The results presented demonstrate that erythropoiesis in the mouse spleen was associated with cyclic changes in cholesterol and dolichol synthesis which were independent of each other. Changes in the rate of cholesterol synthesis were correlated with the level of 3-hydroxy-3-methylglutaryl-CoA reductase activity and with the rate of cell division (thymidine incorporation into DNA), but a cycle of dolichol synthesis appeared to be independent of both these parameters. Consequently the incorporation of $[^{14}C]$acetate into dolichol increased 20- to 30-fold compared to the rate of cholesterol synthesis 5 to 6 days following phenylhydrazine treatment. The pathway for dolichol synthesis branches from that leading to cholesterol at the level of the intermediate farnesyl pyrophosphate, subsequent to the reaction catalyzed by the regulatory enzyme 3-hydroxy-3-methylglutaryl-CoA reductase. Previous studies showed that in several cell culture lines, and in liver, dolichol synthesis and cholesterol synthesis are regulated in a coordinated way by reductase activity. However, during the process of spermatogenesis and in developing mouse brain the regulation of dolichol synthesis appeared to be independent of cholesterol synthesis. Independent regulation of dolichol synthesis is now demonstrated in a third differentiating tissue, the erythropoietic spleen, and the results suggest that a cycle of dolichol synthesis is specifically associated with one or more stages of erythroid differentiation.

During the assembly of N-glycosidically linked proteins, dolichyl phosphate and dolichyl pyrophosphate function as carriers for saccharide residues (1-3). Dolichols are a group of polyprenol lipids which contain from 16 to 22 isoprene units (1). The pathway of dolichol synthesis involves the joining of two molecules which are also intermediates in the cholesterol synthetic pathway, isopentenyl pyrophosphate and farnesyl pyrophosphate (4-6). These molecules are derived in turn by a pathway which includes the metabolism of acetyl-CoA to mevalonic acid. Investigations of the regulation of dolichol synthesis have been limited by the very low rate at which radiolabeled precursors are incorporated into dolichol in most tissues studied to date. In pig liver the rate of [2-14C]mevalonate incorporation into dolichol was 0.09-0.2% of the rate of incorporation into total nonsaponifiable lipids (6). Measurements of $[^{14}C]$acetate incorporation into sterols and dolichol in mammalian cell cultures grown in lipid-free medium and in mouse liver slices incubated in vitro showed that the rates of incorporation into dolichol were 0.02-0.1% of the incorporation rates into sterols (7, 8, 16).

In cultured mouse fibroblasts, a subline of NCTC 929 strain L-cells, high concentrations of 25-hydroxycholesterol suppress 3-hydroxy-3-methylglutaryl-CoA reductase and inhibit sterol and dolichol synthesis; lower concentrations of 25-hydroxycholesterol also inhibit cholesterol synthesis but have less of an effect on dolichol synthesis (7). These observations have led to the suggestion that there is a rate-limiting enzyme in the dolichol synthetic branch of the pathway which is saturated by a lower level of intermediates than is necessary to saturate the next rate-limiting step after 3-hydroxy-3-methylglutaryl-CoA reductase in the cholesterol synthetic branch (7). The regulation of dolichol synthesis in mouse liver appears to be similar to that in L-cells (16).

In contrast to the observations made with L-cells and liver, in mouse testes incubated in vitro the rate of $[^{14}C]$acetate incorporation into dolichol was 1.8-2.4% of the rate of incorporation into cholesterol (8). The synthesis of dolichol at this relatively high rate appeared to be specific to spermatogenesis, since the rate of $[^{14}C]$acetate incorporation into dolichol in testes deficient in spermatogenic cells was only 0.02-0.2% of the rate of incorporation into cholesterol, although the rate of cholesterol synthesis was the same as in normal testes (8). In developing mouse brain, a peak of dolichol synthesis occurred at approximately 7 days after birth, during a time when the rate of cholesterol synthesis was falling rapidly (9). These results suggest that the regulation of dolichol synthesis in two differentiating tissues is independent of cholesterol synthesis and differs from that seen in cultured cells, in fully differentiated testes cells, and in liver.

In order to examine further the relationship between dolichol synthesis and cellular differentiation, we investigated dolichol synthesis in another differentiating tissue, the erythropoietic spleen, which contains a large population of cells of one lineage which are dividing and undergoing differentiation. Erythropoiesis can be induced in the mouse spleen by injecting phenylhydrazine (10, 11). Rencricca et al. (10) reported that phenylhydrazine treatment of mice caused severe hemolysis and decreased the hematocrit from 40 to 20%; this was followed by an increase in the number of erythroid elements in the spleen, from a normal pretreatment level of $0.07 \times 10^8$ cells to $3.5 \times 10^8$ cells 5 days after beginning treatment. The hematocrit returned to the normal level of 40% at day 7. Based on these and other results, Rencricca et al. (10) suggested that hemopoietic stem cells migrate from the bone marrow to the spleen during this period of severe hemopoietic stress, and that differentiation of these cells provides for recovery from the anemic state.

The biochemical events which occur during the early stages

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of erythroid differentiation are not well understood, although membrane changes are presumed to be important. Glycophorin A, the major red cell membrane glycoprotein, is present in basophilic normoblasts but not in earlier erythroid cells, the pronormoblasts (12). Dolichol is involved in the assembly of glycophorin A (13). Erythroid differentiation may involve the sequential loss and acquisition of specific membrane receptors. Iscove (14) showed that early erythroid precursors, burst-forming units become more sensitive to erythropoietin and less dependent on burst-promoting activity as the cells mature to the erythroid colony-forming unit stage.

Here we report that splenic erythropoiesis resulting from severe phenylhydrazine-induced anemia is accompanied by sequential changes in the synthesis of dolichol and dolichol in the mouse spleen, and that a dramatic increase in the ratio of dolichol to cholesterol synthesis occurs during erythropoiesis.

EXPERIMENTAL PROCEDURES

Materials — The following compounds were obtained from the companies indicated: R,S-3-hydroxy-3-methyl-[3-14C]glutaryl-CoA (55 Ci/mol), [1-14C]acetic acid, sodium salt (57 Ci/mol), [1,2-3H]cholesterol (40-60 Ci/mmol), [1-3H]dolichol (12.5 Ci/mmol), [methyl-2-14H]thymidine (20 Ci/mmol), d-[1-3H]mannose (5-15 Ci/mmol), [2-14C]mevalonolactone (40-60 Ci/mol), New England Nuclear; phenylhydrazine hydrochloride, Sigma. Burdick and Jackson (Muskegon, MI) glass-distilled solvents were used for high pressure liquid chromatography. Lyophilized human urinary erythropoietin with activity of 10 units/mg was prepared by Geoffrey Keighley and obtained from Dr. Seldon E. Bernstein of The Jackson Laboratory.

Mice — Male B6D2F1 and C57BL/6J mice from the Animal Resources Department of The Jackson Laboratory were used at 8-10 weeks of age. WBB6F1/W and WBB6F1 +/- mice (F, hybrids of the inbred strains C57BL/6J-W/+ and WB/Re-W/+ ) were obtained from Dr. Seldon E. Bernstein of The Jackson Laboratory. Phenylhydrazine hydrochloride, 60 mg/kg in saline, was injected subcutaneously at intervals of 24 h, starting on day 0. Injections were staggered so that on the same day spleens could be obtained at 0 through 8 days postinjection. For assaying the synthesis of dolichol and cholesterol, 10 mice were injected for each day; for assaying DNA synthesis, 5 mice were injected for each day. C57BL/6J mice were given one intraperitoneal injection of 10 units of erythropoietin, and injections were timed so that on the same day, spleens could be obtained from 3 mice each at 6 and 12 h, and 1 through 5 days after injection.

High Pressure Liquid Chromatography — The high pressure liquid chromatography systems used in this study, comprising a reverse-phase column (Waters Associates, Milford, MA) were previously described (7). Two solvent systems were used: high pressure liquid chromatography system A, 18 aqueous methanol for 30 min, then a linear gradient over 25 min to a final concentration of 35% methylene chloride in 100% methanol; system B, concave gradient No. 10 (Waters Associates model 600 solvent programmer) from 100% methanol to 100% methylene chloride over 48 min. In each system the flow rate was 2 ml/min and 1-ml fractions were collected.

Assaying Dolichol and Cholesterol Synthesis — On the same day, spleens were removed from groups of 8 mice each, representing the control (day 0) and days 1 through 5 after phenylhydrazine injection. The spleens were weighed and teased apart to release the cells. Two 25-ml Erlenmeyer flasks were used per experimental group, each containing 4 spleens in 5 ml of Waymouth's MB 752/1 chemically defined medium, with [1-3H]acetate (200 pCi); this medium was used previously for measuring cholesterol and dolichol synthesis (7). Incubations were for 3 or 4 h. The contents of the 2 flasks for each experimental group were then pooled. Incorporation of [1-3H]acetate into dolichol and cholesterol was determined as previously described (7). Briefly, the tissue was collected by centrifugation, [1-3H]cholesterol and pig liver [1-3H]dolichol standards were added, and the mixture was saponified at 80°C for 2½ h in 30% ethanol KOH containing pyrogallol and butylated hydroxytoluene. Hydrolysis of dolichyl phosphate and dolicholphosphoryl saccharides to free dolichol may be incomplete under these saponification conditions (15). The nonvolatile lipids were fractionated using high pressure liquid chromatography system B, and the cholesterol- and dolichol-containing fractions were collected. The dolichol fraction was acetylated and chromatographed on thin layer chromatography silica gel plates with toluene as solvent; the plates were divided into 1-cm bands, and 14C and 3H in acetylated dolichol were determined. Overlap of 3H into the 3C counting channel was less than 0.005%. Radioabeled cholesterol was detected as digitonin-stable sterol and was quantitated by using a high pressure liquid chromatography system with cells cultures the standard error of the mean values for [1-3H]acetate incorporation into cholesterol and dolichol in replicate assays of a single sample were less than ±5% (7). In the present studies standard error of mean values obtained in replicate experiments with pooled spleens from mice before treatment and 3 days after treatment with phenylhydrazine were within a range of ±10% for dolichol and ±20% for cholesterol.

Assaying DNA Synthesis — Normal and erythropoietic spleens were prepared as described above, and incubated in 5 ml of L-cell medium containing 20 μg of [3H]thymidine. After 1 h, the tissues were filtered through Nitex (HC-3-110, Toko, Inc., Elmford, NY) to obtain single-cell suspensions, and the cells were collected on GF/C filters (25 mm, Whatman) using a sample collection chamber (Millipore) and washed 3 times with ice-cold saline. Salines were treated 3 times with cold 6% perchloric acid to precipitate DNA, then 3 times with 95% ethanol. Blank values were obtained (1) from cells kept cold during the incubation period and collected on filters 5 min after addition of [3H]thymidine. The filters were placed in scintillation vials and 15 ml of toluene scintillation fluid were added.

Chain Lengths and Polariesties of Spleen Dolichols — Using high pressure liquid chromatography system A, [1-14C]dolichols synthesized from acetate and mevalonate by normal spleens and spleens from mice injected 3 and 4 days previously with phenylhydrazine were separated according to chain length. In Fig. 1, chromatograms of [1-14C]dolichols are compared to those of tritiated pig liver standard dolichol, in which the major band contains 19 isoprenoid units (18). The predominant dolichol band synthesized from mevalonate by mouse spleen tissue (Fig. 1c) contains 18 isoprenoid units. The major dolichol bands synthesized from mevalonate by erythropoietic spleens (Fig. 1b) contain 17 and 18 isoprene units. Dolichols synthesized by erythropoietic spleens from acetate (Fig. 1c) chromatographed in a similar manner to those synthesized by erythropoietic spleens from mevalonate, which is incorporated
plates and chromatographed using system described in the text. Acetylated dolichols were eluted from thin layer were pooled, extracted, and chromatographed using system B as dotted lines collected and separated using high pressure liquid chromatography.

It was shown previously (8) that the individual peaks of dolichol (representing different chain lengths) synthesized by mouse testes (8) were dolichols. These results also show the similarity between the species of dolichols synthesized by C57BL/6J mouse spleens and mouse fibroblast cell cultures (7) and mouse testes (8).

Phenylhydrazine-treated Mice—C57BL/6J mice were injected on days 0 and 1 with phenylhydrazine and the spleens were removed from 8 mice each on days 0 through 6 (Fig. 2). A marked increase in spleen weight, from 73 to 484 mg, occurred between day 0 and 5, after which the spleen weight decreased. [14C]Dolichol synthesis increased from 270 dpm/3 h/spleen at day 0 to 1952 dpm/3 h/spleen at day 4, then decreased; whereas cholesterol synthesis increased between day 0 and 3 then decreased. Consequently, the ratio of the disintegrations per min in dolichol to the disintegrations per min in cholesterol increased 30-fold, from 0.93% at day 0 to 31.8% at day 5. The ratio of dolichol to cholesterol synthesis at day 5 was higher than that seen so far in any other mouse tissues.

Measurements of dolichol and cholesterol synthesis in spleens from phenylhydrazine-treated B6D2F1 mice gave results very similar to those obtained with C57BL/6J mice. In B6D2F1 mice the spleen weight increased from 63 to 465 mg between day 0 and 6, then decreased. Dolichol synthesis increased from 177 dpm/3 h/spleen at day 0 to 1209 dpm/3 h/spleen at day 3. In two similar experiments, the value for [14C]acetate incorporation into dolichol by B6D2F1 erythropoietic spleens 4 days after treatment was 922 ± 9 dpm/3 h/spleen. The ratio of dolichol to cholesterol synthesis increased from 0.89% at day 0 to 18.7% at day 6. In both strains of mice the peak in spleen weight coincided with the peak in the ratio of dolichol synthesis to cholesterol synthesis.

It has been reported that in genetically anemic W/ W" mice the erythropoietic response to irradiation (19) and to phenylhydrazine treatment 1 is delayed, in comparison with the response in normal mice. To determine whether the mutation would be expressed as a temporal change in the normal pattern for dolichol and cholesterol synthesis, WBB6F1+/+ and W/ W" mice were injected with phenylhydrazine on days

1 E. S. Russell, unpublished observations.
0 and 1, and dolichol and cholesterol syntheses in the spleens were measured several days later (Table I). At day 3, the increases in spleen weight, dolichol synthesis, and ratio of dolichol to cholesterol synthesis in +/+ mice were similar to the changes at day 3 in C57BL/6J and B6D2F1 mice. In contrast, the phenylhydrazine-treated W/W mice showed less than 2-fold increases in dolichol synthesis and spleen weight at days 3 and 4. However, at day 8 dolichol synthesis was 1038 dpm/3 h/spleen, a 5-fold increase over the day 0 rate of 223 dpm/3 h/spleen. This increase is comparable to that seen at day 3 in normal mice. Based on these results it seems likely that the alterations in dolichol synthesis seen in spleens of phenylhydrazine-treated mice are specific to erythropoiesis, because the time course and magnitude of the changes in three normal strains were very similar, whereas the delayed erythropoietic response of W/W mice was accompanied by a delayed increase in dolichol synthesis.

**Correlation between Cholesterol Synthesis and DNA Synthesis**—In the erythropoietic spleen there is a requirement for cellular proliferation to account for increases in the number of erythroid elements following phenylhydrazine injections (10). The observed increase in the synthesis of cholesterol following phenylhydrazine treatment may be associated with this cellular proliferation. To investigate this possibility, [3H]thymidine incorporation into perchloric acid-precipitable material and cholesterol synthesis from [14C]acetate were measured in samples from the same population of cells. Results of determinations using spleens taken from mice at 0 through 8 days after phenylhydrazine injection are shown in Fig. 3a. Phenylhydrazine treatment resulted in an immediate increase in cholesterol synthesis, followed closely by an increase in DNA synthesis. Both rates of synthesis remained at a delayed increase in dolichol synthesis.

**Correlation between Cholesterol Synthesis and 3-Hydroxy-3-methylglutaryl-CoA Reductase**—Incorporation of [14C]acetate into cholesterol and dolichol synthesis in spleens of untreated and phenylhydrazine-treated C57BL/6J, B6D2F1, and WBB6F1 +/+ and W/W mice

| Strain | Spleen weight (mg) | [14C]Acetate incorporation into Dolichol | [14C]Acetate incorporation into Cholesterol | (dpm dolichol)/(dpm cholesterol) × 100 |
|--------|--------------------|-----------------------------------------|------------------------------------------|-----------------------------------|
| C57BL/6J | Day 0 | 73 | 270 | 28,946 | 0.93 |
|         | Day 3 | 390 | 1,302 | 50,466 | 2.58 |
| B6D2F1 | Day 0 | 63 | 177 | 19,925 | 0.89 |
|         | Day 3 | 322 | 1,209 | 33,762 | 3.58 |
| WBB6F1 +/+ | Day 0 | 99 | 233 | 45,376 | 0.51 |
|         | Day 3 | 434 | 1,067 | 30,969 | 3.45 |
| WBB6F1-W/W | Day 0 | 80 | 223 | 43,970 | 0.51 |
|         | Day 4 | 158 | 401 | 35,461 | 1.20 |
|         | Day 8 | 110 | 1,038 | 30,150 | 10.03 |
| WBB6F1-W/W | Day 0 | 91 | 485 | 59,926 | 0.81 |
|         | Day 3 | 159 | 831 | 49,057 | 1.69 |

Severe hemolysis induced by phenylhydrazine is compensated for in the mouse spleen by a period of intense erythro-
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poiesis. Stem cells migrate from the marrow to the spleen, where differentiation and proliferation of erythroid precursors leads to a rise in the peripheral reticulocyte count 6 days later, and the hematocrit returns to normal at day 7 (10). Although the results presented here do not reflect biochemical changes in a pure population of cells, they do indicate that a cycle of cholesterol synthesis was associated with erythroid cell proliferation and that the synthesis of dolichol may be an important event during one or more stages of erythroid differentiation.

There was a marked increase in dolichol synthesis at a time when cholesterol synthesis was declining in the spleen prior to the appearance of reticulocytes in the blood. The time course and magnitude of the increase in dolichol synthesis were similar in C57BL/6J and B6D2F1 mice.

The dolichol synthesized by normal mouse spleens may be more polar than the dolichol synthesized by erythropoietic mouse spleens. It has been postulated (8) that the higher polarity of mouse testes dolichol relative to pig liver dolichol is due to the presence of an unsaturated α-isoprene unit in testes dolichol; this α-unit is saturated in pig liver dolichol (1). If this is the reason for differing polarities among dolichols, then the presence of saturated α-isoprene units in the dolichols from erythropoietic spleens may indicate that not only is the overall rate at which isoprene units are added to the lengthening chain increased compared to normal spleens, but that the final step in dolichol synthesis, i.e. saturation of the α-unit, is also accelerated.

Evidence has been presented before for independent regulation of dolichol and sterol synthesis. The relationship found between rates of dolichol synthesis, cholesterol synthesis, and levels of 3-hydroxy-3-methylglutaryl-CoA reductase activity in L-cell cultures (7) and in mouse liver (16) suggest that in these cells an enzyme in the dolichol branch of the pathway is saturated at a lower concentration of an isoprene intermediate than is necessary to saturate the next rate-limiting enzyme of cholesterol synthesis after the formation of farnesyl pyrophosphate. Thus, when 3-hydroxy-3-methylglutaryl-CoA reductase activity was high, levels of isoprenyl substrates (farnesyl pyrophosphate and/or isopentenyl pyrophosphate) supplied were saturating for dolichol synthesis but not for cholesterol synthesis. The rates at which the two products were synthesized could therefore be regulated differentially by a single enzyme, 3-hydroxy-3-methylglutaryl-CoA reductase, acting before the branch point.

Low rates of dolichol synthesis relative to cholesterol synthesis seen in testes devoid of active spermatogenesis and in liver seem consistent with this kind of regulation. However, in spermatogenetic testes the ratio of dolichol synthesis to cholesterol synthesis was 1.8 to 2.4%, which is approximately 10 times the ratios seen in liver, various cell cultures, and non-spermatogenetic testes. This increased ratio appeared to be due to an elevation in the rate of dolichol synthesis suggesting that the relationship between the two branches of the pathway was altered in the differentiating cells. Independent regulation of the two branches of the pathway by a mechanism which may be qualitatively different from that in L-cell cultures and in liver was also observed in developing mouse brain (9). The rate of dolichol synthesis increased from 0 to 7 days after birth while the rate of cholesterol synthesis declined rapidly.

The present results demonstrate that independent peaks of cholesterol and dolichol synthesis followed treatment with phenylhydrazine. The changes in the rates of cholesterol and dolichol synthesis as percentages of the control (day 0) value were similar in erythropoietic spleens from C57BL/6J, B6D2F1, and WBB6F1-/+ mice. In addition, the ratio of dpm in dolichol to dpm in cholesterol in control spleens varied only slightly, from 0.51 to 0.93%, among the three strains of mice. During the period of intense erythropoiesis following phenylhydrazine treatment, the ratio increased 20- to 30-fold, representing both a severalfold increase in the rate of dolichol synthesis and a later decrease in the rate of cholesterol synthesis. That this altered ratio is due to erythropoiesis is shown by the relatively slow response of genetically anemic W/W" mice to phenylhydrazine. The changes in spleen weight, rate of dolichol synthesis, and the ratio of dpm in dolichol to dpm in cholesterol seen at day 3 and 4 in normal mice were not seen until day 8 in W/W" mice (Table 1).

The increase in the rate of cholesterol synthesis that occurred soon after treatment of the mice with phenylhydrazine was closely correlated with corresponding increases in the rate of thymidine incorporation into DNA. Thus cholesterol synthesis appears to be increased as part of the process of cellular proliferation. The association of de novo cholesterol synthesis with lymphocyte mitogenesis in vitro and with cell division in vivo has been noted previously (20, 23). A correlation between DNA synthesis and cholesterol synthesis was demonstrated in vitro by Chen et al. (23) who showed that exposure of lymphocytes to phytohemagglutinin was followed by a cycle of cholesterol synthesis which preceded a cycle of DNA synthesis. Inhibition of the cholesterol synthesis with 25-hydroxycholesterol prevented the cells from entering the phase of DNA synthesis. It has been hypothesized that cell division in vivo also requires cholesterol synthesis, as high rates of cholesterol synthesis are found in intestinal crypt cells (21) and epidermis (22). The results reported here clearly demonstrate coincidental cycles of increased cholesterol synthesis and DNA synthesis in normal cells stimulated in vitro to enter a phase of proliferation and differentiation.

The observed changes in dolichol synthesis during erythropoiesis, on the other hand, appeared to be unrelated to the rate of DNA synthesis. This increased rate of dolichol synthesis was therefore not simply a function of enhanced erythroid cell proliferation, nor was it due to overall enhancement of the cholesterol synthetic pathway. On the contrary, the evidence suggests that there is a separate regulatory mechanism which provides for increased dolichol synthesis in the absence of increased cholesterol synthesis and which does not involve increased 3-hydroxy-3-methylglutaryl-CoA reductase activity. Presumably, increased dolichol synthesis is associated with a need for glycoproteins during the differentiation process. Preliminary results (unpublished) show that the rate of tunicamycin-inhibitable [14C]mannose incorporation into spleen glycoproteins reaches a peak at day 4 to 5 after phenylhydrazine injections.

These results demonstrate a third example of increased dolichol synthesis associated with the differentiation of mammalian cells. Investigations by Schneider et al. (24) indicate that dolichol synthesis is required for differentiation of sea urchin embryos. Thus the synthesis of dolichol during the differentiation of several different kinds of cells may be of considerable importance. Identification of the stages in erythropoietic differentiation at which the rate of dolichol synthesis increases and investigation of glycoprotein synthesis during erythropoiesis may allow insight into biochemical events involved in and regulating erythroid differentiation.

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