Synthesis of Riboflavin Nucleotides by Mature Human Erythrocytes

By BARBARA MANDULA AND ERNEST BEUTLER

Intact human red blood cells can synthesize FAD and FMN from riboflavin. The rate of synthesis of FAD is linearly proportional to the concentration of riboflavin in the medium at levels below 0.9 μM. With 0.9 μM riboflavin, the rate of synthesis is about 0.1 mμmole FAD/ml. red blood cells/hour. Incubation of red blood cells with riboflavin can result in increased red cell glutathione reductase activity when the enzyme is measured in the absence of added FAD. This indicates that the FAD concentration in the red cells increased during the incubation. The rate of incorporation of radioactive riboflavin into red blood cells is the same whether the cells are suspended in plasma or in a phosphate-saline-glucose medium. The time it takes for half the FAD in normal human red blood cells to turn over is calculated to be about 6 days, assuming a single mixing pool of red cell FAD.

R ED CELL GLUTATHIONE REDUCTASE has been shown to be a flavin enzyme,[1-3] and recent studies have demonstrated that in normal subjects it is not saturated with its prosthetic group, FAD.[4,5] Administration of riboflavin to normal subjects causes a significant increase in red cell glutathione reductase activity within 1 or 2 days, an increase which is associated with a rise in red cell FAD concentration.[4,5] These results suggested that red blood cells (RBC) may be able to convert riboflavin to FAD in vivo, although the possibility that the liver or other organs participate could not be ruled out. It was therefore of interest to us to determine whether intact red cells can convert riboflavin to FAD in vitro and to study the conditions under which such synthesis may occur.

The biosynthesis of FAD from riboflavin has been shown to occur in two steps:[6,7]
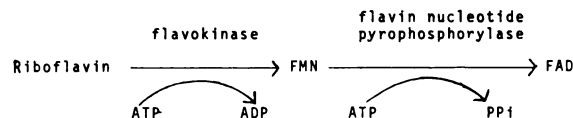
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We have studied the incorporation of radioactive riboflavin into intact red cells and find that exogenous riboflavin is readily incorporated into FMN and FAD. We also calculate a tentative turnover rate for FAD in red cells under physiologic conditions.

MATERIALS AND METHODS

Incubation of RBC with Radioactive Riboflavin

Thirty ml. freshly drawn heparinized human blood was centrifuged at 1000 × g. for 15 minutes. After withdrawing and saving the plasma, the buffy coat was removed and discarded. The RBC were washed twice in four volumes of ice-cold 0.9 per cent NaCl with removal of residual buffy coat between washings.

A mixture of equal volumes of washed RBC and either plasma or phosphate-saline-glucose solution (250 mg. glucose added to 100 ml. of a solution consisting of one part 0.15 M potassium phosphate buffer, pH 7.4, plus nine parts 0.15 M NaCl) was used for incubations in the presence of radioactive riboflavin. Plasma was used unless noted otherwise. Incubations were carried out at 37° C with constant shaking. In the case of plasma, an atmosphere of 95 per cent air, 5 per cent CO_2 was used to achieve a pH of 7.4. Radioactive riboflavin was added to the incubation mixture at ice temperature just before incubation was started. The concentration of riboflavin is expressed in terms of its concentration in the medium in which the RBC were suspended. Samples, generally 1 ml., were withdrawn at various times into 39 ml. ice-cold 0.9 per cent NaCl. After centrifugation, the RBC were washed by resuspension in 39 ml. of ice-cold 0.9 per cent NaCl, and recentrifugation. A portion of the supernatant solution from the second washing was counted to insure that essentially no residual radioactivity was present in the supernatant. Two-tenths ml. washed RBC were hemolyzed by freeze-thawing after addition of an equal volume of water. Samples of this hemolysate were used for radioactivity determination and for hemoglobin estimation in order to know the total radioactivity incorporated. The calculations made assume that each milliliter of RBC contains 333 mg. hemoglobin.

Isolation of RBC Flavins

Two and one-half ml. distilled H_2O were added to 0.5 ml. RBC which had been incubated with radioactive riboflavin and then washed twice. Henceforth, procedures were carried out as quickly as possible and the solution was kept as cold and dark as possible. After removal of 0.05 ml. hemolysate for hemoglobin determination, 0.05 ml. of 3 mM FAD was added to the hemolysate to serve as carrier, and as a means of following losses occurring during the procedure. Three ml. 20 per cent trichloroacetic acid (TCA) were then added to the hemolysate, the solution was mixed with a stirring rod, and was kept in the dark on ice for 15 minutes. Protein was removed by centrifugation for 15 minutes at 1000 × g. Three ml. of the TCA supernatant were extracted with four 4 ml. portions of cold diethyl ether to remove TCA. Two and one-half ml. of the extracted solution were frozen in an acetone-dry ice bath and lyophilized in the dark. The residue was dissolved in 0.3 ml. cold H_2O. Duplicate samples of 0.075 ml. of solution were spotted 10 cm. from the cathode end of a piece of Whatman 3MM paper, 32 cm. × 28.5 cm., which had been immersed in buffer and then blotted. Electrophoresis was then carried out for 2 hours at approximately 1500 volts, with a current flow of 80 milliamperes, or 2.5 milliamperes/cm., in a continuous system of sodium acetate, pH 5.1, I = 0.05[8], on a Brinkmann Pherograph Mini 68. Under these conditions, riboflavin, FMN and FAD are well separated. After drying the paper in the dark, the fluorescent compounds were located under long-wave ultraviolet light, and the appropriate area for each compound cut into narrow strips and placed in 10 ml. 10 per cent TCA at 37° C to elute the flavins. The material eluted from the paper was analyzed for flavin fluorescence by the modification of Lowry's method[9],[10] described by Beutler.[5] A nonfluorescent part of the paper served as a blank. Seven ml. of the remaining paper eluate were extracted twice with 10 ml. cold diethyl ether and, after removing the residual ether with a stream of air, 5 ml. of the TCA-free solution were placed

Table 1.—Distribution of [¹⁴C]-riboflavin Between Plasma and RBC

| | Per Cent of Total Recovered | | Per Cent Expected for Free Diffusion | |
Suspending Medium	Medium	RBC	Medium	RBC
Plasma	88	12	59	41
Phosphate-saline glucose	85	15	59	41

Equal volumes of RBC and suspending medium were mixed and kept on ice for 15 minutes with occasional shaking in the presence of 1.5 μm [¹⁴C]-riboflavin. The suspension was then centrifuged and radioactivity was measured in the RBC and in the medium. It is assumed that 70 per cent of the RBC volume is water. Total recovery of radioactivity was 94 per cent of the initial amount.

in a scintillation vial and evaporated to dryness at 95° C. Counting solution was added and radioactivity determinations were made. For the duplicate electrophoretic sample, the entire migration pathway on the paper was cut into strips 2 cm. × 4 cm. and counted in a scintillation counter. In this way it was possible to correct for incomplete elution off the paper.

Radioactivity

Radioactivity was generally determined on a Packard scintillation counter using 1 ml. of aqueous solution plus 10 ml. counting solution,[11] with an efficiency of 70 per cent for ¹⁴C. Occasionally 1 ml. Soluene (Packard Instruments) was substituted for water. Quench correction with an external standard was used when necessary. Paper strips were counted in the presence of 20 ml. counting solution. Background in this system was about 45 cpm. One mμmole riboflavin incorporated per ml. of red cells represented between 750 and 900 cpm. All samples were counted in duplicate for 50 or 100 minutes. Hemolysates were counted with a Nuclear-Chicago gas flow counter with an efficiency of 9.5 per cent. Background in this system was approximately 17 cpm, and the incorporation of 1 mμmole riboflavin per ml. of red cells was represented by 120 cpm. All samples were counted in duplicate to a total of 2560 counts.

Glutathione Reductase Activity

The enzyme assay was performed as described previously.[4]

Reagents

[2-¹⁴C]-D-Riboflavin, 34.1 mCi./mmole, was purchased from Nuclear-Chicago. The purity was verified by descending paper chromatography in collidine saturated with water,[12] and by paper electrophoresis. In both cases, the radioactivity was found in a single spot with the same mobility as authentic riboflavin. No radioactivity moved with FMN or FAD. GSSG, NADPH, FAD, FMN and nonradioactive riboflavin were obtained from Sigma Chemical Co. FAD was shown to be free of FMN or riboflavin by fluorescence measurements and by paper electrophoresis. All other chemicals were reagent grade, and all solutions were made with distilled water.

RESULTS

To determine whether riboflavin freely diffuses into RBC from the medium, 0.5 ml. RBC was added to a test tube containing 0.5 ml. plasma or 0.5 ml. phosphate-saline-glucose. Radioactive riboflavin was added to each tube to give a final concentration of 1.5 μM in the medium. The tubes were kept in an ice bath for 15 minutes with occasional shaking, and were then centrifuged. A portion of supernatant and of RBC was then counted. As shown in Table 1, riboflavin does not freely diffuse into red cells. The small amount of
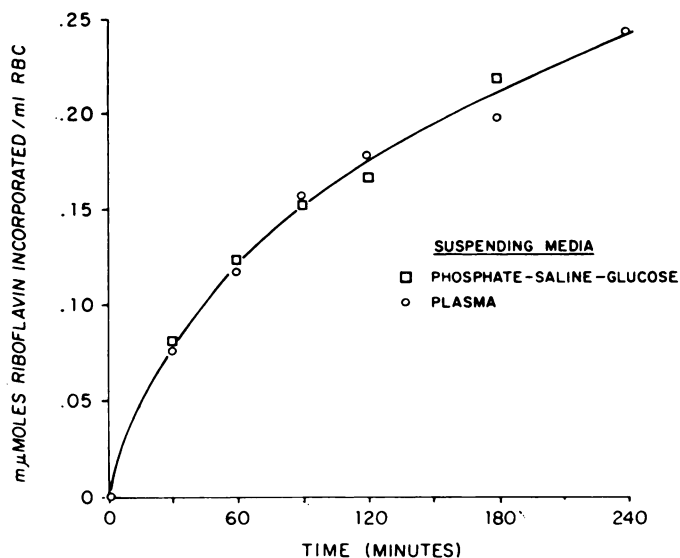
Fig. 1.—Incorporation of [^{14}C]-riboflavin into RBC suspended in plasma (○) or phosphate-saline-glucose (□). The concentration of riboflavin in the media was 1 μM. At the times indicated, the cells were washed twice, hemolyzed and then radioactivity and hemoglobin were determined.

radioactivity associated with the RBC was probably largely due to trapped supernatant.

Figure 1 compares the rates of entrance to radioactive riboflavin into RBC when the RBC are incubated either with plasma or with phosphate-saline-glucose at 37°C for up to 4 hours. Since riboflavin enters the red cells as effectively with either suspending medium, plasma apparently does not contain factor(s) necessary for riboflavin transport into red cells. In five experiments with 0.9–1 μM riboflavin in the plasma, the incorporation rates were between 0.14 and 0.25 mμmoles/ml. RBC/90 minutes.

To determine the relative contribution of leukocytes and platelets to the incorporation of riboflavin into blood cells, blood was collected in 5 per cent polyvinylpyrrolidone–3.2 per cent sodium citrate, and the red cells were permitted to sediment at 37°C for 30 minutes. The leukocyte–platelet-poor red cell fraction and the leukocyte–platelet-rich supernatant fraction were each washed two times in 0.9 per cent saline and the rate of riboflavin incorporation into the cells measured for 3 hours. The incorporation of riboflavin was 30 times as active, on a per cell basis, in white cells than in red cells. On a per protein basis, incorporation was 10 times as active in the leukocyte–platelet-rich preparation than in red cells. Since the washed packed red cells used in these studies contained only approximately 1700 white cells per cubic mm., giving a 1:6000 white cell:red cell ratio, it can be estimated that only approximately ½ per cent of riboflavin incorporation was due to leukocyte activity.

Figure 2 shows the separation of riboflavin, FMN and FAD obtained by paper electrophoresis, using authentic compounds. Red cell extracts treated
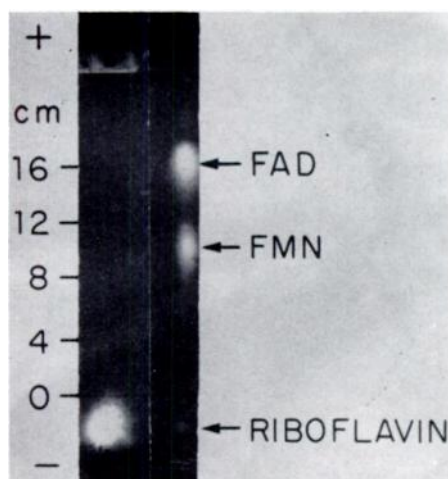
Fig. 2.—Pattern of electrophoresis of riboflavin, FMN and FAD. Details of the procedure are in the Methods section.

Table 2.—Recovery of Fluorescence and Radioactivity During Isolation of Flavins from RBC

	Per Cent of Initial	
	Fluorescence	Radioactivity
TCA supernatant	75	76
TCA supernatant after ether extraction	75	80
Solution after lyophilization	57	59
Elution off paper	36	35

Equal volumes of RBC and plasma were incubated with 1 μM [^{14}C]-riboflavin for 90 minutes. The flavins were then separated electrophoretically as described in the Methods Section. 100 per cent radioactivity is based on the total radioactivity in the washed RBC. 100 per cent fluorescence is based on the amount of FAD added to the hemolysate.

as described in the Materials and Methods section gave equally good resolution. The recoveries of fluorescence, which had been added as carrier FAD, and of radioactivity, from the FMN, FAD and riboflavin spots after paper electrophoresis were used to calculate the amount of radioactivity incorporated into each fraction at the end of the incubation. It was assumed that all fluorescence in the three spots originated as FAD because of the large amount of carrier FAD added. Therefore, the values for radioactivity incorporated into each flavin could be corrected for FAD breakdown during the isolation procedure. The total fluorescence in the FMN spot was about 20 per cent of that in the FAD spot (after conversion of the FAD spot to FMN) and virtually no fluorescence was found in the riboflavin spot. The total yield of fluorescent material was used to correct for losses occurring during the procedure for separating the flavins. The fluorescence yield and the radioactivity yield were parallel throughout the procedure (Table 2).

Incorporation of radioactivity into red cell FAD, FMN and riboflavin was measured after 30, 60 and 90 minutes of incubation with [^{14}C]-riboflavin (Fig. 3). The incorporation into FAD was linear with time for 90 minutes, although total incorporation fell off somewhat after 60 minutes. The total radioactivity recovered in the three flavins is seen to agree closely with the amount of
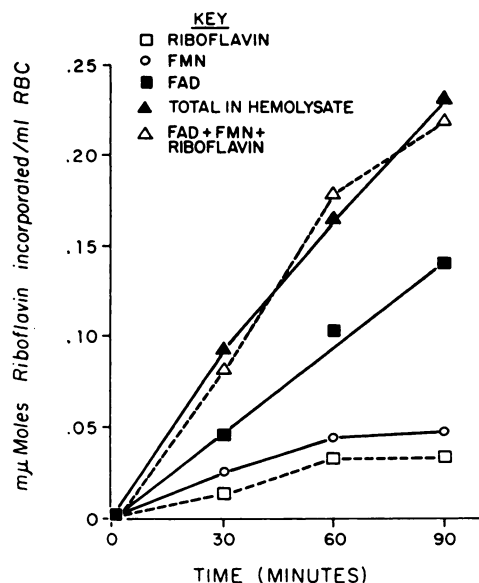
Fig. 3.—Incorporation of [^{14}C]-riboflavin into RBC riboflavin (□), FMN (○) and FAD (■) at various times. The incubation and isolation procedures are described in the Methods section. The concentration of riboflavin in the plasma was 0.9 μM. The total radioactivity incorporated into RBC is shown here both as the sum of recovered riboflavin + FMN + FAD (△), and as the total radioactivity in the hemolysates (▲).

radioactivity initially present in the hemolysate, thus showing that little, if any, radioactivity could have been incorporated into an unidentified compound. When a portion of the incubation mixture with radioactive riboflavin was kept on ice for 90 minutes with occasional shaking, no incorporation of radioactivity into red blood cells occurred.

To study the effect of concentration of riboflavin on its incorporation into red blood cell flavins, red blood cells were incubated for 90 minutes with 0.45, 0.9 or 2.7 μM radioactive riboflavin. Total incorporation was proportional to the concentration of riboflavin at the two lower concentrations, but with 2.7 μM robiflavin, the incorporation rate was no longer proportional to riboflavin concentration (Fig. 4).

After red cells were incubated with radioactive riboflavin, a small net increase in red blood cell flavin concentration could be demonstrated fluorometrically, but the results were not clearly outside the range of error of the method used. However, an increase of the activity of glutathione reductase, a flavin enzyme, could be demonstrated. Freshly drawn blood was incubated with nonradioactive riboflavin, and at various times, samples were removed and glutathione reductase activity was measured in the red blood cell hemolysates. The enzyme activity in hemolysates is known to be stimulated by preincubation with FAD.[5] After appropriate incubation of red cells with riboflavin, their glutathione reductase activity, without additional FAD in the assay cuvette, was raised to the same level that the zero-time sample achieved with FAD in the assay cuvette (Fig. 5). This suggests that FAD can be synthesized in red cells incubated with riboflavin.

DISCUSSION

Although mature erythrocytes do not synthesize protein,[13] they are capable of synthesizing a variety of small molecules. Red cells synthesize glutathione
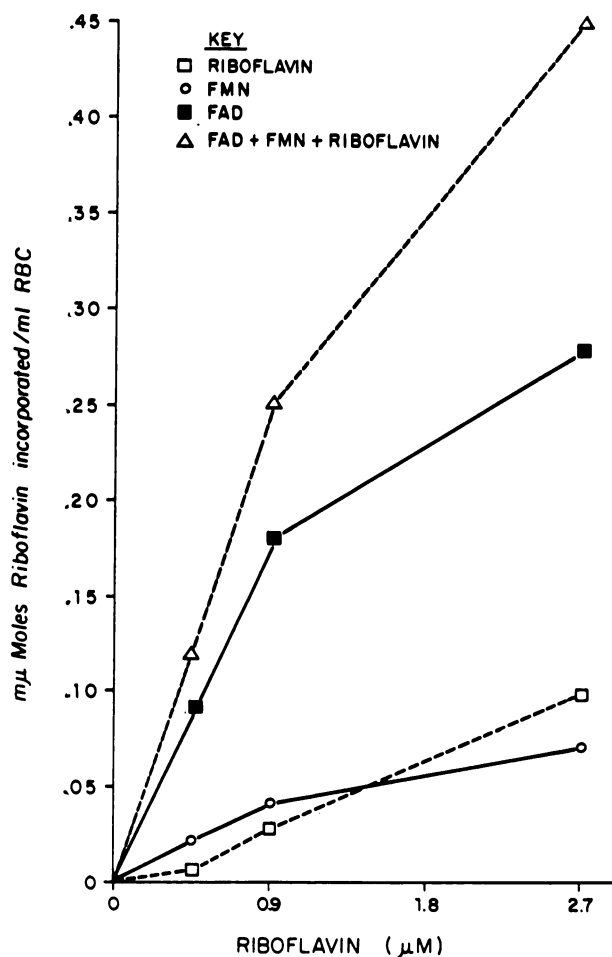
Fig. 4.—Incorporation of [^{14}C]-riboflavin into RBC riboflavin (□), FMN (○), and FAD (■) at various concentrations of plasma riboflavin. Incubations were for 90 minutes. Details of the procedure are described in the Methods section. The total incorporation is shown as the sum of recovered RBC riboflavin + FMN + FAD (△).

from the component amino acids,[14] they can synthesize NAD and NADP from nicotinic acid;[15-17] and can synthesize adenine nucleotides if they are provided with adenine.[16] The synthesis of flavin coenzymes has received only scant attention. The present studies clearly show that erythrocytes have the capacity to incorporate riboflavin rapidly into FMN and FAD.

Klein and Kohn[18] presented evidence of net synthesis of FAD from riboflavin by blood cells, but the authors made no attempt to differentiate the synthetic abilities of platelets, white cells or erythrocytes. To measure FAD concentrations, the authors utilized D-amino acid oxidase, whose activity had been shown to be proportional to the concentration of FAD.

More recently, Pispa and Huttenen[19] showed that reticulocytes and erythrocytes from rats incorporate radioactive riboflavin into FAD. Reticulocytes were found to be more effective than were erythrocytes.

From the data presented here, we can approximate the turnover of FAD in human red cells. Our studies with intact human red blood cells show that approximately 0.1 mμmole riboflavin is incorporated into FAD/ml. RBC/hour
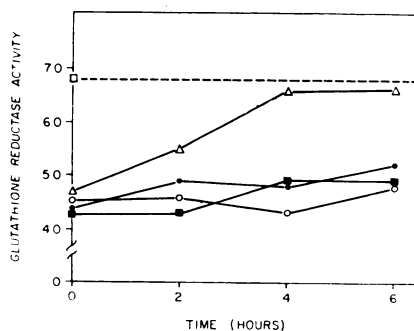
Fig. 5.—Glutathione reductase activity in RBC incubated with various concentrations of riboflavin. Whole blood to which riboflavin had been added was incubated at 37° C under 95 per cent air, 5 per cent carbon dioxide with constant shaking. At the times indicated, samples were taken for measurement of RBC glutathione reductase activity. FAD was omitted from the assay cuvettes, except in the case of the control (□) which was assayed with FAD. Enzyme activity is expressed as μmoles/minute/gm. hemoglobin. Concentrations of added riboflavin in plasma were none (■), 0.033 μM (○), 0.17 μM (●), and 1.7 μM (△).

at 37°C, starting with 0.9 μM riboflavin in the medium (Fig. 3). The in vivo concentration of riboflavin in normal human plasma is about 0.02 μM,[9] although the levels may increase up to 0.3 μM several hours after administration of 5 mg. riboflavin.[20] Since our data indicate that incorporation of riboflavin into FAD in vitro is proportional to plasma riboflavin concentrations below 0.9 μM (Fig. 4), then at 0.02 μM riboflavin, the incorporation rate of riboflavin into FAD would be approximately 0.0022 mμmole/ml. RBC/hour. If the normal FAD concentration is taken as 0.5 mμmole/ml. RBC,[4] then the $t_{1/2}$ for FAD turnover in vivo is calculated to be about 6 days, assuming the in vitro studies are an adequate model for the in vivo situation, and that a single pool of FAD exists. It is recognized that there is, in all probability, not such a single mixing pool: FAD exists in various combinations with proteins, and those forms of FAD may well differ in the rate at which they are turned over. Nonetheless, our average estimate does give a general idea of the rate at which FAD may be turned over in vivo in man. The finding that the rate of FAD synthesis is directly proportional to the riboflavin content of the medium would lead to the prediction that raising plasma riboflavin levels should increase the steady-state RBC FAD concentration if the turnover time remains the same. This, indeed seems to be the case. Riboflavin administration raised red cell FAD levels in man[5] and has provided us with a useful tool for the manipulation of red cell metabolism in vivo.

ACKNOWLEDGMENT

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