Direct observation of protein synthesis in vitro is made difficult by the fact that the total protein content of tissue slices decreases upon incubation, owing to the predominance of proteolytic reactions. This has necessitated the use of isotopes to demonstrate that protein formation or "turn-over" is taking place under such conditions.

Experiments with radioactive carbon, reported in a previous paper (1), demonstrated the incorporation of amino acids into serum albumin by chicken liver slices. This incorporation was found to proceed at a rapid rate, which suggested that the formation of this specific protein might be demonstrated in these liver slice systems without the use of isotopes.

An immunological method was developed by which the small amounts of serum albumin present in liver slices could be determined. The total amount of serum albumin was found to increase in each of twenty experiments, at an average rate which corresponded roughly to observed rates of albumin production in vivo. Studies were made of the effects on this rate of production of changes in the ionic environment and of addition to the incubation medium of substrates, inhibitors, and hormones.

EXPERIMENTAL

Methods

Several investigators have reported the application of quantitative immunological techniques to the determination of serum albumin in serum, urine, and edematous and cerebrospinal fluids (2-4). These methods are extremely sensitive and give results which have been found to agree well with electrophoretic determinations.

Preparation of Standard Albumin—Albumin was isolated from chicken
plasma by a procedure which included two series of fractionations with ammonium sulfate, and three fractionations with alcohol under various conditions. Electrophoretic analysis of the final product at pH 8.6 showed only one peak, which was estimated to contain not more than 0.9 percent of impurity. A test for immunological homogeneity was performed according to the antibody gel method of Oudin (3), in which bands of precipitate representing each antigenic component in the solution tested are observed to diffuse into gelled antiserum. Although such a procedure shows only a minimal number of components with certainty, the albumin fraction appeared to be homogeneous by this test.

Fig. 1. The relation between the amount of standard albumin added to a fixed volume of antiserum and the amount of precipitate resulting.

Rabbit Antiserum—Portions of this albumin were injected into five rabbits three times weekly for 4 weeks. The weekly doses were 1.5, 2.2, 2.6, and 3.6 mg. 5 days after the last injection, blood was drawn, under sterile conditions, from all of the rabbits and the sera were pooled. This combined antiserum gave a positive ring test with the standard albumin diluted 1:800,000 and contained approximately 0.5 mg. of antibody nitrogen per cc.

Microdetermination of Serum Albumin—The relation between the amount of standard albumin added to a fixed volume of antiserum and the amount of precipitate resulting is plotted in Fig. 1. In order to conserve antiserum, a microprocedure was used for determination of the amount of precipitate. This consisted of a modification of the method described by
Johnson (6), in which the amount of dichromate reduced in the presence of 66 per cent H$_2$SO$_4$ at 100° is taken as a measure of the amount of organic material present. The results are consistent when only one type of substance, such as protein, is analyzed.

For determination of serum albumin, slices were homogenized for 2 minutes at 0° with 4 volumes of saline, or with the medium with which they had been incubated, in a Potter-Elvehjem glass homogenizer. The slice homogenates, or in some cases the isolated incubation media, were centrifuged at 5° at 10,000 X g for 10 minutes. The layer of lipide which rose to the top was removed, and the centrifugation repeated.

Two aliquots of the clear supernatant were added by constriction pipette to 0.083 cc. of antiserum, under the same conditions used in determining the standard curve. These aliquots were chosen to give precipitates of widely differing sizes and the results were averaged in order to minimize errors in the manner in which the standard curve was drawn. Pyrex test-tubes, 1 X 12 cm. with tapered bottoms, were employed. The final volume was adjusted to 0.26 cc. by the addition of 0.9 per cent NaCl.

After 24 hours the antibody precipitates were centrifuged for 5 minutes at 1100 X g and washed twice with 0.11 cc. of saline. A third washing of the precipitate and the walls of the tube with water was employed in order to remove chloride, which was found to interfere in the dichromate reaction.

To each tube was added 0.187 cc. of 0.251 N K$_2$Cr$_2$O$_7$, followed by 0.47 cc. of 95 per cent H$_2$SO$_4$. The contents were stirred occasionally with a glass rod during the 30 minute period at 100°. After cooling, 4 cc. of water were added and the solution was mixed. The density was read in a Beckman spectrophotometer at 445 m$\mu$ against a similar solution to which sodium hyposulfite had been added to reduce the dichromate completely. The densities of test solutions were subtracted from the density of an unreduced standard, and the microequivalents of dichromate reduced by the precipitate were calculated by proportion.

By this method 10 to 30 $\gamma$ of serum albumin could be determined with a precision of ±5 per cent. Analyses for albumin in samples of chicken serum gave 90 per cent of the value obtained by the salt fractionation method of Howe (7). Determinations made on saline liver extracts to which known amounts of albumin had been added gave the expected analytical results.

Incubation Procedure—Immediately after killing the animal, chicken livers were cut into 0.5 mm. slices with a Stadie slicer and incubated in Erlenmeyer flasks containing medium with the following composition: Na 135, K 10, Ca 10, Cl 125, HCO$_3$ 40 mm per liter, in conjunction with a gas phase containing 5 per cent CO$_2$-95 per cent O$_2$. 


Unwashed liver slices were found to contain about 1.5 mg. of serum albumin per gm. of wet liver. In order to lower the initial level, the slices were routinely washed for an hour by rocking in a 500 cc. flask containing the above medium. The slices were then blotted on filter paper, and portions of about 2 gm. were accurately weighed and placed in 125 cc. flasks containing 10 cc. of medium. A sample was also taken for determination of initial albumin level, which had been lowered to about 0.3 mg. per gm. by the washing procedure.

![Graph showing change in serum albumin content of liver slices during incubation.](http://www.jbc.org/)

**Fig. 2.** The increase in the amount of serum albumin during incubation. Both the medium and slices were analyzed separately for the albumin level.

**Results**

In all of the experiments, increases in the amount of serum albumin present in the system were found after 4 hours of incubation. The average rate of increase in twenty experiments was 0.12 mg. per gm. of liver per hour. Only 20 per cent of the livers gave increases of less than 0.08 mg. per gm. per hour.

Fig. 2 shows the manner in which this increase occurred during a typical experiment. In this experiment both medium and slices were analyzed separately for the albumin level. Each time which is plotted represents a separate flask. Fig. 2 shows that albumin continues to be washed from the slices as the incubation proceeds, and appears in the medium at a rate which is more rapid during the 1st hour than in the latter stages of incubation.

**Effect of Ions and Inhibitors**—Table I summarizes the changes in the
The net increase of serum albumin caused by variation in the ionic composition of the incubation medium and by addition of various metabolic inhibitors. The presence of phosphate was inhibitory, with or without the presence of bicarbonate. Either calcium or magnesium was required for maximal albumin production. While this requirement for divalent cation may simply reflect the dependence of albumin formation on general intra-

Table I

<table>
<thead>
<tr>
<th>Condition</th>
<th>Production in 4 hrs. compared to control flask (per cent)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mm per liter PO₄ added</td>
<td>30</td>
</tr>
<tr>
<td>Ca replaced by Mg</td>
<td>94</td>
</tr>
<tr>
<td>Ca replaced by Mg, reduced to 2.5 mm per liter</td>
<td>105</td>
</tr>
<tr>
<td>Divalent cation omitted</td>
<td>74</td>
</tr>
<tr>
<td>K replaced by Na</td>
<td>95</td>
</tr>
<tr>
<td>Na “ “ K</td>
<td>60</td>
</tr>
<tr>
<td>260 mOsm per liter</td>
<td>99</td>
</tr>
<tr>
<td>260 “ “ “</td>
<td>100</td>
</tr>
<tr>
<td>350 “ “ “</td>
<td>102</td>
</tr>
<tr>
<td>O₂ replaced by N₂</td>
<td>23</td>
</tr>
<tr>
<td>Cyanide, 0.002 M</td>
<td>0</td>
</tr>
<tr>
<td>“ 0.0002 M</td>
<td>53</td>
</tr>
<tr>
<td>Azide, 0.005 M</td>
<td>6</td>
</tr>
<tr>
<td>Arsenite, 0.001 M</td>
<td>10</td>
</tr>
<tr>
<td>“ 0.0001 “</td>
<td>27</td>
</tr>
<tr>
<td>Iodoacetate, 0.001 M</td>
<td>10</td>
</tr>
<tr>
<td>“ 0.0001 “</td>
<td>52</td>
</tr>
<tr>
<td>Dinitrophenol, 0.0008 M</td>
<td>2</td>
</tr>
<tr>
<td>“ 0.0001 “</td>
<td>54</td>
</tr>
<tr>
<td>Arsenate, 0.01 M</td>
<td>26</td>
</tr>
<tr>
<td>“ 0.001 “</td>
<td>25</td>
</tr>
<tr>
<td>Fluoride, 0.005 M</td>
<td>44</td>
</tr>
<tr>
<td>Malonate, 0.01 “</td>
<td>94</td>
</tr>
</tbody>
</table>

cellular enzymatic activity, it is of interest to note that magnesium has been found necessary in model systems which effect the formation of peptide bonds (8–11). The effects caused by the different inhibitors tested show that the appearance of albumin is a process dependent upon a supply of energy generated within the cell. The inhibitions caused by the two concentrations of dinitrophenol used are similar to the effects which this compound has on the incorporation of labeled alanine into liver slice proteins (12).
Since the dinitrophenol appears to affect cellular reactions by blocking the transfer of phosphate bond energy (13), the results found here imply that high energy phosphate is involved in the formation of proteins. Other evidence for the importance of phosphate bond energy is found in the functioning of adenosinetriphosphate in the formation of peptide bonds in model systems (8-11).

It is interesting to note that high concentrations of malonate, which were without effect in the present system, also failed to inhibit the active

### Table II

*Simultaneous Appearance of Albumin and Incorporation of Radioactivity*

<table>
<thead>
<tr>
<th>Condition</th>
<th>Initial level</th>
<th>Level after 6 hrs. incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Observed data</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amount of serum albumin present, mg. per gm. liver</td>
<td>0.28</td>
<td>0.70</td>
</tr>
<tr>
<td>Total radioactivity in serum albumin, c.p.m. per gm. liver</td>
<td>0</td>
<td>724</td>
</tr>
<tr>
<td>Calculated data</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Specific activity of albumin carboxyl groups, c.p.m. per mg. albumin</td>
<td>1,030</td>
<td></td>
</tr>
<tr>
<td>Specific activity of albumin carboxyl groups, c.p.m. per mm CO$_2$</td>
<td>125,000</td>
<td></td>
</tr>
<tr>
<td>Specific activity of dicarboxylic amino acid carboxyl groups, c.p.m. per mm CO$_2$</td>
<td>375,000</td>
<td></td>
</tr>
</tbody>
</table>

* Determined by counting CO$_2$ released from hydrolysate by treatment with ninhydrin, based on specific activity of bicarbonate of 1,000,000 c.p.m. per mm of CO$_2$.

† Calculated by dividing the total radioactivity by the amount of albumin.

‡ Calculated by multiplying the figure of the previous line by 121, the average residue weight found for serum albumin.

§ Calculated by multiplying the figure of the previous line by 3.0, the ratio of specific activities found (1) for the CO$_2$ released by ninhydrin from the dicarboxylic amino acids to that released from the total hydrolysate.

intracellular concentration of amino acids by rat diaphragms (14), although this system was sensitive to all other inhibitors tested. This comparison suggests that the intracellular concentration of amino acids is an important factor in the synthesis of proteins.

*Effect of Substrates and Hormones*—No consistent effects were observed upon the addition of amino acid mixtures or oxidizable substrates to the media during incubations. The liver slice system appears to be complete in itself with regard to the supply of amino acids and of energy-yielding compounds required.

No significant change in the rate of albumin production was found upon
addition to the incubation medium of insulin, thyroxine, growth hormone, adrenal extract, cortisone, or desoxycorticosterone. This does not preclude hormonal influences in the control of the formation of albumin, since the demonstration of hormonal effects by addition in vitro has rarely been accomplished, possibly owing to such factors as destruction of the hormone, failure of penetration, or insufficient time for action.

Incorporation of Radioactive Carbon into Albumin—The incorporation of large amounts of labeled amino acids into serum albumin concomitant with a net increase would serve to indicate that this albumin is actually newly formed protein, and not merely preformed albumin which had been released from some complex within the cell. Accordingly, an experiment was performed in which were determined both the total amount of albumin present and the amount of incorporation of carbon from C\(^{14}\)O\(_2\) into the dicarboxylic amino acids of the albumin molecule. The incubation and counting procedures have been described previously (1). The amount of radioactivity incorporated was determined by precipitating aliquots of albumin with antiserum, washing and hydrolyzing the precipitates, and counting the CO\(_2\) released from the hydrolysate by treatment with ninhydrin.

From the measurements on the serum albumin present after 4 hours of incubation, it could be calculated that 37.5 per cent of the carboxyl groups of aspartic and glutamic acids which are released by ninhydrin contained labeled carbon (Table II). If it were assumed that the albumin present at the start of incubation underwent no turnover, this figure would be raised to 63 per cent for the dicarboxylic amino acids of the albumin produced during the incubation. This high degree of incorporation strongly suggests that the appearance of serum albumin as observed in the experiments reported here does represent formation of new protein.

DISCUSSION

A number of earlier reports have been concerned with the incorporation in vitro of labeled compounds into the proteins of tissues and homogenates. These studies furnished suggestive evidence for the synthesis of new protein molecules. In the present experiments, the application of immunological techniques combined with radioactivity measurements has yielded more direct proof for this synthetic process.

By using the average rate observed for albumin production by slices, a rough calculation can be made comparing the rate of production in vitro with the rate of albumin replacement indicated for the intact animal by isotope studies in vivo. The average observed liver size was 1.8 per cent of the body weight. Hence a 2.5 kilo chicken would have a liver weighing 45 gm. Based on the average rate of serum albumin production of 0.12
mg. per gm. of liver per hour, a liver of this size would form 5.4 mg. of serum albumin per hour, or 129 mg. per day.

A 2.5 kilo chicken would have a total circulating albumin content of 1730 mg., assuming a blood volume of 7 per cent, a hematocrit of 0.45, and an albumin level in serum of 1.8 per cent. Hence the liver could replace the total circulating albumin in 1730 / 129 or 13.4 days. This calculation gives a half life for serum albumin in the chicken in substantial agreement with the turnover rates determined for various animals by observation of the rates of incorporation of isotopes in vivo (15–17).

While this calculation can give only an order of magnitude, the demonstration that the liver can produce albumin at a rate comparable to the rate of albumin replacement in the intact animal suggests that the liver, unaided, can make all or a large part of the body's requirement of albumin. This concept has previously been indicated by experiments involving operative procedures (18) and isotope studies on perfused livers (19). A further implication of this calculation is that the incorporation of labeled amino acids observed in vivo actually represents the formation of new molecules and not merely replacement of amino acids in existing molecules, as suggested earlier by the studies of Heidelberger et al. on antibodies of passive immunization (20).

SUMMARY

1. An immunological technique was developed by which small amounts of serum albumin in chicken liver slices and their incubation media could be determined. The standard albumin for this procedure was obtained by isolation from chicken serum.

2. It was shown that chicken liver slices, upon incubation in bicarbonate medium, produce a net quantity of serum albumin of about 0.12 mg. per gm. of liver per hour.

3. Investigations were made of the effect on albumin production of variations in the ionic composition of the incubation medium and of addition to the medium of inhibitors, substrates, and hormones.

4. Demonstration of incorporation of large amounts of C¹⁴O₂ into the dicarboxylic amino acids of this albumin is further evidence that the observed appearance of albumin actually represents the formation of new protein.

The authors wish to thank Mr. M. J. Budka for carrying out electrophoretic analyses on the standard albumin.

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NET PRODUCTION OF SERUM ALBUMIN BY LIVER SLICES
Theodore Peters, Jr. and Christian B. Anfinsen


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