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THE SYNTHESIS OF HEMOGLOBIN IN A CELL-FREE SYSTEM

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The microsomal particles[1] have been implicated as the major sites of protein synthesis within the cells of a variety of tissues. The studies of Borsook, Zamecnik, Hultin, and others, with intact animals and various types of whole cell systems (see review by Askonas *et al.*[2]), as well as electron-microscope studies,[3] provided the original evidence for this conclusion. Zamecnik and Keller[4] have, in addition, developed cell-free systems which incorporate C^{14}-labeled amino acids into protein. These include microsomes as well as various soluble enzymes.[5] Further studies have partially defined the intermediate stages involved.[6] However, it has not yet been possible to equate incorporation of labeled amino acid into protein with actual protein synthesis (see review by Campbell[7]).

Kruh and Borsook[8] have demonstrated that rabbit reticulocytes synthesize hemoglobin in vitro and that approximately 85 per cent of the soluble protein made by such cells is of this one species. Studies with whole cells by Rabinovitz and Olson[9] have shown that microsomes participate in the synthesis of hemoglobin.

These authors have also demonstrated incorporation of iron into hemoglobin in a cell-free system containing reticulocyte microsomes.[10]

It is clear, then, that reticulocytes offer a favorable system for study of the synthesis of a specific protein. This paper describes a cell-free system consisting of reticulocyte microsomes and soluble enzymes which possesses the ability to produce hemoglobin.

METHODS AND MATERIALS

Preparation of Microsomes and Enzymes.—Rabbit reticulocytes were prepared and washed as described by Borsook *et al.*[11] All operations were performed at 4° C. unless otherwise indicated. The packed cells (usually 50 ml. from 2 rabbits) were lysed by the addition of 4 volumes of 0.005 M magnesium chloride, and the mixture was stirred gently for 10 minutes. One volume of 1.5 M sucrose containing 0.15 M potassium chloride was added slowly, and the mixture centrifuged at 10,000 $\times$ g for 10 minutes. The precipitate, containing cell membranes and unbroken cells, was discarded. The solution was then centrifuged for 1 hour at 105,000 $\times$ g, yielding a precipitate and supernatant (super I). The precipitate (microsomes) was washed by suspending in 100 ml. of Medium A[5] and was centrifuged for 1 hour at 105,000 $\times$ g. The washed microsomes were suspended in Medium A, yielding a pale-yellow, opalescent solution (20–28 mg. of ribonucleoprotein per ml.). The supernatant (super II) was discarded.

The dark-red supernatant from the first high-speed centrifugation (super I) was adjusted to pH 5.15 with 1 N acetic acid, centrifuged, and the precipitated protein dissolved in 0.1 M Tris buffer, pH 7.5 (pH 5 enzyme, 12 mg. of protein per ml.). Guinea-pig liver pH 5 enzyme was prepared in a similar way[12] but was reprecipitated at pH 5.15 before use.

Assay Procedure.—The complete reaction mixture contained 0.5 ml. of microsomes; 0.4 ml. of pH 5 enzyme; 150 μg. of creatine kinase purified through the alcohol fractionation;[13] 1 μmole of potassium adenosine triphosphate (ATP), adjusted to pH 7.5 with potassium hydroxide; 20 μmoles of creatine phosphate (CrP), adjusted to pH 7.0 with hydrochloric acid; 0.25 μmole of guanosine triphosphate (GTP); 0.1 μmole of C^{14}-labeled amino acid (leucine, isoleucine, and valine were the uniformly labeled L-isomers obtained from Nuclear-Chicago, Inc., diluted to a specific activity of 3,800 counts/min/mμmole); and the complete amino acid mixture, in a final volume of 1.4 ml. Creatine phosphate was obtained from the California Corporation for Biochemical Research, Los Angeles, California, and other nucleotides were products of the Pabst Laboratories. The complete amino acid mixture contained the 18 amino acids in the proportions, but at one-sixth the final concentration, described by Borsook and co-workers.[14] The C^{14}-labeled amino acid under study was omitted from the mixture of unlabeled amino acids. In some experiments, 0.2 ml. of super I, containing approximately 30 mg. of hemoglobin per ml., was added.

After incubation for the indicated time (15–60 minutes) at 37° C. with shaking, the proteins were precipitated and washed once with 3.5 per cent trichloracetic acid, dissolved in 0.5 ml. of 1 N sodium hydroxide, reprecipitated, and washed once with trichloracetic acid, then heated at 60° C. for 10 minutes in 3 ml. of 95 per cent ethanol. Two volumes of ether were added after heating, to insure precipitation

of protein, and the precipitate was finally washed twice with ether. The dried precipitates were weighed and counted with a Nuclear "Micromil" window gas-flow counter. All results are corrected for self-absorption. The results are given as counts per minute (c.p.m.) per milligram of microsomes, based on the actual dry weight of a sample of microsomes washed as described above. This precipitate was found by ribose analysis to contain 30–40 per cent ribonucleic acid (RNA).

Isolation of Hemoglobin.—The large-scale incubation for the isolation of hemo-globin contained (in one beaker) the usual assay constituents at a sixfold level, using reticulocyte pH 5 enzyme, and with 1.2 ml. of super I added. The mixture was incubated for 60 minutes and then cooled in an ice bath. An additional 1.3 ml. of super I was then added, to bring the total carrier hemoglobin to about 60 mg. The mixture was centrifuged for 1 hour in the cold at 105,000 × *g* to remove microsomes. The supernatant proteins contained approximately 103,000 c.p.m. This solution was adjusted to pH 5.15 and the precipitate removed. The super-natant was immediately readjusted to pH 7.0. The proteins precipitated at pH 5 contained 36,000 c.p.m. This precipitate probably includes, in addition to pH 5 enzyme, aggregated ribonucleoprotein from microsomal degradation and ab-sorbed hemoglobin (estimated as 10 per cent of the supernatant hemoglobin). The supernatant solution was dialyzed overnight against 0.1 *M* Tris buffer, pH 7.5, and the hemoglobin then precipitated by addition of solid ammonium sulfate to 90 per cent saturation. The precipitate was dissolved in water and dialyzed against developer No. 2, as described by Allen and co-workers.[15] An aliquot of the dialyzed solution, containing 31,000 c.p.m., was then chromatographed on Amberlite IRC-50 resin, using this same developer. Since rabbit hemoglobin is adsorbed on the resin more strongly than human hemoglobin, the final elution was done at room tem-perature.[16] The optical densities at 280 and 415 mμ were recorded for each fraction. For determination of radioactivity, the protein was precipitated with trichloracetic acid, followed by the ethanol and ether washes as described above. Carrier hemo-globin was added where needed to provide 10–15 mg. of dry protein per sample for counting.

EXPERIMENTS AND RESULTS

Properties of Incorporation System.—The results in Table 1 show incorporation of C[14]-labeled leucine into protein, using microsomes from rabbit reticulocytes, plus

TABLE 1

INCORPORATION OF C[14]-LEUCINE INTO PROTEIN

Reaction Mixture	C.p.m./Mg Microsomes
Complete system,* g. pig pH 5 enzyme	670
Complete system,* minus amino acid mix	366
Complete system,* minus pH 5 enzyme	192
Complete system,* minus pH 5 enzyme minus amino acid mix	110
Complete system,* minus ATP, minus CrP	45
Complete system,* with reticulocyte pH 5 enzyme	806

* The complete system is described in the text. The incubation time was 30 minutes.

pH 5 enzymes from either guinea-pig liver or reticulocytes. The process is energy-dependent, as originally observed by Zamecnik and Keller.[4] No incorporation occurred in the absence of microsomes, with boiled microsomes, or with cell debris substituted for microsomes. Five μg. of crystalline ribonuclease reduced incorpora-

tion more than 90 per cent. Substitution of 10 μmoles of ATP for the CrP inhibited incorporation almost completely. When GTP was omitted, the activity was reduced to half that of the complete system, even without removal of nucleotides.[5] In contrast to preparations of liver microsomes,[17] the results with different batches of reticulocytes were reproducible under standard conditions, agreeing with one another to within 15–20 per cent.

Addition of the complete amino acid mixture developed by Borsook and coworkers[14] doubled the incorporation of C^{14}-leucine, as shown in Table 1, and had a similar effect on C^{14}-valine incorporation in other experiments. This amino acid mixture was designed to yield optimal synthesis of hemoglobin by whole cell suspensions of rabbit reticulocytes. In this respect amino acid "incorporation" in the cell-free system resembles protein synthesis by whole cells.[8] Addition of an amino acid mixture containing each amino acid at a uniform concentration (0.25 μmoles per beaker) was inhibitory.

The finding that only freshly-prepared pH 5 enzyme was effective in stimulating incorporation in the cell-free system suggests that labile enzymes play a role in this stimulation. Studies with guinea-pig liver pH 5 enzyme have shown that certain activating enzymes which are present in very low amounts in fresh preparations are inactive after overnight storage,[18] either at 4° or at −20° C. Thus it is possible that all the activating enzymes are needed for incorporation in the cell-free system, although other labile enzymes may be important also. More detailed investigation of these points is required, however, since the optimal assay conditions have not been studied in detail. For example, the addition of glutathione (5 μmoles per beaker) and extra magnesium ion (3 μmoles per beaker) each increased the incorporation of C^{14}-leucine by 15 per cent and were additive when both were present. The addition of 0.2 ml. of super I increased incorporation sometimes as much as twofold, but this effect varied with different microsome preparations.

Isolation of Radioactive Hemoglobin.—In most cell-free amino acid incorporation systems[4, 17] the reaction ceases after 15–20 minutes of incubation. Progress curves with reticulocyte microsomes (complete system) show an almost constant increase in incorporation for at least 45 minutes and occasionally for 60 minutes. It is probable that amino acids are assembled into protein in the ribonucleoprotein particles and then secondarily released into the cytoplasm as completed proteins.[19] A failure to release completed proteins from the microsomes in other cell-free systems might account for the cessation of the reaction after a short time.

The continued incorporation for longer time periods with reticulocyte microsomes suggested that soluble proteins were being produced. To study this, the usual mixture, after incubation, was centrifuged for 1 hour at 105,000 × g, and microsomes and soluble proteins were washed separately as described above. After a 30-minute incubation time, 10–15 per cent of the radioactivity was present in soluble proteins. When the microsomes from these experiments were frozen and thawed or treated with desoxycholate,[20] the radioactivity in soluble protein was increased only slightly. After 1 hour of incubation, however, 50–60 per cent of the radioactivity was present in soluble proteins. The following experiment demonstrates that the C^{14}-labeled soluble proteins consist mainly of labeled hemoglobin.

A large-scale incubation was carried out as described in "Methods and Materials,"

and the soluble proteins were chromatographed.   The coincidence of radioactivity with the hemoglobin peak (Fig. 1) demonstrates that C^{14}-labeled hemoglobin had been formed.   Little, if any, non-heme protein was present in the major peak, as shown by the constant ratio of optical density at 415mμ to that at 280mμ through-

out the greater portion of the peak.   The specific activity (ratio of counts to optical density at 280 mμ) remained constant throughout the hemoglobin peak and was also similar to the specific activity of the mixture of proteins in the first small peak.   The recovery of hemoglobin and protein (based on the optical densities) was over 90 per cent of the amount put on the column, including a small protein peak, which was eluted with higher buffer concentrations (not shown in Fig. 1).   The recovery of radioactivity was 86 per cent, e.g., 3,000 c.p.m. in the first peak, 21,500 c.p.m. in the hemoglobin peak, and approximately 2,000 c.p.m. with the stronger buffer elution, out of a total of 31,000 c.p.m. originally put on the column.   The distribution of radioactivity suggests that all

FIG. 1.—Chromatogram of soluble proteins on IRC-50. The column was eluted at 4° until tube 32, and then at room temperature.

the soluble proteins are being synthesized in the cell-free system.   According to the results given above, hemoglobin synthesis accounts for about 82 per cent of the total protein recovered from the column.

Incorporation of Various Amino Acids.—Further information, which indicates that "incorporation" in this system is synonymous with synthesis of normal cell proteins, is provided by the ratios of the amounts of C^{14}-labeled leucine, isoleucine, and valine incorporated.   As shown in Table 2, these ratios coincide closely with the proportions of these amino acids in crude globin (the total soluble proteins of the reticulocyte).   This agreement is further evidence that these proteins, of which hemoglobin is the largest constituent, are being synthesized.   The data also permit a distinction to be made between the "structural" protein of the microsome and the protein being synthesized.   Since 50 per cent of the radioactivity remained in the microsomes and the isoleucine and leucine content of microsomal protein are nearly the same,[21] incorporation into microsomal protein would have changed the observed ratios.   Therefore, it is probable that the radioactivity in the microsomes largely represents hemoglobin precursors.   A similar conclusion has been
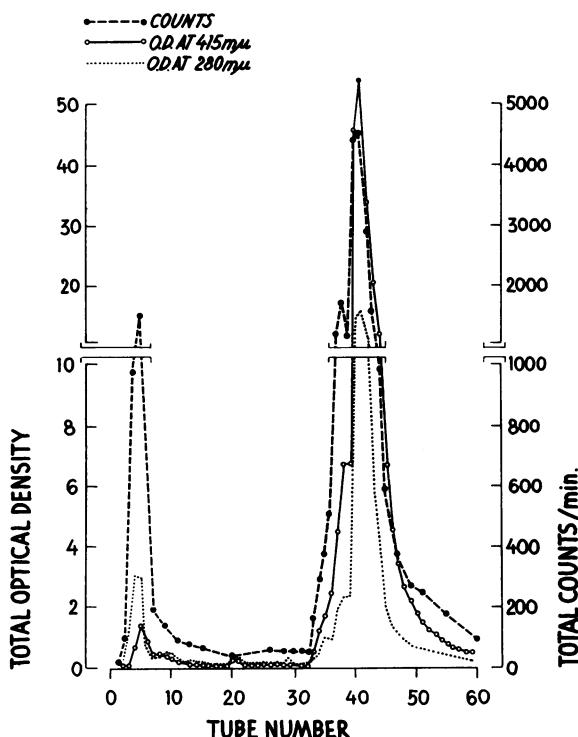
TABLE 2

INCORPORATION OF C[14]-LABELED AMINO ACIDS COMPARED TO
AMINO-ACID COMPOSITION OF RABBIT GLOBIN

Reaction Mixture	C.p.m./ Mg Micro- somes	Ratio	Amino Acid Composi- tion†	Ratio
Complete system,* g. pig pH 5 enzyme, leucine	748	1.00	11.7	1.00
Complete system,* g. pig pH 5 enzyme, isoleucine	90	0.12	1.3	0.11
Complete system,* g. pig pH 5 enzyme, valine	538	0.72	8.1	0.77

† From analyses of crude rabbit globin (P. Ts'o, J. Bonner, and H. Dintzis, unpublished data). The amino acid composition is given as gm. per 100 gm. of protein, and the ratios are given as molar ratios for comparison with the radioactivity data.

* The complete system is described in the text. The incubation time was 60 minutes and the total protein of the reaction mixture was washed and counted.

reached by Dintzis *et al.*[22] based on studies with whole reticulocytes. In general, this would imply that the ribonucleoprotein serves as a template for protein synthesis but not as a precursor of the proteins synthesized.[23, 24]

DISCUSSION AND SUMMARY

The incorporation of C[14]-labeled amino acids into protein in a system containing microsomal particles from rabbit reticulocytes plus soluble enzymes has been described. Although the study of this system is still in its early stages, the evidence indicates that the process under investigation is the synthesis of soluble cell protein which consists mainly of hemoglobin. The evidence which leads to this conclusion is summarized as follows. Incorporation of labeled amino acid was stimulated by addition of a complete mixture of amino acids of a particular composition. Soluble proteins containing radioactivity were formed, and radioactive hemoglobin was isolated. Leucine, isoleucine, and valine were incorporated in the ratios in which they are present in crude hemoglobin.

At this stage of the investigation, the factors required for maximum synthesis appear similar to those reported for incorporation into microsomes of other animal tissues.[7] These findings strengthen the concept of Hoagland *et al.*[25] that the intermediate stages in protein synthesis involve amino acid activation followed by formation of amino acid-RNA.[12, 26] The results also provide evidence that the amino acid sequence of the final protein is determined in the microsomal particle. The evidence for this conclusion is that, although the pH 5 enzyme of guinea-pig liver forms leucine-RNA and isoleucine-RNA at similar rates,[18] these amino acids are incorporated into protein in the proportions in which they exist in hemoglobin.

We wish to express our appreciation to Dr. Henry Borsook, whose encouragement and numerous contributions in this field have greatly aided these studies. It is a pleasure to thank Dr. James Bonner and Dr. Howard Dintzis for many stimulating discussions.

[1] The term "microsomes" or microsomal particles as used in this paper includes the "microsomes" of tissues such as liver, which consist of ribonucleoprotein particles plus endoplasmic reticulum, as well as "microsomes" from tissues such as reticulocytes, which consist mainly of

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DELAYED LIGHT EMISSION IN GREEN PLANT MATERIALS: TEMPERATURE-DEPENDENCE AND QUANTUM YIELD*

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Introduction.—The discovery of the delayed light emission of plant materials by Strehler and Arnold in 1951[1] has stimulated a good deal of interest in this rather remarkable property. The emitted light has been shown to be due to an electronic transition between the first excited singlet state of chlorophyll and the ground state.[2, 3] At room temperature, a luminescence is observable from about 0.01 second[4] to several minutes[5] after excitation. Thus the electronic transition cannot be rate-determining, and the process represents neither normal fluorescence nor normal phosphorescence. Indeed, there is some evidence[4, 6] that the decay curve of the luminescence is the resultant of more than one rate-limiting process. Strehler