STUDIES ON THE BIOSYNTHESIS OF OVALBUMIN*

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Linderstrøm-Lang and Ottesen have reported the discovery of a bacterial enzyme which brings about the transformation of ovalbumin to a new, easily crystallizable protein, plakalbumin. Unlike other proteolytic processes, this reaction seems to involve the rupture of a limited number of peptide bonds, with the release of only slightly over 1 per cent of the total ovalbumin nitrogen (1, 2). Villee, Linderstrøm-Lang, and Ottesen report that this nitrogen corresponds to 6 amino acid residues (3 alanine, 1 glycine, 1 valine, 1 aspartic acid) which appear as a "hexapeptide" and its split-products (3).¹

In the present experiments this proteolytic reaction has been applied to samples of radioactive ovalbumin prepared by the incubation of minced oviduct tissue with C¹⁴O₂ (4). Aspartic acid from the hexapeptide (non-protein nitrogen) fraction could then be isolated and the specific activity of its carboxyl carbons compared with the average specific activity of all the aspartic acid residues from the plakalbumin fraction. In every case, the specific activity of the hexapeptide aspartic acid residue was much higher than that of the plakalbumin aspartic acid residues (approximately 31 residues (5)). The ratios of these specific activities varied from 1.3:1 to 3.5:1.

These data, showing a marked difference in the way in which amino acid residues are introduced into different points in the molecule, might be interpreted as indicating the synthesis of peptide intermediates prior to the biosynthesis of ovalbumin molecules. Other possible interpretations will be discussed in a later section.

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¹ 92 per cent of the non-protein nitrogen split off is accounted for by three peptide fractions: (a) a hexapeptide containing the 6 residues listed above, (b) a tetrapeptide (1 alanine, 1 glycine, 1 valine, 1 aspartic residue), and (c) a dipeptide (alanylalanine) (3). For convenience, the term hexapeptide will be used in this paper to refer to the entire non-protein nitrogen split off.
Methods and Results

Biosynthesis and Purification of C¹⁴-Ovalbumin—Laying hens were sacrificed by decapitation and the oviduct was removed. The tissue was finely minced with scissors and 20 gm. were transferred to 500 cc. flasks containing 50 cc. of inorganic medium (Na 77.5, K 77.5, Cl 115 mM per liter, 5 per cent C¹⁴O₂⁻O₂). After incubation for periods varying from 1 to 6 hours, the flasks were chilled and the contents homogenized and centrifuged. The clear supernatant was adjusted to pH 5 and taken to 40 per cent saturation with ammonium sulfate, and the precipitate discarded. By adjusting to the isoelectric point (pH 4.7) and increasing the ammonium sulfate concentration, ovalbumin was crystallized directly from the supernatant. The material was recrystallized three times after the addition of carrier ovalbumin. The electrophoretic patterns obtained on the final crystalline preparations showed only the characteristic A₁-A₂ complex of ovalbumin. The presence of small amounts of radioactive protein contaminants not detectable by electrophoretic analysis cannot be ruled out. However, the high degree of specificity for ovalbumin of the enzyme preparation employed (2) makes it unlikely that such contaminants could contribute significantly to the radioactivity of the non-protein nitrogen released during the digestion.

These final preparations, although free of detectable amounts of protein impurities, contained some radioactivity which could be released as C¹⁴O₂ by direct treatment with ninhydrin of aliquots of the unhydrolyzed protein. This absorbed radioactivity was easily removed, however, by the routine dialysis against distilled water for 48 to 72 hours to which all the radioactive ovalbumin samples were exposed to render them salt-free. As will be seen in Table I, this adsorbed radioactivity was reduced to less than 0.5 per cent of the total counts released by ninhydrin from the hydrolyzed protein after only 6 hours dialysis and, after 96 hours dialysis, to less than 0.2 per cent. Alternate alkali and acid treatment (as suggested by A. Keston) did not alter the specific activity of this dialyzed material.

Table I

Controls on Adsorbed Radioactivity

<table>
<thead>
<tr>
<th>Treatment of sample</th>
<th>Total counts released by ninhydrin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crystals, unwashed, wet with mother liquor</td>
<td>208</td>
</tr>
<tr>
<td>After 6 hrs. dialysis (vs. 2 liters H₂O)</td>
<td>6</td>
</tr>
<tr>
<td>&quot; 96 &quot; &quot; 4 &quot; &quot;</td>
<td>3</td>
</tr>
<tr>
<td>&quot; hydrolysis</td>
<td>1537</td>
</tr>
<tr>
<td></td>
<td>1580</td>
</tr>
</tbody>
</table>

* C¹⁴O₂ was released from undiluted BaC¹⁴O₄ received from Oak Ridge, Tennessee.
Incubation of C^{14}-Ovalbumin with Bacillus subtilis Enzyme—The proteolytic reaction described in the introduction was brought about by the use of an exoenzyme of *B. subtilis* precipitated from the culture medium with alcohol. The amount of enzyme used in each digestion was equal to 0.5 per cent of the ovalbumin being digested. As previously found by Linderstrøm-Lang and Ottesen (2) and confirmed in this laboratory, the use of high enzyme concentrations and prolonged incubation produces secondary, non-specific reactions, with the release of additional residues. However, these secondary reactions are much slower and in the incubations as performed here represent a very small increment in non-protein nitrogen. Furthermore, the essential qualitative finding reported here, namely, that different aspartic acid residues are not equivalent, is not dependent on the specificity of the enzyme reaction.

Except for two experiments, the paper chromatograms showed only the expected four amino acids in the hydrolyzed hexapeptide fraction. A faint extra chromatographic band was noted in these two experiments which was identified as glutamic acid. In one of these experiments the quantity was sufficient to permit a comparison of the specific activity of this glutamic acid with that isolated from the plakalbumin portion. As in the case of aspartic acid, the hexapeptide glutamic acid residue was much more active than the average of the plakalbumin glutamic acid residues (3.4:1).

The enzyme preparation was added to the C^{14}-ovalbumin solutions at room temperature, after the pH was adjusted to 6.4 with ammonium hydroxide. The progress of the digestion was followed by the salt titration method of Linderstrøm-Lang and Ottesen (2). Digestion was stopped by the addition of an equal volume of 10 per cent trichloroacetic acid (TCA).

Since the enzymatic conversion and the TCA precipitation bring about marked changes in the protein molecule, the possibility had to be considered that adsorbed radioactive amino acids, while not detected on the intact ovalbumin, might have been released by these processes. Tests on the TCA supernatant, however, showed no counts released by ninhydrin before hydrolysis, thus ruling out this source of contamination.

Isolation of Aspartic Acid from Plakalbumin—The plakalbumin was washed four times with 5 per cent TCA, three times with alcohol-ether, once with ether, and hydrolyzed with 6 N HCl. The hydrolysate was decolorized by boiling with acid washed norit, and, after removal of excess HCl by evaporation, the dicarboxylic amino acids were precipitated as the barium salts (4). This material was purified by reprecipitation and the aspartic acid, after neutralization to about pH 8 with sodium hydroxide, was separated by descending paper chromatography with phenol
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as a solvent. Special heavy paper\(^4\) was used to permit good separation of the relatively large quantities of aspartic acid needed for these experiments. Guide strips cut from the larger paper were developed with ninhydrin to locate the aspartic acid band, which was then eluted with H\(_2\)O. The aspartic acid was determined by the gasometric ninhydrin method (6) and the C\(^{14}\)O\(_2\) released was collected through the side arm into Ba(OH)\(_2\). The BaC\(^{14}\)O\(_3\) was plated and counted on an inside flow counter (7). Specific activity was calculated from the amount of amino acid determined with the Van Slyke apparatus, and the total counts per minute in the C\(^{14}\)O\(_2\) collected. Control samples of radioactive alanine determined by this method showed an over-all standard deviation of 3 per cent.

**Isolation of Aspartic Acid from Hexapeptide**—The first trichloroacetic acid supernatants were combined and freed from TCA by continuous ether extraction and hydrolyzed with 6 N HCl, and the acid was removed as above. The hydrolysate was neutralized and chromatographed on paper without further treatment. The aspartic acid band was eluted and the carboxyl carbons released and counted as described above.

**Isolation of Aspartic Acid As Copper Salt**—As a check on the validity of the paper method, the aspartic acid of the plakalbumin fraction in Experiment 28 (Table II) was isolated and twice recrystallized as copper aspartate. The specific activity of these crystals was determined by direct counting and subsequently by the gasometric ninhydrin method as above. The specific activity of this fraction, as obtained by three independent methods, showed a standard deviation of 10 per cent.

**Hexapeptide-Plakalbumin Ratios**—As shown in Table II, the hexapeptide aspartic acid residue in every experiment showed a higher specific activity than the average of the plakalbumin aspartic acid residues. The ratios of these specific activities varied from 1.3 to 3.5.

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\(^4\) Schleicher and Schuell, No. 470-A filler, White S-8939 (13).
DISCUSSION

Aspartic acid residues derived from different positions in the ovalbumin molecule have different specific activities under the conditions of these experiments. Any mechanism postulated for ovalbumin synthesis must account for this "asymmetry" in the synthetic process.

The present results are not compatible with a mechanism of synthesis involving the formation of ovalbumin molecules directly from free amino acids by means of a template process. Such a mechanism might be inferred from the results of "delayed supplement" feeding experiments in which it has been shown that amino acids must be fed simultaneously for optimal utilization in protein synthesis (8). It is important to recognize that the results of these feeding experiments fit equally well with a mechanism involving protein synthesis by way of intermediates. In a system in mass equilibrium the absence of any one of the intermediate "building stones" would be expected to inhibit the forward reactions leading to net synthesis.

Protein synthesis by way of peptide intermediates differing in rates of replacement or pool size is compatible with the data presented here. In the light of other evidence (9–12) pointing to such a mechanism, this seems most likely. However, other interpretations are possible and final conclusions must await further data.

SUMMARY

1. Crystalline radioactive ovalbumin has been prepared by in vitro incubation of oviduct minces from hens with C\textsuperscript{14}O\textsubscript{2}.

2. By using a bacterial proteolytic enzyme discovered by Linderstrøm-Lang, it has been possible to split off less than 2 per cent of this labeled protein molecule in the form of small peptides for comparison with the remainder.

3. It has been shown that, under the conditions of these experiments, aspartic acid isolated from the peptide fraction has a much higher specific activity than the average specific activity of aspartic acid residues in the remaining protein fraction.

4. It is suggested that these results support a theory of ovalbumin synthesis by way of peptide intermediates. Other possible interpretations cannot yet be ruled out.

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