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ION-EXCHANGE CHROMATOGRAPHIC STUDY OF AMINO ACID DEGRADATION DURING HYDROLYSIS OF AVIAN PROTEIN

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SUMMARY

The losses of amino acid during acid hydrolysis of oviduct magnum protein from the domestic fowl have been measured using a Technicon TSM-1 amino acid analyser. The results were subjected to statistical analysis and a linear regression was obtained for each amino acid investigated. Methionine, in the presence of oxygen, did not exhibit significant degradation. A loss of 5.5% was found for phenylalanine after 24 h of hydrolysis under nitrogen. It is suggested that the results could be applied to animal proteins from tissue of similar composition but not to analysis of protein from plant materials.

INTRODUCTION

Initial studies in this department on the composition of the oviduct tissue of the domestic fowl used microbiological methods of amino acid analysis[1,2]. The relatively small number of amino acids assayed in these studies —a maximum of ten— does not allow for satisfactory comparison with more complete contemporary data of the composition of oviduct proteins. The microbiological methods used to determine amino acid content have been superseded by chromatographic procedures including gas–liquid and ion-exchange chromatography[3]. Thus Jackson and Anderson[4], in a more recent study of the amino acid composition of oviduct tissue in the immature fowl, used the ion-exchange chromatographic method of analysis after acidic hydrolysis of the oviduct protein.

The hydrolysis of protein materials, even under controlled conditions, can lead to the alteration or destruction of some amino acids, while the hydrolysis of others

may be incomplete as demonstrated by Hirs *et al.*[5], who used the sealed-tube method, and by Mondino and Bongiovanni[6], who used open-flask reflux techniques. The degree of destruction may depend on the nature of the protein molecule, the conditions of hydrolysis and the non-protein components present[7–9].

Other problems encountered in the preparation of protein hydrolysates that truly reflect the amino acid composition of the original protein include the production of artifacts after the removal of hydrochloric acid from the hydrolysates[10] and the racemization of amino acids during hydrolysis[11]. The first difficulty has been overcome by the rotary evaporation of hydrolysates under reduced pressure at about 40° as described by Weidner and Eggum[12]. The racemization of amino acids does not affect their chromatographic separation and is relevant only when microbiological assay methods of analysis are used.

Tristram[13] has questioned the value of amino acid analyses published without consideration being given to the losses sustained during hydrolysis which are specific to the hydrolytic method used and the nature of the protein samples.

The incomplete release of amino acids combined with the necessity to determine degradative losses of amino acids in oviduct tissue has provided the reason for the present study.

EXPERIMENTAL

Preparation of hydrolysates

Triplicate 65-mg samples of powdered, freeze-dried magnum from the oviduct of a laying Brown Leghorn hen were refluxed in 1-l, three-necked, round-bottomed flasks with 350 ml of constant boiling 6 *N* hydrochloric acid (AnalaR) by a modification of the method of Mondino and Bongiovanni[6]. This high volume:weight ratio was considered necessary to prevent humin build-up, which had been found to indicate progressive decomposition of some amino acids. Then 35 ml of a 0.1 *M* phenol solution were added to act as an antioxidant[3]. The hydrolysis was carried out at 110° and oxygen-free nitrogen was bubbled through the reaction mixture continuously. After refluxing for each of the following times, 18, 24, 36, 48, 72 and 96 h, a 25-ml aliquot of reaction mixture was removed from the flask and rapidly cooled.

A 20-ml aliquot of each of the cooled samples was evaporated on a rotary evaporator at 40 $\pm$ 1° with repeated washing with glass-distilled, deionized water to remove all traces of hydrochloric acid.

The final concentrate was then taken just to dryness and transferred, with citrate buffer (pH 2.00), to a 5-ml volumetric flask containing 0.5 ml of norleucine solution (4 μmoles/ml in pH 2.00 citrate buffer) and made to volume.

A similar procedure, differing only in the absence of nitrogen inflow during hydrolysis of the protein, was also carried out in triplicate. Its purpose was to determine the kinetics of the destructive oxidation of cystine and methionine.

Amino acid analysis

This was performed on a Technicon TSM-1 amino acid analyser with two columns, 350 $\times$ 6.3 mm and 50 $\times$ 6.3 mm, Type C resin and the standard 132-min buffer system but with the following modifications. The recommended buffer flow-rate of 0.55 ml/min was increased to 0.6 ml/min for both columns and a pH 6.13

citrate buffer containing 10.2 g NaCl/l buffer was used instead of the recommended buffer of pH 6.00. This latter modification improved the resolution of histidine and lysine. One standard chromatogram per six to twelve sample chromatograms was used and the measurement of peak areas accomplished by means of the manual planimeter method.

Results are calculated in μg of amino acid present in the sample per μmole of norleucine standard, according to the equation:

$$\text{amino acid } (\mu g) = s/a \times A/S \times \text{MW}$$

where:

s = area of norleucine in standard chromatogram
a = area of amino acid in standard chromatogram
A = area of amino acid in sample chromatogram
S = area of norleucine in sample chromatogram
MW = molecular weight of amino acid.

Analysis of variance of the recorded values produced a linear regression line for each amino acid investigated.

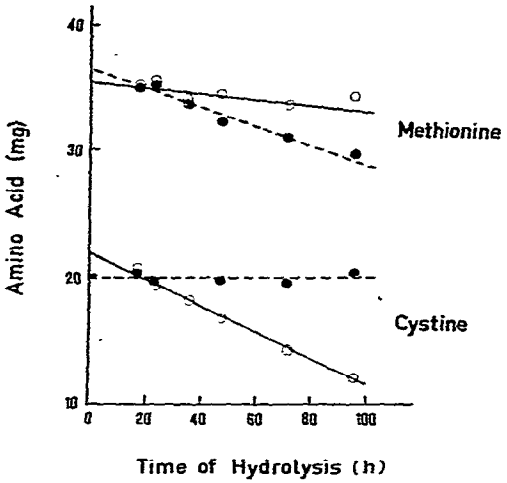
RESULTS

The equations from the analyses of variance for the amino acids investigated are presented in Table I. The values for aspartic acid refer to the asparagine plus aspartic acid content and those for glumatic acid refer to the glutamine plus glutamic acid content of the protein since asparagine and glutamine are converted quantitatively

TABLE I

LINEAR REGRESSION ANALYSIS OF THE DATA RELATING TO AMINO ACID DEGRADATION DURING THE ACID HYDROLYSIS OF OVIDUCT MAGNUM PROTEIN

Amino acid	*Regression equation*	*Significance of slope*
Under nitrogen		
Alanine	$y = -0.01x + 70.64$	NS
Arginine	$y = 0.02x + 74.80$	NS
Aspartic acid	$y = -0.05x + 109.96$	NS
Cystine	$y = 0.00x + 19.98$	NS
Glycine	$y = 0.01x + 59.80$	NS
Glutamic acid	$y = 0.01x + 148.17$	NS
Histidine	$y = -0.10x + 53.23$	$P < 0.01$
Isoleucine	No equation	—
Leucine	$y = 0.01x + 94.31$	NS
Lysine	$y = -0.002x + 84.00$	NS
Methionine	$y = -0.07x + 36.42$	$P < 0.001$
Phenylalanine	$y = -0.19x + 80.78$	$P < 0.001$
Proline	$y = 0.001x + 48.81$	NS
Serine	$y = -0.32x + 77.91$	$P < 0.001$
Threonine	$y = -0.02x + 54.49$	$P < 0.001$
Tyrosine	$y = -0.02x + 58.60$	NS
Valine	No equation	—
Aerobic conditions		
Cystine	$y = -0.10x + 21.80$	$P < 0.001$
Methionine	$y = -0.01x + 35.16$	NS



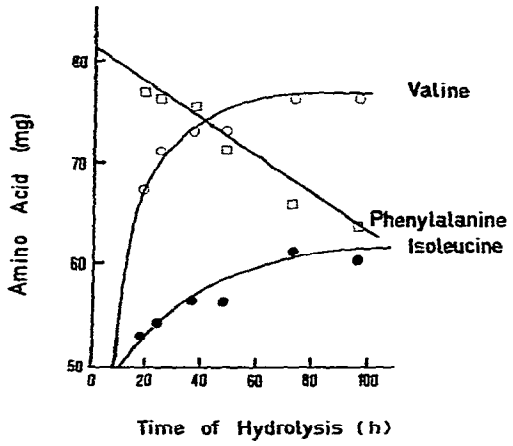


Fig. 1. The effect of time of acid hydrolysis on the recovery of cystine and methionine under nitrogen (●) and aerobic conditions (○).

Fig. 2. The effect of time of acid hydrolysis under nitrogen on the recovery of isoleucine, phenylalanine and valine.

to aspartic and glutamic acid, respectively, during the first few minutes of hydrolysis[14]. An examination of the table shows that histidine, methionine (Fig. 1), phenylalanine (Fig. 2), serine and threonine (Fig. 3) under nitrogen and cystine in the presence of oxygen (Fig. 1) are degraded to a significant degree after 24 h of hydrolysis. The percentage losses of these six amino acids as a result of acid hydrolysis are given in Table II. The remaining amino acids, with the exception of isoleucine and valine, are stable after 96 h of hydrolysis under nitrogen. Methionine in the presence of oxygen did not exhibit significant degradation.

The concentrations of both isoleucine and valine increased to a maximum after 72 h of hydrolysis when lysis of all peptide bonds appeared complete. Therefore the

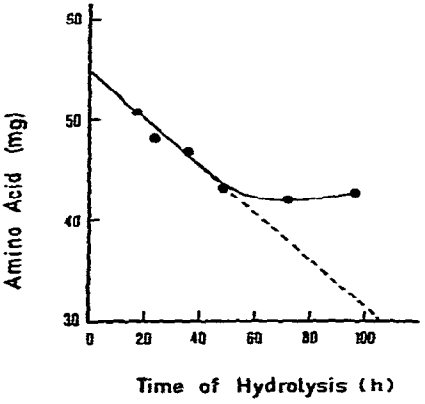


Fig. 3. The effect of time of acid hydrolysis under nitrogen on the recovery of threonine.

TABLE II

LOSSES OF AMINO ACIDS DETECTED AFTER 24 h OF ACID HYDROLYSIS OF OVIDUCT MAGNUM PROTEIN

Amino acid	*Percentage destruction after 24 h hydrolysis*
Under nitrogen	
Histidine	4.45
Methionine	4.72
Phenylalanine	5.52
Serine	9.86
Threonine	9.97
Aerobic conditions	
Cystine	11.14

maxima of the curves for these amino acids were obtained from the mean of the 72-h and 96-h results. The 24-h results were calculated as percentages of these maxima and gave 88.8 and 92.6% for isoleucine and valine, respectively.

DISCUSSION

Alanine, arginine, glycine, leucine, lysine and proline were all found to be stable under the conditions of hydrolysis used in the study since their regression equations exhibited no statistically significant slopes.

Tyrosine is particularly noted for its extensive destruction during hydrolysis[15]. Hirs *et al.*[16] suspected that a catalyst, perhaps aspartic acid, was involved in the removal of tyrosine while Moritz and Wade[17] demonstrated a loss of approximately 80% in the presence of nitroarginine. Munier[18] reported a halogenation mechanism as being responsible for tyrosine destruction. In the present study, the presence of phenol in each hydrolysis flask, as suggested by Sanger and Thompson[19], was probably effective in preventing the possible formation of halotyrosines since no loss of tyrosine was observed.

Serine and threonine (Fig. 3) were progressively destroyed with increasing time of hydrolysis even in the absence of oxygen. After 24 h of hydrolysis, assuming a zero order reaction, losses were estimated at approximately 10% for both acids, a result which is in accord with the findings of other workers who found losses between 0 and 15% with various proteins[13].

Smith and Stockell[20] assumed a linear decrease of serine and threonine with respect to time. The serine values obtained conform well to the regression line indicating a zero order reaction. The degradation kinetics for threonine would conform to that of a first or second order reaction since the factor or factors causing threonine degradation have apparently disappeared from solution after 48 h of hydrolysis. Kassel and Laskowski[21] calculated second order kinetics for the degradation of serine and threonine from chymotrypsinogen B. Hirs *et al*[5]. applied first order kinetics in the study of serine and threonine losses. However, the amount of threonine present attains a "steady state" at 48 h of hydrolysis or longer (Fig. 3) and the regression line was based on the results for 18, 24, 36 and 48 h. These produced a fairly precise re-

gression line and the assumptions inherent in their use was therefore thought justifiable.

The results shown in Fig. 2 agree well with those of Harfenist[22] who found maximum yields for isoleucine and valine after 70 h of hydrolysis. The slope of the histidine line was significant only at the 5% level.

Cystine is degraded post-fissionally in oxidising conditions and the results obtained (Fig. 1) show that it was removed from solution to the extent of 11.1% at 24 h of hydrolysis. Zero time extrapolation to determine cystine content is complicated by the wide variations frequently found in recoveries of cystine. This is due partly to the sensitivity of cystine to oxidation under aerobic conditions and partly to its degradation in the presence of carbohydrates or tryptophan[9,23]. Cysteine may be present as a result of the reaction of cystine with tryptophan and numerous methods to determine the total cystine plus cysteine content of proteins have been derived[24,25]. However, in the present study, hydrolysis of the protein under nitrogen resulted in no cystine loss over a period of 96 h. The intercept of the regression line on the ordinate obtained contained, within its defined confidence limits, the intercept of the regression line on the ordinate calculated from analyses performed under air. Therefore the cystine values obtained, relating to the cystine content of the original protein, were judged sufficiently precise for calculation of the degree of destruction after 24 h of hydrolysis in oxidising conditions. Furthermore, the regression line fitted to the results of analyses performed under oxidising conditions was a precise enough fit to confirm the validity of zero time extrapolation analysis in the present study.

A search of the literature failed to reveal reports on the destruction of phenylalanine during open-flask hydrolysis of proteins although the work of Moritz and Wade[17] indicated that phenylalanine, in the presence of nitroarginine, was converted to chlorophenylalanine. A decrease was observed in the phenylalanine present after 24 h of hydrolysis carried out under nitrogen, the loss being 5.5% (Fig. 2). The reaction would appear to be zero order although the values obtained do not conform well to the regression line. Owing to baseline irregularities in the tyrosine–phenylalanine area of the chromatograms, the imprecision in the measurement of peak areas may be responsible for the variability of the figures as calculated rather than to the variability in the degree of destruction of phenylalanine. Nevertheless, these baseline difficulties would be reflected in the tyrosine values and since this is not so, the observed decrease must be regarded as independent of imprecise experimentation.

In the present work, it is unlikely that the nitroarginine was present in sufficient quantity to account for the observed phenylalanine degradation. Further, it is unlikely that chlorination of phenylalanine or other amino acids would proceed in the presence of phenol, which is readily chlorinated.

The results presented do not indicate significant degradation of methionine (Fig. 1) under open-flask conditions where oxygen was present. However, when hydrolysis was effected under nitrogen, methionine degradation proceeded at a significant rate (4.7% of the intercept on the ordinate at 24 h). The intercept of the regression lines on the ordinate for analyses carried out under aerobic conditions and under nitrogen were different, though within each other's probability intervals.

The hydrolysis procedure, based on the observations of Moore and Stein[26] should take into consideration the losses of amino acids which may occur due to air oxidation. In fact, methionine sulphoxide, although not a proven constituent of native

proteins, is known to be readily formed by oxidation of methionine residues of proteins under aerobic conditions. Neumann *et al.*[27] reported that acid hydrolysis of proteins containing methionine sulphoxide was accompanied by losses of cystine and tyrosine. However, it was found that the addition of phenol prior to hydrolysis protected tyrosine but the oxidation of the sulphur-containing amino acids still occurred[28]. This would appear to be borne out by the present study. The fact that Ray and Koshland[29] found that methionine sulphoxide reverts largely to methionine under reducing conditions would suggest that degradation of methionine in the absence of oxygen should not be expected. Nevertheless, during the chromatography of amino acids, if methionine is not protected from oxidation by the inclusion of thiodiglycol in the buffers as an antioxidant[30] it is gradually converted to the sulphoxide. Smith and Stockell[20] suggested that, with short hydrolysis (20 to 30 h), a precise estimation of methionine was impossible since a number of peptides were eluted during chromatography between isoleucine and valine. The apparent decrease of methionine between 36 and 70 h of hydrolysis associated with increases of isoleucine and valine may indicate that isoleucine and valine emerged from the column with methionine and were hydrolyzed during the longer time of hydrolysis.

Since oviduct magnum tissue is approximately 90% protein and 8% lipid in the dry matter[1] it is unlikely that carbohydrate was responsible for methionine degradation[23]. However, it is possible that, owing to the composition of the tissue, lipid hydroperoxides could be formed during sample preparation[31]. O'Brien[32] found that, when pure linoleic acid hydroperoxide was reacted with cytochrome C decreases of methionine were obtained in an acid hydrolysate. It is possible that a free-radical reaction may take place during acid hydrolysis, causing a loss of methionine when lipid and protein are present in the hydrolyzing sample.

Although the results presented could probably be used as an indicator of the hydrolytic losses of amino acids found in animal tissues of similar composition to oviduct magnum, they would not be of value in interpreting results obtained from many plant materials where the presence of considerable amounts of carbohydrate is known to result in humin formation with a consequent loss of amino acids.

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