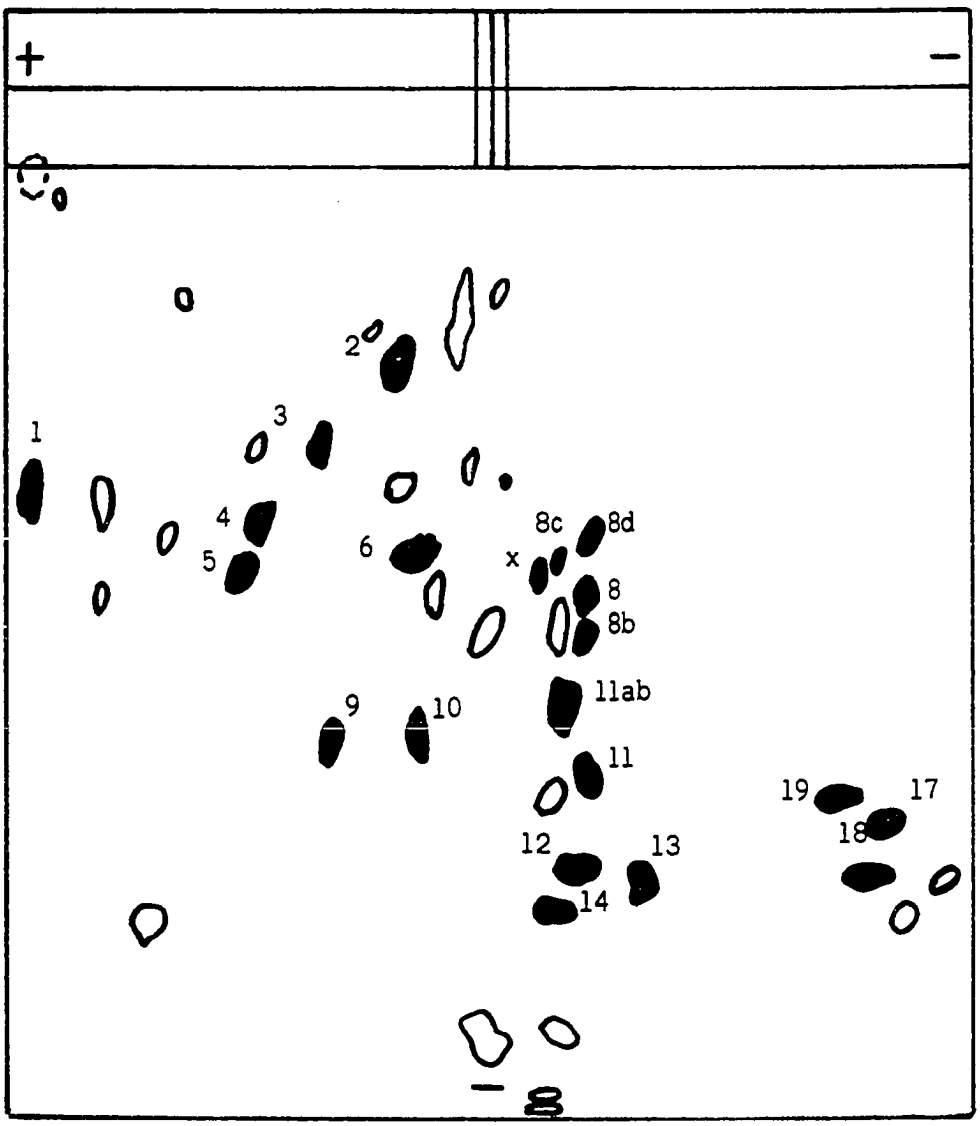


Figure 23 - Fingerprint pattern of oxidized tryptic peptides from mixture of pure ^{3}H b-a', plus crude ^{14}C protein. Darkened spots are those which correspond to the standard map.

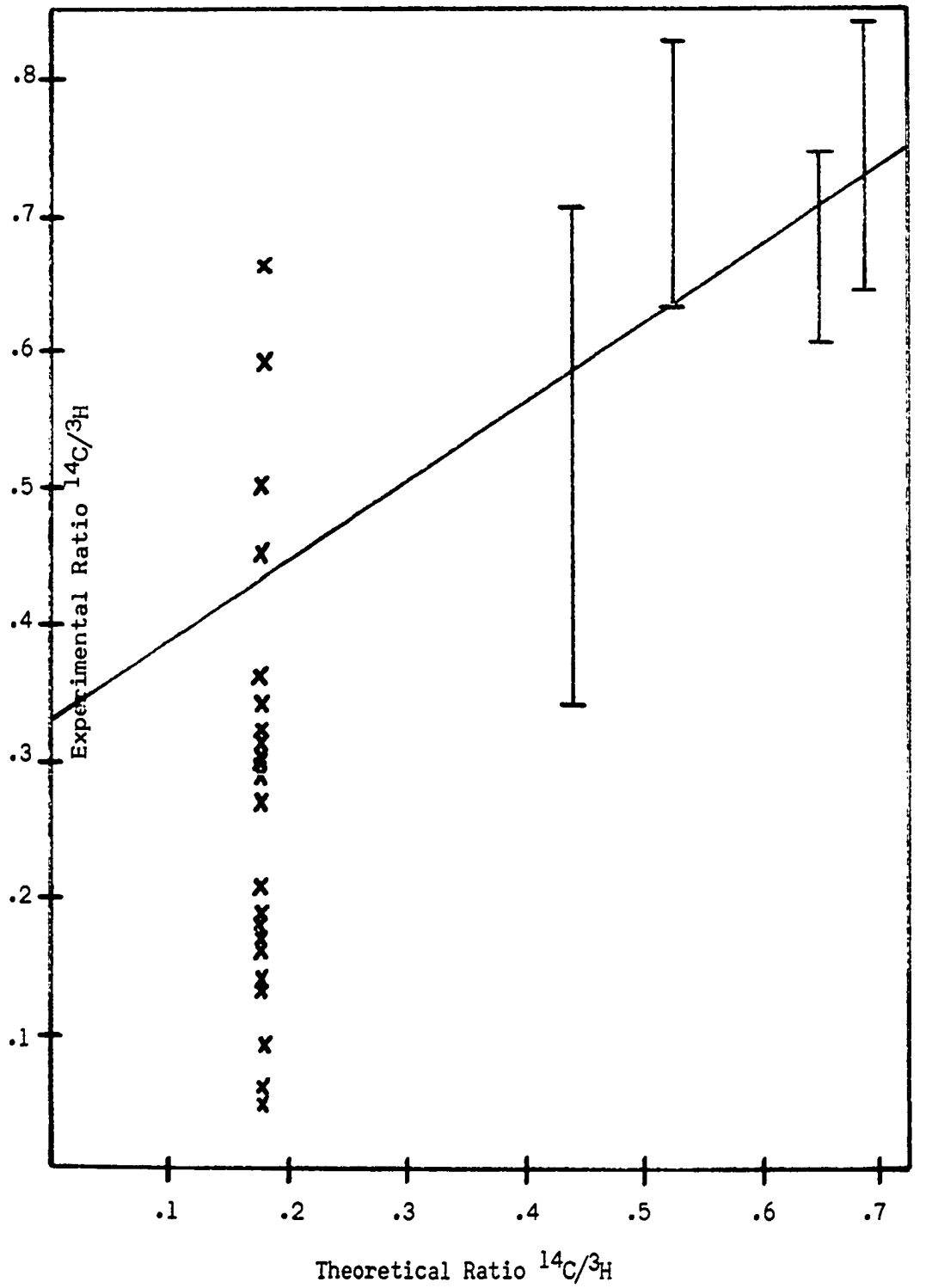


Figure 24 - Regression line of digest of b-a'. Slope equals 0.59, degree of correspondence, r, equals 0.71, X equals the unknown peptides from crude digest, plotted against the theoretical ratio of that mixture.

CHAPTER IV

DISCUSSION

The application of the peptide fingerprinting technique to the present study of the protein ovalbumin, a relatively unexplored molecule, has been useful in clarifying certain aspects of the structure and synthesis of this protein. Firstly, the number of disulfide and sulfhydryl groups have been ascertained, as well as partial sequences identifying some of them. Secondly, the order of phosphorylation of two phosphoserine sites has been established. Thirdly, two phosphothreonine containing ovalbumin fractions have been found. There is apparently no phosphoserine content in these fractions. Finally, a radioassay method has been devised, which is suitable for the study of biosynthesis of this protein in various systems. These aspects of this study will be discussed separately.

Discussion of the Cysteic Peptides

Some of the earlier work done by Fothergill (10) and Akabori (2), indicated that there were a total of 6 or 7 respectively, cysteic residues in ovalbumin. Later analysis by Weintraub and Schlamowitz (4), based on values obtained from three hydrolysis times, gave a value of eight. This is in agreement with the value reported here. The present study showed 4 sulfhydryl peptides, which is in agreement with the studies

of Diez et al. (9). It is apparent from his studies that some of the controversial values reported for sulfhydryl groups have been due to the fact that the sulfhydryls are partially inaccessible to reaction in the native protein conformation. After extensive denaturation, they obtained 4 sulfhydryls, using four separate reactive agents. The presence of two disulfide linkages is suggested by the total of eight residues for the molecule. As mentioned in the Results, peptides six and eight may form one such linkage. They are not sulfhydryl peptides as they do not disappear following carboxymethylation. Fothergill has also identified at least one disulfide (10) near the carboxy-terminal of the molecule.

Although the present work does not prove the existence of two disulfides, it does strongly support that probability. It is quite possible that one of the disulfide linkages is very inaccessible to proteolytic digestion due to steric hindrance, and is thus undetected within the diagonal. This possibility is already suggested by the necessity of strenuous denaturation to unmask all the sulfhydryl groups. A very intriguing suggestion was made by Delitzer and Lobachevskaya (2). They had obtained values of 5 sulfhydryls and 1.5 disulfides. To explain this they speculated that ovalbumin actually consists of an equimolar mixture of two proteins, each with eight sulfur containing residues. They reasoned that if one chain contained 4 sulfhydryl and 2 disulfide, and the other contained 6 sulfhydryl and 1 disulfide, this would give average values of 5 and 1.5. Although a single carboxy-terminal only has been detected (2), the possibility of two chains containing identical carboxy-terminal peptides, but differing internally, must not be dismissed. This observation may be pertinent to the heterogeneity found in

phosphorylation and in fingerprint maps of the protein. It may also have a bearing on the finding of phosphothreonine species of ovalbumin, in addition to the known phosphoserine species. Also, as was reported by Baker (8), there are two genetic species present in egg white of some White Leghorn flocks. This was even further suggested by the work of Lush (2), in which he detected electrophoretic heterogeneity in the A_3 form of ovalbumin. Since this form contains no phosphorus, it indicates some other structural heterogeneity, perhaps in some amino acids, or in the carbohydrate structure, or both.

Discussion of the Phosphopeptides

Two unique phosphoserine sequences have been found: a) Asp-Ser(P)-Ile-Glu-Ala-Glu; and b) Val-Val-Gly-Ser(P)-Ala-Glu-Ala-Gly-Glx. These sequences confirm the results obtained independently by Milstein (14).

The object of this study however, was not merely the elucidation of the amino acid sequences surrounding the Ser(P) residues, but to determine whether the individual sites were phosphorylated in a random manner, or, due to some structural or biological cause, obeyed a compulsory order of phosphorylation.

Perlmann (12) found evidence of the esterification of at least one of the phosphates of the molecule to a serine residue. She isolated a peptide containing the amino acid composition: Asp, Glu, Ala, Leu, Ser, and about 50% of the phosphate from a proteolytic digest of ovalbumin. This peptide probably belonged to sequence (a) above. Since at the time of her study, the second site of phosphorylation of ovalbumin was unknown, Perlmann studied the specificities of the enzymes which

could dephosphorylate this protein. She found that prostatic phosphatase, which will remove 46% of the phosphorus from ovalbumin, was most active at pH 5.3. Its specificity was only for -O-P- bonds of monoester linkages. On the other hand both intestinal phosphatase, and potato phosphatase release 46% of the ovalbumin phosphate within about one-half hour. They then commence to release the remaining phosphorus content of ovalbumin over a period of about 14 hours. The pH optimum of potato phosphatase is 5.6, but intestinal phosphatase has two optimas, one at pH 5.3 and one at pH 9.0. Hydrolysis is very slight at pH 7.0. Both of these enzymes will cleave both -O-P- and -N-P- bonds of monoester linkages. Since the prostatic enzyme will cleave only one phosphate, and the other enzymes will cleave both, Perlmann conceived the possibility that the second linkage was an -N-P- linkage to the guanido group of an arginine residue, or to a free amino group. She further suggested that this C-N-P- bond could be shifted to a -C-O-P- bond during acid hydrolysis therefore giving the appearance of both phosphates being bound to hydroxyl groups.

The work of Flavin (13), Milstein (14), and the present work, all show that both phosphates in ovalbumin are bound to serine residues. To account for the fact that prostatic phosphatase will only cleave at one of these points of attachment, it may be feasible to postulate that the three dimensional structure of the protein surrounding these Ser(P) sequences may be sufficiently different as to block the approach of the prostatic enzyme to one site. This conformational difference might also account for the very slow cleavage at this site by the intestinal and potato enzymes. The length of the polypeptide chain and the position of

the phosphate in relation to the chain length might also influence sensitivity to phosphatases.

Flavin (13) reported finding short peptides with compositions similar to that of sequence (a) reported in this work. His peptides were present both before and after treatment with prostatic phosphatase. He therefore concluded that this peptide belonged to the resistant phosphorylation site remaining after dephosphorylation of ovalbumin A_1. Flavin also found the peptide Ser(P)-Ala, which he showed to disappear after prostatic phosphatase treatment. Ser(P)-Ala belongs to sequence (b) above, and is proposed by Flavin to be the phosphatase labile moiety of ovalbumin A_1.

Despite the fact that dephosphorylation of ovalbumin A_1 leads to an increased appearance of a species of ovalbumin with an electrophoretic mobility similar to that of ovalbumin A_2, it could not be certain that phosphorylation might not be a random process. Two possibilities could lead to this result. First, although dephosphorylation of A_1 gives a peak in the position of A_2, this does not rule out the possibility that in a natural heterogeneous mixture, some species of ovalbumin which contain only one phosphate, and so would appear in the same electrophoretic peak position as A_2, might have their single phosphate at the other site. This would be the case if there are, as the literature has suggested (12, 8), more than one genetically distinct ovalbumin species. The A2 species produced by Perlmann was found by cleavage of one phosphorylated site. This was probably due to sensitivity of that site to dephosphorylation. However when biosynthesis of the molecules occurs, the rate of phosphorylation may not be influenced in the same way (or order) as de-

phosphorylation.

To help clarify this question the three species of ovalbumin were partially purified in this laboratory, from a heterogeneous mixture purchased commercially. The results of fractionation with DEAE-Sephadex appear to be conclusive. Although fractions b and c contained both A_2 and A_1, as seen from disc gel electrophoresis, they contained both Ser(P) peptide sequences, as would be expected from any fraction containing A_1. Fraction a, however, although slightly contaminated, contains about 90% A_2. This fraction contained only one sequence, sequence (b). This indicates that phosphorylation is not random. Had the two sequences been present, one could say that within the same fraction, different protein molecules had their only phosphopeptide in one sequence, and others in the other sequence. This was not the case. However, if phosphorylation of the two sites are accomplished by two separate enzymes, then the possibility cannot be excluded that the enzyme phosphorylating sequence b is kinetically more active than the other enzyme. This would also result in an ovalbumin fraction containing predominantly this sequence.

An interesting feature of these results is that they are contradictory to those of Flavin (13), who found that the Ser(P)-Ala peptide of sequence b was not present in A_2. This may be due to a specificity difference between the dephosphorylating enzymes and the natural phosphorylating enzymes. Also, if a structural conformation is responsible for slow dephosphorylation of sequence a, this may not mean that this sequence wasn't phosphorylated first.

The work reported here would have been enhanced had enzymatic dephosphorylation of the two sequences been performed to test the sensi-

tivity of each sequence to prostatic and intestinal phosphatases. However this specificity might not be the same on a small peptide substrate, as on the complete protein.

After purification of fractions b-a, b-a', and b-d, by hydroxyapatite, it was shown that fractions b-a' (A_2), and b-d (A_2 plus A_1), contain phosphothreonine but no phosphoserine. A possible explanation of this is that threonine is the first moiety to be phosphorylated, and that later, as the molecules assume their correct three dimensional structure, transacylation occurs from the threonine to adjacent or nearby serine residues. It could also be that the protein isolated in this work may be a minor genetic variant or a contaminant. A minor contaminant would be normally eliminated during commercial purification procedures, and so phosphoserine only is observed in commercial preparations. If this protein were a minor genetic variant of ovalbumin, it would imply that the conditions of incubation used in this work caused a shift in the normal ratio of variants synthesized so that the usually minor species were synthesized as the major species in this system.

It is unlikely that the fractions are actually contaminants and not ovalbumins, for they gave the same peptides as commercial ovalbumin in fingerprint maps. Also a cysteic diagonal was made of the radioactive protein, and the pattern produced was identical to that obtained using commercial ovalbumin. Therefore the cysteic peptides of the two proteins are the same. This does not rule out the possibility that minor genetic variants of ovalbumin were synthesized and isolated, which contain phosphothreonine rather than phosphoserine. An alternative explanation might be that these proteins are actually ovalbumin fractions of

the usual species; but that since the protein conformation is probably not the same, or not completed in tissue incubation, the normal specificity of phosphorylating enzymes was affected differently, leading to phosphorylation of threonine rather than serine moieties. The altered conformational properties of the synthesized proteins may also have increased the lability of phosphoserine residues to dephosphorylation, therefore phosphoserines may be formed and then dephosphorylated faster than phosphothreonines.

Discussion of the Assay

The purpose of this assay was to provide a reliable, reproducible means of identifying the synthesis of a specific protein, by means of its peptide components. If a full, three dimensionally completed protein structure were not identifiable, then an assay, such as will be discussed, would provide a method of following the partial synthesis of the protein by identifying and, or, quantifying the presence of specific peptides belonging only to this protein. The assays used to date, as outlined in the Introduction, have included identification of a protein by disc gel electrophoresis, comparison of elution patterns of peptides from digested proteins passed through columns, and most specifically, immunoprecipitation by a specific antibody. These methods are useful, in conjunction with testing of isolated RNA fractions in a cell-free system, for the detection of mRNA activity. The immunoprecipitation technique is especially well adapted to this purpose (25). The assay presented in this work, is uniquely applicable to a study of the kinetics of the translational process of protein biosynthesis, for which the author is unaware of an existing assay. By assaying, at varied time in-

tervals, the specific peptides synthesized, one could study the kinetics of translation along the length of a protein, and determine the degree of uniformity or variability in rate of translation of each protein segment.

Theoretically, this assay is designed to specifically identify peptides from a mixture of synthesized proteins or polypeptides by three means: a) the location of the peptide in fingerprint maps as compared to standard peptides; b) the coincidence of this peptide spot with a positive spot in a radioautograph; and c) the ratio of ^{14}C label in the unknown peptide with ^{3}H label from the standard peptide. If a peptide was truly from the protein under study then the ratio of $^{14}C/^{3}H$ for this peptide should closely correspond to the slope of the curve established from ratios of pure preparations of this peptide in various proportions of ^{14}C- and ^{3}H-labeled material. Although the yield of ^{14}C in the unknown peptide might vary, its ratio to a known amount of ^{3}H should fall on the line, if it is a peptide from this protein. If not, the ratio should give a spurious value, as any ^{3}H present would represent background picked up from tailing on the paper maps or some similar artifact.

Several problems, however, remain inherent in the assay at the present time, although with better procedures, and more time, these could probably be minimized. The first problem was in producing an evenly and completely labeled protein for purification and assay procedures. The time of incubation of oviduct minces was three hours, with ^{14}C-amino acids, and with ^{3}H-amino acids. In this time period some total protein was surely labeled. However, it would have been much better to establish

the optimal time period for labeling of ovalbumin compared to total protein. This optimal time period most likely also differs when using ^{3}H-amino acids from that using ^{14}C-amino acids. This might occur due to a difference in the kinetics of the chain elongation step due to the isotope effect on the reaction at the carboxyl- and amino-groups of the joining amino acids. The rate of incorporation into ovalbumin also varied due to physiological conditions. The keeping of chickens in the animal wing was found to cause degeneration of the oviduct of actively laying hens within 3 to 4 days. This was most likely due to trauma and shock from close association with barking dogs. To bypass keeping hens in the animal wing, they were purchased from the farm immediately before use. However the condition of the oviduct was still not uniformly swollen as it is in actively laying hens.

To minimize these problems in the future, two alternative methods of labeling would be preferable. Firstly, if one used the same oviduct, divided in half, for incubation with each label, then the physiological factor would at least be identical in both incubations. Also if the minced tissue were allowed to preincubate, without labeled amino acids for a period of time, this would allow completion of nascent polypeptide chains with cold endogenous amino acids. When the labeled amino acids were then added, a higher proportion of completely labeled chains should be synthesized. A second alternative would be to inject labeled amino acids into live laying hens kept under conditions conducive to contentedness of the animals. Injection might also be made into immature chicks which have been previously injected with estrogen to induce ovalbumin synthesis (22, 23, 25). Either of these procedures should lead to

more even and complete labeling of protein in the oviduct, as protein synthesis would occur in the natural physiological environment and optimal hormonal stimulation.

To check the degree of uneven labeling of peptides obtained in this work, the peptides were plotted in order of their increasing isotope ratios (Figures 25, 26). As the degree and evenness of label of the two isotopes increases (approaching 1) the peptides must be closer to the carboxyl-terminus of the protein, as nascent polypeptide chains would be completed with labeled amino acids. These graphs were made in a manner similar to that used by Campbell, Sargent and Brew (37, 38) to establish the synthesis of proteins from the amino- to the carboxyl-terminals. A clear, but not steep gradient was seen in ratios of peptides from fraction b-a (Figure 25). The gradient is also consistent and alike for each different proportion of isotopes. Those peptide ratios which fall grossly off the gradient were also those peptides which had very low count recovery, and so this effect might be blamed on non-statistical counting. The low recovery of counts from peptides of this fraction and digest may be due to two factors. First, the peptides did not separate cleanly into discrete areas on the filter paper during electrophoresis. There was therefore some tailing of both radioactivity and of ninhydrin sensitive material. For this reason, when peptide spots, identified by ninhydrin staining, were cut from the paper, some loss of radioactivity belonging to this peptide occurred due to tailing. There must have been some additional loss due to absorption of counts on the paper. Since the total radioactivity applied to the paper was low, the combination of loss due to tailing of peptides plus absorption by the

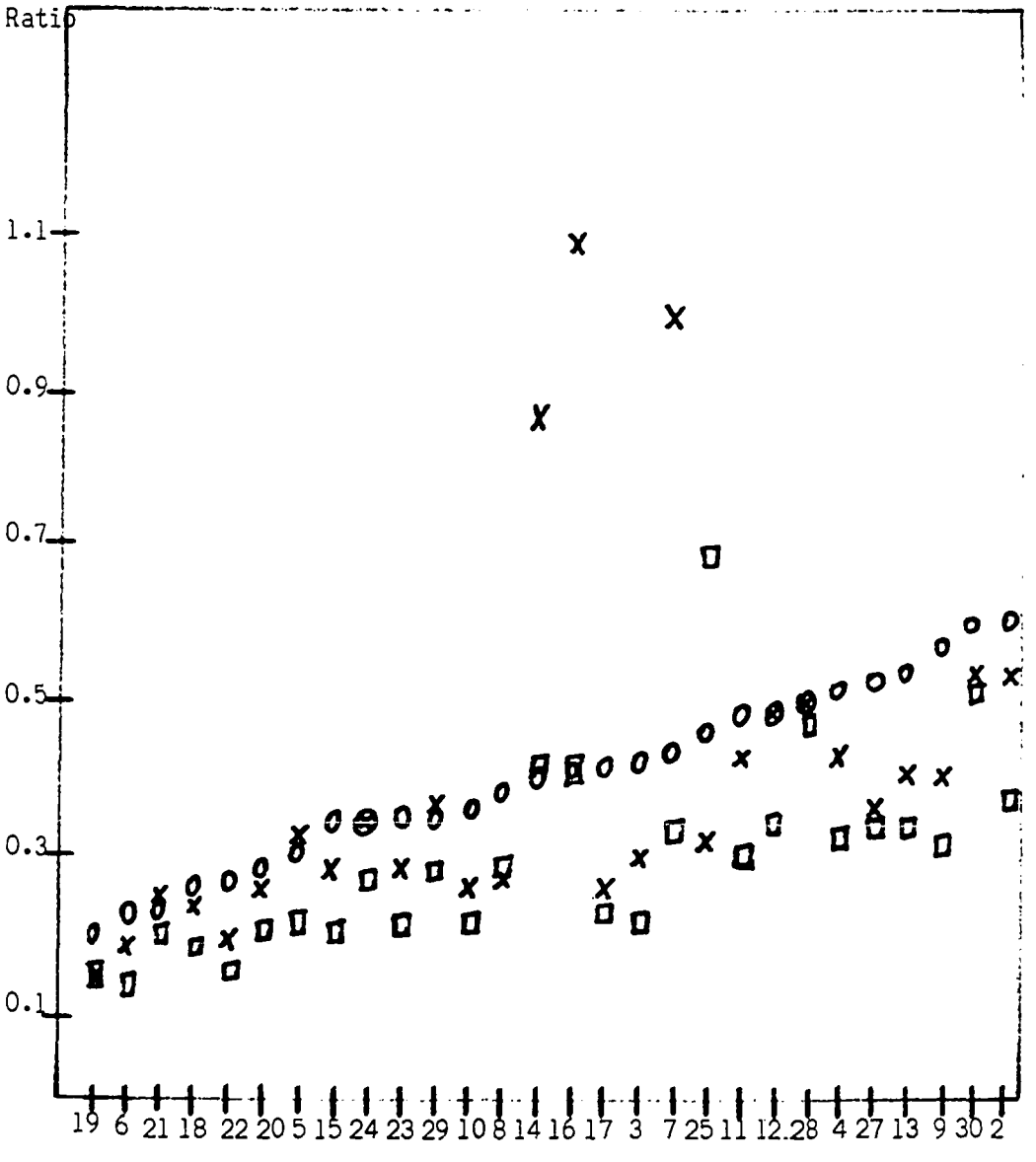


Figure 25 - Graph of gradient of ratios ($^{14}C/^{3}H$) in peptides from digest of fraction b-a. Peptides are arranged along abscissa in order if increasing ratio at a 1 to 1 concentration of isotopes. O equals a 1 to 1 mixture of isotopes. X equals 1-.8-.2 mixture of ^{3}H, ^{14}C, and commercial ovalbumin, respectively. ☐ equals a 1-.6-.4 mixture of the same order.

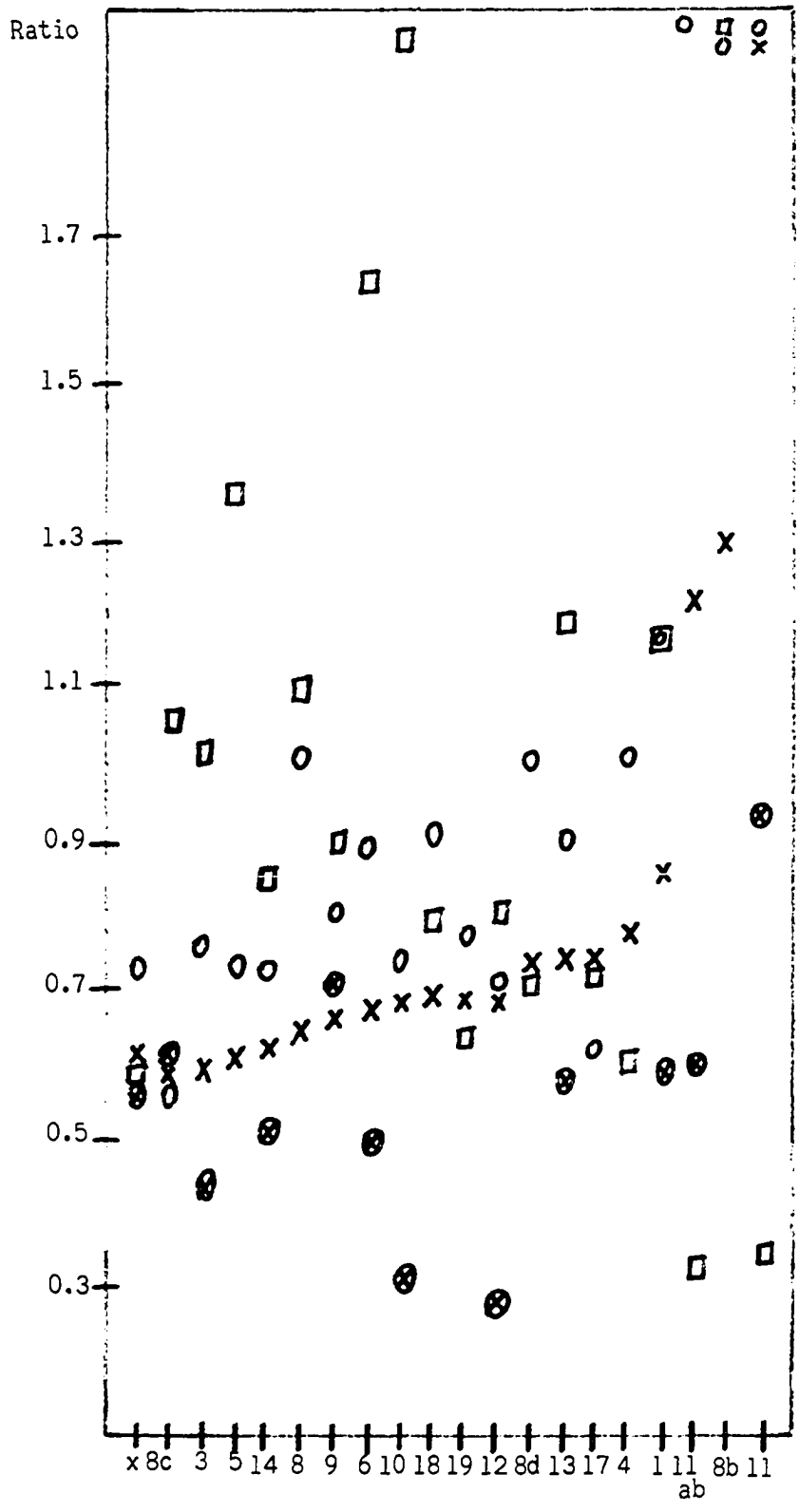


Figure 26 - Graph of gradient of ratios in peptides from digest of fraction b-a'. Peptides are arranged along the abscissa in order of increasing ratio at a concentration mixture of 0.4-0.5-0.1, ^{3}H, ^{14}C, and commercial ovalbumin, respectively. ⊗ equals .4-.3-.3 mixture as above. □ equals .4-.4-.2 mixture. X equals .4-.5-.1 mixture. ⊖ equals .4-.6 mixture.

paper could give lower counts in some peptides since not all peptides tailed equally badly. When low counts are measured, even though efficiency of counting is corrected for, the statistical accuracy is lowered.

The gradient of labels in the peptides could be used, as suggested by Campbell et al. (37, 38) to give an indication of where peptides are located in the polypeptide chain in relation to the terminals. The fact that the gradient was fairly uniform for each isotope mixture in Figure 25 accounts for the high degree of correlation in the regression line for this digest, despite some uneven labeling of the molecule.

On graphing the peptides from tryptic digestion of fraction b-a', however, a steeper gradient was seen. Moreover, the gradients for each isotope mixture were very inconsistent (Figure 26). This agrees with the general lack of correlation in the ratio graphs of peptides from this digest. Several things may have influenced these results. A number of peptides from this digestion had very low counts and so there is more statistical error in counting in this digest. The reason for this was that a smaller amount of material was applied to the filter paper in an effort to reduce smearing and tailing of radioactivity (which accounted for some error in the first digest). However, although the pattern was much clearer, the yield was also low. Another possible explanation is that fraction b-a' may be a genetically distinct species from b-a, and therefore its synthesis may be regulated differently, or be more influenced by physiological factors (8).

One further, and very serious problem remains inherent in this assay. Some peptides which were eluted from the fingerprint of combined pure ^{3}H-labeled and crude ^{14}C-labeled protein, were identical in position

to pure peptides. Yet these peptides gave isotope ratios which fell away from the statistical range of the regression line. On the other hand, several peptides which never appeared in standard maps, and so might not belong to ovalbumin, fell close to the regression line. Therefore a more positive means of identification of these peptides is necessary. For example, a comparison of the amino acid compositions to see if the same amino acids are labeled with each isotope. Also, the range of error within the regression line would need to be lowered, especially in the second digest. This could be accomplished by incorporating the improvements in methodology discussed above, i.e., labeling of pure proteins more evenly and completely, using optimal time and conditions, improving the physiological parameters involved; and using more labeled material on the cleaner separating map.

In conclusion the assay described here is useful in the identification of specific peptide synthesis. This identification is based on electrophoretic mobility, correspondence of ninhydrin positive spots to radioactive exposure of radioautographs, and correspondence of isotope ratio to that of pure mixtures. The use of the regression line is still problematic as the methodology needs to be improved. As an assay for protein synthesis directed by an isolated mRNA in a cell-free system, it is far inferior to such refined techniques as immunoprecipitation. However, its usefulness as an assay of the kinetics of the translational step of protein biosynthesis would seem to be worth developing.

CHAPTER V

SUMMARY

Five unique cysteic acid peptides were partially sequenced. Four of these were determined to be sulfhydryl containing peptides, due to their reactivity with iodoacetate. The remaining sequence, plus three others, not determined, are most likely involved in disulfide linkages. This would account for a maximum number of eight cysteic residues determined by this laboratory, and at least one other (4).

Two phosphoserine containing sequences were determined, accounting for the two phosphorylated moieties of ovalbumin A_1. Identification of the sequences in fractionated material indicated that phosphorylation is probably not random. This assumes that there is only one amino acid chain of ovalbumin, and that heterogeneity is due only to the differences in phosphates. There is good evidence that this may not be the case since heterogeneity has been found in the dephosphorylated fraction (2). In this laboratory, differences in many peptides were observed on fingerprint patterns compairing the fractions. Therefore, the possibility remains that there is structural heterogeneity due to genetic or physiological controls or both.

The use of hydroxyapatite was found to be an excellent means of fractionating heterogeneous proteins by means of their phosphorus content

(42).

The ovalbumin fractions A_2 and A_1, formed by incubation of minced oviduct in tissue culture media, with radioactive amino acids, displayed the presence of phosphothreonine. It is suggested that phosphothreonine may be transacylated to phosphoserine at later steps. Ovalbumins A_3 and A_2 were both observed as intermediates in the synthesis of A_1.

The use of fingerprint mapping techniques to identify peptides synthesized in vitro may be useful in corroboration with other means of identification. It is not a positive identification however, as different peptides may have very similar, or even identical mobilities during electrophoresis. The usefulness of dual isotope ratios as an identification of specific protein peptides has not been conclusively demonstrated in this work. However it should not be dismissed as a valuable assay procedure, if technical problems can be worked out. The technique is useful in qualitatively determining the positions of synthesized peptides along the growing polypeptide chain. The usefulness of this assay in studying the kinetics of translation is proposed for future study.

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