Protein Synthesis by Human Platelets

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SUMMARY

The present studies show that human platelets can incorporate uniformly labeled $^{14}$C-$L$-leucine into trichloroacetic acid-precipitable material. This material is digested by Pronase but not by deoxyribonuclease or ribonuclease. Acid hydrolysis of the material liberates a compound with chromatographic properties identical with those of authentic $L$-leucine. The incorporation of uniformly labeled $^{14}$C-$L$-leucine by platelets is inhibited by puromycin but not by actinomycin D. To our knowledge, this study constitutes the first definite demonstration that mammalian platelets can synthesize protein.

EXPERIMENTAL PROCEDURE

Materials—Blood platelets were isolated (4) from normal human volunteers by differential centrifugation of blood containing EDTA as an anticoagulant. The platelets were washed and resuspended in 0.85% NaCl and used within 3 hours of venesection. Platelet counts were performed by phase microscopy. Contamination of the platelets by erythrocytes and leukocytes was always less than 1 cell/4000 platelets.

$L$-Leucine-$^{14}$C (New England Nuclear) was tested for radiochemical purity by means of paper chromatography, and we found with each of two solvent systems that the radioactivity migrated as a single substance with an $R_s$ identical with that of authentic $L$-leucine. Amino acids of the configuration and of the highest purity available were purchased from Mann; puromycin dihydrochloride was obtained from Nutritional Biochemicals, Pronase from Calbiochem, DNase and RNase from Worthington, and NCS tissue solvent from Nuclear-Chicago. Actinomycin D was a gift from Merck.

Methods—Protein synthesis was studied by use of a modification of the method of Manchester and Young (5). The standard incubation mixture included 4 mmoles of $L$-leucine-$^{14}$C (1 $\mu$Ci), 13.75 mmoles of glucose, 109 platelets suspended in 0.5 ml of 0.85% NaCl, 1.5 ml of Krebs-Ringer bicarbonate buffer (6) modified to contain 50% of the recommended calcium concentration, and 0.5 ml of 0.85% NaCl containing substances to be tested for their effects on protein synthesis, all in a total volume of 2.5 ml. The mixture was incubated for 30 min in a shaker bath at 37$^\circ$ in an atmosphere of 95% O$_2$-5% CO$_2$. Reactions were stopped by addition of 2.5 ml of 20% TCA containing 10 mM L-leucine. The resulting precipitate was separated by centrifugation, resuspended in 10% TCA containing 5 mM L-leucine, and heated at 90$^\circ$ for 15 min. It was washed three times with the 10% TCA-leucine solution and once each with ethanol-ether (1:1) and ether. The protein precipitate was then dissolved in 1 ml of NCS solvent at room temperature and added to 15 ml of 0.4% diphenyloxazole in toluene. The radioactivity of the samples was assayed in a Packard Tri-Carb liquid scintillation spectrometer and was never less than twice the background level. Counting time was selected to yield at least 10,000 counts so that the standard error of the count was less than 1%. Values for counts per min were converted to disintegrations per min by use of the channels-ratio method (7). Each incubation was carried out in duplicate, and the results, which usually agreed within 5% and never differed by more than 10%, were averaged. To calculate the amount of $L$-leucine incorporated into protein, we divided the value for radioactivity recovered in the TCA-precipitated material by the value for the specific activity of the $L$-leucine in the incubation medium. The amount of $L$-leucine-$^{14}$C incorporated by 109 platelets varied from donor to donor over a 5-fold range with the maximum incorporation being about 1% of the L-leucine available. In each experimental platelet from a single donor were used.

RESULTS

Conditions of Assay—Incubation of platelets with L-leucine-$^{14}$C led to incorporation of radioactivity into the TCA-precipitable fraction of the platelets. In a representative experiment, heating the platelet suspension for 3 min in a boiling water bath reduced the net counts per min of L-leucine-$^{14}$C incorporated from 2235 to 87. The extent of incorporation of L-leu-
cine-U-^{14}C was proportional to the duration of incubation (Fig. 1). Increasing the concentration of L-leucine in the incubation mixture resulted in increased incorporation of the amino acid (Fig. 2).

A linear relationship between the concentration of platelets in the incubation mixture and the incorporation of L-leucine-U-^{14}C was not observed until the conditions of incubation were optimal. When the only amino acid added was L-leucine-U-^{14}C at 1.6 \mu M, as in the standard reaction mixture, incorporation of \(^{14}C\) was proportional to the concentration of platelets at the lower end of the range tested, but incorporation reached a maximum value which could not be exceeded at still greater platelet concentrations (Fig. 3A). When L-leucine-U-^{14}C was added at 1 \text{ mm}, the incorporation of \(^{14}C\) varied with platelet concentration over the entire range tested as shown in Fig. 3B. When a mixture of 20 amino acids, including L-leucine, was added at 20 \mu M of each amino acid, L-leucine-U-^{14}C incorporation varied in a linear relationship to platelet concentration over the entire range (Fig. 3C).

The significance of the contribution by leukocytes contaminating the platelet suspension to the incorporation of L-leucine-U-^{14}C was studied by varying the leukocyte concentration in the incubation mixture while holding the platelet concentration constant. The amount of L-leucine-U-^{14}C incorporated into TCA-precipitable material changed little when the leukocyte concentration was varied over a 30-fold range, whereas leucine incorporation was quite sensitive to alterations in platelet concentration (Table I). On the basis of these findings we feel that leukocytes in the platelet suspension did not contribute significantly to the observed L-leucine-U-^{14}C incorporation.

**Identification of Product**—The nature of the labeled product was investigated in two ways. First, a sample of the labeled TCA-precipitated fraction was dissolved in 0.05 \text{n NaOH and
Platelets were incubated with L-Leucine-U-\(^{14}\)C under the conditions of the standard assay, and the TCA-precipitable material was isolated according to the procedure described in the text. This material was dissolved in 0.05 N NaOH and neutralized. Aliquots of the solution, each containing approximately 2200 cpm of \(^{14}\)C, were incubated at 37° in a total volume of 1.0 ml with additions as indicated. Incubations were stopped by addition of 1 ml of 20% TCA containing 10 mm L-Leucine. The resulting precipitate and the supernatant solution were treated and assayed for radioactivity as described in the text. All incubations were performed in duplicate.

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\begin{array}{|c|c|c|}
\hline
\text{Additions} & \text{Incubation time (hrs)} & \text{Radioactivity (cpm)} \\
\hline
\text{None} & 6 & 25 \hspace{1cm} 2166 \\
\text{DNase (20 \mu g), MgSO}_4 (5 \text{ \mu moles}) & 1 & 34 \hspace{1cm} 2677 \\
\text{RNase (20 \mu g)} & 1 & 40 \hspace{1cm} 1905 \\
\text{Pronase (1 mg)} & 6 & 1999 \hspace{1cm} 76 \\
\hline
\end{array}
\]

Experimental details were as described for the standard assay except that mannitol, 10 mg, was added to each flask in Experiment 2 to aid in dissolving the actinomycin D. Different donors provided the platelets for the two experiments. Each experiment is representative of three performed.

Inhibitors of Protein Synthesis—The effect of inhibitors of protein synthesis on the incorporation of L-Leucine-U-\(^{14}\)C by platelets is summarized in Table III. Puromycin inhibited the incorporation of L-Leucine-U-\(^{14}\)C into the TCA-precipitable fraction of the platelets by as much as 95%. Actinomycin D had little or no effect under the same conditions.

\[
\begin{array}{|c|c|c|}
\hline
\text{Inhibitor} & \text{Incorporation (cpm)} & \text{Inhibition (\%)} \\
\hline
\text{None} & 20.4 & 0 \\
\text{Puromycin, 0.1 mM} & 3.36 & 84 \\
\text{Puromycin, 1.0 mM} & 0.04 & 95 \\
\text{Actinomycin D, 25 \mu g per ml} & 4.37 & 4 \\
\text{Actinomycin D, 100 \mu g per ml} & 4.22 & 7 \\
\hline
\end{array}
\]

In these experiments we have shown that human blood platelets incorporate L-Leucine into a product which is digestible by a proteolytic enzyme and which yields free L-Leucine upon acid hydrolysis. The incorporation is (a) proportional to time, substrate concentration, and amount of tissue, (b) prevented by heating the platelets before incubation, and (c) inhibited by puromycin. These findings indicate that human platelets can synthesize new protein from amino acids. To our knowledge, this phenomenon in mammalian platelets has not been proved before.

Human platelets have no nuclei and have not been found to contain DNA (3, 8). Although there might be undetected cyto-
plasmic DNA in platelets, the failure of actinomycin D to inhibit protein synthesis is, perhaps, evidence against this possibility. The ability of platelets to synthesize protein thus implies that messenger RNA derived from the nucleated precursor, the megalakaryocyte, persists in the platelet and is metabolically relatively stable. A similar situation has been shown to exist in the reticulocyte (9), which synthesizes hemoglobin long after the nucleus of the cell has been extruded.

The identity of the protein made by platelets remains to be determined. It may be that a varied complement of structural proteins and enzymes are being replenished, but, as in the case of the reticulocyte, it is also possible that the platelets make a predominant protein, such as fibrinogen or thrombosthenin, the contractile protein of platelets. Experiments are in progress in this laboratory to explore these possibilities.

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REFERENCES
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