THE AMINO ACID COMPOSITION AND NH_2-TERMINAL AMINO ACIDS OF COMPONENT 6 OF DIETHYLSTILBESTEROL TREATED COCKERELS

by

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INTRODUCTION

The lipoproteins of serum contain protein components in combination with phospholipides, cholesterol and its esters, triglycerides and other lipides (5). The importance of lipoproteins in lipide transport has created considerable interest in their structure resulting in the present attempt to add a little to the solution of this problem.

Most lipoproteins occuring in nature probably have to be classified as secondary valence complexes which are held by van der Waals forces. However, the phosphorous and protein may be bound through -O-P and -N-P esters. These linkages involve alcoholic or aromatic hydroxyl groups of amino acids. In order to determine the N-terminal amino acid serving as a linkage between a protein and the phosphorous, the amino acid compostion of the delipidized component 6 was determined by two dimensional chromatographic technique. Further, the NH_2-terminal amino acids of component 6 of chicken serum lipoproteins have been determined. An attempt has been made to compare the NH_2-terminal amino acid (29) of component 6 before and after delipidization. In order to accomplish the above, the DNP amino acid derivative of the non-delipidized component 6 and the DNP amino acid derivative of the delipidized component 6 were determined. Thus a qualitative difference in the NH_2-terminal amino acid of the non-delipidized component 6 and of the delipidized component 6 was evaluated.

REVIEW OF LITERATURE

Sandor (28) has reviewed the occurence of the lipides in living matter. Lovern (18) has given chemical information regarding the same. Lipoproteins are combinations of proteins and lipids, and the importance of lipoproteins in lipids transport has created considerable interest in their structure. The present investigation is an attempt to probe into the structure of a lipoprotein. Lipoproteins connotes a group of compounds with properties (biological reactivity, solubility, colour, optical and other physical constants) different from those of the sum of their components. The lipoproteins of serum contain protein components in combination with phospholipides, cholesterol and its esters, triglycerides and other lipides and it has been widely speculated that lipids are carried in plasma as a special lipid-protein complex.

It has been observed by Deutsch and Goodloe (9) that the electrophoretic pattern of plasma of male chicken were different from that of female chicken and that this was due to the female species containing more globulin fraction than the male. Kibrich and Blonstein (12) noted that, as the birds matured, the total protein and the b-globulin fraction of the sera increased. Variations in total protein, phosphorous and calcium has been repeatedly reported. Brandt et al (4) confirmed that the total protein, a-globulin and b-globulin increased with age. Clegg et al (8) showed that when a diethylstilbesterol solution was injected into cockerels it brought about changes in serum of these birds

which were similar to the changes that occur when hens began egg production. Moore (22) compared the electrophoretic pattern of chicken sera before and after ether extraction and showed that it contained an enormous quantity of lipoid substance. Ether extraction also removed the sex differences observed in the electrophoretic pattern of chicken sera. However, the ether extraction method of McFatlane (19) caused some of the electrophoretic components to disappear completely. This indicated that chicken sera contained firmly bound lipoprotein complexes which were mainly responsible for the electrophoretically observed differences. Various studies on the source of phosphorous found in the egg have shown that extra phosphorous appeared in the blood of birds during egg production. According to Chargoff (5) phosphorous plays an important role in the binding of lipids to protein and this speculation was indicated by the amount of phosphorous found in various lipoproteins.

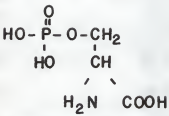
Clegg and Hein (6) and Clegg et al (7), in order to determine the P^{32} activity associated with the blood serum and egg proteins of the chicken after giving them radioactive phosphorous, combined moving boundary electrophoresis and radioactive tracer technique, and they noted that electrophoretic components one and five of diethylstilbesterol injected rooster blood serum exhibited high phosphorous activity. Similar results were obtained for activities associated with the electrophoretic components of laying hen blood serum. They also observed that component one showed very high P^{32} activity. Ericson (11) observed that component three contained the largest amount of total phosphorous associated with serum

protein of the non-laying hen. Component 2, albumin and Components 3, 5 and 6 exhibited little phosphorous activity. Ericson (11) observed that these components increased in amount in the diethylstilbesterol injected chicken serum and contained a high amount of total phosphorous and lipid and may be considered lipoproteins. The present evidence indicates that when the hen starts egg production and when the cockerel was injected with diethylstilbesterol solution, certain components increased in amount and some new components appear. It has been shown that these proteins had very high amounts of phosphorous and lipids and hence may be assumed to be phospho-lipoproteins. Serum lipids do not occur in the free form but in combination with one another and with protein as a high molecular weight lipoprotein complexes, bound together by secondary valences held together by van der Waal's forces. Isolation of high lipid and phosphorous containing protein fractions has been performed. Using salt-fractionation ultracentrifuge and preparatory electrophoresis Misra (21) has purified component 6 to the extent that it is homogenous to electrophoretic and ultracentrifuge analysis although gradient ion exchange chromatography indicated a slight heterogenity. Ericson (11) has evaluated the lipid and phosphorous contents of serum samples of laying hens, diethylstilbesterol injected chicken serum contained a high amount of total phosphorous and lipid and may be considered as lipoproteins.

As regards to the bonding between a protein and phosphorous both -O-P and -N-P esters present themselves as possibilities. Such linkages would involve either alcoholic or aromatic hydroxyls,

on the one hand, or free amino groups or the guanido group of arginine, on the other. To date, only three phosphoamino acids have been isolated from biological materials: O-phosphorylserine and O-phosphrylthreonine, both representatives of an -O-P ester and phosphoarginine, which has a -N-P bond. In addition it has been possible to prepare phosphorylated derivatives of other amino acids, e.g., tyrosine, oxyproline (14), glycine, alanine, glutamic acid, leucine and glycylglycine (31) and the methylester of N-phosphorylphenylalanine (17).

O-Phosphorylserine: This amino acid $C_3H_8O_6NP$ (P = 16.7%) and with $[\alpha] = +7.2$ (1) was first obtained by Lipmann and Levene (16) from vitellinic acid. Lipmann (15) subsequently succeeded in isolating this substance from an acid hydrolyzate of casein, and thus established that phosphoserine may occur as a constituent in phosphoproteins. More recently Ågren, de Verdier, and Glomset (1) crystallized O-Phosphorylserine. The following structure is usually assigned to this amino acid.



Here one has to keep in mind the experiments of Bergman and Miekely (2) who showed that in the case of benzoylserine the acidity of the medium determines whether the benzoyl residue is linked to the hydroxyl or to the a- amino group of the amino acid. That a similar situation exists in the case of phosphoserine

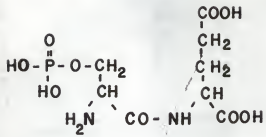
follows from the work of Plapinger and Wagner-Jauregg (25). These investigators found that on treatment of the N-diisopropylphosphoryl derivatives of the DL-serine methyl ester with boiling aqueous hydrochloric acid O-phosphorylserine is formed from the N-compound. Therefore, the existence of phosphoserine with a -N-P bond in a native protein is feasible, e.g. as the N-terminal amino acid of a peptide chain.

O-Phosphorylthreonine: This phosphoamino acid with an O-P linkage, phosphothreonine $C_4H_{10}O_6NP$ (P = 15.5%), $[\alpha] = -7.37$, was isolated from an acid hydrolyzate of casein by de Verdier (10). Plapinger and Wagner-Jauregg (25) showed that as in the case of phosphoserine, migration of the phosphate group from the N- to the O-position occurs in the case of this compound.

Phosphoarginine: $C_6H_{15}O_6N_4P$ (P = 11.5%) is the only amino acid with a -N-P bond thus far encountered in biological material (20). Its presence in the muscles of invertebrates suggests that this substance fulfills a role similar to that of creatinephosphate in vertebrates. However, it is still unknown whether or not phosphoarginine occurs in phosphoproteins. One of the characteristic features of these three phosphoamino acid is that in contrast to the intact proteins, the phosphate group is stable in 0.25 N sodium hydroxide solution. The N-P bond of phosphoarginine, however, is acid labile.

O-Phosphorylserylglutamic acid: In 1933, shortly following the discovery of phosphoserine (16), Levene and Hill (14) isolated from a casein hydrolyzate a dipeptide consisting of serine, glutamic acid and phosphoric acid. Posternak and Pollaczeck (26)

demonstrated the presence of a free a-amino group in the serine moiety of the molecule and assigned the following structure to the dipeptide:



However, these investigators showed that the peptide bond of phosphorylserylglutamic acid was resistant to the action of a dipeptidase from pig intestine. However, after removal of the phosphate group with the aid of kidney phosphatase the dipeptidase readily hydrolyzed the peptide bond. It is thus clear that the phosphate group has a protective action on the peptide linkage, and one can conclude that not only the type of linkage but also the surrounding molecular configuration determines whether an enzyme will act.

In order to probe these amino acid-phosphorous-lipid relationship in component 6 the amino acid composition of the component 6 was investigated. The subsequent discussion is a description of our attempt to find a relationship between the amino acid composition of component 6 and the lipid portion.

MATERIALS AND METHODS

Blood Serum Sample

The serum in this work was obtained from the blood of White Rock cockerels. They were about one to two years old and were fed a commercial chick starter or grower at the Kansas State University Poultry Farm. The cockerels were injected intramuscularly with 3.0 mgm diethylstilbesterol per one ml propylene glycol for seven days. On the eighth day the roosters were sacrificed and the blood collected in 50 ml centrifuge tubes. The blood was allowed to clot at 38°C for two to three hours and then centrifuged at 3,000 revolutions per minute for 20 minutes at 0°C. The serum was decanted and stored overnight at 7°C.

Component 6

In this investigation ultracentrifuge[1] techniques were employed in the initial isolation of component 6. Cellulose nitrate tubes were filled with 13 ml of serum and capped tightly with aluminium caps. The tubes were placed in a 40,000 r.p.m. rotor and the serum samples were centrifuged for 16 hours at 40,000 r.p.m. at 0°C. At the conclusion of centrifugation, the tubes were very carefully removed from the rotor. The top layer was a pale yellow, semi-solid substance of the consistency of chilled cream. The bottom layer was hard, sticky, semi-solid and red in colour. The

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[1] Spinco Preparative Ultracentrifuge Model L.

middle layer was a clear solution very pale yellow in colour. The top layer was removed very carefully by means of a spatula. This top fraction as found by Misra (21) was essentially component 6.

Delipidization. (23) Component 6 was transferred into a 150 ml beaker and treated with 83 ml of absolute methanol with constant stirring for 30 minutes. Then 83 ml of chloroform were added and the contents were transferred into a 250 ml volumetric flask which was kept in a water bath at 60°C for 15 minutes. The flask was then cooled and the volume made up to 250 ml with chloroform. The precipitated protein was separated from the solvent by means of a Buchner funnel under suction and dried in the oven at 105°C for half-hour and weighed. The component 6, thus obtained, was the starting material for subsequent NH_2-terminal Amino Acids analyses.

Hydrolysis of Component 6. (3) Half-gram of the sample of component 6 obtained as mentioned above was hydrolyzed under reflux with 10 ml of 6N hydrochloric acid for 20 hours. The excess hydrochloric acid was removed on a steam bath. The hydrolyzate was taken up with warm water, filtered and again evaporated to dryness and then dissolved in 1.0 ml of 10% 2-propanol which is an effective preservative and does not cause esterification.

Spotting. (13) 1.0 λ to 2.5 λ aliquot was used for spotting the chromatogram. The chromatograms were run by the two dimensional descending chromatographic method for 16 hours, employing 22.5 x 18.25-inch sheet of Watman No. 1 chromatographic paper. The first solvent was n-Butyl alcohol, glacial acetic acid and water in the ratio of 4:1:5. The paper was then sprayed with a

borate buffer of pH 8.3 consisting of 200 ml of 0.1 M boric acid plus 113.5 ml of 0.1N sodium hydroxide solution. The second solvent was made by mixing 25 gms of phenol, 25 gms of m-cresol and 75 ml of the borate buffer, of pH 8.3. The aqueous phase for equilibrating the chambers was prepared by mixing 250 ml of the pH 8.3 borate buffer with 8.0 ml of the second solvent and was placed in a beaker at the bottom of the chromatographic cabinet. The ninhydrine mixture employed for the analysis of the chromatogram was composed of 50 ml of 0.1% ninhydrine in ethyl alcohol, 2 ml of collidine and 15 ml of glacial acetic acid.

Standard mixture A was prepared by dissolving 200 micromoles each of lysine, aspartic acid, glycine, threonine, proline, valine, tryptophan, phenyl alanine and leucine in 2 ml of 1N hydrochloric acid and making up to 10 ml with 0.1N hydrochloric acid.

Standard mixture B was prepared by dissolving 200 micromoles each of cystine, histidine, arginine, serine, glutamic acid, alanine, tyrosine, methionine and isoleucine in 2.4 ml of 1N hydrochloric acid and making up to 10 ml with 0.1N hydrochloric acid.

By dissolving the amino acid solutions in 0.1N hydrochloric acid, complete dissolution of the relatively insoluble amino acids such as systine and tyrosine was effected and bacterial contamination was inhibited.

Analysis of Chromatograph. Five λ aliquots (equal to 45.5 ugms of each amino acid) of standard solutions A and B were applied

to a spot 4 inches from each edge of a 22.5 x 18.25-inch sheet of special Whatman No. 1 chromatographic paper and allowed to dry. The paper was then run overnight (16 hours) with butanol-acetic acid-water (4:1:5) in the long direction. The next morning the paper was removed from the chromatocab and suspended from horizontal glass rods in the hood and dried in a current of air drawn from an York, air cooler. The dried paper was then sprayed with borate buffer of pH 8.3 and allowed to dry. The chromatogram was then run during the second night (16 hours) with the second solvent (phenol, meta-cresol) along its short direction. The next morning it was removed from the chromatocab and dried. The dried chromatogram was placed in an oven at 70°C for 10 minutes to expel the excess of the adhering solvent. The chromatogram was sprayed with the ninhydrine solution, allowed to air-dry and kept in the drying oven at 105°C for half hour to develop the colour. The component 6 was chromatogrammed in a similar manner and the two were compared visually and by measuring the relative distance tranversed by the individual spots of the standards A and B and the constituent amino acids of component 6.

EXPERIMENTAL

Diethylstilbesterol Injected Cockerels Blood Serum

White Rock cockerels were injected intramuscularly with 3.0 mgms of diethylstilbesterol per one ml of propylene glycol for seven days. On the eighth day the cockerels were sacrificed, the blood collected in 50 ml centrifuge tubes and placed in a water

bath at 38°C. The blood was allowed to clot at 38°C for three hours and then centrifuged at 3,000 revolutions per minute for 20 minutes at 0°C in a refrigerated centrifuge.[1] The serum was decanted into a graduate cylinder and stored overnight at 7°C.

To isolate component 6, ultracentrifugation[2] techniques were employed. The serum was placed in cellulose nitrate tubes of 13 ml capacity and capped tightly with aluminium caps. The tubes were placed in a 40,000 r.p.m. rotor and the serum sample was centrifuged for 16 hours at 40,000 r.p.m. at 0°C. After the lapse of the specified period of centrifugation, the tubes were carefully removed from the rotor and kept in ice. The top layer was pale yellow, semi-solid substance of the consistency of chilled cream. This top fraction was designated component 6 by Misra (14). The aluminium caps were carefully removed from the cellulose nitrate tubes, and component 6 was removed from the tubes with the help of a spatula and placed in a beaker kept in ice. Component 6 was then transferred into a dialysis bag and dialysed against distilled water for 24 hours.

The material in the cellulose sac was transferred into a 150 ml beaker and treated with 83 ml of absolute methanol with constant stirring with a glass rod for 30 minutes. Then 83 ml of chloroform was added and the contents were transferred into a

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[1] Refrigerated Centrifuge - SerVall Centrifuge by the General Radio Corporation, Cambridge, Mass.

[2] Ultracentrifuge - Spinco Preparative Ultracentrifuge Model L. by Beckman Instrument Inc., Spinco Division, Belmont, California.

250 ml volumetric flask which was kept in a water bath at 60°C for 15 minutes. The volumetric flask was shaken gently, care being taken not to shake the flask vigorously so that the solvent would not spurt out of the flask. The flask was then cooled under running water from the tap and the volume made up to 250 ml with chloroform. The precipitated protein was filtered off in a Buchner funnel and then resulting protein precipitate was placed between Whatman No. 1, size 9.0 cm filter paper to squeeze out as much of the solvent as possible. It was then placed on a small watch glass and dried in the oven at 105°C for half-hour and weighed.

One-half gram of the sample of component 6 obtained as mentioned above was placed in a flat bottomed refluxing flask. A few pieces of pumice stone were added to prevent bumping and 10 ml of 6 (N) hydrochloric acid was added into the flask. The condenser was fitted into the flask and refluxed gently for 20 hours. After the period of the refluxing was over, the heating was discontinued and when the flask cooled down to room temperature its contents were filtered through a Whatman No. 1 filter paper into a 100 ml pyrex beaker. The beaker was kept on a steam bath and the excess of hydrochloric acid was removed on the steam bath. When the volume of the hydrolyzate was about $1\frac{1}{2}$ ml the beaker was removed and allowed to cool to room temperature. Then 1.0 ml of 10% 2-propanol was added which acted as a preservative. The 10% 2-propanol solution of the hydrolyzate so obtained was stored in the refrigerator at 7°C. A 1.0λ to 2.5λ aliquot was used for spotting the chromatogram.

Chromatographic Technique

The paper used for chromatographic work was a special Whatman No. 1, chromatographic paper of the size 22.5 x 18.25-inch. Two lines were drawn, at a distance of $2\frac{3}{4}$ inches from each edge of the longer side of the paper and two lines were drawn at a distance of $2\frac{1}{2}$ inches from the shorter length of the paper. These lines were drawn so that the starting point of each chromatogram was the same. The solution was applied to a spot 4 inches from each edge of 22.5 x 18.25-inch sheet of a special Whatman No. 1 chromatographic paper by means of a micro pipette. About 200 ml of the butanol-acetic acid-water (4:1:5) solvent was taken in each of the two troughs at the top and 200 ml of the butanol-acetic acid-water (4:1:5) solvent was placed in two trays at the floor of the chromatocab. Two chromatographic papers of size 22 x 18.25-inch were suspended into these troughs, so that the shorter edge of the paper remain dipped in this solvent. This procedure was adopted to equilibrate the chromatocab chamber. The spotted paper was then hung from two glass rods in the chromatocab for four hours to equilibrate the paper. After the lapse of four hours for the period of equilibration the papers were dipped in the solvent, care being taken that the pencil marks on the paper were in alignment with the glass rods supporting the paper. The chromatogram was then run overnight for 16 hours. The next morning the paper was removed from the chromatocab and suspended from horizontal glass rods in the hood. The solvent front was marked with a pencil. The chromatogram was then allowed to dry

in a current of air drawn from an York, air cooler. The dried paper was then sprayed with borate buffer of pH 8.3 with the help of an atomizer sprayer. The first solvent of butanol-acetic acid-water (4:1:5) was removed and the chromatocab was cleaned and aerated and then the second solvent of phenolmeta cresol (1:2) was put in two trays. The dried chromatogram was then put inside the chromatocab to equilibrate for four hours prior to the actual running of the solvent. After the lapse of the specified period of four hours for equilibration the chromatogram was dipped in the solvent so that the pencil marks drawn on the longer side of the chromatogram were in alignment with the glass rods supporting the paper. The next morning the paper was removed from the chromatocab and suspended from horizontal galss rods in the hood. The solvent front was marked with a pencil and the chromatogram was then allowed to dry in a current of air. The dried paper was then kept in the drying oven at 70°C for 10 minutes to expel the excess solvent adhering to the chromatogram. The chromatogram was next sprayed with a 0.2% ninhydrine solution in alcohol containing 2,4,6-trimethylpyridine (Collidine) by means of an atomizer sprayer. The chromatogram was then allowed to air-dry for 10 minutes and then kept in the drying oven at 105°C for half-hour to develop the colour. The spots of the developed chromatogram were encircled with a pencil mark. The spots were then matched with the standard spots.

Standard Amino Acid Mixtures
A_1, A_2, B_1 and B_2

The standard mixtures A_1, A_2, B_1 and B_2 were prepared by dissolving 200 micromoles of the following acid respectively as shown in the Table 1 below.

Table 1. Standard mixture.

Standard mixtures	Amino acid present	Amount of each present in the mixture
A_1	Threonine	200 micromoles
	Lysine	200 micromoles
	Proline	200 micromoles
	Valine	200 micromoles
	Tryptophan	200 micromoles
	Phenyl Alanine	200 micromoles
A_2	Aspartic acid	200 micromoles
	Leucine	200 micromoles
B_1	Cystine	200 micromoles
	Alanine	200 micromoles
	Histidine	200 micromoles
	Tyrosine	200 micromoles
	Isoleucine	200 micromoles
B_2	Serine	200 micromoles
	Arginine	200 micromoles
	Methionine	200 micromoles
	Glutamic acid	200 micromoles

Mixture A_1 was prepared by dissolving 200 micromoles each of threonine, cysine, proline, valine, tryptophan and phenylalanine in 2.0 ml of 1(N) hydrochloric acid in a 50 ml beaker and then making up the volume in a 10 ml volumetric flask with 0.1(N) hydrochloric acid. Similarly, mixture A_2 was prepared with 200 micromoles each of aspartic acid and leucine and following the same procedure adopted for the preparation of mixture A_1. The

standard mixture B_1 was prepared by dissolving 200 micromoles each of alanine, cystine, histidine, tyrosine and isoleucine in 2.4 ml of 1(N) hydrochloric acid in a 50 ml beaker making up the volume in a 10 ml volumetric flask with 0.1 N hydrochloric acid. Similarly, mixture B_2 was prepared with 200 micromoles each of serine, arginine, methionine and glutamic acid, and following the same procedure adopted for the preparation of mixture B_1. The beaker containing the solution of amino acids was kept on a steam bath and evaporated to about $1\frac{1}{2}$ ml. Bacterial contamination was inhibited by making up the solution in hydrochloric acid. One ml of 10% propanol was added with constant stirring to prevent recrystallization. The standards were run by the same procedure as mentioned before for the determination of the amino acids of the unknown component 6. The spots of the developed chromatogram were encircled with a pencil. These spots were the standard reference with which the constituent amino acids of component 6 were compared with and subsequently identified.

DNP Amino Acids of Component 6
(Whole, i.e. Non-Delipidized)

The component 6, obtained as mentioned before, was dialyzed against distilled water for 24 hours. The material in the cellulose sac was dissolved in 50 ml of 2% sodium bicarbonate solution in a 150 ml beaker with constant stirring by means of a glass rod for one hour (29). Fifty ml of the above solution was placed in a 250 ml Erlenmyer flask and mixed with 100 ml of 2.5 percent 2,4-dinitrofluorobenzene (DNP) in alcohol and shaken for 120

minutes at room temperature. The reaction mixture was treated with 6(N) hydrochloric acid with thorough shaking and the pH adjusted to 2. The acidic solution containing the insoluble DNP derivative of component 6 was extracted thrice with 25 ml portions of ether in a separatory funnel and each time the ether layer was carefully removed from the aqueous layer. After each addition of 25 ml portions of the ether layer the suspension was shaken carefully in a separatory funnel opening the stop-cock to release the pressure and allowed to settle. The insoluble DNP derivative of component 6 was then filtered, washed with water, alcohol and finally with ether. The aqueous solution obtained as the filtrate from the insoluble DNP derivative was dried in the oven at 105°C for 30 minutes and weighed. The dried DNP derivative was placed in a flat bottomed refluxing flask, a few pieces of pumice stone were added to prevent bumping and 10 ml of 6(N) hydrochloric acid was added into the flask. The condenser was fitted into the flask and refluxed gently for 20 hours. During the period of refluxing, 15 ml portion of the concentrated aqueous phase mentioned above was added to the main hydrolyzate in the refluxing flask. Then the acid was removed and the hydrolyzate concentrated on a steam bath and the chromatographic analysis carried out as mentioned previously in the previous section.

DNP Amino Acids of Delipidized Component 6
(i.e. Without Lipids)

The component 6 obtained as mentioned before was dialyzed against distilled water for 24 hours. The material in the

cellulose sac was transferred into a 150 ml beaker and treated with 83 ml of absolute methanol with constant stirring for 30 minutes. Then 83 ml of chloroform was added and the contents were transferred into a 250 ml volumetric flask which was kept in a water bath at 60°C for 15 minutes. The volumetric flask was shaken gently, care being taken not to shake the flask vigorously in which case the solvent would spurt out of the flask. The flask was then cooled under running water from the tap and the volume made up to 250 ml with chloroform. The precipitated protein was filtered off in a Buchner funnel and the resulting protein precipitate was placed between a Whatman No. 1, size 9.0 cm filter paper to squeeze out as much of the solvent as possible. The precipitate was placed on a small watch glass and dried in the oven at 50°C for 30 minutes. One-half gram of the dried sample was placed inbetween a glazed paper and finely ground with the help of a glass rod. The ground component 6 was thoroughly mixed with 50 ml of 2% sodium bicarbonate solution in a 150 ml beaker with constant stirring with a glass rod for 30 minutes. Fifty ml of the solution was taken in a 250 ml Erlenmyer flask and mixed with 100 ml of 2.5% 2,4-dinitrofluorobenzene (DNP) in alcohol and shaken for 120 minutes at room temperature. The rest of the procedure is the same as mentioned earlier for the non-delipidized preparation of component 6.

RESULTS AND DISCUSSIONS

To evaluate qualitatively, the amino acid composition of component 6, standard chromatograms of the various amino acids were run. Further, since running the chromatogram with a mixture of several different amino acids resulted in a highly blurred and overlapped chromatogram, the standard reference chromatogram of the amino acids was divided into four groups, viz.- A_1, A_2, B_1 and B_2, respectively, in such a way that the amino acids with maximum chromatographic separation relative to one another were included in the same group as indicated in Table 1. The four different groups of the standard amino acid mixture were run separately and the developed chromatograms are shown in Plate I, II, III and IV respectively. Then each of these four chromatograms was labelled and compared with the standard reference as given in the literature (13). Thus, each amino acid was located on the chromatogram. From these four chromatograms, a fifth chromatogram was prepared which indicated all the different amino acids used as reference standards at their respective positions as shown in Plate V. This chromatogram (Plate V) served as the reference standard for the observed spots in the chromatogram of the unknown. The experimental procedure was repeated several times to test the reliability of the results. However, the overlapping position of the following pairs of amino acid, viz., leucine-isoleucine, methionine-tryptophan and alanine-glutamic acid were evaluated after a series of runs by comparing the colour produced and the distance traversed by the respective amino acid.

EXPLANATION OF PLATE I

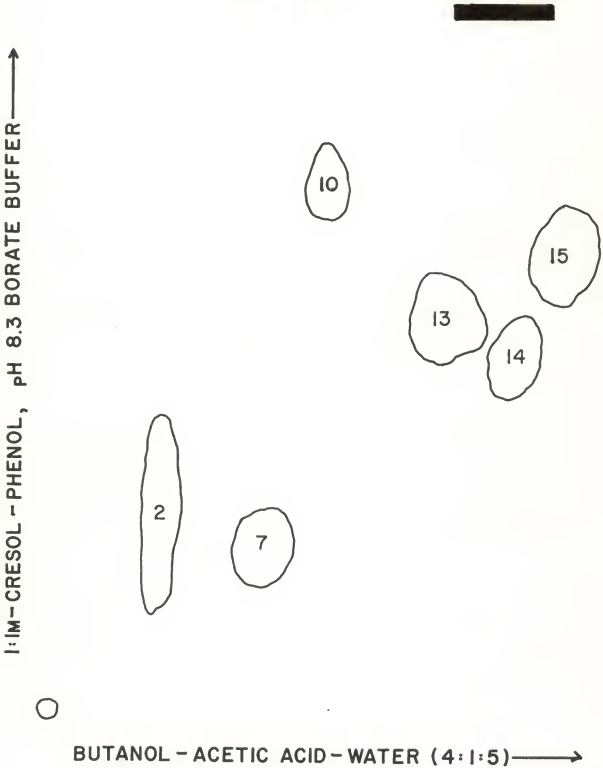
Two dimensional chromatogram of standard amino acid mixture A_1 containing threonine, lysine, proline, valine, tryptophan and phenylalanine. Five λ aliquot of 0.02M amino acid solution in 0.1 N hydrochloric acid containing 10 percent isopropyl alcohol was used for spotting the chromatogram indicated by the unnumbered circle in the Plate.

The first solvent used in the long direction was butanol-acetic acid-water in the ratio of 4:1:5, respectively. Borate buffer of pH 8.3 was used for spraying the chromatogram after running the first solvent.

The second solvent used in the short direction of the chromatogram was phenol-m-cresol in the ratio of 1:2 containing measured amount of pH 8.3 borate buffer. The chromatogram was developed with 0.1 percent ninhydrin solution in alcohol containing collidine, 2,3,6-trimethylpyridine. Time for running the chromatogram in either direction was 16 hours.

The encircled spot 2 correspond to threonine, spot 7 lysine, spot 10 proline, spot 13 valine, spot 14 tryptophan and spot 15 phenylalanine, respectively.

PLATE I


10
15
13
14
2
7
1:1M-CRESOL-PHENOL, pH 8.3 BORATE BUFFER
BUTANOL-ACETIC ACID-WATER (4:1:5)

EXPLANATION OF PLATE II

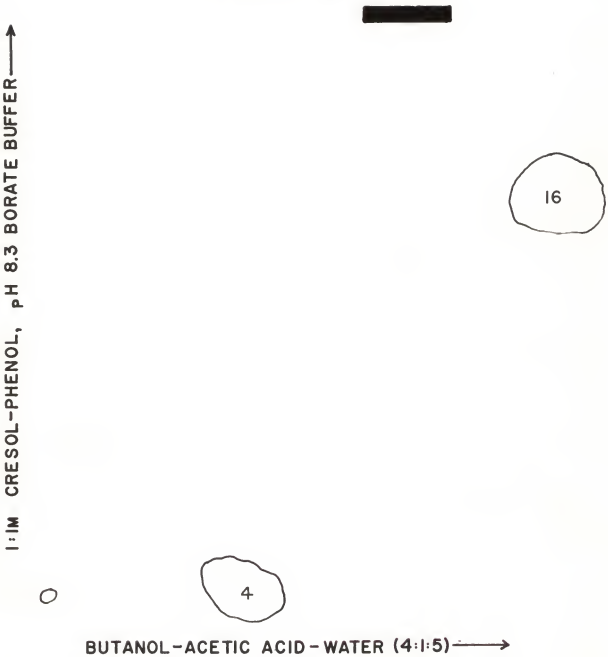
Two dimensional chromatogram of standard amino acid mixture A_2 containing aspartic acid and leucine. Five λ aliquot of 0.02M amino acid solution in 0.1 N hydrochloric acid containing 10 percent isopropyl alcohol was used for spotting the chromatogram indicated by the unnumbered circle in the Plate.

The first solvent used in the long direction was butanol-acetic acid-water in the ratio of 4:1:5, respectively. Borate buffer of pH 8.3 was used for spraying the chromatogram after running the first solvent.

The second solvent used in the short direction of the chromatogram was phenol-m-cresol in the ratio of 1:2 containing measured amount of pH 8.3 borate buffer. The chromatogram was developed with 0.1 percent ninhydrin solution in alcohol containing collidine, 2,4,6-trimethylpyridine. Time for running the chromatogram in either direction was 16 hours.

The encircled spot 4 corresponds to aspartic acid and spot 16 corresponds to leucine.

PLATE II


1:1M CRESOL-PHENOL, pH 8.3 BORATE BUFFER
16
4
O
BUTANOL-ACETIC ACID-WATER (4:1:5)

EXPLANATION OF PLATE III

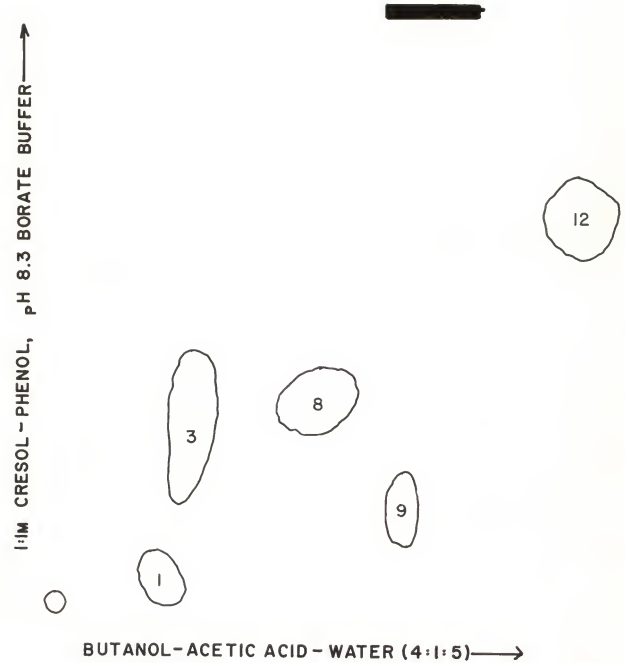
Two dimensional chromatogram of standard amino acid mixture B_1 containing cystine, alanine, histidine, tyrosine and isoleucine. Five λ aliquot of 0.02M amino acid solution of 0.1 N hydrochloric acid containing 10 percent isopropyl alcohol was used for spotting the chromatogram indicated by the unnumbered circle in the Plate.

The first solvent used in the long direction was butanol-acetic acid-water in the ratio of 4:1:5, respectively. Borate buffer of pH 8.3 was used for spraying the chromatogram after running the first solvent.

The second solvent used in the short direction of the chromatogram was phenol-m-cresol in the ratio of 1:2 containing measured of pH 8.3 borate buffer. The chromatogram was developed with 0.1 percent ninhydrin solution in alcohol containing collidine, 2,4,6-trimethylpyridine. Time for running the chromatogram in either direction was 16 hours.

The encircled spot 1 corresponds to cystine, spot 3 alanine, spot 8 histidine, spot 9 tyrosine and spot 12 isoleucine.

PLATE III


1:1M CRESOL-PHENOL, pH 8.3 BORATE BUFFER
12
8
3
9
1
BUTANOL-ACETIC ACID-WATER (4:1:5)

EXPLANATION OF PLATE IV

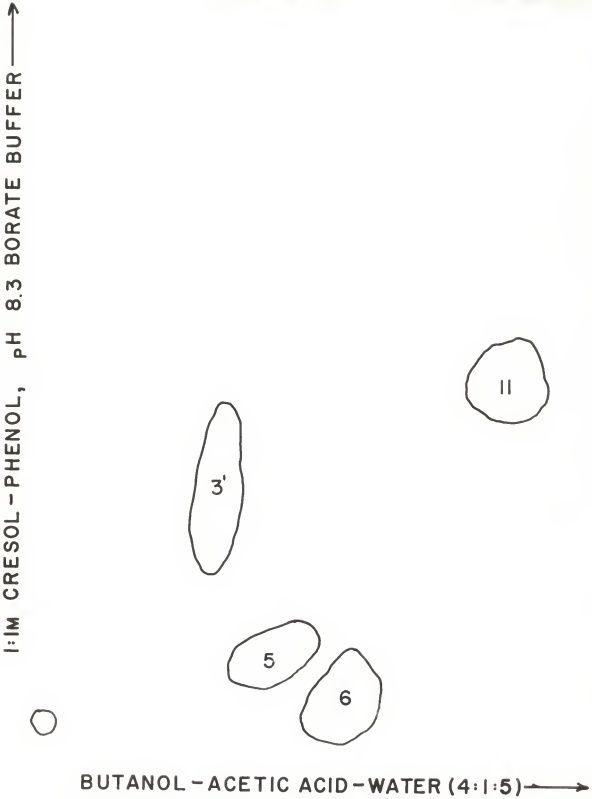
Two dimensional chromatogram of standard amino acid mixture B_2 containing serine, glutamic acid, arginine and methionine. Five λ aliquot of 0.02M amino acid solution in 0.1 N hydrochloric acid containing 10 percent isopropyl alcohol was used for spotting the chromatogram indicated by the unnumbered circle in the Plate.

The first solvent used in the long direction was butanol-acetic acid-water in the ratio of 4:1:5, respectively. Borate buffer of pH 8.3 was used for spraying the chromatogram after running the first solvent.

The second solvent used in the short direction of the chromatogram was phenol-m-cresol in the ratio of 1:2 containing measured amount of pH 8.3 borate buffer. The chromatogram was developed with 0.1 percent ninhydrin solution in alcohol containing collidine, 2,4,6-trimethylpyridine. Time for running the chromatogram in either direction was 16 hours.

The encircled spot 3 corresponds to glutamic acid, spot 5 serine, spot 6 arginine and spot 11 methionine, respectively.

PLATE IV


1:1M CRESOL-PHENOL, pH 8.3 BORATE BUFFER
BUTANOL-ACETIC ACID-WATER (4:1:5)
11
3'
5
6

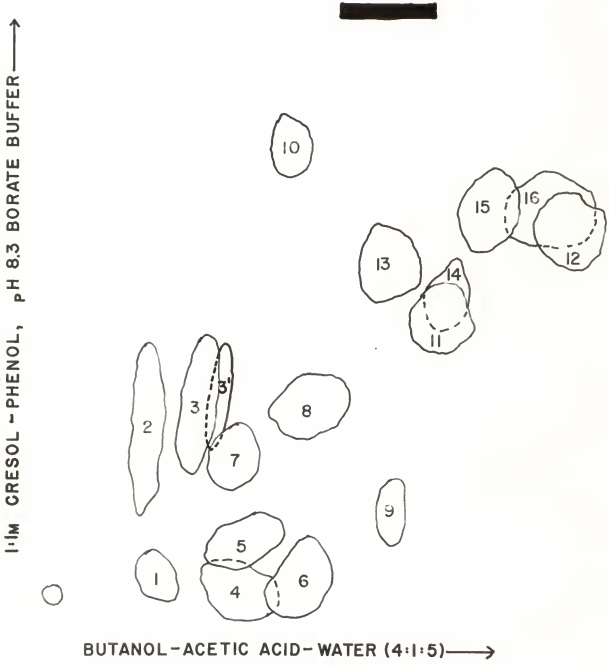
EXPLANATION OF PLATE V

Plate V was prepared by combining Plates I, II, III and IV respectively, which indicated all the different amino acids used as reference standard at their respective positions. Plate V served as the reference standard for the observed spots in the chromatogram of the unknown.

The arrow in the figure indicates the direction of the flow of the particular solvent and the unnumbered circle is the starting point of the chromatogram where spotting was done. It is at a distance of four inches from each edge of the 22 X 18.25-inch Whatman No. 1 chromatographic paper.

The encircled spot 1 corresponds to cystine, spot 2 threonine, spot 3 alanine, spot 3' glutamic acid, spot 4 aspartic acid, spot 5 serine, spot 6 arginine, spot 7 lysine, spot 8 histidine, spot 9 tyrosine, spot 10 proline, spot 11 methionine, spot 12 isoleucine, spot 13 valine, spot 14 tryptophan, spot 15 phenylalanine, and spot 16 leucine.

PLATE V


1:1M CRESOL - PHENOL, pH 8.3 BORATE BUFFER
BUTANOL - ACETIC ACID - WATER (4:1:5)
1
2
3
3'
4
5
6
7
8
9
10
11
12
13
14
15
16

The colour developed with ninhydrin and the relative distance traversed by each of the amino acids in these standard mixture and in the component 6 were observed and evaluated. The arrow in the Plate indicates the direction of the flow of the particular solvent and the unnumbered circle is the starting point of the chromatogram where spotting was done. It is at a distance of four inches from each edge of the 22 x 18.25-inch Whatman No. 1 chromatographic paper. The encircled spots bearing numerical figures indicate an individual amino acid as described in the explanation of various plates facing each respective plate. The arrow indicates the flow of the solvents as indicated in the respective plates. Since the position of alanine (3 in Plate III) and that of glutamic acid (3' in Plate IV) are nearly the same, alanine was retained, as it was observed to agree more closely with that of the amino acid in Component 6. The numerical figures representing a particular amino acid shown in Plate V has been retained through out the series of the chromatographic investigations, to facilitate explanation of the different chromatograms described hereinafter.

In this investigation of the amino acid composition and NH_2-terminal amino acids of Component 6 of diethylstilbesterol treated cockerels, ultracentrifugation was employed in the initial isolation of Component 6. Component 6 was delipidized with methanol and chloroform and the precipitated protein was filtered off, dried in the oven and weighed. One-half gram of the sample was hydrolyzed with 6 (N) hydrochloric acid gently under reflux for 20 hours. The hydrolyzate was then filtered through a Whatman

No. 1 filter paper into a 100 ml beaker. The excess hydrochloric acid was removed and the hydrolyzate concentrated on a steam bath to about $1\frac{1}{2}$ ml. The concentrated hydrolyzate was cooled and 1 ml of 10 percent 2-propanol was added as a preservative. One λ to $1\frac{1}{2}$ λ aliquot were used for spotting the chromatogram. The unnumbered circle shown in Plate VI is the starting point of the chromatogram. The encircled spot in the developed chromatogram shown in Plate VI represents the position of the various amino acids as obtained with the delipidized component 6. The numerical numbers in Plate VI correspond to a particular amino acid as described in the explanation of Plate VI.

It was observed that with an amount of the hydrolyzate larger than 1 λ to $1\frac{1}{2}$ λ the individual amino acid spots were blurred and overlapped. On the other hand an aliquot less than one-half lambda resulted in a chromatogram where several amino acids spots were found to be missing. Thus 1 λ to $1\frac{1}{2}$ λ aliquot was found to be the most suitable concentration for spotting the chromatogram. It may be noticed that the position of lysine is slightly deviated from that of the standard. This spot was observed consistently at the same place and after a review of literature (31) no other amino acid but lysine could be placed there. The Plate VI shown was the result of several repeatative chromatographic runs and all resulted in the same spots at the same relative distance. The amino acids were identified by a comparison with the standard chromatogram shown in Plate V. This gives the total amino acids composition of delipidized component 6.

EXPLANATION OF PLATE VI

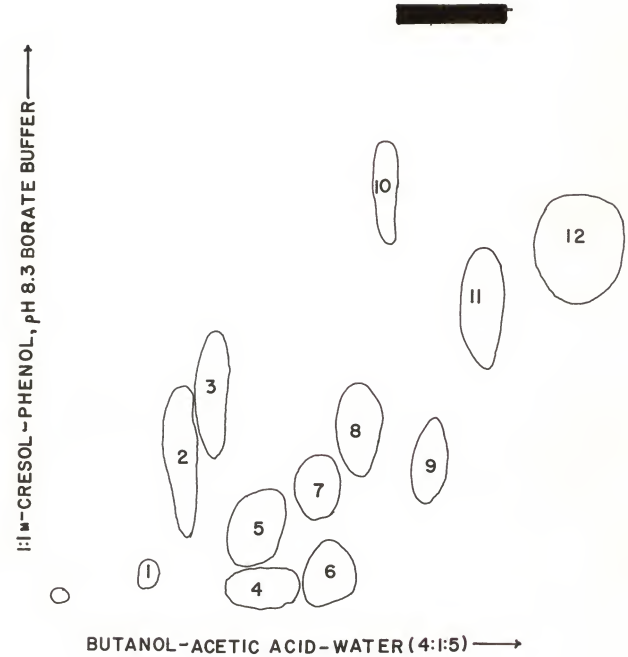
Two dimensional chromatogram of delipidized component 6 of diethylstilbesterol treated cockerels. $1\frac{1}{2}$ λ aliquot of the hydrolyzate in 10 percent isopropyl alcohol was used for spotting the chromatogram indicated by the unnumbered circle in the Plate.

The first solvent used in the long direction was butanol-acetic acid-water in the ratio of 4:1:5, respectively. Borate buffer of pH 8.3 was used for spraying the chromatogram after running the first solvent.

The second solvent used in the short direction of the chromatogram was phenol-m-cresol in the ratio of 1:2 containing measured amount of pH 8.3 borate buffer. The chromatogram was developed with 0.1 percent ninhydrin in alcohol containing collidine, 2,4,6-trimethylpyridine. Time for running the chromatogram in either direction was 16 hours.

The encircled spot 1 corresponds to cystine, spot 2 threonine, spot 3 alanine, spot 4 aspartic acid, spot 5 serine, spot 6 arginine, spot 7 lysine, spot 8 histidine, spot 9 tyrosine, spot 10 proline, spot 11 methionine and spot 12 isoleucine, respectively.

PLATE VI


1:1 m-CRESOL-PHENOL, pH 8.3 BORATE BUFFER
BUTANOL-ACETIC ACID-WATER (4:1:5)
1
2
3
4
5
6
7
8
9
10
11
12

In order to investigate the NH_2-terminal amino acid of the whole component 6 (the non-delipidized component 6) of diethylstilbesterol treated cockerels, the method of B. Shore (29), as modified by us, was adopted. The dialyzed material in the cellulose sac was dissolved in 50 ml of 2 percent sodium bicarbonate solution with constant stirring. Fifty ml of the solution was then mixed with 100 ml of 2.5 percent 2,4-dinitrofluorobenzene (DNP) solution in alcohol for two hours as described previously. The insoluble DNP derivative of the whole component 6 was hydrolyzed and the final solution was mixed with 1 ml of 10 percent 2-propanol as a preservative. Ten to 20 solution was used for the two dimensional chromatographic procedure as mentioned previously. The developed chromatogram is shown in Plate VII. The numerical number in Plate VII corresponds to a particular amino acid as described in the explanation of Plate VII.

The chromatogram shown in Plate VII was compared with the standard chromatogram shown in Plate V, as well as with the chromatogram obtained with the delipidized component 6 shown in Plate VI and also with the delipidized DNP derivative of component 6 shown in Plate VIII. It will be observed from Plate VII that the amino acids missing from the chromatogram of DNP with lipid were alanine and isoleucine.

To evaluate the chromatogram of DNP amino acid of the delipidized component 6 the material in the cellulose sac was delipidized with methanol and chloroform and the precipitated protein was filtered off, dried in the oven and weighed. One-half

EXPLANATION OF PLATE VII

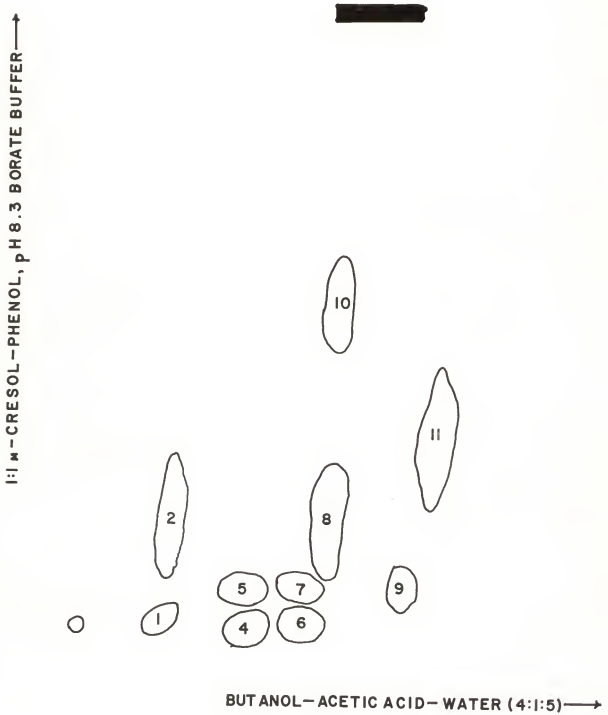
Two dimensional chromatogram of the DNP derivative of the non-delipidized component 6 of diethylstilbesterol treated cockerels. Ten λ aliquot of the DNP hydrolyzate in 10 percent isopropyl alcohol was used for spotting the chromatogram indicated by the unnumbered circle in the Plate.

The first solvent used in the long direction was butanol-acetic acid-water in the ratio of 4:1:5, respectively. Borate buffer of pH 8.3 was used for spraying the chromatogram after running the first solvent.

The second solvent used in the short direction of the chromatogram was phenol-m-cresol in the ratio of 1:2 containing measured amount of pH 8.3 borate buffer. The chromatogram was developed with 0.1 percent ninhydrin in alcohol containing collidine, 2,4,6-trimethylpyridine. Time for running the chromatogram in either direction was 15 hours.

The encircled spot 1 corresponds to cystine, spot 2 threonine, spot 4 aspartic acid, spot 5 serine, spot 6 arginine, spot 7 lysine, spot 8 histidine, spot 9 tyrosine, spot 10 proline and spot 11 methionine, respectively.

PLATE VII


1:1 m-CRESOL-PHENOL, pH 8.3 BORATE BUFFER
10
11
2
8
5
7
9
1
4
6
BUTANOL-ACETIC ACID-WATER (4:1:5)

EXPLANATION OF PLATE VIII

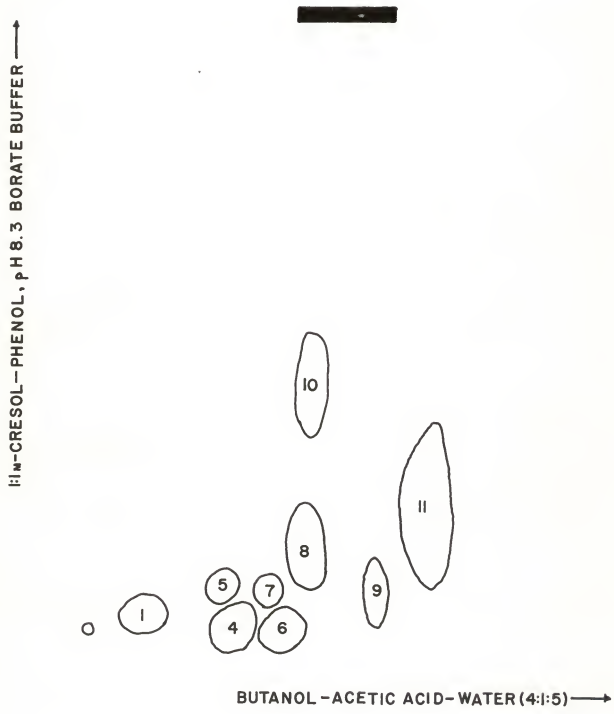
Two dimensional chromatogram of the DNP derivative of delipidized component 6 of diethylstilbesterol treated cockerels. Twenty λ aliquot of the DNP hydrolyzate in 10 percent isopropyl alcohol was used for spotting the chromatogram indicated by the unnumbered circle in the figure.

The first solvent used in the long direction was butanol-acetic acid-water in the ratio of 4:1:5, respectively. Borate buffer of pH 8.3 was used for spraying the chromatogram after running the first solvent.

The second solvent used in the short direction of the chromatogram was phenol-m-cresol in the ratio of 1:2 containing measured amount of pH 8.3 borate buffer. The chromatogram was developed with 0.1 percent ninhydrin in alcohol containing collidine, 2,4,6-trimethylpyridine. Time for running the chromatogram in either direction was 16 hours.

The encircled spot 1 corresponds to cystine, spot 4 aspartic acid, spot 5 serine, spot 6 arginine, spot 7 lysine, spot 8 histidine, spot 9 tyrosine, spot 10 proline and spot 11 methionine, respectively.

PLATE VIII


1:1 M-CRESOL-PHENOL, pH 8.3 BORATE BUFFER
O
1
4
5
6
7
8
9
10
11
BUTANOL-ACETIC ACID-WATER (4:1:5)

gram of the protein was ground and dissolved in 50 ml of 2 percent sodium bicarbonate solution with constant stirring. Fifty ml of the solution was then mixed with 100 ml of 2.5 percent 2,4-dinitrofluorobenzene solution in alcohol for two hours, as described previously. The insoluble DNP derivative of the delipidized component 6 was hydrolyzed and the final solution was used for the two dimensional chromatographic procedure as mentioned previously. The developed chromatogram is shown in Plate VIII. The numerical numbers in Plate VIII correspond to a particular amino acid as described in the explanation of Plate VIII.

When a comparison of Plate VIII obtained with the DNP derivative of the delipidized component 6 was made with Plate VII obtained with the DNP derivative of the whole component 6 it was observed that Plate VIII contained three amino acids less, viz.--alanine, threonine and isoleucine, whereas in Plate VII, only two amino acids were found missing, viz.--alanine and isoleucine. Thus, threonine would appear to be some way involved in the linkage of the lipid portion.

Variation in NH_2-terminal groups may be a reflection of a linkage between the protein and the lipids in lipoproteins. It has been suggested that comparison of ultracentrifugal patterns and NH_2-terminal amino acid content is a first step in distinguishing among closely related lipoproteins (30).

Amino acid analysis revealed that the portion of the peptide chain which contain the phosphorous are rich in isoleucine, leucine, aspartic acid and glutamic acid (26, 27). These results indicate that certain type of amino acid sequence can recur in all

phosphorous containing proteins. Our findings indicated that component 6 contained isoleucine and aspartic acid.

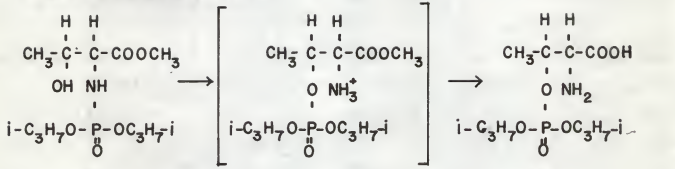
Since these amino acids occur in phospholipids, one must be concerned whether extensive washings with organic solvents were effective in removing phospholipids from the DNP-protein before hydrolysis. It was experimentally observed by Shore et al (30) that phospholipids, except possibly those tightly bound, were effectively removed. On treatment with certain organic solvents, the linkage between lipid and protein tends to break. The action of alcohol in destroying the links between protein and lipid appears to be irreversible (5).

As regards the manner in which the phosphorous is bound to a protein, both -O-P and -N-P esters present themselves as possibilities. These linkages involve alcoholic or aromatic hydroxyl, on the one hand or free amino groups or the guanidine group of arginine on the other (24).

To date only three phosphoamino acids have been isolated from biological material. O-phosphorylserine, O-phosphorylthreonine, both are representative of an -O-P esters and Phosphoarginine, which has a -N-P bond.

Phosphothreonine, $C_4H_{10}O_6NP$ (P = 15.5%), $[\alpha]$ = -7.37 was isolated from an acid hydrolyzate of casein by de Verdier in 1953 (10). Plapinger and Wagner-Jauregg (25) showed that migration of the phosphate group from the N- to O-position also occurs in the case of this compound, as described below. It was advisable to determine whether O-phosphorylated hydroxy amino acids can be formed by migration of phosphoryl group from nitrogen to oxygen.

It is a well established fact that certain N-acyl 1,2-amino alcohols rearrange on treatment with mineral acids to the corresponding amine salt of the O-acyl-1,2 amino alcohol. It was observed that similar transformation was accomplished easily with N-diisopropyl phosphoryl derivative of dl-threonine. These findings provide a simple method for preparation of threonine phosphoric acid in 50 percent yield. Treatment of the N-diisopropyl phosphoryl derivative of dl-threonine methyl ester with boiling aqueous hydrochloric acid gave 50 percent yield of threonine phosphoric acid.



I II III

The intermediate II could not be isolated. When I was first treated with dry hydrochloric acid and then hydrolyzed, 50 percent of III was obtained.

Thus, from the foregoing observations, it can be concluded that the whole component 6 (i.e. non-delipidized component 6) contained isoleucine and alanine as the NH_2-terminal amino acids whereas the delipidized component 6 contained isoleucine, alanine and threonine as the NH_2-terminal amino acids. These results indicate that threonine may be involved in the linkage between the amino acid-phosphorous-lipid moiety in lipoproteins.

SUMMARY

1. The amino acid composition of the delipidized component 6 was determined by the two dimensional chromatographic technique.

2. In order to determine the NH_2-terminal amino acid of the whole component 6, the DNP amino acid derivative of whole component 6 was prepared and the chromatogram was obtained as described earlier.

3. Similarly, the DNP amino acid of the delipidized component 6 was determined and the chromatogram was obtained as described earlier.

4. It was observed that in the whole component 6, the NH_2-terminal amino acids were isoleucine and alanine, whereas in the delipidized component 6, the NH_2-terminal amino acids were isoleucine, alanine and threonine.

5. From the foregoing observations it could be concluded that threonine was in someway involved in the linkage between the amino acid-phosphorous-lipid moiety in lipoproteins.

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The author wishes to express his sincere appreciation to his major professor, Dr. R. E. Clegg, of the Department of Biochemistry, for his guidance and assistance throughout this investigation. Gratitude is expressed to Mr. John W. Teas, of the Animal Nutrition division of the Department of Biochemistry, Kansas State University for his assistance in this investigation. Special thanks are extended to Mr. M. M. Simlot, Mr. J. Kohr, Miss R. C. Chen, Mrs. B. K. Myers, Miss J. Wells and Miss S. J. Nelson for their help during the course of the preparation of the dissertation.

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THE AMINO ACID COMPOSITION AND NH_2-TERMINAL AMINO ACIDS OF COMPONENT 6 OF DIETHYLSTILBESTEROL TREATED COCKERELS

by

SUNIT KUMAR MALIK

B. S., University of Calcutta, 1948

AN ABSTRACT OF A THESIS

submitted in partial fulfillment of the

requirements for the degree of

MASTER OF SCIENCE

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KANSAS STATE UNIVERSITY
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1961

The lipoproteins of serum contain protein components in combination with phospholipides, cholesterol and its esters, triglycerides and other lipides. Most lipoproteins occuring in nature probably have to be classified as secondary valence complexes which are held by van der Waals forces. The phosphorous and protein may be found through -O-P and -N-P esters. These linkages involve alcoholic or aromatic hydroxyls groups of amino acids. In order to probe these the amino acid-phosphorous-lipid relationship in component 6 the amino acid component 6 was investigated. The serum in this work was obtained from the blood of diethylstilbesterol treated White Rock Cockerels. In this investigation ultracentrifuge techniques were employed in the initial isolation of component 6.

The amino acid composition of component 6 was determined by the two dimensional chromatographic technique. The solvent system described previously consisted of butanol-acetic acid-water in the ratio of 4:1:5 for the first run and meta-cresol-phenol in the ratio of 2:1 for the second run. Borate buffer of pH 8.3 was used for spraying the chromatograms inbetween the two solvent runs. In order to determine the NH_2-terminal amino acids of the whole component 6 the DNP amino acid derivative of whole component 6 was prepared and the chromatogram was obtained as described earlier. Similarly the DNP amino acid of the delipidized component 6 was determined and the chromatograms obtained.

It was observed that in the whole component 6, the NH_2-terminal amino acids were Isoleucine, and Alanine whereas in the delipidized component 6, the NH_2-terminal amino acids were

Isoleucine, Alanine and Threonine. From the foregoing observations it could be concluded that Threonine was in someway involved in the linkage between the amino acid-phosphorous-lipid moiety in lipoproteins.

Relationships of these findings has been in accordance with the observance that threonine phosphate has been envisaged as one of the possible linkages in the amino acid-phosphorous-lipid bondings.