EVIDENCE OF INTERMEDIATE COMPOUNDS IN SERUM
ALBUMIN SYNTHESIS

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The processes which convert amino acids into proteins have been studied
mainly from the aspect of intracellular location, energy and substrate re-
quirements, or action of inhibitors. The great bulk of these data probably
concerns the initial combination of the amino acids, the step termed "pep-
tidization" by Lipmann (1), and tells us little about subsequent reactions
or possible intermediate compounds.

It has been proposed (Borsook (2), Janssen (3), Langmuir and Schaefer
(4), Northrop (5)) that the intermediates may be peptides, templates,
protein films, or non-specific proteinogens termed "urproteins" by Nor-
throp. Borsook et al. (6) have studied the composition of several large
peptides in tissues which may represent stages in the formation of proteins.
Steinberg and Anfinsen (7, 8) have presented data on non-uniform labeling
of different residues of the same amino acid in ovalbumin which favor the
concept of peptide intermediates.

Tracer studies of serum albumin formation are presented here, which
indicate that the amino acids are in intermediate compounds for about 20
minutes before forming the final protein. These intermediates are found
not to resemble serum albumin in either serological, electrophoretic, or
solubility behavior, but are apparently converted into albumin by a process
which causes all three of these properties to appear simultaneously.

EXPERIMENTAL

Incorporation of C\(^{14}\)O\(_2\) and labeled alanine into serum albumin has been
shown to proceed in chicken liver slices incubated in a bicarbonate medium
(9). Subsequently a net increase was demonstrated in the amount of
serum albumin present during incubation, by a quantitative immunological
determination of the albumin (10).

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† At present on leave of absence with the United States Navy. Opinions or
assertions contained herein are those of the author and are not to be construed as
official or reflecting the views of the Navy Department or the naval service at large.
Demonstration of Lag in CO₂ Incorporation

It was suggested in the former paper that there is a delay in the incorporation of carbon dioxide into the serum albumin found in the medium. More complete studies have now been made of the time relationships of the C¹⁴O₂ incorporation into the total serum albumin, the C¹⁴O₂ incorporation into the total liver slice protein, and of the quantity of serum albumin present.

Conventional Isotope Experiments—Chicken liver slices were washed for 1 hour in bicarbonate medium (10) to lower the content of preformed albumin and then divided among several flasks containing C¹⁴O₂ in the gas and buffer phases. At intervals during incubation at 38° flasks were removed, and determinations were made of the specific activity of the inorganic carbon dioxide and of the carbon dioxide released by ninhydrin treatment of samples of washed, defatted, hydrolyzed slice proteins (precipitated by 1 per cent picric acid). The combined slices and medium were analyzed for serum albumin as previously described (10). Larger aliquots were added to 1 cc. portions of an antiserum prepared against a highly purified sample of chicken serum albumin, and tests were made to insure antibody excess. The precipitates were washed twice with saline and hydrolyzed. The total radioactivity released by ninhydrin was determined on these hydrolysates (9).

Fig. 1 shows the type of relationship which was found in three experiments. Both the production of new serum albumin and the incorporation of carbon dioxide into the aspartic and glutamic acids of the liver proteins begin within 5 minutes of the start of incubation, while labeled carbon does not appear in the serum albumin for 15 to 20 minutes. Approximately the same delay was observed with glycine-1-C¹⁴ as substrate, as shown later in this article (Fig. 4, c).

The delay in incorporation into albumin suggests that the small molecules are first incorporated into some "precursor" material, which then acquires the antigenic properties of serum albumin.

The in vivo work of Borsook et al. (11) likewise describes a delay in appearance of labeled amino acids in serum proteins which is not observed with visceral proteins.

"Reverse" Isotope Experiments—More direct information was obtained from "reverse" isotope experiments, in which the slices were washed for 1 hour in flasks containing C¹⁴O₂ and then transferred, after vigorous rinsing for 5 minutes, to flasks containing non-radioactive medium. The flasks were flushed at 15 to 30 minute intervals with 5 per cent CO₂-95 per cent O₂ to remove traces of radioactive carbon dioxide which might accumulate. Slices and medium were analyzed as described above.

It is seen in Fig. 2 that the labeled slices continue to form radioactive
Fig. 1. Time relations in the liver slice system. The serum albumin data refer to total serum albumin in medium plus slices. Serum albumin was separated by immunological precipitation for determination of both its amount and its radioactivity.

Fig. 2. "Reverse" isotope experiment. Slices were first incubated in radioactive medium for 60 minutes and were then washed and incubated in non-radioactive medium for the times indicated. Observed radioactivities were expressed as micromoles of labeled carbon remaining in 1 gm. of liver protein, or in the serum albumin contained in an equivalent amount of slices. The serum albumin values were then increased by a factor of 100 in plotting in order to show the shape of the curve.
serum albumin, although the total radioactivity of the liver slice protein does not rise. A similar effect was observed when labeled alanine was used as the substrate. Loss of radioactivity by the liver protein is not appreciable, since only a small fraction of the carbon atoms is replaced per hour. The carbon atoms of the newly formed serum albumin must arise from compounds which are contained in the slice and which are not readily removed by washing.

Appearance of Serum Albumin Properties

The possibility was considered that the "precursor" material represents a molecule with some of the physical properties of serum albumin, but which has not yet acquired the antigenic specificity upon which the above determinations were based. To test this possibility, incubations were conducted with increased substrate radioactivity. After the incorporation into serum albumin was determined with antiserum, a large known amount of carrier serum albumin was added, and the specific radioactivity in the resulting albumin determined on samples separated both by electrophoresis and by a series of alcohol and ammonium sulfate fractionation steps.

Incubation—5 gm. portions of wet chicken liver slices were incubated for 15, 30, or 60 minutes with 15 cc. of medium (Na 135, Ca 15, K 10, Cl 120, HCO₃ 40 m.eq. per liter, pH 7.5), which had previously been equilibrated with 5 per cent CO₂-95 per cent O₂. NaHCO₃ was included in preparing the medium in the C¹⁴O₂ experiments. Glycine containing varying proportions of glycine-¹-C¹⁴ (Tracerlab) was substituted for 20 mM per liter of NaCl in the glycine experiments. It was not feasible to use a shorter time than 15 minutes, owing to the high concentration of isotope required to give measurable incorporation.

Crude Albumin Preparation—The contents of each flask were then chilled, homogenized, and centrifuged for 20 minutes at 50,000 × g at 3° after addition of dilute acetic acid to pH 6.8. A crude albumin fraction was separated from the supernatant by addition of solid ammonium sulfate to 50 per cent saturation, centrifuging, then saturating the supernatant with ammonium sulfate at pH 4.9, and centrifuging. While some loss was involved, this procedure served to purify somewhat the albumins from the radioactive substrates and from the contaminating liver proteins.

Removal of substrate and contaminating proteins was further accomplished by dialyzing the saturated ammonium sulfate precipitates against distilled water. Radioactive glycine was washed out by adding non-isotopic glycine before precipitation and before dialysis.

Four immunological samples, representing 0.25 gm. of incubated liver slices each, were obtained by precipitation with antiserum as before.

Electrophoretic Separation—To the remainder of the crude albumin solu-
tions, representing about 4 gm. of liver slices per flask, were added 150 mg. of purified chicken serum albumin (10) to act as carrier. Since the amount of albumin added was known, the total radioactivity in the serum albumin of the crude preparation could be determined by finding the specific radioactivity of the resulting diluted albumin.

Electrophoresis of the mixtures (Fig. 3) showed a large homogenous peak, with a mobility of 7.1, and several small, red, liver protein peaks with mobilities of 2 to 6. The highest mobility encountered for any non-albumin peak was 6.3. Occasionally the ascending albumin peak was split. By means of suitable compensation a separation was made at mobility 6.5, so that in the upper cell of the ascending side a sample of protein with mobility 6.5 or greater was obtained. In their studies of the mobility of liver pro-

![Fig. 3. Electrophoresis of crude slice preparation plus carrier serum albumin.](image)

The solution was dialyzed overnight against Veronal buffer, pH 8.6, ionic strength 0.1, and subjected to a current of 25 ma. in a standard Klett-Tiselius apparatus. The curve is the descending cell boundaries of a typical run. Direction of migration is indicated by the arrow. The author wishes to thank Dr. Britton Chance of the Johnson Research Foundation for the use of the electrophoresis apparatus, and Mr. E. F. Gould for his advice and assistance in the use of this equipment.

Separation Based on Solubility—The solution from the bottom and two lower electrophoresis cells was dialyzed against distilled water overnight and subjected to low temperature alcohol fractionation. The basic steps are shown in the accompanying scheme.

The final albumin was a pure white protein which precipitated sharply upon a 3 to 5 per cent increase in alcohol concentration. This was then divided into five equal fractions at 25° by 1 to 2 per cent increases in ammonium sulfate saturation in the range of 57 to 65 per cent saturation.

Counting of Samples—All of the protein samples were boiled, defatted, hydrolyzed, treated with ninhydrin, and the carbon dioxide counted as BaC₁₄O₃ (9). Slice picric acid precipitates containing radioactive glycine were first dialyzed exhaustively to remove substrate. Total radioactivity
was measured on the immunological samples. Specific radioactivity was measured on all other samples and converted to total counts by calculation.

Initial and final concentrations of glycine-1-C\textsuperscript{14} were determined by ninhydrin treatment, and the observed incorporation of glycine into protein then corrected to correspond to a steady glycine concentration of 16 mM per liter. The assumption was made that the effect of change in substrate concentration on incorporation followed a logarithmic curve (14–17).

Results

Numerical data given in Table I show the degree of agreement found among the serum albumin samples. The incorporation in liver protein and serum albumin is plotted in Fig. 4 in order to portray the time relationships.

The 15 to 20 minute lag in incorporation of C\textsuperscript{14} into serum albumin is clearly evident in the curves of Fig. 4, and is seen to be independent of the method by which the albumin is separated. The similarity of the three
albumin curves of any one experiment signifies that the protein in which the C\textsuperscript{14} is appearing acquires the serological, electrophoretic, and solubility characteristics of serum albumin simultaneously.

**Table I**

*Incorporation of C\textsuperscript{14} into Serum Albumin*

<table>
<thead>
<tr>
<th>Substrate used</th>
<th>Time</th>
<th>By antibody†</th>
<th>By electrophoresis‡</th>
<th>By solubility§</th>
</tr>
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<tr>
<td></td>
<td></td>
<td>Fraction 1</td>
<td>Fraction 2</td>
<td>Fraction 3</td>
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<tr>
<td>CO\textsubscript{2}</td>
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<td>8.4</td>
<td>6.6</td>
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<tr>
<td>CO\textsubscript{2}</td>
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<tr>
<td>15 min.</td>
<td>5.0</td>
<td>12.5</td>
<td>10.7</td>
<td>7.7</td>
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<td>Glycine</td>
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<td>1.3</td>
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<td>360</td>
<td>345</td>
<td>360</td>
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* In micromoles × 10\textsuperscript{-3} carbon per gm. of liver protein.
† The immunological values are the average of four samples.
‡ The electrophoretic values are single samples. Diffusion of slight amounts of proteins with mobility less than 6.5 into the upper cell during electrophoresis might tend to raise the apparent incorporation into these samples.
§ The lowest value in the series of ammonium sulfate cuts is plotted in Fig. 4 as representing the incorporation into the protein with solubility properties of serum albumin. Since the added carrier diluted the albumin present (about 1 mg. per gm. of liver (10)) by approximately 40:1, the specific radioactivity of the resulting albumin would be appreciably lower than that of contaminating liver proteins.

**Discussion**

The intermediate compounds whose existence is implied by these experiments can be described as either protein in nature or so bound to proteins as not to be removed by repeated aqueous and lipid solvent washings. They will not diffuse from slices during 5 minutes of vigorous washing in bicarbonate medium. The serum albumin "precursor" material exists for 15 to 20 minutes, during which time it does not display either physical or serological properties of that protein. It is then converted by an "all at once" process into a serum albumin molecule complete with regard to all of these properties.
Absence of a significant delay in the incorporation into the liver slice proteins does not, however, preclude the function of intermediates in the formation of these proteins. It is entirely possible that protein synthesis in general proceeds via intermediates of a form precipitated by protein reagents such as 1 per cent picric acid. Hence it is only by studying specific proteins isolated by other means that such intermediates would be detected.

Intermediates of the above characteristics can be consistent with several of the current theories of protein synthesis. The peptide mechanism favored by Borsook (2) and Anfinsen and Steinberg (7, 8) could apply if the peptides do not exist in the free state, but attached to heavier molecules.
and indistinguishable from them by the usual techniques. The radioactive compounds which Winnick (18) and Peterson and Greenberg (19) have demonstrated to be removable from protein precipitates by alkali or mercaptoethanol treatment may represent such intermediates. The data of Steinberg and Anfinsen (8) indicate that the intermediate peptides may exist in pools of varying size and turnover rate.

The template theory also appears capable of satisfying the above requirements. The time delay observed in the case of serum albumin would represent the period during which the embryo protein is held insoluble on the template while its amino acids are accumulated. All of the properties of the protein would be expected to appear simultaneously, coinciding with the time of release of the protein from the mold. Labeled carbon atoms held on templates would be counted along with the labeled carbon atoms of the liver proteins.

Several workers (11, 20–23) have reported that the microsomes incorporate carbon before it appears in the other fractions of liver cells. It is possible that these particles, high in content of ribonucleic acid, which is often associated with the synthesis of proteins, contain the "templates" on which the proteins of the cell are formed.

Evidence has previously been presented (9, 10) that the incorporation observed actually measures synthesis of new protein and not adsorption or the replacing of amino acids in existing proteins. A recent paper by Luck and Brunish (24) points out the need for constant precautions against interpreting adsorption of isotopes as incorporation. Dependence of C\textsubscript{14}O\textsubscript{2} incorporation on the presence of oxygen (25) and, more important, the large proportion of serum albumin molecules which becomes labeled (10) are additional points for validity of observations made with the C\textsubscript{14}O\textsubscript{2} system. Other evidence is discussed by Frantz and Zamecnik (16).

**SUMMARY**

1. The appearance of radioactive carbon in serum albumin has been found to commence 15 to 20 minutes later than does the uptake of radioactive carbon into the total protein of chicken liver slices. Non-labeled albumin continues to appear during this period.

2. Labeled serum albumin has been separated from liver slice incubations by methods based on three different properties: antigenic activity, electrophoretic mobility, and solubility. C\textsubscript{14}O\textsubscript{2} and glycine-1-C\textsubscript{14} were employed as tracers. In either case, serum albumin appears to acquire all three of the above properties simultaneously.

3. The results suggest that the conversion of amino acids into complete proteins requires a measurable period of time, and that the final step in this conversion is one which affects both the biological and physical properties of the molecule.
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