Separation of and cholesterol synthesis by human lymphocytes and monocytes

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Abstract We have devised techniques for the isolation of human monocytes which do not require the adherence of the cells to a surface. In 15 consecutive experiments using density-gradient and counterflow centrifugations, a population of mononuclear cells that was 75 ± 11% monocytes was obtained within 2 hours of venipuncture. These cells had never been pelleted and represented approximately three-fourths of the monocytes that had been present in the whole blood. In another 22 consecutive experiments using sedimentation in gelatin followed by counterflow and density-gradient centrifugations, a population of lymphocytes that was 99.5 ± 0.5% pure and a population of monocytes that was 94 ± 3% pure were obtained within 3 hours of venipuncture. When these freshly isolated cells were incubated in the lipoprotein-deficient fraction of serum (d > 1.21 g/ml) or in solvent-extracted serum, the monocytes incorporated 10–20 times more [2-^{14}C]acetate into sterols than did the lymphocytes. Monocytes were seen to constitute between 6 and 46% of the mononuclear cells isolated from normal individuals by the usual density-gradient centrifugation of whole blood on Ficoll–Hypaque. We conclude that future studies of cholesterol metabolism utilizing human mononuclear cells must take into account this large variation in the percentage of monocytes and their disproportionately greater activity during short-term incubations in media that induce sterol synthesis.—**Fogelman, A. M., J. Seager, M. Hokom, and P. A. Edwards.** Separation of and cholesterol synthesis by human lymphocytes and monocytes. *J. Lipid Res.* 1979. **20:** 379–388.

Supplementary key words leukocytes · density-gradient centrifugation · counterflow centrifugation

We previously utilized mixed populations of leukocytes to study cholesterol metabolism in humans (1–3). In an effort to determine the relative contributions of the individual cell types, we separated human leukocytes into three fractions using counterflow centrifugation (4). The first fraction contained virtually pure lymphocytes. The second fraction was enriched in monocytes but the majority of the cells in this fraction were granulocytes. The third fraction contained virtually pure granulocytes. It was surprising to find that the granulocytes in both the second and third fractions were unable to synthesize sterols from either acetate or mevalonate. This finding allowed us to set up simultaneous equations for the purpose of comparing the activities of the lymphocytes and monocytes. The solutions to these equations revealed that during the fifth to seventh hours of incubation, the monocytes incorporated approximately five times more [2-^{14}C]acetate into sterols than did the lymphocytes in full serum medium and approximately 20 times more than the lymphocytes in lipid-depleted medium. The monocytes also incorporated approximately 20 times more [2-^{14}C]mevalonate into sterols than did the lymphocytes. Subsequently, Ho et al. (5) reported that they found no difference in the ability of human monocytes and lymphocytes to utilize radioactive acetate for sterol biosynthesis. More recently, Bilheimer et al. (6) reported that when mononuclear cells prepared by density-gradient centrifugation were cultured in the lipoprotein-deficient fraction of serum for 57 hr, the adherent cells (70% of which were macrophages) incorporated only about one fifth as much [^{14}C]acetate into sterols as did the nonadherent cells which were mainly lymphocytes (see Table II in reference 6).

We have now developed new techniques for the rapid separation of human lymphocytes and monocytes that do not rely on the adherence of the cells to a surface and hence should avoid the substantial changes in cellular metabolism that are known to occur within 2 hr of adherence (7, 8). These methods consistently produced populations of cells with purities in excess and recoveries comparable to or in excess of those previously reported (9–13). These techniques have made it possible to directly compare the incorporation of acetate into sterols in lymphocytes and monocytes during the initial hours after their isolation. Our present results completely confirm our earlier conclusions and demonstrate unequivocally the greater activity of the monocytes in short-term incubations in media that induce sterol synthesis.

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TABLE 1. Summary of clinical and biochemical characteristics of normal subjects

Sex	Number of Subjects	Age	Serum Cholesterol Concentration	Serum Triglyceride Concentration
		yr	*mg/dl*	*mg/dl*
Female	10	29 ± 8[a] (21–51)[b]	191 ± 35 (143–250)	57 ± 24 (29–102)
Male	36	30 ± 8 (18–57)	195 ± 34 (125–257)	72 ± 29 (30–147)

[a] Mean ± 1 SD.
[b] Range.

METHODS

Materials

[2-^{14}C]Acetic acid, sodium salt (59 Ci/mol) was purchased from Amersham/Searle Corp. (Arlington Heights, IL). Plasmagel was purchased from the HTI Corporation (Buffalo, NY); Hypaque sodium 50% (w/v) (sodium diatrizoate injection USP = sodium 3,5-diacetamido-2,4,6-triiodobenzoate) was purchased from Winthrop Laboratories (New York, NY); Ficoll and bovine serum albumin (Fraction V) were obtained from Sigma Chemical Co. (St. Louis, MO). Heparin USP 1000 units/ml was supplied by the UCLA Pharmacy. Bacto-Latex particles, 0.81 μm in diameter, were purchased from Difco Laboratories (Detroit, MI). Cultures of *Candida albicans* were a gift from Dr. R. I. Lehrer, Department of Medicine, UCLA. Other supplies and chemicals were obtained from sources previously reported (2, 3).

Subjects

Forty-six normal subjects and one heterozygous familial hypercholesterolemic male participated in these studies. The normal subjects were students or staff at UCLA and their pertinent clinical and biochemical characteristics are presented in **Table 1.** The clinical and biochemical details of the heterozygote have previously been reported (14). No one received drugs that might have affected serum cholesterol levels or lipid metabolism. All had normal hematocrits, white blood cell and differential counts, and normal serum values on a multiphasic screening panel that included calcium, phosphorus, glucose, blood urea nitrogen, uric acid, total protein, albumin, total bilirubin, alkaline phosphatase, lactic dehydrogenase, and glutamic oxalacetic transaminase activity. Informed consent was obtained in writing from each person.

Collection of blood

After a 12–14 hr fast, 120 ml of blood was drawn aseptically from a cubital vein using a siliconized needle and plastic syringes as previously described (2). The blood was anticoagulated with heparin (5 units/ml) in all experiments unless otherwise stated.

Separation of cells by method A

Ficoll–Hypaque was prepared by dissolving 11.8 g of Ficoll in 150 ml of warm water and Hypaque (50%, w/v) was added (about 36 ml) to give a density of 1.080 g/ml. The Ficoll–Hypaque and all other solutions were sterilized through a 0.22 μm millipore filter. The heparinized blood was mixed with half its volume of phosphate-buffer saline and each 25 ml was underlayered with 15 ml of the Ficoll–Hypaque solution in 40-ml siliconized tubes and centrifuged at 400 *g* for 40 min at 18°C as described by Boyum (15). The clear plasma layer above the interface was aspirated and discarded. Most of the cells in the band at the plasma Ficoll–Hypaque interface were harvested with a 10-ml plastic pipet and the remaining cells at the interface were collected with a siliconized Pasteur pipet. The total volume containing the mononuclear cells and platelets from all of the tubes was 65–75 ml. This volume was slowly injected into the 70-ml loading chamber of a Beckman Elutriator System which consisted of a Beckman JE-6 rotor in a Beckman J21B high speed refrigerated centrifuge equipped with a strobe unit which illuminated the separation chamber within the rotor and allowed precise measurement of the rotor speed. Flow through the system was controlled with a Cole Parmer 7545 Masterflex pump and 7013 pump head which was turned off during the addition of the cells to the loading chamber. The rotor temperature was 15°C and the rotor speed during the loading procedure was 4000 rpm in the first six experiments, but in later experiments this was reduced to 3740 rpm. The cells were loaded into the rotor with 200 ml of ice-cold Krebs–Ringer phosphate buffer, pH 7.4, containing 15 mM glucose and 1% bovine serum albumin (buffer A) at an initial flow rate of 3.4 ml/min. After 10 min flow was increased to 5.0 ml/min. The loading chamber was clamped off and the rotor speed was decreased to 2030 rpm over a 4-min period and, after an additional 5 min, the flow rate was increased to 8.8 ml/min and the first 40-ml fraction was collected. Fractions 2, 3, 4, and 5 (40 ml each) were collected at 10.2, 11.5, 12.5, and 13.4 ml/min, respectively. The pump and the rotor were then stopped, the cells were removed from the separation chamber and studied directly or incubated after aliquots were taken for cell counts, morphologic, and cytochemical examination.

Separation of cells by method B

Thirty-ml aliquots of the heparinized blood were mixed with 15 ml of gelatin (Plasmagel) in 60-ml plastic syringes and allowed to settle at room temperature

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for 30 min as described previously (2). The supernatants were centrifuged at 400 *g* for 10 min at 5°C. The cells were resuspended in 25 ml of buffer A and injected into the 30-ml loading chamber with the pump off and with the rotor speed and temperature at 2030 rpm and 15°C, respectively. The cells were loaded into the rotor with 120 ml of ice-cold buffer A at a flow rate of 7.6 ml/min. The loading chamber was clamped off and the first 40-ml fraction was collected at 8.8 ml/min. Fractions 2, 3, 4, 5, 6, and 7 (40 ml each) were collected at 10.2, 11.5, 12.5, 13.4, 14.6, and 15.8 ml/min, respectively. Repeated analysis of the cells contained in these fractions revealed that fractions 2 and 3 contained the purest populations of lymphocytes and fractions 6 and 7 contained the purest populations of monocytes. The cells remaining in the separation chamber were found to be mainly granulocytes. Fractions 2 and 3 were combined and 6 and 7 were combined after centrifugation at 400 *g* for 10 min and each new combination was resuspended in 25 ml of buffer A, underlayered with 15 ml of Ficoll–Hypaque (d = 1.080 g/ml), and centrifuged at 400 *g* for 40 min at 18°C. The cells at the interface were removed with siliconized Pasteur pipets and washed three times with centrifugation in 40 ml of buffer A, resuspended in buffer A, and studied directly or incubated after aliquots were taken for cell counts, morphologic, and cytochemical examination.

Classification and viability of cells

The cells were classified by their morphology on Wright-stained smears, reaction with α-naphthyl butyrate esterase (lipase) stain (16), ability to ingest latex particles (17), and ability to ingest heat-killed *Candida albicans* (18). Smears for morphologic and cytochemical examination were prepared with a Shandon–Southern cytospin centrifuge (Model SCA-0031, Shandon Southern Products, Ltd., Runcorn, Cheshire, England), and a minimum of 1000 cells were counted for each determination. Cell counts were performed by standard techniques on six separate aliquots from each sample. The lymphocytes were classified in Dr. John L. Fahey's laboratory according to the percent lymphocytes forming rosettes with sheep red blood cells, E rosettes (19); the percent forming rosettes with sheep red blood cells coated with rabbit anti-sheep immunoglobulin, EA rosettes (20); and the percent with surface membrane immunoglobulin (21). The reaction of the lymphocytes to phytohemagglutinin was determined as described by Elhilali et al. (19). The ability of the cells to exclude supravital stains was determined as described previously (2).

Incubation of cells

The cells were incubated in duplicate in 10-ml stoppered siliconized flasks under 95% O_2–5% CO_2 at 37°C as previously described (2). The flasks contained buffer A fortified with 100 units of penicillin/ml, 100 μg of streptomycin/ml, 0.33 mM sodium acetate, and, in some experiments, 40% serum (36 mg protein/ml), full or lipid-depleted; in other experiments the lipoprotein-deficient fraction of serum (d > 1.21 g/ml) was used (17.6 mg protein/ml). Sodium acetate was used in a concentration 0.33 mM when it was found that identical results were obtained with 0.66 mM or 1.32 mM. The incorporation of [2-^{14}C]acetate into sterols was found to be linear with respect to cell number in incubations containing 1 to 10 × 10^6 cells/ml. We routinely incubated 2 to 4 × 10^6 cells/ml in a total volume of 2 ml. Duplicate flasks without cells were included as controls for the experiments utilizing [2-^{14}C]acetate.

Preparation of sera and the lipoprotein-deficient fraction of serum

Human AB-negative, complement inactivated serum (full serum), solvent-extracted serum (lipid-depleted serum), and the lipoprotein-deficient fraction of serum (d > 1.21 g/ml) were prepared as previously described (2, 3).

Determination of radioactive sterols

The flasks were removed from the incubator, immediately placed in ice, and ethanol and carrier cholesterol (1 mg/ml) were added to the whole incubation mixture before saponification with 2 N KOH in 50% ethanol as described previously (2). The nonsaponifiable fraction was extracted with petroleum ether and the digitonin-precipitable sterols were determined as previously described (2). No radioactivity was present in the digitonin-precipitable sterol fractions from the control flasks incubated without cells.

Determination of protein

Cells prepared by Method B were washed successively in 40 ml, 20 ml, and 10 ml of phosphate-buffered saline in siliconized tubes which were centrifuged at 400 *g* for 10 min at 5°C. After the third wash the cell pellets were resuspended in 0.3 ml of water. The protein content of all specimens was determined by the method of Lowry et al. (22).

Serum cholesterol and triglyceride determinations

The serum cholesterol concentration was determined by the method of Allain et al. (23) and the serum triglyceride concentration was determined by the method of Eggstein and Kuhlmann (24).

Statistical analysis

Statistical analysis was carried out as previously described (25) using a Hewlett-Packard 97 calculator and programs supplied in the Hewlett-Packard Stat Pac I.

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TABLE 2. Separation of monocytes by density-gradient and counterflow centrifugations (Method A), and separation of lymphocytes and monocytes by sedimentation in gelatin followed by counterflow and density-gradient centrifugations (Method B)

Method	Fraction	Number of Cells Obtained	Composition of Cells		
			Granulocytes	Lymphocytes	Monocytes
		$\times 10^6$	%	%	%
A[a]	Separation chamber	57 ± 20[b] (39–84)[c]	0.5 ± 0.6 (0–1)	36 ± 7 (28–48)	64 ± 7[d] (51–71)
A[e]	Separation chamber	50 ± 21 (24–80)	1 ± 2 (0–4)	17 ± 7 (6–30)	82 ± 6[d] (70–93)
B[f]	Lymphocyte	59 ± 26 (10–115)	0.1 ± 0.1 (0–0.4)	99.5 ± 0.5 (98–100)	0.4 ± 0.5 (0–2)
B[f]	Monocyte	24 ± 8 (10–38)	2 ± 1 (0–4)	4 ± 2 (0–9)	94 ± 3 (89–100)

[a] In six consecutive experiments the cells were prepared by Method A as described in Methods utilizing an initial rotor speed of 4000 rpm.
[b] Mean ± 1 SD.
[c] Range.
[d] $P < 0.001$.
[e] In nine consecutive experiments the cells were prepared by Method A as described in Methods utilizing an initial rotor speed of 3740 rpm.
[f] These data represent the results of 22 consecutive experiments in which the cells were prepared by Method B as described in Methods.

RESULTS

Separation of cells

The monocyte content of the mononuclear cells produced by density-gradient centrifugation of whole blood using Ficoll–Hypaque in 31 experiments was 20 ± 10% (mean ± SD, range 6–46%) and the recovery of the monocytes from whole blood was essentially complete. As shown in **Table 2,** density-gradient centrifugation of whole blood followed by counterflow centrifugation (Method A) produced a population of cells that averaged 75 ± 11% monocytes in 15 consecutive experiments. When the initial rotor speed was 4000 rpm, the cells in the separation chamber were 64 ± 7% monocytes in the first six consecutive experiments. When the initial rotor speed was decreased to 3740 rpm in the next nine consecutive experiments, the monocytes increased to 82 ± 6% ($P < 0.001$). In addition to the increased purity, there was a slight increase in the recovery of the cells (69 ± 16% vs. 75 ± 21%, mean ± SD) which was not statistically significant. Method A had the advantage of yielding cells that had never been pelleted. The cells were simply removed from the separation chamber and placed directly into the incubation flasks. The time required from venipuncture to the point of incubating the cells was approximately 2 hr. There were no platelets and virtually no red blood cells seen in these preparations.

As shown in Table 2, the separation of lymphocytes and monocytes in 22 consecutive experiments utilizing the sedimentation of whole blood in gelatin followed by counterflow and density-gradient centrifugations (Method B) produced a population of lymphocytes that was 99.5 ± 0.5% (mean ± SD) pure and a population of monocytes that was 94 ± 3% pure. There were no platelets and virtually no red blood cells seen in these preparations. The recovery of lymphocytes and monocytes from the gelatin supernatant was 52 ± 18% (mean ± SD) and 47 ± 12% (mean ± SD), respectively. However, the gelatin supernatant contained only 48 ± 12% (mean ± SD; range 26–64%) of the lymphocytes that had been present in the whole blood, while it contained 95 ± 12% (mean ± SD; range 69–104%) of the monocytes that had been present in whole blood. Hence, the overall recovery of lymphocytes from whole blood was approximately 25% while the overall recovery of monocytes from whole blood was approximately 45%.

In order to determine if the pure lymphocytes obtained by Method B were representative of the lymphocytes present in whole blood, the lymphocytes obtained in six experiments using Method B were classified into subclasses and were found not to be significantly different from those prepared from whole blood on Ficoll–Hypaque gradients. Specifically, the percent lymphocytes forming rosettes with sheep red blood cells (E rosettes, 64 ± 8% vs. 66 ± 10%, mean ± SD), the percent forming rosettes with sheep red blood cells coated with rabbit anti-sheep immunoglobulin (EA rosettes, 10 ± 5% vs. 16 ± 9%, mean ± SD), and

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the percent with surface membrane immunoglobulin (12 ± 7% vs. 10 ± 6%, mean ± SD) were not significantly different. The time required from venipuncture to the point of incubating the cells was approximately 3 hr with Method B.

Viability of cells

More than 99% of the cells separated by Methods A and B excluded supravital stains after incubation, and 95 ± 1% (mean ± SD; range 94–96%) and 94 ± 4% (mean ± SD; range 90–98%) of the cells classified as monocytes on the basis of morphology and reaction with the α-naphthyl butyrate esterase stain, phagocytized latex particles and heat-killed *Candida albicans*, respectively. The lymphocytes exhibited a normal reaction to phytohemagglutinin.

Time course of the incorporation of [2-^{14}C]acetate into sterols

We have shown previously that the incubation of leukocytes in buffer alone, devoid of lipoproteins, did not lead to an increase in sterol synthesis, but incubation of these cells in solvent-extracted serum (lipid-depleted medium), abetalipoproteinemic serum, or the lipoprotein-deficient fraction of serum (d > 1.21 g/ml) led to sterol loss from the cells and an increase in the rate of sterol synthesis from acetate (3). In these earlier experiments we added [2-^{14}C]acetate during the 5th to 7th hr of incubation (3, 4). The experiments shown in **Fig. 1** reveal that a substantial increase in the rate of sterol synthesis occurred after the 3rd hr of incubation in the lipid-depleted medium. We concluded from these data that we could utilize an earlier and hence more convenient time for the addition of the [2-^{14}C]acetate. Therefore, in most of our subsequent experiments we studied sterol synthesis between the 3rd to 5th hr of incubation.

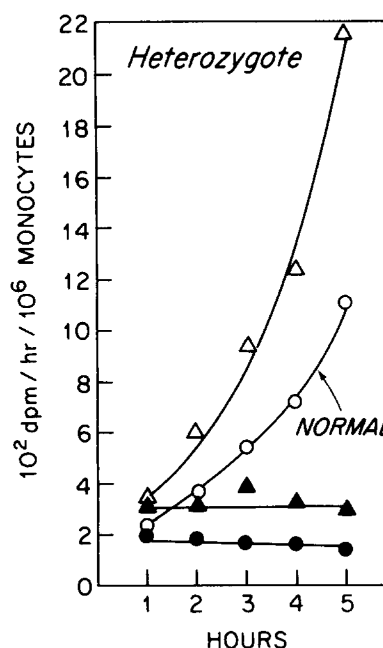

Fig. 1. Time course of the incorporation of [2-^{14}C]acetate into sterols. Monocytes were prepared by Method A as described in Methods from a heterozygote familial hypercholesterolemic male (triangles) and an age, race, and sex matched normal subject (circles). The cells were incubated in duplicate in full serum medium (closed symbols) or lipid-depleted medium (open symbols) as described in Methods. Each time point represents the midpoint of a 2-hr period during which 19.2 μCi of [2-^{14}C]acetate (30 Ci/mol) was present. At the conclusion of each 2-hr period the incubation mixture was saponified and analyzed for radioactive sterols as described in Methods.

Sterol synthesis in cells prepared with and without counterflow centrifugation

In order to determine the effects of counterflow centrifugation on sterol synthesis, we prepared mononuclear cells by density-gradient centrifugation from five normal subjects and then washed half of each person's cells with and without counterflow centrifugation (**Table 3**). The monocyte content of the cells washed with counterflow centrifugation was slightly but significantly higher than that of the cells washed without counterflow centrifugation (30 ± 6%, mean ± SD, range 20–37% vs. 27 ± 7%, mean ± SD, range 16–33%; $P < 0.05$). When the cells were incubated in full serum medium there was no difference in the incorporation of [2-^{14}C]acetate into sterols whether the cells had been subjected to counterflow centrifugation or not (145 ± 38 vs. 163 ± 42 dpm/hr per 10^6 monocytes). However, in the lipid-depleted medium the cells washed by counterflow centrifugation incorporated slightly more [2-^{14}C]acetate into sterols than did the cells washed without counterflow centrifugation (794 ± 154 vs. 673 ± 195 dpm/hr per 10^6 monocytes; $P < 0.01$).

The incorporation of [2-^{14}C]acetate into sterols by mononuclear cells as a function of the percent monocytes and lymphocytes

We next proposed the following hypothesis. If the activity of lymphocytes and monocytes were equal with respect to the incorporation of [2-^{14}C]acetate into sterols, then populations of mononuclear cells containing various proportions of monocytes should have had the same activity when the results were expressed as dpm/hr per 10^6 cells. On the other hand, if our previous conclusions were correct (4), then the activity of the mononuclear cells should have increased as the percent monocytes increased. In order to test this hypothesis, we bled 12 normal subjects and isolated their mononuclear cells from whole blood by density-gradient centrifugation. These cells were then injected

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TABLE 3. Incorporation of [2-^{14}C]acetate into sterols by cells washed with or without counterflow centrifugation

With Counterflow		Without Counterflow	
Full Serum Medium	Lipid-Depleted Medium	Full Serum Medium	Lipid-Depleted Medium
dpm/hour/10^6 mononuclear cells			
42 ± 7[a] (34–54)[b]	241 ± 77[c] (164–350)	43 ± 9 (35–56)	182 ± 66[c] (118–278)
dpm/hour/10^6 monocytes			
145 ± 38 (113–210)	794 ± 154[d] (616–1029)	163 ± 42 (123–229)	673 ± 195[d] (442–960)

Blood was drawn from five normal individuals and anticoagulated with sodium citrate (final concentration 0.35%) and centrifuged at 150 *g* for 15 min at 15°C. The platelet-rich plasma was removed and centrifuged at 3000 *g* for 15 min to remove the platelets.[e] The platelet-poor plasma was returned to the cells and one-half volume of phosphate-buffered saline was added. The diluted platelet-poor blood was underlayered with Ficoll–Hypaque and centrifuged at 400 *g* for 40 min at 18°C. The mononuclear bands were removed and half of the cells from each band were injected into the loading chamber of the counterflow centrifuge. These cells were processed according to Method A as described in Methods, except that the pump and rotor were stopped just before the collection of the first fraction and the cells were removed from the separation chamber. The other half of the cells from each mononuclear band were diluted (1:8, v/v) with buffer A and centrifuged at 400 *g* for 10 min at 5°C in 40-ml siliconized tubes. The supernatants were decanted and the cells from the various tubes were recombined into one tube, diluted to 40 ml with buffer A and centrifuged at 400 *g* for 10 min. The cells were washed twice more in 40 ml of buffer A and then these cells and those washed by counterflow centrifugation were incubated in duplicate in full serum medium or lipid-depleted medium as described in Methods. After 3 hr, 19.2 μCi of [2-^{14}C]acetate (30 Ci/mol) was added. The incubation mixture was saponified 2 hr later and analyzed for radioactive sterols as described in Methods.

[a] Mean ± 1 SD.
[b] Range.
[c] $P < 0.01$.
[d] $P < 0.01$.
[e] The platelets were removed prior to the washing of the cells, otherwise they would have been promptly removed from the cells washed with counterflow centrifugation but not from those washed without counterflow centrifugation.

into the counterflow centrifuge and fractionated so that there were subpopulations of mononuclear cells that contained virtually no monocytes and subpopulations with increasing proportions of monocytes. The sterol synthesizing ability of each fraction was determined and the results are shown in **Fig. 2.** The activity increased linearly as the percent monocytes increased ($r = 0.91$; $P < 0.0005$).

Sterol synthesis in the lipoprotein-deficient fraction of serum

The experiments described thus far utilized the lipid-depleted medium to induce an increase in the rate of sterol synthesis from acetate. Ho et al. (5) and Bilheimer et al. (6) utilized the lipoprotein-deficient fraction of serum for this purpose and found no difference in sterol synthesis between lymphocytes and monocytes (5) or found the lymphocytes (nonadherent cells) to be five times more active than the monocytes (adherent cells) (6). In order to test whether the greater activity of the monocytes in the present series of experiments might have been due to the different incubation media, we prepared lymphocytes and monocytes from eight normal subjects and incubated them in the lipoprotein-deficient fraction of serum (**Table 4**). The monocytes incorporated approximately 10 times more [2-^{14}C]acetate into sterols than did the lymphocytes (283 ± 78 vs. 27 ± 7 dpm/hr per 10^6 cells; $P < 0.001$).

Since Ho et al. (5) and Bilheimer et al. (6) calculated their data in terms of the activity per mg of protein, we determined the protein content of the cells and recalculated the data in order to obtain the activity per mg of protein. The monocytes contained twice as much protein as did the lymphocytes (61 ± 7 μg

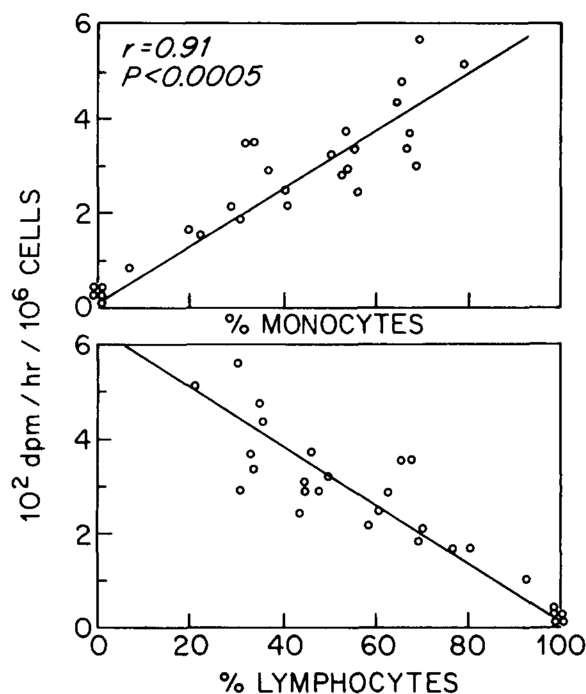

Fig. 2. The incorporation of [2-^{14}C]acetate into sterols by mononuclear cells as a function of the percent monocytes and lymphocytes. Mononuclear cells from 12 normal subjects were prepared by density-gradient centrifugation, injected into the counterflow centrifuge, and processed as described for Method A in Methods. The cells were eluted from the rotor into 40-ml siliconized tubes as the flow rate was increased as described in Methods. The tubes were centrifuged at 400 *g* for 10 min at 5°C. The supernatants were decanted, the cells resuspended, aliquots taken for cell count and morphologic and cytochemical examination, and the cells were incubated in duplicate in the lipid-depleted medium as described in Methods. After 3 hr 19.2 μCi of [2-^{14}C]acetate (30 Ci/mol) was added. Two hours later the incubation mixture was saponified and analyzed for radioactive sterols as described in Methods.

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protein/10^6 cells, mean ± SD; range 51–75 μg protein/10^6 cells vs. 32 ± 4 μg protein/10^6 cells, mean ± SD; range 26–37 μg protein/10^6 cells). However, as shown in Table 4, the lymphocyte activity was only 20% of that of the monocytes (0.9 ± 0.3 vs. 4.7 ± 1 × 10^3 dpm/hr per mg protein; $P < 0.001$). We conclude from these data that the different results of Ho et al. (5) and Bilheimer et al. (6) to those reported here are probably not due to differences in the incubation media or the method of calculating data.

Sterol synthesis in buffer vs. lipid-depleted medium

Lymphocytes and monocytes were prepared by Method B as described in Methods from four normal subjects. The incorporation of [2-^{14}C]acetate into sterols was measured in duplicate between 0 and 1 hr by adding 19.2 μCi of [2-^{14}C]acetate (59 Ci/mol) at the start of the incubation to cells in buffer A (a medium that previously had been shown not to alter the rate of sterol synthesis from acetate [3]) or by adding 19.2 μCi of [2-^{14}C]acetate (59 Ci/mol) after 5.5 hr of incubation to duplicate flasks containing cells in lipid-depleted medium. After a 1-hr incubation with the [2-^{14}C] acetate, the incubation mixture was saponified and analyzed for radioactive sterols as described in Methods. The values obtained for the cells incubated in buffer A were 50 ± 33 and 158 ± 40 dpm/hr per 10^6 cells for lymphocytes and monocytes, respectively ($P < 0.05$). The rate of incorporation of [2-^{14}C]acetate into sterols in the lipid-depleted medium with lymphocytes and monocytes increased to 73 ± 46 and 1643 ± 743 dpm/hr per 10^6 cells, respectively ($P < 0.05$), during the period of 5.5–6.5 hr.

TABLE 4. Incorporation of [2-^{14}C]acetate into sterols by lymphocytes and monocytes incubated in the lipoprotein-deficient fraction of serum

Lymphocytes		Monocytes	
dpm/hr/10^6 cells	*10^3 dpm/hr/mg protein*	*dpm/hr/10^6 cells*	*10^3 dpm/hr/mg protein*
27 ± 7[a,c] (16–38)[b]	0.9 ± 0.3[d] (0.4–1.3)	283 ± 78[c] (158–415)	4.7 ± 1[d] (2.7–6.9)

Lymphocytes and monocytes from eight normal subjects were prepared by Method B and the cells were incubated in duplicate in the lipoprotein-deficient fraction of serum (17.6 mg protein/ml) as described in Methods. After 3 hr, 19.2 μCi of [2-^{14}C]acetate (30 Ci/mol) was added. The incubation mixture was saponified 2 hr later and analyzed for radioactive sterols as described in Methods.
[a] Mean ± 1 SD.
[b] Range.
[c] $P < 0.001$.
[d] $P < 0.001$.

DISCUSSION

The usual technique for obtaining human monocytes involves the separation of mononuclear cells from blood by density-gradient centrifugation followed by adherence of the monocytes to glass or plastic substrates (26). Recent evidence from two laboratories suggests that substantial alterations in monocyte metabolism occur within hours after human monocytes are allowed to adhere to a surface. Bodel, Nichols, and Bainton (7) found that as early as 2 hr after human monocytes adhered to a surface, they developed peroxidase activity in the perinuclear cisterna and rough endoplasmic reticulum. Van Ginkel et al. (8) found that human monocytes developed procoagulant activity within 1 hr after they adhered to glass. This activity was not present initially and could be prevented by incubating the cells on a surface to which they could not adhere. The separation techniques described in this paper should avoid the alterations in monocyte metabolism that occur as a consequence of adherence.

Other investigators have also tried to avoid the necessity of employing adherence for the isolation of human monocytes. Loos et al. (9) prepared mononuclear cells on Ficoll–Isopaque gradients, resuspended the cells in a medium containing AB serum, and incubated the cells at 37°C for 30 min. The lymphocytes and monocytes were then separated on discontinuous density gradients containing Ficoll–Isopaque and human serum albumin. The purity of their monocytes averaged 75% and 82% from defibrinated blood and anticoagulated blood, respectively, and the recoveries averaged 34% and 28%, respectively.

Nathanson et al. (10) prepared mononuclear cells on Ficoll–Hypaque gradients and then separated the lymphocytes and monocytes by isopycnic centrifugation on discontinuous gradients of colloidal silica polyvinylpyrrolidinone. The purity of the monocytes averaged 82% and the recovery averaged 66%. The major disadvantage of this method was the inherent toxicity of colloidal silica which was taken up into secondary lysosomes. However, polyvinylpyrrolidinone was also taken up into secondary lysosomes and may have neutralized the effects of the colloidal silica.

Barr, Whang-Peng, and Perry (11) prepared leukocyte concentrates by sedimentation of whole blood in dextrose–dextran and mononuclear cell concentrates were then prepared by density-gradient centrifugation on Ficoll–Hypaque. The cells were allowed to react with sheep erythrocytes and those forming rosettes were removed by further Ficoll–Hypaque sedimentation. This step was repeated. The remaining cells were allowed to react with sheep erythrocytes coated with rabbit anti-sheep immunoglobulin and those cells forming rosettes were removed by density sedimenta-

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tion in Ficoll–Hypaque. The remaining cells were then subjected to velocity sedimentation which yielded a population of monocytes that was on the average 78% pure.

Johnson, Mei, and Cohn (12) prepared leukocyte-rich plasma by sedimentation of whole blood with dextran and then separated the monocytes in a continuous albumin gradient. The purest monocyte fraction contained 61% monocytes and 74% of the monocytes were recovered from the albumin gradient.

Sanderson et al. (13) utilized density-gradient centrifugation on Ficoll–Hypaque to separate the mononuclear cells from 10 ml of blood taken from each of six normal subjects. The mononuclear cells were washed by centrifugation, resuspended, and then injected into their counterflow centrifuge which contained a specially built separation chamber (27). The cells were sequentially washed from the chamber and monitored with a Coulter counter connected to a pulse height analyzer for measuring cell volumes and an xy-recorder. This system enabled the centrifuge operator to determine when a given cell fraction had been completely eluted. They obtained a monocyte purity of 90.4 ± 1.6% with a range of 86.5–97.6% and with a recovery of 40–50% in the six experiments.

Method A as described in this paper was similar to the technique of Sanderson et al. (13), but differed in that the mononuclear band from the Ficoll–Hypaque gradient was injected directly into the system without prior washing and the monocytes were simply harvested from the separation chamber. The purity in the experiments utilizing an initial rotor speed of 3740 rpm averaged 82% and the recovery averaged 75% (Table 2). Method B produced a population of monocytes with purities in excess of those obtained by Sanderson et al. (94 ± 3%, range 89–100%) and with a comparable recovery (Table 2). A major advantage of Method B as compared to the method of Sanderson et al. (13), was that a separation chamber was used that holds considerably more cells than the Sanderson chamber (27), and consequently substantially more blood (120 ml vs. 10 ml) could be processed. Moreover, Method B yielded a pure population of lymphocytes in addition to the monocytes and the method did not require a Coulter counter, pulse height analyzer, or xy-recorder. The reproducibility of the method is attested to by the purities obtained in 22 consecutive experiments. We believe that our results and those of Sanderson et al. clearly demonstrate the superiority of the counterflow centrifuge in the separation of human monocytes.

Sterol synthesis does not appear to be impaired in cells subjected to counterflow centrifugation (Table 3). Indeed, the cells washed with counterflow centrifugation and incubated in the lipid-depleted medium were slightly more active than those washed without counterflow centrifugation. This slight increase in activity may reflect a more thorough washing of the cells which resulted in a more efficient removal of adherent lipoproteins than occurred when the cells were washed without counterflow centrifugation.

The incorporation of [2-^{14}C]acetate into sterols by the monocytes was only 3-fold greater than the incorporation by the lymphocytes when the cells were incubated for the first hour after isolation in a medium that has previously been shown not to produce sterol loss or alter sterol synthesis from acetate (3). However, when the cells were incubated in the lipoprotein-deficient fraction of serum (d > 1.21 g/ml) or lipid-depleted medium, media that have previously been shown to produce sterol loss and an increase in the rate of sterol synthesis (3), the monocytes incorporated 5, 10, or 20 times more [2-^{14}C]acetate into sterols than did the lymphocytes (Table 4, and the experiments described in the text comparing sterol synthesis in buffer vs. lipid-depleted medium). It is not possible to decide from these experiments whether the increased induction of sterol synthesis in the monocytes occurred as a result of greater sterol loss or for some other reason(s). However, it is possible to unequivocally state that when mononuclear cells prepared by the usual density-gradient centrifugation of whole blood on Ficoll–Hypaque (15) are incubated in these media, the monocytes will account for a substantial portion if not for almost all of the [^{14}C]acetate incorporated into sterols. The data in Table 3 and Fig. 2 are evidence in favor of this statement. The data in Fig. 2 were obtained from populations of mononuclear cells that varied from populations containing virtually no monocytes to populations with approximately 80% monocytes which were studied under conditions similar to those in the experiments described in Table 3. The line generated by the data in Fig. 2 would predict that a population of mononuclear cells that was 100% monocytes would have had an activity in excess of 600 dpm/hr per 10^6 cells. The incorporation of [2-^{14}C]acetate into sterols by the mononuclear cells incubated in lipid-depleted medium was 794 ± 154 dpm/hr per 10^6 monocytes for the cells washed with counterflow centrifugation and 673 ± 195 dpm/hr per 10^6 monocytes for the cells washed without counterflow centrifugation (Table 3). These calculations were made for populations of mononuclear cells that contained 30% and 27% monocytes, respectively, and assumed that all of the [2-^{14}C]acetate incorporated into sterols could be attributed to the activity of the monocytes. The

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agreement between these sets of data, taken together with the data from Table 4, lend strong support to the argument that the monocytes will account for a substantial portion if not for virtually all of the [2-^{14}C] acetate incorporated into sterols by mononuclear cells obtained by density-gradient centrifugation on Ficoll–Hypaque (15) and incubated in media that induce sterol synthesis. Since most of our previous studies were limited to 7-hr incubations (1–4), we kept within this period in this present series of experiments. However, we have also studied cells incubated for up to 22 hr and have found similar results with respect to the relative activities of the lymphocytes and monocytes in both the lipoprotein-deficient fraction of serum (d > 1.21 g/ml) and in lipid-depleted medium.[3] The differing results of Ho et al. (5) and Bilheimer et al. (6) to those reported here may relate to changes that occur in longer incubations (e.g., 57 hr) or may be due to the inability to quantitatively remove monocytes from a population of mononuclear cells by adherence to a dish (see Fig. 2[a] in reference 8) and/or may be due to changes in the monocytes that are induced by adherence of the cells to a surface. Our results are in good agreement with those reported by Young and Rodwell (28) for 3-hydroxy-3-methylglutaryl coenzyme A reductase activity in freshly isolated rat leukocytes. One can calculate from the data presented in their Table 2 that the monocytes were approximately five times more active than the lymphocytes.

Mononuclear cells prepared from normal subjects by density-gradient centrifugation of whole blood on Ficoll–Hypaque contained from 6 to 46% monocytes (20 ± 10%, mean ± SD). These results are in agreement with those reported by Lehrer (29). An increasing number of investigators are using mononuclear cells prepared by density-gradient centrifugation (5, 6, 30–32) or mixed leukocytes (33–36) for short-term incubations in the lipoprotein-deficient fraction of serum or in solvent-extracted serum. We conclude that in the future such studies of cholesterol metabolism utilizing mononuclear cells must take into account the large variation in the percentage of monocytes that may occur, and studies using either mononuclear cells or mixed leukocytes must consider the disproportionately greater activity of the monocytes during short-term incubations in media that induce sterol synthesis.

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[3] Fogelman, A. M., J. Seager, M. Hokom, and P. A. Edwards. Unpublished data.

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