A deficiency of mixed function oxidase activities in the cholesterol biosynthetic pathway of human granulocytes

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Abstract Highly purified human granulocytes synthesize [^{14}C]farnesol and [^{14}C]squalene but not [^{14}C]sterols from [^{14}C]mevalonic acid. Dimethylsulfoxide was found to be an excellent vehicle for carrying [^{3}H]squalene-2, 3-oxide into the intact cells. The granulocytes synthesized [^{3}H]lanosterol from this substrate, but were unable to further process the newly synthesized lanosterol along the cholesterol biosynthetic pathway. In contrast, intact lymphocytes and monocytes were able to synthesize radioactive cholesterol from either [^{14}C]mevalonic acid or [^{3}H]squalene-2,3-oxide. These results indicate that normal human granulocytes have retained squalene-2,3-oxide–lanosterol cyclase activity but have lost squalene epoxidase activity and at least one other mixed function oxidase activity that is required to transform lanosterol into cholesterol. These results may provide an explanation for the accumulation of farnesol and squalene that has been previously observed in populations of mixed leukocytes (Fogelman, A. M., Edmond, J., Seager, J., and Popják, G. (1975) *J. Biol. Chem.* **250:** 2045–2055 (1); Burns, C. P., Welshman, I. R., Edmond, J., and Spector, A. A. (1979) *Biochim. Biophys. Acta* **572:** 345–351) (12).—**Shechter, I., A. M. Fogelman, and G. Popják.** A deficiency of mixed function oxidase activities in the cholesterol biosynthetic pathway of human granulocytes. *J. Lipid Res.* **1980. 21:** 277–283.

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It was reported by Fogelman et al. (1) that intact mixed leukocytes and the 10,000 *g* supernatant of the disrupted cells had a limited capacity to synthesize sterols from [^{14}C]mevalonate as compared to their ability to synthesize farnesol and squalene. Later, when the leukocytes were fractionated, it was found that the granulocytes, which constitute the majority of leukocytes, were unable to synthesize sterols from either [^{14}C]acetate or [^{14}C]mevalonate (2). In contrast, the lymphocytes and monocytes, which comprise only 30% and 5% respectively of the leukocytes, can synthesize sterols, the monocytes being much more active than the lymphocytes (2, 3). Although the granulocytes cannot synthesize sterols, they synthesize [^{14}C]farnesol and [^{14}C]squalene from [2-^{14}C]mevalonate (2). We now report that the failure of the granulocytes to synthesize sterols can be attributed to their lack of squalene epoxidase. We show further that the granulocytes have retained squalene-2,3-oxide:lanosterol cyclase activity, but apparently do not contain enzymes needed for the conversion of lanosterol into cholesterol.

MATERIALS AND METHODS

Human subjects

Ten normal volunteers from the student body and staff of UCLA donated blood for these studies after written informed consent was obtained. All had normal hematocrits, white blood cell and differential counts, and normal plasma cholesterol and triglyceride levels. No one received drugs that might have affected serum cholesterol levels, lipid metabolism, or leukocyte function.

Collection, separation and incubation of leukocytes

Blood was collected after a 12–14 hr fast and the leukocytes were separated by counterflow centrifugation as previously described (3). The purity of the granulocyte fractions exceeded 99.5%, and the purity of the monocyte and lymphocyte fractions was greater than 95% and 99%, respectively. The leukocytes were incubated in Krebs-Ringer phosphate buffer pH 7.4, fortified with 15 mM glucose and 1% bovine serum albumin, in a total volume of 1 ml in siliconized tubes that were shaken in air at 200 rpm at 37°C. In experiments with [2-^{14}C]mevalonate the substrate was added

Abbreviations: TLC, thin-layer chromatography.

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to the tubes just before the addition of the cells. In preliminary experiments no difference was seen at the concentrations used in these experiments whether [2-^{14}C]mevalonic acid DBED salt or the sodium salt of [2-^{14}C]mevalonic acid was used. Therefore, the DBED salt was used (labeled either with ^{14}C or ^{3}H) because it was more convenient.

Incubation of cells with radioactive squalene or squalene-2,3-oxide was done as described above except that the incubations also contained 10% dimethylsulfoxide (Me_2SO). The incorporation of radioactive substrates into radioactive products was linear with respect to time (up to 4 hr) and was linear with respect to cell numbers (up to 10^8 cells). Benzene solutions of radioactive substrates were placed in a tube, the solvent evaporated under a stream of N_2 and 0.1 ml of Me_2SO added. After vigorous mixing buffer was added and the incubations were started by the addition of cells. More than 95% of the cells excluded trypan blue and phagocytized latex particles at the conclusion of the incubations.

Extraction and analysis of radioactive products

The reactions were stopped by the addition of 0.5 ml of 10% KOH in methanol. After 30 min at 37°C, 0.5 ml of acetone was added. Each incubation was then extracted three times with 1 ml of hexane. The hexane extracts were combined, evaporated under a stream of N_2 and applied to thin-layer chromatography (TLC) plates. Silver nitrate-impregnated plates were made by dipping them into 1.5% $AgNO_3$ in acetonitrile for 2 min. The plates were dried in a hood for 15 min and, after application of the samples, developed with hexane–ethyl acetate–benzene 380:120:1. The following R_f values were obtained: squalene, 0.94; squalene-2,3-oxide, 0.86; lanosterol, 0.74; cholesterol, 0.46; and farnesol, 0.21 (cf. also Fig. 4). Co-chromatography was done by mixing standards with radioactive materials before the application to the TLC plates. Substances were detected on silica gel TLC plates after exposure to iodine vapors, and on $AgNO_3$-impregnated plates with a spray of 3% (w/v) phosphomolybdic acid in propan-2-ol followed by heating over a hot plate. The plates were scanned for radioactivity in a Packard Radiochromatogram scanner model 7201. Quantitative measurements of radioactivity were made by scraping zones of the gel into counting vials containing 10 ml of scintillation fluid. The scintillation fluid was made by adding 10% complete counting cocktail (RPI 3a70B) to a 0.5% (w/v) solution of 2,5-diphenyloxazole in toluene. The vials were counted in a Packard Tri-Carb liquid scintillation spectrometer model 3320. The counting efficiencies were 86% for ^{14}C and 52% for ^{3}H. In the double label experiments using both ^{14}C and ^{3}H the counting efficiency for ^{14}C was 44% and 20% for ^{3}H. Duplicate assays differed by less than 5%.

Biosynthesis of [^{14}C]squalene

[^{14}C]Squalene was biosynthesized by a slight modification of the procedure described by Popják (4). NADPH was substituted for $NADP^+$ and 0.5 unit of glucose-6-phosphate dehydrogenase was added per ml of incubation. Each incubation contained 100 μCi of the dibenzylethylenediamine (DBED) salt of *RS*-[2-^{14}C]mevalonic acid (51 Ci/mol). The purified product gave a single radioactive spot on silica gel TLC plates developed with hexane and on 1.5% $AgNO_3$-impregnated silica gel TLC plates developed with hexane–ethyl acetate–benzene 380:120:1. A typical preparation yielded 13 μCi of [^{14}C]squalene which was stored at −29°C in benzene at a concentration of 10^6 cpm/ml.

Biosynthesis of [^{14}C] and [^{3}H]squalene-2,3-oxide

Radioactive squalene-2,3-oxide was biosynthesized from radioactive mevalonate in rat liver homogenates that were prepared and incubated as previously described (4) except that the plant growth retardant 2′-isopropyl-4′-(trimethylammoniumchloride)-5′-methylphenyl-piperidine-1-carboxylate (AMO 1618) was added to inhibit squalene-2,3-oxide:lanosterol cyclase (5). Flavineadeninedinucleotide (FAD) was also added as a necessary cofactor for squalene epoxidase (6). The concentrations of AMO 1618 and FAD were 0.3 mM and 0.1 mM, respectively (7). The incubation mixtures (10 ml) were shaken in 25 ml conical flasks at 200 rpm in a gyrotory water bath at 37°C for 4 hr. The reaction was stopped by the addition of 5 ml of 10% KOH in methanol. After 30 min at 37°C the mixtures were extracted five times with 5 ml of hexane. The radioactive products were separated on 0.5 mm silica gel plates developed to 10 cm with 0.5% ethyl acetate in benzene (6). The gel containing the radioactive spot corresponding to squalene-2,3-oxide (R_f 0.59) was scraped off and extracted with ethyl acetate. The ethyl acetate was subsequently removed under a stream of nitrogen. As shown in **Fig. 1,** radioactive squalene-2,3-oxide was by far the major product of the synthesis. A typical yield was 7% to 9% of the radioactivity added as either [^{14}C] or [^{3}H]*RS*-mevalonate. The squalene-2,3-oxide was stored at −29°C in alkaline benzene (1 ml of concentrated NH_4OH in 100 ml of benzene) at a concentration of 10^6 cpm/ml.

Animals, biochemicals, radioactive supplies, reagents

Liver enzyme preparations were made from female Sprague–Dawley rats weighing 190–220 g. AMO 1618

was supplied by Calbiochem, San Diego, CA, and glucose-6-phosphate dehydrogenase by Sigma, St. Louis, MO. *RS*-[2-^{14}C]Mevalonic acid DBED salt (51 Ci/mol) was purchased from Amersham/Searle Corp., Arlington Heights, IL. *RS*-[5-^{3}H]Melavonic acid DBED salt (5 Ci/mmol) was obtained from New England Nuclear Corp., Boston, MA. Thin-layer silica gel plates (F-25H) were purchased from Merck, Rahway, NJ. Scintillation counting fluid, 3a70B, was purchased from Research Products International Corp., Elk Grove Village, IL. All other chemicals and reagents were obtained from sources previously cited (1, 3).

RESULTS

The previous observations by Fogelman et al. (2) that human granulocytes could synthesize squalene, but not sterols, from either [^{14}C]acetate or [^{14}C]mevalonate, suggested that these cells either lost the ability to convert squalene to the 2,3-oxide, or the ability to cyclize the latter to lanosterol, or were devoid of these activities. We therefore tested pure preparations of granulocytes for their ability to cyclize labeled squalene-2,3-oxide to lanosterol.

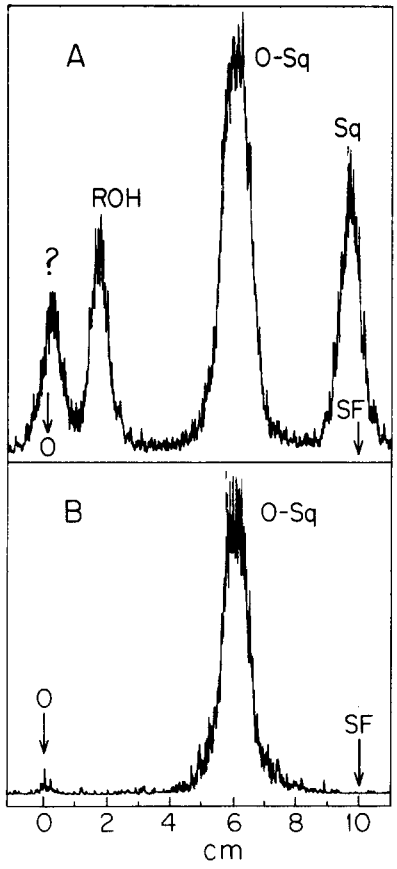

Fig. 1. Biosynthesis and purification of radioactive squalene-2,3-oxide. *RS*-[2-^{14}C]Mevalonic acid DBED salt (100 μCi) was incubated with rat liver homogenates in the presence of AMO 1618 and purified as described in Materials and Methods. Panel A is the radiochromatogram of the products of the incubation. The R_fs were as follows: squalene (Sq), 0.98; squalene-2,3-oxide (O-Sq) 0.59; alcohols (ROH), 0.16; polar unidentified products remained at the origin. Panel B is the radiochromatogram of the squalene-2,3-oxide purified as described in Materials and Methods.

TABLE 1. Effect of solvents on the formation of [^{14}C]lanosterol from [^{14}C]squalene-2,3-oxide in human polymorphonuclear leukocytes (granulocytes)

Solvent for Oxido-squalene	Concentration of Solvent, % (v/v)				
	0.0	1.0	4.0	10.0	15.0
	^{14}C *cpm in lanosterol*				
Acetone	270[a]	626	1103	371	219
Ethanol	270	514	512	186	74
Me_2SO	270	974	4008	6116	5763
Control[b]	16				

[a] Mean of three incubations without solvent.
[b] Suspension of cells killed with KOH added to substrate.
[^{14}C]Squalene-2,3-oxide (10,000 cpm) was dissolved in 0.1 ml of a solvent and then 15 × 10^6 granulocytes, suspended in 0.9 ml Krebs-Ringer phosphate buffer, pH 7.4, containing 15 mM glucose and 1% (w/v) bovine serum albumin, were added to it. The cells were incubated at 37°C in a shaking water bath. After 20 min the reaction was stopped with KOH, the unsaponifiable material was extracted from the mixtures and analyzed on the $AgNO_3$-impregnated TLC plates as described in Materials and Methods. All the experiments were done with the same batch of granulocytes.

Effects of detergents and solvents on cyclization of squalene-2,3-oxide by granulocytes

In preliminary experiments we found that squalene-2,3-oxide coated onto the walls of incubation test tubes (cf. Methods) was converted by granulocytes into chromatographically identifiable lanosterol in 2 to 3% yield in the absence of a solvent or a detergent. Three detergents, Triton X-100, KYRO EOB (Procter & Gamble), and sodium deoxycholate with and without 0.7 M KCL (8, 9) were tried at 0.2 to 0.8% concentrations to deliver squalene-2,3-oxide to the cells. We observed a 1% to 2% conversion of the squalene-2,3-oxide into lanosterol at 0.2% concentrations of Triton X-100 and KYRO EOB. Higher concentrations of these detergents and all concentrations of deoxycholate gave very doubtful conversions (17–180 cpm/10,000 cpm of added oxidosqualene). Examination of these cells by microscope showed that even the lowest concentration of these detergents tested (0.2%) caused the complete disintegration of the granulocytes.

In contrast, the solvents ethanol, acetone, and Me_2SO used for solubilizing the squalene-2,3-oxide increased the conversion of this substrate into lanosterol by the granulocytes. Me_2SO was particularly effective, as in its presence (10 to 15%) in the incubation about 60% conversions to lanosterol were observed (**Table 1**).

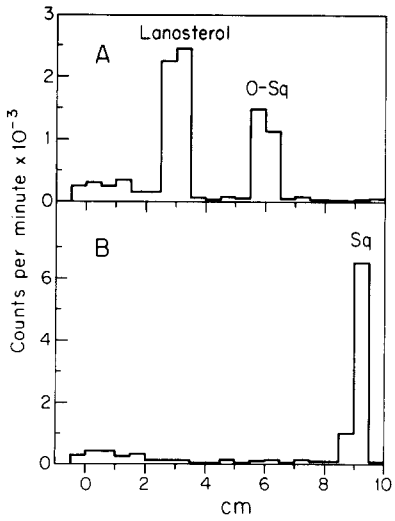

Fig. 2. Utilization of [^{14}C]squalene and [^{14}C]squalene-2,3-oxide by granulocytes. [^{14}C]Squalene (10,000 cpm) or [^{14}C]squalene-2,3-oxide (10,000 cpm) were incubated with 3.0×10^7 granulocytes in the presence of 10% Me_2SO as described in Materials and Methods. After 20 min the reaction was stopped and the radioactive materials were extracted as described. The radioactive materials were chromatographed on thin-layer plates of silica gel G developed to 10 cm with 7% ethylacetate in benzene. Panel A represents the radioactivity found after incubation with [^{14}C]squalene-2,3-oxide (O-Sq) and Panel B, the materials obtained after incubation with [^{14}C]squalene (Sq).

Me_2SO had apparently no deleterious effects on the viability of the granulocytes. After incubations containing 10% Me_2SO, over 90% of the cells were microscopically intact and excluded Trypan Blue and were fully able to ingest latex particles. In all subsequent experiments we used Me_2SO (10% final concentration) for the delivery of squalene-2,3-oxide and squalene to the granulocytes. In contrast to the efficient conversion of [^{14}C]squalene-2,3-oxide into lanosterol by granulocytes, [^{14}C]squalene, presented to the cells in Me_2SO, was not metabolized further by these cells (**Fig. 2**).

To test whether squalene-2,3-oxide was indeed taken up by granulocytes in the presence of Me_2SO, eight incubations were set up in which 17×10^6 cells suspended in 900 μl of Krebs-Ringer phosphate buffer (cf. note to Table 1) were added to [^{14}C]squalene-oxide (30,000 cpm) dissolved in 100 μl of Me_2SO and incubated at 37°C. At times varying from 1 to 120 min the ^{14}C remaining in the medium and that taken up by the cells were measured. As shown in **Fig. 3,** within 1 min there was a significant association of the squalene-oxide with the granulocytes and this was followed by a continued, nearly linear, uptake of the substrate by the cells. The uptake by the cells in this experiment was relatively low, e.g., from the experiments recorded in Table 1, and others not recorded here, conversions of as much as 80% of [^{14}C]squalene-oxide into lanosterol by the granulocytes were observed.

Localization of products synthesized by granulocytes from [2-^{14}C]mevalonate (and [^{14}C]squalene-2,3-oxide)

We have examined once again the utilization of [2-^{14}C]mevalonate by the granulocytes and found, in confirmation of previous findings (2), that the granulocytes were unable to complete the sterol biosynthetic pathway beyond squalene. The only unsaponifiable products of the synthesis from mevalonate by the granulocytes were free farnesol and squalene. We examined the location of these two products and found that the squalene was entirely intracellular, whereas all the farnesol was found in the incubation medium (**Fig. 4**).

Simultaneous utilization of [2-^{14}C]mevalonate and [^{3}H]squalene-2,3-oxide by human leukocytes

Since the conversion of squalene-2,3-oxide into lanosterol by granulocytes occurred in the presence of Me_2SO we needed to ascertain whether this solvent or the squalene-2,3-oxide itself may not have had some "inductive" effects on some "repressed" enzyme(s) in the granulocytes. We found that 10% Me_2SO in the incubation medium of granulocytes with [2-^{14}C]mevalonate caused only an approximately 20% decrease in the incorporation of the precursor into free farnesol and squalene in 2-hr incubations without any evidence of appearance of either squalene-2,3-oxide or of sterols in either the cells or the medium (data not shown).

In further experiments, pure preparations of granulocytes, lymphocytes and monocytes were incubated simultaneously with [2-^{14}C]mevalonate and [^{3}H]squalene-2,3-oxide in the presence of 10% Me_2SO as de-

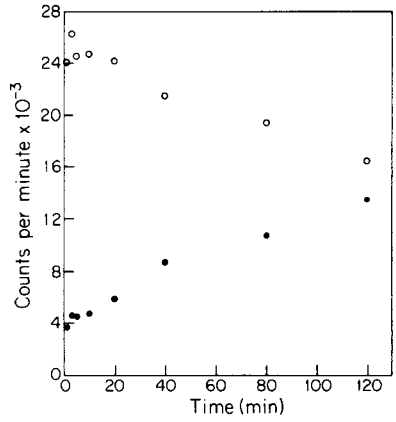

Fig. 3. Uptake of [^{14}C]squalene-2,3-oxide by granulocytes. 17×10^6 cells suspended in 0.9 ml Krebs-Ringer phosphate buffer were added to a 0.1 ml solution of [^{14}C]squalene-2,3-oxide (30,000 cpm) in Me_2SO and were incubated with shaking in siliconized tubes at 37°C. At various intervals the cells were centrifuged down, the medium removed, the cells washed with fresh incubation buffer, and the radioactivity of the cells (●) and of the medium (○) was then determined.

tailed in the notes to **Fig. 5.** The data of that figure show that the lymphocytes and monocytes converted not only the [^{3}H]squalene-2,3-oxide into lanosterol and, to a lesser extent, into cholesterol, but also [2-^{14}C]-mevalonate into lanosterol and squalene without the intermediary accumulation of squalene-2,3-oxide. There was a just detectable amount of [^{14}C]farnesol

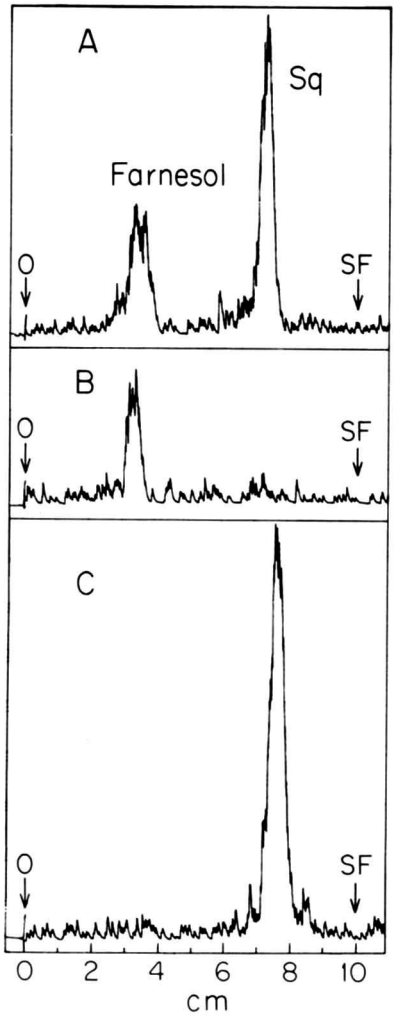

Fig. 4. Localization of radioactive products synthesized by granulocytes from *RS*-[2-^{14}C]mevalonate. Granulocytes (3.0×10^7) were incubated with *RS*-[2-^{14}C]mevalonic acid DBED salt (100,000 cpm) for 2 hr as described in Materials and Methods. From one half of the incubation, the products were extracted from the medium and cells together and analyzed on $AgNO_3$-impregnated TLC plates as described in Materials and Methods (Panel A). The other half was centrifuged at 300 *g* for 10 min at 25°C. The supernatant was removed and the cells were washed three times with 1 ml of the incubation buffer. The washes were combined with the supernatant, extracted, and analyzed on $AgNO_3$–TLC plates as described in Materials and Methods (Panel B). The cells were extracted and analyzed on TLC plates as described in Materials and Methods (Panel C). The faster-moving radioactive peak migrated with authentic squalene (Sq) and the slower-moving peak migrated with authentic *trans*-farnesol. (Authentic squalene and authentic *trans*-farnesol were included as internal standards on each TLC plate.)

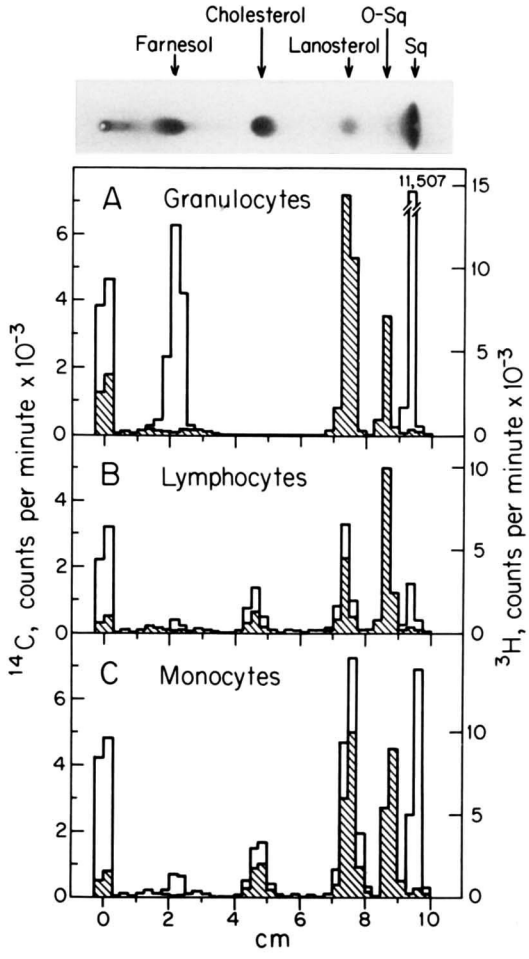

Fig. 5. The incorporation of [2-^{14}C]mevalonate and [^{3}H]squalene-2,3-oxide by granulocytes, lymphocytes, and monocytes. Cells were incubated with *RS*-[2-^{14}C]mevalonic acid DBED salt (1.9×10^6 cpm) and [^{3}H]squalene-2,3-oxide (20,000 cpm) for 1 hr at 37°C in the presence of 10% Me_2SO as described in Materials and Methods. Products were extracted and chromatographed on $AgNO_3$–TLC plates and 2.5 mm zones of the gel were scraped off and counted for [^{14}C] (▭) and [^{3}H] (▨). The position of authentic standards of farnesol, cholesterol, lanosterol, squalene-2,3-oxide (O-Sq), and squalene (Sq) are shown at the top. Panel A represents the radioactive products obtained from 3.01×10^7 granulocytes. Panel B represents the radioactive products obtained from 1.56×10^7 lymphocytes. Panel C represents the radioactive products obtained from 1.11×10^7 monocytes.

in the incubations of the lymphocytes and monocytes. In contrast, the granulocytes provided only squalene and free farnesol from [2-^{14}C]mevalonate and [^{3}H]-lanosterol from [^{3}H]squalene-2,3-oxide. The nature of the ^{14}C- and ^{3}H-labeled substance(s) remaining at the origin of the chromatograms shown in Fig. 5 is not known.

DISCUSSION

Our main observations explain why human polymorphonuclear leukocytes (granulocytes) do not syn-

thesize sterols. They do not contain, apparently, the mixed function oxidase needed for the formation of squalene-2,3-oxide from squalene even though they contain the enzyme that cyclizes the oxide to lanosterol. Astruc et al. (10) demonstrated that squalene-2,3-oxide:lanosterol cyclase activity was much greater than that of the squalene epoxidase activity in non-cholesterogenic rat tissues and in human placental tissue. Because they studied microsomal activities, they were unable to decide whether this difference in activities was due to an artifact of their preparations, or was representative of true differences in the intact tissues which would indicate that squalene epoxidation in vivo might be a rate limiting step in cholesterol biosynthesis. Our results support the latter conclusion. A further deficiency of the granulocytes, which might also be attributed to a deficiency of some other mixed function oxidase(s), is their inability to convert lanosterol into cholesterol. These deficiencies are not apparent in lymphocytes and monocytes even though their ability to convert lanosterol into cholesterol is limited (cf. Fig. 5). The lack of enzyme activities sufficient to convert lanosterol to cholesterol has previously been reported in platelets, reticulocytes, buffy coat (mixed leukocytes), bone marrow, saphenous vein, coronary artery, and aorta (11). However, the failure of such enzyme activities in these preparations may have been due to technical artifacts, since it has subsequently been established that mixed leukocytes (1, 12) can synthesize cholesterol from acetate or mevalonate. Our results cannot be attributed to an artifact since the monocytes and lymphocytes which were treated identically to the granulocytes were able to synthesize cholesterol (Fig. 5). Moreover, we have previously presented evidence to suggest that 3-hydroxy-3-methylglutaryl coenzyme A reductase activity is preserved in pure granulocytes despite their inability to synthesize sterols (2). The presence of rate limiting steps beyond 3-hydroxy-3-methylglutaryl coenzyme A reductase has been demonstrated previously in mixed leukocytes (1, 12), but this is the first demonstration that the accumulation of farnesol and squalene, which had been seen in such preparations, was probably due to the lack of squalene epoxidase activity in the granulocytes.

The presentation of a water-insoluble substrate to any enzyme system, let alone to intact cells, is a major technical problem in research on lipids. Our experience with Me_2SO commends its use where detergents fail.

Our observations as to the inability of granulocytes to synthesize sterols are at variance with the data of Loveless and Slater (13) who claimed that granulocytes could synthesize sterols albeit only at a rate one-fourth the rate of mononuclear cells (13). Examination of the data of Loveless and Slater (13) and their methods of preparation of the fractions of leukocytes forces us to conclude that their finding of sterol synthesis by granulocytes could be accounted for by contamination of the granulocyte preparations by as little as 5% of monocytes. In our experience the failure of sterol synthesis from either acetate or mevalonate can be demonstrated only in granulocyte preparations which are more than 99% pure. Our results in this respect agree with those of Young and Rodwell (14).

Kandutsch, Chen, and Heiniger (15) have demonstrated that de novo sterol synthesis is required for and precedes DNA synthesis and cell division by phytohemagglutinin-stimulated lymphoid cells in culture. It is attractive to speculate whether the inability of the mature human granulocytes to divide and their short life (1 to 2 days) may not be related to their defect in sterol synthesis.

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