Phospholipid Biosynthesis in the Membranes of Immature and Mature Red Blood Cells†

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ABSTRACT: Reticulocytes were isolated from rabbits in which reticulocytosis was induced by phenylhydrazine injection. Reticulocytes, as well as erythrocytes from untreated rabbits, were lysed and the resulting membranes were washed and purified by isopycnic centrifugation. The purified membranes from reticulocytes were found to contain enzymes that catalyze the synthesis of phosphatidylcholine from CDP-choline and 1,2-diglyceride, and the synthesis of phosphatidylinositol from CDP-diglyceride and inositol. In contrast, erythrocyte membranes are devoid of these enzymes. Thus, during the maturation process whereby reticulocytes are converted to erythrocytes *de novo* synthesis of these two lipids ceases.

It is well established that in prokaryotic cells the site of synthesis of the membrane phospholipids is the cell membrane (Patterson and Lennarz, 1971; White *et al.*, 1971; Bell *et al.*, 1971). In the more complex eukaryotic cells, the endoplasmic reticulum appears to be the major site of synthesis of the membrane phospholipids (Wilgram and Kennedy, 1963), although apparently the plasma membrane does have the capacity to synthesize at least one class of phospholipids (Victoria *et al.*, 1971).

The erythrocyte and its precursor, the reticulocyte, represent cell types that are intermediate in complexity, at least in terms of the presence of subcellular organelles, between eukaryotic and prokaryotic cells. For this reason it was of interest to determine whether or not the plasma membrane of the reticulocyte, and its mature form, the erythrocyte, contained the enzymes necessary for synthesis of the phospholipids of the membrane.

There is a wealth of information regarding the morphological changes that occur during the maturation of reticulocytes to erythrocytes. Both cell types lack a nucleus, and the mature erythrocyte is devoid of cytoplasmic organelles (Simpson and Kling, 1968). The situation concerning the presence of cytoplasmic organelles that might be involved in lipid synthesis in reticulocytes is less clear, and appears to be dependent on the given species. Clearly rabbit reticulocytes contain ribosomes, which appear to exist in two major pools, one of which is cytoplasmic and appears to function in the biosynthesis of hemoglobulin, and another which is associated with the plasma membrane and may be responsible for synthesis of membrane proteins (Burka and Bulova, 1971). Endoplasmic reticulum and the Golgi apparatus have not been reported in rabbit reticulocytes, and are clearly absent in canine reticulocytes (Simpson and Kling, 1968). In contrast, both of those intracellular components, or remnants thereof, have been detected in mouse reticulocytes (Bessis and Breton-Gorius, 1964; Orlic *et al.*, 1965).

Earlier studies on lipid biosynthesis have been performed using intact rabbit reticulocytes and water-soluble precursors. Studies using either [^{14}C]acetate (O'Donnell *et al.*, 1958) or $^{32}P_i$ (Raderecht *et al.*, 1962) provided little information regarding the question of *de novo* synthesis. More recent studies by Solviter and Tanaka (1967) using [^{14}C]glycerol, as well as labeled choline and inositol, strongly suggest that rabbit reticulocytes do have the capacity for *de novo* synthesis of lipids, but, again, these studies were performed with intact cells. The absence of published information regarding *de novo* synthesis in cell-free extracts of rabbit reticulocytes prompted us to investigate two enzymatic reactions that are components of two distinct pathways for phospholipid synthesis in mammalian cells, namely, CDP-choline:1,2-diglyceride phosphocholinetransferase (Weiss *et al.*, 1958), which catalyzes the reaction shown in eq a and CDP-diglyceride:inositol phosphatidyltransferase (Paulus and Kennedy, 1960), which catalyzes the reaction shown in eq b.

$$\text{CDP-choline} + \text{1,2-diglyceride} \longrightarrow \text{phosphatidylcholine} + \text{CMP} \quad \text{(a)}$$

$$\text{CDP-diglyceride} + \text{inositol} \longrightarrow \text{phosphatidylinositol} + \text{CMP} \quad \text{(b)}$$

Materials and Methods

Radioactive Compounds and Lipids. Radioactive compounds were obtained from New England Nuclear Corp. Lipid substrates and standards were purchased from Serdary Research Laboratories, London, Ontario, Can. The fatty acyl groups of the 1,2-diglyceride and the CDP-diglyceride were palmitoyl moieties.

Reticulocyte and Erythrocyte Membranes. Blood was obtained from New Zealand white rabbits. Erythrocyte membranes were prepared as previously described (Burka *et al.*, 1967). Reticulocytosis was induced by a daily, subcutaneous injection of 1 ml of 2.5% phenylhydrazine in 0.1 M sodium phosphate buffer, pH 7.4 (10–15 mg/kg body weight per day), for 4 days. Forty-eight hours after the final injection blood was obtained *via* cardiac puncture using a heparinized syringe. Reticulocyte membranes were prepared in the same manner as the erythrocyte membranes (Burka *et al.*, 1967). Reticulocytes were quantitated by microscopy after staining with a solution containing 0.5 g of new Methylene Blue and 1.6 g of potassium oxalate in 100 ml of water. Reticulocytes in untreated rabbits

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represented 1–2% of the red cell population, whereas in phenylhydrazine-treated rabbits 90–95% of the red cells were reticulocytes.

Erythrocyte and reticulocyte membranes were purified by discontinuous sucrose gradient centrifugation. Erythrocyte membranes from one rabbit were suspended in 30 ml of 0.001 M Tris-HCl–0.0015 M $MgCl_2$ (pH 7.4) and the suspension was divided into six equal portions. Each 5-ml portion was layered over a stepwise gradient containing 10 ml each of 0.32, 0.80, and 1.2 M sucrose containing 0.0015 M $MgCl_2$. Reticulocyte membranes from one rabbit were suspended in 30 ml of 0.32 M sucrose containing 0.0015 M $MgCl_2$, and were also divided into 5-ml portions. Each 5-ml portion of the suspension was layered onto a gradient containing 8 ml each of 0.8, 1.2, 1.5, and 1.8 M sucrose–$MgCl_2$. Gradients were then centrifuged at 0° in an SW-27 rotor for 60 min at 25,000 rpm in a Beckman L3-40 ultracentrifuge. Erythrocyte membranes collected at the 0.8–1.2 M sucrose interface as a white diffuse band. Reticulocyte membranes collected at the 1.2–1.5 sucrose interface, and were tan and coalescent in appearance. No leucocytes were evident in either fraction when they were examined in a Spencer hemocytometer by light microscopy.

Membranes were isolated from the sucrose band by dilution with two volumes of the Tris–$MgCl_2$ buffer followed by centrifugation. Pellets were suspended in Tris–$MgCl_2$ buffer at a final protein concentration of 2.5–5.0 mg/ml and stored at −20°.

In some preparations of reticulocyte membranes, in addition to the usual band of reticulocytes (fraction II) at the 1.2–1.5 M sucrose interface, a white diffuse band (fraction I) was observed at the 0.8–1.2 M sucrose interface (see Results).

Electron Microscopy. Samples from erythrocyte and reticulocyte membrane preparations were fixed in 4.5% glutaraldehyde buffered with 0.1 M sodium cacodylate (pH 7.2). After fixation, the membrane preparations were postfixed in 1% osmium tetroxide, dehydrated, and embedded in Epon. Thin sections were cut on a Reichert ultramicrotome, stained with lead citrate and uranyl acetate, and examined in a Phillips 200 electron microscope (Figure 1). The erythrocyte membranes (Figure 1A) appeared characteristic of mammalian red cell ghosts (Marchesi *et al.*, 1969); the reticulocyte membranes (Figure 1B) were indistinguishable from them at several magnifications.

Enzyme Assays. CDP-choline:1,2-diglyceride phosphocholine transferase was assayed in a final volume of 100 μl containing Tris-HCl (pH 8.0), 50 mM; 1,2-diglyceride, 3 mM, suspended in sodium taurocholate, final concentration 0.2%, $MnCl_2$, 10 mM; CDP-[*methyl*-^{14}C]choline (specific activity 2500 cpm/nmol), 0.135 mM; and membranes (up to 320 μg of protein). The mixture was incubated in a shaking water bath for 30 min at 37° after which time the reaction was stopped by addition of 2.0 ml of $CHCl_3$–CH_3OH (2:1). After adding 0.4 ml of 0.9% saline (pH 3) and mixing thoroughly, the resulting two phases were clarified by centrifugation. The upper phase was removed and the lower phase was washed twice with $CHCl_3$–CH_3OH–0.9% saline (3:48:47) each time removing the resulting water phase. The lower phase was taken to dryness in a scintillation vial, and after adding 10 ml of toluene–Triton scintillation fluid, chloroform–methanol-extractable radioactivity was measured in a Packard Tri-Carb scintillation counter.

CDP-diglyceride:inositol phosphatidyltransferase was assayed in a final volume of 100 μl containing Tris-HCl (pH 8.0), 25 mM; CDP-diglyceride, 0.75 mM, suspended in sodium taurocholate, final concentration 0.15%; $MnCl_2$ 3.0 mM; [2-^{3}H]inositol (specific activity 600 or 1680 cpm per nmol), 4.5 mM; and membranes (up to 420 μg of protein). Incubation and assay conditions were identical with those described above for phosphatidylcholine synthesis. In both assays, the lipid substrate was suspended in sodium taurocholate (7.5 mg/ml) and dispersed by a Bronwill Biosonik sonicator.

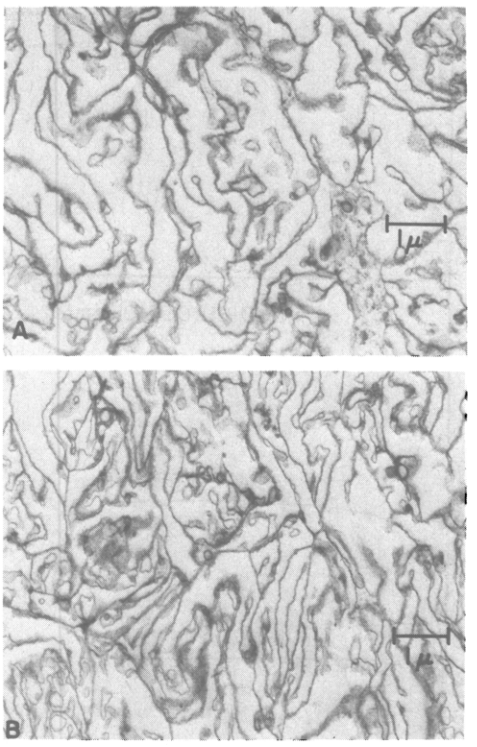

FIGURE 1: Electron micrographs of (A) erythrocyte membranes, and (B) reticulocyte membranes. Magnification 15,000 ×.

Results

CDP-choline:1,2-Diglyceride Phosphocholine Transferase. Substrate Requirements and Product Characterization. Maximal incorporation of [^{14}C]phosphorylcholine from CDP-choline into phosphatidylcholine was dependent on addition of exogenous 1,2-diglyceride, which stimulated the incorporation nearly 3-fold. In contrast to the results of Weiss *et al.* (1960) using rat liver preparations, saturation was achieved with diglyceride at a final concentration of 3 mM (Figure 2A). Although the apparent K_m for diglyceride was calculated at 7.8×10^{-4} M, this value has only operational significance because of the insolubility of the lipid. The substrate saturation curve for CDP-choline is shown in Figure 2B. The K_m for CDP-choline is 2.5×10^{-5} M. The products of both diglyceride-dependent and -independent synthesis of radioactive lipid were chromatographed with authentic phosphatidylcholine in the following thin-layer chromatographic systems: silica gel G, $CHCl_3$–CH_3OH–H_2O (65:25:4); silica gel H, $CHCl_3$–CH_3OH–H_2O (95:35:5); and silica gel H (Na_2CO_3), $CHCl_3$–CH_3OH–CH_3CO_2H–H_2O (25:15:4:2). In each system, at least 90% of the incorporated radioactivity was associated with authentic phosphatidylcholine.

Delipidation of the membrane preparation with acetone (−20°) abolished the diglyceride-independent synthesis of phosphatidylcholine. Synthesis could be restored by addition of exogenous diglyceride but the level of restored activity was low. Addition of CMP (final concentration, 2 mM) inhibited

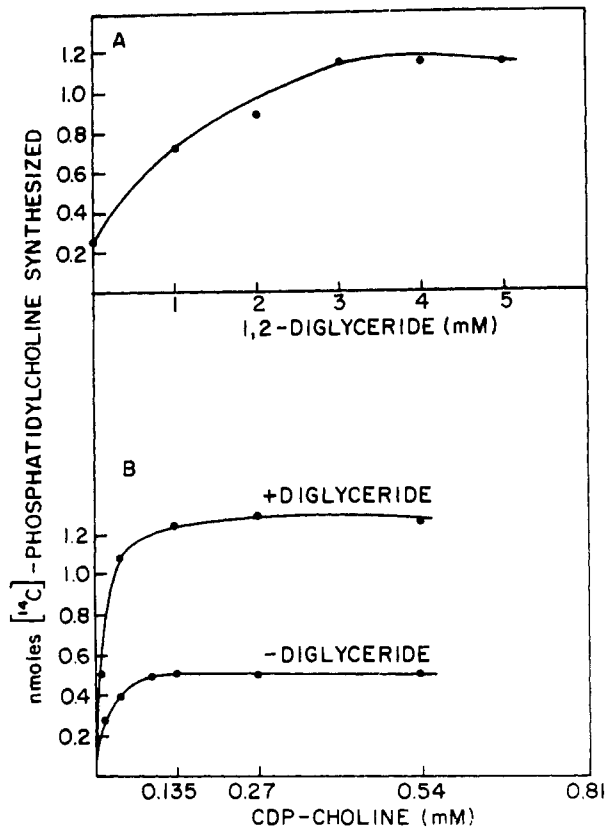

FIGURE 2: (A) The dependence of phosphatidylcholine synthesis on 1,2-diglyceride concentration. In both parts A and B the incubation conditions were as described in Methods except that 0.312 mg of membrane was used. (B) The dependence of phosphatidylcholine synthesis on CDP-choline concentration in the presence and absence of 1,2-diglyceride.

both the diglyceride-independent and diglyceride-stimulated synthesis of phosphatidylcholine by 60–70%. The dependence of the reaction of diglyceride and the inhibitory effect of CMP are both consistent with the direct participation of diglyceride and CDP-choline in the formation of phosphatidylcholine according to the reaction shown in eq a.

EFFECTS OF pH AND CATIONS. Diglyceride-dependent and -independent synthesis of phosphatidylcholine were optimal at pH 8.0 While Mn^{2+} produced a 12-fold stimulation of diglyceride-dependent phosphatidylcholine formation (Figure 3), Mg^{2+} was virtually without effect. The optimum Mn^{2+} concentration was about 10 mM.

EFFECT OF TIME AND PROTEIN CONCENTRATION. The incorporation of [^{14}C]phosphorylcholine into phosphatidylcholine was linear with protein up to 165 μg at 37° in the presence and absence of diglyceride (Figure 4A). Both reactions were also linear with time up to 60 min (Figure 4B).

CDP-diglyceride:Inositol Phosphatidyltransferase. Substrate Requirements and Product Characterization. In most membrane preparations the incorporation of [^{3}H]inositol into lipid was not absolutely dependent on exogenous liponucleotide, but the addition of CDP-diglyceride-stimulated inositol incorporation 3-fold. The saturation curve for CDP-diglyceride is shown in Figure 5A. The apparent K_m is 9.3×10^{-5} M, but due to the relative water insolubility of CDP-diglyceride this calculation represents an approximation. From the saturation curves for inositol shown in Figure 5B an apparent K_m of 5.7×10^{-4} M was estimated. Albumin (10 mg/ml) did not stimulate incorporation of [^{3}H]inositol as in pancreas (Prottey and Hawthrone, 1967) and brain extracts (Benjamin and Agranoff, 1969). In fact, albumin actually reduced CDP-diglyceride-independent activity by 60% and CDP-diglyceride-stimulated activity by 20%. The addition, individually of phosphatidylglycerol, phosphatidylethanolamine, or phosphatidylcholine (all at 1 mM) did not stimulate [^{3}H]lipid synthesis. In fact, in the absence of exogenous CDP-diglyceride they inhibited incorporation of [^{3}H]inositol by approximately 25%.

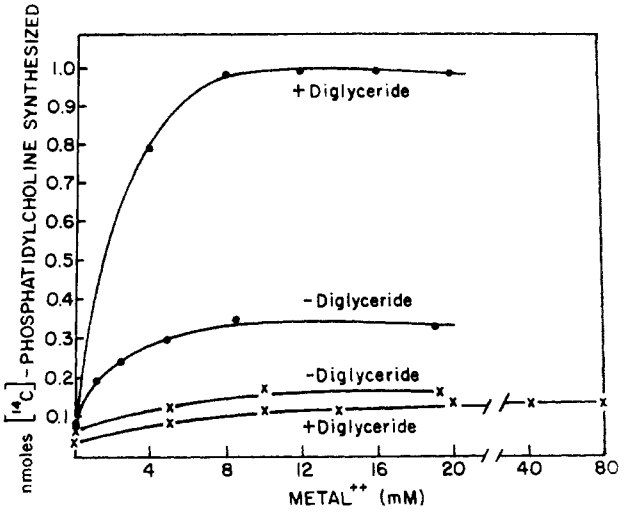

FIGURE 3: Effect of metal ions on phosphatidylcholine formation. Mn^{2+} (●) and Mg^{2+} (X). Incubation conditions were as in Figure 2 except that the amounts of protein used with and without 1,2-diglyceride were 0.055 and 0.095 mg, respectively.

The products of both the CDP-diglyceride-independent and

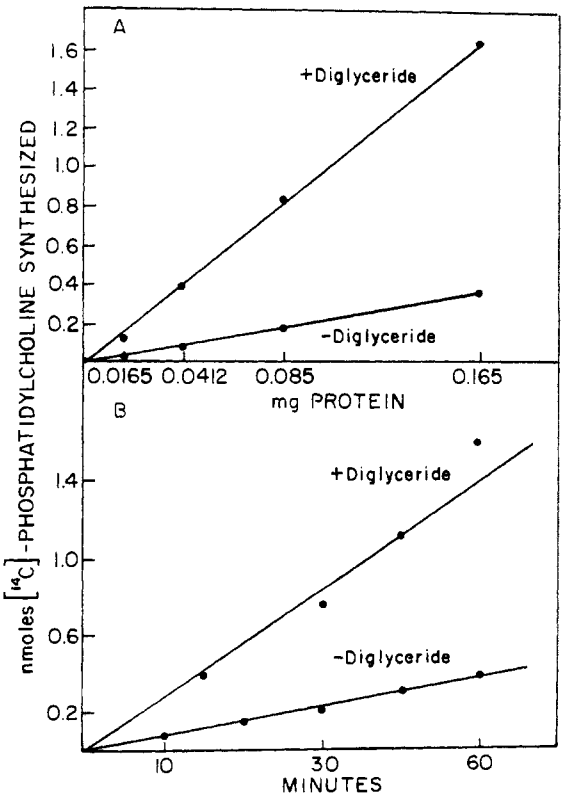

FIGURE 4: (A) Effect of protein concentration on phosphatidylcholine formation. (B) Formation of phosphatidylcholine as a function of time. In both parts A and B the assay conditions were as described in Figure 2.

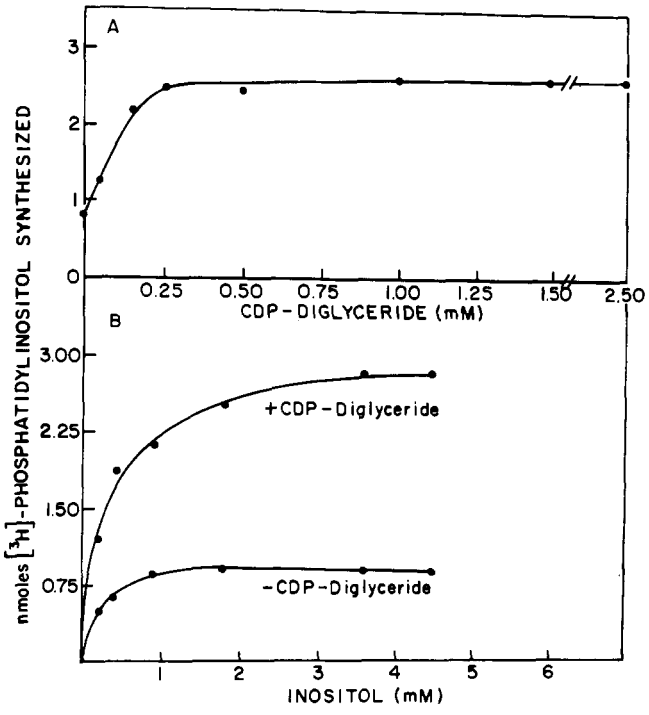

FIGURE 5: (A) The dependence of phosphatidylinositol synthesis on CDP-diglyceride concentration. The incubation conditions were as described in Methods, except that 0.312 mg of protein was added. (B) Dependence of phosphatidylinositol synthesis on inositol concentration in the presence and absence of CDP-diglyceride. Incubations conditions were the same as in part A.

CDP-diglyceride-stimulated reactions were chromatographed with authentic phosphatidylinositol in the following two thin-layer chromatographic systems using silica gel H (Na_2CO_3): $CHCl_3$–CH_3OH–CH_3CO_2H–H_2O (25:15:4:2) and $CHCl_3$–CH_3OH–H_2O (65:25:4). In both systems, 95% of the incorporated radioactivity was associated with authentic phosphatidylinositol. Similar results were obtained with SG-81 paper using $CHCl_3$–CH_3OH–H_2O (65:24:4) as the developing solvent.

EFFECTS OF pH AND CATIONS. The pH optimum of both CDP-diglyceride-independent and CDP-diglyceride-stimulated activity was 8.0. Whereas Mn^{2+} stimulated both activities, Mg^{2+}, even at relatively high concentration, had minimal stimulatory effect (Figure 6).

EFFECTS OF TIME AND PROTEIN CONTENT. Both CDP-diglyceride-independent and CDP-diglyceride-stimulated activities were linear with increasing amounts of protein up to 412 μg (Figure 7A), and linear with time up to 60 min (Figure 7B).

SEPARATION OF CDP-DIGLYCERIDE-DEPENDENT AND -INDEPENDENT PHOSPHATIDYLINOSITOL SYNTHETASE ACTIVITY. As previously mentioned (*cf.* Methods), in some reticulocyte preparations a second, minor band was observed on the sucrose gradients. The less dense band (fraction I) collected at the 0.8–1.2 M sucrose interface (where erythrocyte membranes collected on control gradients), whereas the more dense band (fraction II) of reticulocyte membranes collected at the 1.2–1.5 M sucrose interface. It is unlikely that fraction I was due to erythrocyte membrane contamination, as it contained considerable activity for phosphatidylinositol synthesis, whereas control erythrocyte membranes contain little if any activity.

Upon further analysis it could be demonstrated that when fractions I and II were resolved, fraction II (reticulocyte membranes) had an absolute dependence on CDP-diglyceride for phosphatidylinositol synthesis (Figure 8A). In contrast fraction I showed considerable activity for phosphatidylinositol synthesis in the absence of CDP-diglyceride, although synthesis was stimulated by the liponucleotide, especially at early time points (Figure 8B). When fraction I could not be resolved from the reticulocyte membranes and only one band (fraction II) was present, both the CDP-diglyceride-dependent and -independent activities appeared to be present. The sum of the CDP-diglyceride-independent activity in fraction I and the CDP-diglyceride-dependent activity in fraction II is shown in Figure 8C. The value obtained is comparable to the

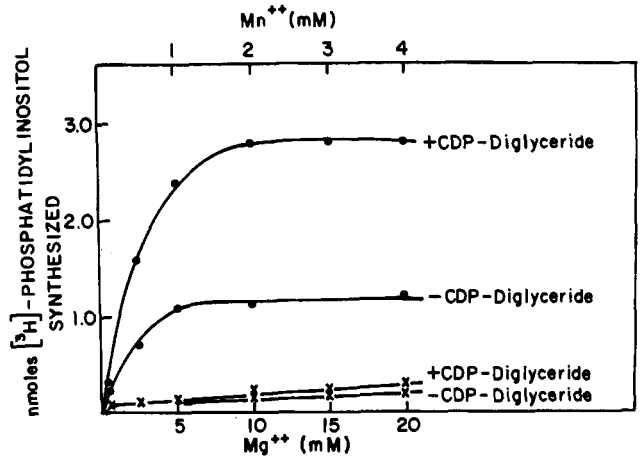

FIGURE 6: Effect of metal ions on phosphatidylinositol formation. Incubation conditions were as in Figure 5 except that 0.237 mg of protein was used: Mn^{2+}, ●—●; Mg^{2+}, ×—×.

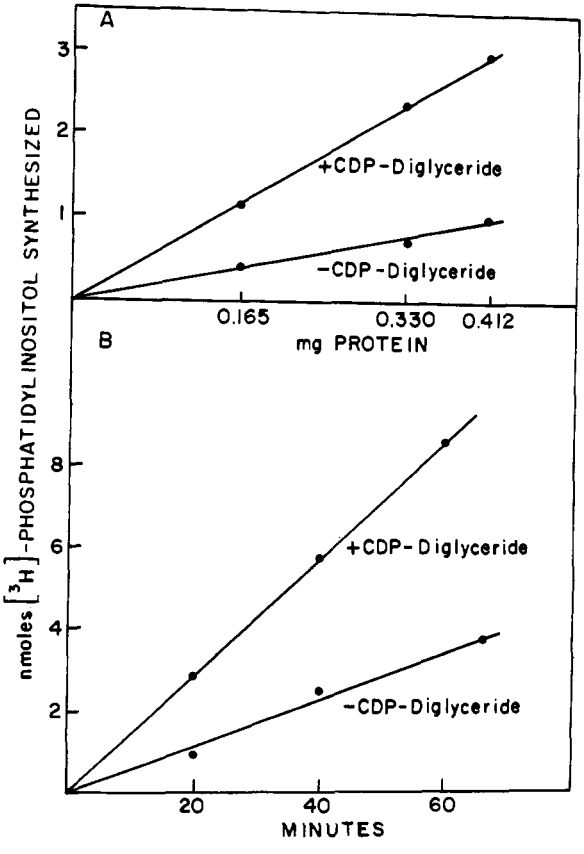

FIGURE 7: (A) Effect of protein concentration on phosphatidylinositol formation. (B) Phosphatidylinositol formation as a function of time. In both parts A and B conditions were as described in Methods except that in part B 0.412 mg of protein was used.

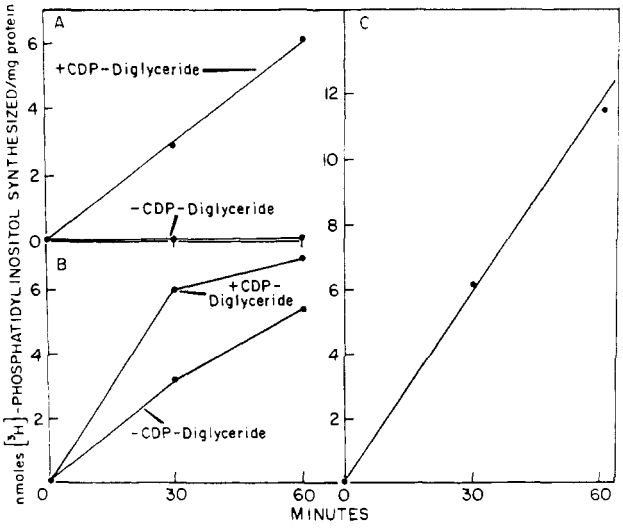

FIGURE 8: (A) Time course of phosphatidylinositol synthesis in purified reticulocyte membranes (fraction II) and (B) in fraction I. Incubation conditions in A and B were as described in Methods. (C) Calculated sum of synthetic activity in fraction I (without CDP-diglyceride) plus fraction II (with CDP-diglyceride).

activity found in most preparations in which fractions I and II are not resolved (*i.e.*, 12–17 nmol of phosphatidylinositol synthesis/mg per hr). Thus, it is evident that the CDP-diglyceride-dependent and -independent activities for phosphatidylinositol synthesis result from the action of enzymes in different membrane fractions, and that under certain conditions the membrane-associated enzyme that catalyzes the synthesis of phosphatidylinositol in the absence of CDP-diglyceride can be separated from the reticulocyte membranes. However, it remains to be established why in some preparations this component can be separated from the reticulocyte membranes, whereas in others it cannot.

COMPARISON OF THE ACTIVITIES OF RETICULOCYTE AND ERYTHROCYTE MEMBRANES IN SYNTHESIS OF PHOSPHATIDYLCHOLINE AND PHOSPHATIDYLINOSITOL. In Table I the activities of erythrocyte and reticulocyte membranes in the synthesis of phosphatidylcholine and phosphatidylinositol under the optimal conditions established for reticulocyte membranes are compared. The specific activity of reticulocyte membranes in synthesis of the two lipids is at least 15- to 20-fold greater than that of erythrocyte membranes.

Discussion

In mammalian cells the overall process of erythropoiesis is, in general terms, believed to proceed in the following manner. (1) The erythroblast, originating in the bone marrow, extrudes its nucleus in the process of being converted to a reticulocyte. (2) The reticulocyte, during a period of a few days extrudes its remaining intracellular organelles, and is converted to the mature erythrocyte. (3) The erythrocyte, laden with hemoglobin, survives in a functional form within the blood stream for several months.

TABLE I: Comparative Activities in Mature Erythrocyte and Reticulocyte Membranes.

	Phosphatidylcholine Synthetase[a]	Phosphatidylinositol Synthetase[a]
Erythrocyte membranes	<0.20	0.2–0.9
Reticulocyte membranes	10.3–15.7	12.2–17.8

[a] Activity expressed in nanomoles of lipid formed per milligram of protein per hour. The range shown was observed in a number of experiments with different membrane preparations. Assay conditions were as described in the text.

A number of studies, usually performed on intact cells, have revealed that the reticulocyte is capable of incorporating radioactive precursors into lipid. However in most cases it is not clear if such incorporation represents *de novo* synthesis or exchange reactions. In order to study more fully the question of *de novo* synthesis of phospholipids in reticulocyte and erythrocyte membranes we have examined two reactions that are representative of separate, major pathways for phospholipid synthesis in mammalian cells; namely, the synthesis of phosphatidylcholine from CDP-choline and 1,2-diglyceride, and the synthesis of phosphatidylinositol from CDP-diglyceride and inositol. Ideally such a study should be performed utilizing reticulocytes isolated from normal blood, but this was precluded because of the extremely low content of reticulocytes in normal blood (2% of the total red blood cells).

Consequently, reticulocytosis was induced by phenylhydrazine treatment, which shortens the life span of erythrocytes and, concomitantly, induces massive production of reticulocytes. Reticulocytes isolated in this manner, as well as erythrocytes isolated from untreated rabbits, were lysed and the washed plasma membranes were further purified by isopycnic centrifugation. The plasma membrane preparation from reticulocytes and erythrocytes were very similar in appearance. However, it should be emphasized that, although the reticulocyte during its 1–3-day maturation period loses virtually all of its intracellular membranous organelles, the *average* population would be expected to contain some intracellular material. Consequently, if these membranous components have the same buoyant density as the plasma membrane they could be contaminants in the final purified preparation of reticulocyte plasma membranes.

With these limitations in mind, the following conclusions can be drawn. Reticulocyte membranes are active in the synthesis of phosphatidylcholine and phosphatidylinositol. Maximal synthesis of phosphatidylcholine requires CDP-choline and exogenous 1,2-diglyceride. The apparent K_m is 2.5×10^{-5} M for CDP-choline and approximately 7.8×10^{-3} M for 1,2-diglyceride. The reaction requires Mn^{2+} ion and the pH optimum is 8.0. Maximal synthesis of phosphatidylinositol requires exogenous CDP-diglyceride and inositol. The apparent K_m is approximately 9.3×10^{-5} M for CDP-diglyceride and 5.7×11^{-4} M for inositol. Optimal activity is observed at pH 8.0 in the presence of Mn^{2+} ion. In most membrane preparations significant synthesis of [^{3}H]phosphatidylinositol from [^{3}H]inositol was observed in the absence of exogenous CDP-diglyceride. This reaction was found to have the same pH and metal ion requirements as the CDP-diglyceride-stimulated reaction. In the latter phases of this study several reticulocyte membrane preparations were found to have an *absolute* dependence on exogenous CDP-diglyceride. In these preparations a second, less dense component observed in isopycnic centrifugation was found to catalyze synthesis of [^{3}H]-phosphatidylinositol in the absence of CDP-diglyceride, although the synthesis was slightly stimulated by the presence of the liponucleotide. These findings suggest that, either the enzymatic component responsible for CDP-diglyceride independent synthesis of phosphatidylinositol in most of the reticulocyte preparations is associated with a membranous

contaminant of the plasma membrane of the reticulocytes that is rich in endogenous CDP-diglyceride, or a separate enzyme, not requiring CDP-diglyceride is present. The nature of such an enzyme is unknown, but in view of recent studies on exchange reactions between the water-soluble polar moieties of several lipids (Raghavan *et al.*, 1972) it may be a simple exchange reaction between [^{3}H]inositol and the polar head group of another lipid in the membrane preparation. It is not likely that the lipid undergoing exchange with free [^{3}H]-inositol is endogenous phosphatidylinositol, since in unpublished experiments exchange between prelabeled [^{3}H]-phosphatidylinositol and unlabeled inositol could not be detected.

In any event, it is clear that the purified reticulocyte membranes contain enzymes that are quite active in the *de novo* synthesis of phosphatidylinositol and phosphatidylcholine. In contrast, the membranes of the mature erythrocyte are virtually devoid of biosynthetic activity for these two lipids. The most interesting question that arises from these findings is what are the factors that control the cessation of phospholipid synthesis during the final stages of maturation of the red blood cell? Either the biosynthetic apparatus for phospholipid synthesis is inactivated or, if it is associated with an intracellular membrane component, it is extruded from the cell. At the present time it is not possible to distinguish between these two alternatives.

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