notes on methodology

A comparison of delipidated sera used in studies of sterol synthesis by human mononuclear leukocytes

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Summary Sterol synthesis in human mononuclear leukocytes is stimulated by delipidated serum. Synthesis in media containing serum delipidated by three different methods is compared. Significant differences between subjects are shown and these differences are maximized by measuring synthesis in serum delipidated by extraction with butanol–diisopropyl ether 40:60 and diethyl ether.—**Slater, H. R., and F. W. Robertson.** A comparison of delipidated sera used in studies of sterol synthesis by human mononuclear leukocytes. *J. Lipid Res.* 1979. **20:** 413–416.

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Studies of sterol synthesis in human mononuclear leukocytes have proved useful in the detection of familial hypercholesterolemia (1–5). In the presence of delipidated serum, these cells increase their endogenous sterol synthesis, a response which is believed to be a result of removal of cellular cholesterol by the medium (2). Two approaches have been made to remove cholesterol from serum, extraction into organic solvents and removal by flotation ultracentrifugation. Special attention must be paid to removal of residual organic liquids to avoid toxic effects on the cells. A new technique based on extraction of serum with mixtures of butanol and diisopropyl ether (6) has been modified for use in cell studies and compared with two other techniques already in use for this purpose.

MATERIALS AND METHODS

All delipidation procedures were carried out on aliquots of a stock of pooled human serum (250 mg cholesterol/100 ml). All delipidated sera were dialyzed together against PBS, pH 7.3.

Abbreviations: PBS, phosphate-buffered saline; LDL, low density lipoprotein.

Delipidation technique A (Ref. 6)

Immediately before the extraction procedure, diisopropyl ether was mixed with powdered aluminium oxide (1 g/10 ml) to remove potentially unstable peroxides. Ten ml of serum was added to 20 ml of a mixture of butanol and diisopropyl ether 40:60 (v/v) and mixed for 30 min at room temperature on a rotary blood mixer. The lower aqueous phase was then removed by syringe and washed with 20 ml of diethyl ether to remove residual butanol. After residual diethyl ether was removed by vacuum evaporation at 37°C, the delipidated serum was dialyzed against three changes of 50 volumes of PBS, pH 7.3. Cholesterol and protein concentrations were then measured.

Delipidation technique B (Ref. 7)

Ten ml of serum was shaken with 3 ml of diethyl ether and the mixture was frozen in liquid nitrogen. After thawing for 6–8 hr, the lower layer was removed by syringe and mixed with another 3 ml of diethyl ether. The mixture was frozen again in liquid nitrogen and, after thawing for 6–8 hr, the lower layer was removed. This cycle was repeated three times more. After residual diethyl ether was removed by vacuum evaporation, the delipidated serum was dialyzed against three changes of 50 volumes of PBS, pH 7.3. Cholesterol and protein concentrations were then measured.

Delipidation technique C (Ref. 8)

The density of 10 ml of serum was adjusted to 1.25 with KBr and the mixture was centrifuged at 114,000 *g* for 48 hr at 15°C. After removal of the upper layer of lipoprotein, the delipidated serum was dialyzed against three changes of 50 volumes of PBS, pH 7.3. Cholesterol and protein concentrations were then measured.

Cholesterol was measured by spectrophotometric assay based on the Liebermann–Burchard reaction (9). Cholesterol concentrations in sera delipidated by methods A and C were less than 1 mg/dl as measured by this assay. Cholesterol concentration in serum delipidated by method B was 4 mg/dl. The protein concentrations (10) of all delipidated sera were adjusted to 50 mg/ml. All sera were sterilized by membrane filtration.

LDL was prepared by ultracentrifugal flotation (8) from serum at d 1.020–1.064 g/ml. This fraction was stabilized in 4% bovine serum albumin and dialyzed against three changes of 50 volumes of PBS, pH 7.3.

Cell separation

Human mononuclear leukocytes were separated from 20 ml of heparinized blood collected after a 12–14 hr fast. The blood was mixed with an equal volume of calcium-free Krebs–Ringer phosphate buf-

TABLE 1. Synthesis of sterols by mononuclear leukocytes from four normal individuals in medium containing serum delipidated by methods A, B, and C

Subject	A			B			C		
	Max	Min	Max/Min	Max	Min	Max/Min	Max	Min	Max/Min
	pmol			*pmol*			*pmol*		
1	306	44	7.0	145	55	2.6	149	23	6.5
2	236	32	7.3	91	31	2.9	75	19	3.9
3	162	16	10.1	77	25	3.1	49	12	4.1
4	164	19	8.6	94	30	3.1	67	11	6.1
Mean	216.9	28	8.3	102	35	2.9	85	16	5.2

Human mononuclear leukocytes were isolated from 20 ml of venous blood collected from each of four healthy donors. Maximum incorporation of [^{14}C]acetate into sterols by cells from each subject was measured simultaneously by adding 20 μl of [^{14}C]acetate (22.4 Ci/mol) to cultures (2×10^6) cells containing 28% serum, delipidated by method A, B, or C, in culture medium (RPMI 1640) and incubated at 37°C for 20 hr. Digitonin-precipitable sterols were isolated and radioactivity was measured as described in Methods. Minimum incorporation of [^{14}C]acetate in the absence of delipidated serum was 30, 20, 17, and 14 pmol for subjects 1, 2, 3, and 4, respectively. The average difference between pairs of replicates is 18% of the mean of the maximum and 7% of the mean of the minimum incorporations.

fer, pH 7.3, and layered on top of 20 ml of Ficoll–Hypaque (11). After centrifugation at 400 *g* for 25 min, the layer of mononuclear cells at the blood–Ficoll–Hypaque interface was removed. The cells were washed twice with Krebs–Ringer phosphate buffer, pH 7.3, and aliquots were taken for differential and total cell counts. No platelets were observed in these preparations.

Cell incubation

Cells (2×10^6) were suspended in RPMI 1640 media (Gibco Biocult Labs Ltd.) containing 100 U of penicillin/ml and 100 μg of streptomycin/ml with 1–1.5 ml of delipidated serum, 0.4 ml of LDL (72 mg cholesterol/dl), and 20 μl of [U-^{14}C]acetate (22.4 Ci/mol). All volumes were made up to 3.52 ml with RPMI 1640. After equilibrating the media with 95% O_2/5% CO_2, the cells were incubated at 37°C for 20 hr.

Isolation of digitonin-precipitable sterols

Cells and media were saponified separately in 50% methanol and 1.3 N NaOH at 70°C for 1 hr. Neutral lipids were extracted into petroleum ether (bp 60–80°C). After evaporation of petroleum ether under nitrogen, extracted lipids were redissolved in 4 ml of ethanol–acetone 1:1 containing 1 mg of carrier cholesterol.

β-Hydroxy-sterols were precipitated by adding 1 ml of digitonin solution (12) and then 1 ml of water. The digitonides were washed twice in 80% ethanol and once in ether, and finally redissolved in 0.5 ml of methanol which was added to 9 ml of Unisolve 1 (Koch-Light Labs Ltd.) scintillation fluid for measurement of radioactivity. Cell-free blanks contained less than 200 cpm per flask and recovery ± SD was 85 ± 3% as measured with [^{3}H]cholesterol. One $\times 10^3$ cpm is equivalent to 20 pmol of [^{14}C]acetate.

RESULTS

Serum delipidated by any of methods A, B, or C has been shown to enhance the rate of sterol synthesis by cells incubated in buffer alone (**Table 1**). The rates of maximum and LDL-inhibited synthesis in a single delipidated serum varied among subjects. For the purposes of variance analysis, the data have been log transformed to remove the correlation between mean and variance.

For maximum incorporation there is significant heterogeneity between treatments ($F = 69.8, P < 0.001$) and between subjects ($F = 24.1, P < 0.001$). For minimum incorporation there is no significant heterogeneity between treatments ($F < 1.0$) but there is heterogeneity between subjects ($F = 6.1, P < 0.01$). The error value was based on the pooled-between-replicates variation separately for either maximum or minimum incorporation (i.e., 0.01 and 0.04 for 12 degrees of freedom, respectively). For neither maximum nor minimum incorporation was there indication of significant treatment–subject interaction.

Incubation of cells in each delipidated serum resulted in a proportion of the newly synthesized sterol appearing in the medium (**Fig. 1**). This proportion was the same for each delipidated serum. Furthermore, if the rate of synthesis was inhibited by LDL, the same proportion of newly synthesized sterol was detected in the medium. With the exception of the minimum incorporation in serum delipidated by method A, the agreement with the data presented in Table 1 is good.

DISCUSSION

After treatment by methods A and C, cholesterol could not be detected in the resultant delipidated serum. However, a trace (4 mg/dl) was not extracted by method B, an observation which has been noted by other workers (2). It is of note however that despite this residual cholesterol, delipidation of serum by this technique produces a serum that is as good an inducer of sterol synthesis as that produced by method C which is cholesterol-free. It is also of note that this residual cholesterol concentration is of the same order as that concentration of LDL cholesterol that is used to inhibit sterol synthesis and, yet, stimulation of synthesis is not prevented.

Delipidation by method C is distinguished from the others in that there is removal of the apoprotein moieties as intact lipoproteins in the centrifugation procedure. Although there is a trace amount of A apoprotein in serum delipidated by this method,[1] there is no detectable B apoprotein.

The variation in rates of leukocyte sterol synthesis is typical of that reported elsewhere (14). However, the effect of delipidation technique on both the maximum rate of synthesis and the ratio of maximum to LDL-inhibited rate has not been reported before and has great relevance to the use of these two parameters in the study of leukocyte sterol synthesis in the normal and hypercholesterolemic state (1–5).

A similar relationship between total incorporation of [^{14}C]acetate into sterols and [^{14}C]sterols in the medium (Fig. 1) has been reported by other workers (2). The observation that this relationship holds for different delipidated sera both in the presence and absence of LDL is a clear indication of the importance of these sera in determining the rate of sterol synthesis.

In studies of familial hypercholesterolemia, Fung et al. (13) have indicated the importance of using a delipidated serum that induces maximum sterol synthesis; incomplete delipidation can lead to apparent lack of inhibition by LDL, resulting in misclassification of this disorder.

Of the delipidation methods described, method A has proved the most satisfactory. Not only has this method produced sera that induce higher rates of sterol synthesis, but is has also proved to be by far the most convenient method to use. This method has the advantage that the whole procedure can be completed quickly and also with high recovery of the volume originally extracted. The method is also flexible in that quantities of serum from one ml to several liters can be processed with ease.

[1] Slater, H. R., and F. W. Robertson. Unpublished observations.

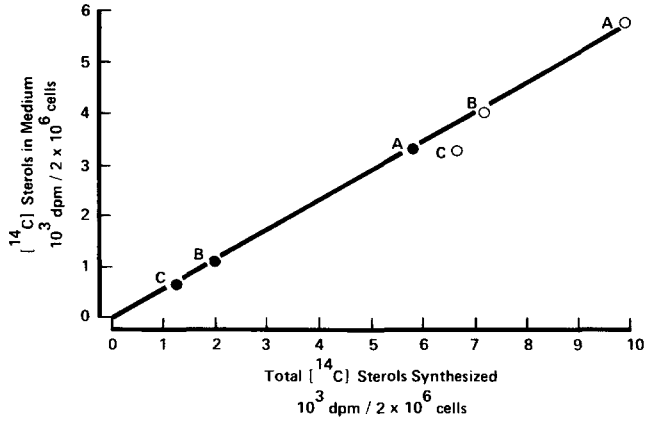

Fig. 1. Relationship between total mononuclear leukocyte sterol synthesis and sterols appearing in the culture medium. Human mononuclear leukocytes were isolated from 20 ml of venous blood collected from a healthy female donor as described in Methods. Flasks containing 2×10^6 cells each were incubated for 20 hr with 4 μCi of [^{14}C]acetate in RPMI 1640 containing 43% serum delipidated by method A, B, or C with (●) or without (○) LDL (8 mg cholesterol/100 ml). Cells and media were then separated, digitonin-precipitable sterols were extracted, and radioactivity was measured. $y = 0.57x - 0.07$; $r = 0.99$; $P < 0.001$.

Studies are at present being made to explain the different cell responses to sera delipidated by different methods and the nature of the transfer of de novo synthesized sterols from cell to medium.

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REFERENCES

1. Fogelman, A. M., J. Edmund, J. Seager, and G. Popják. 1975. Abnormal induction of 3-hydroxy-3-methyl glutaryl coenzyme A reductase in leukocytes from subjects with heterozygous familial hypercholesterolemia. *J. Biol. Chem.* **250:** 2045–2055.
2. Fogelman, A. M., J. Seager, P. A. Edwards, and G. Popják. 1977. Mechanism of induction of 3-hydroxy-3-methyl glutaryl coenzyme A reductase in human leukocytes. *J. Biol. Chem.* **252:** 644–651.
3. Higgins, M. J. P., D. S. Lecamwasam, and D. J. Galton. 1975. A new type of familial hypercholesterolemia. *Lancet.* **2:** 737–740.
4. Higgins, M. J. P., and D. J. Galton. 1977. The regulation of sterol biosynthesis in leukocytes of subjects with familial hypercholesterolemia. *Eur. J. Clin. Invest.* **7:** 301–305.
5. Ho, Y. K., M. S. Brown, D. W. Bilheimer, and J. L. Goldstein. 1976. Regulation of low density lipoprotein receptor activity in freshly isolated human lymphocytes. *J. Clin. Invest.* **58:** 1465–1474.
6. Cham, B. E., and B. R. Knowles. 1976. Changes in

electrophoretic mobilities of α- and β-lipoproteins as a result of plasma delipidation. *Clin. Chem.* **22:** 305–309.
7. McFarlane, A. S. 1942. Behaviour of lipoids in human plasma. *Nature.* **149:** 439.
8. Hatch, F. T., and R. S. Lees. 1968. Practical methods for plasma lipoprotein analysis. *Adv. Lipid Res.* **6:** 1–68.
9. Kim, E., and M. Goldberg. 1969. Serum cholesterol assay using a stable Liebermann–Burchard reagent. *Clin. Chem.* **15:** 1171–1179.
10. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
11. Boyum, A. 1976. Isolation of lymphocytes, granulocytes and macrophages. *Scand. J. Immunol.* **5,** suppl: 5.
12. Sperry, W. M. 1963. Quantitative isolation of sterols. *J. Lipid Res.* **4:** 221–225.
13. Fung, C. H., A. K. Khachadurian, C. Wang, and I. F. Dull. 1977. Regulation of lipid synthesis by low density lipoproteins in cultured skin fibroblasts in homozygous familial hypercholesterolemia. *Biochim. Biophys. Acta.* **487:** 445–457.
14. Fogelman, A. M., J. Edmund, A. Polito, and G. Popják. 1973. Control of lipid metabolism in human leukocytes. *J. Biol. Chem.* **248:** 6928–6929.