Abnormal Induction of 3-Hydroxy-3-methylglutaryl Coenzyme A Reductase in Leukocytes from Subjects with Heterozygous Familial Hypercholesterolemia*

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ALAN M. FOGELMAN,† JOHN EDMOND, JANET SEAGER, AND G. POPIÁK

From the Research and Medical Services, Veterans Administration, Wadsworth Hospital Center, the Departments of Medicine and Biological Chemistry, UCLA School of Medicine and Molecular Biology Institute, University of California, Los Angeles, California 90024

SUMMARY

Human leukocytes isolated from fresh defibrinated blood were shown to utilize acetate and mevalonate for sterol synthesis. The capacity of the leukocytes to synthesize sterols is limited severely as compared to their ability to convert mevalonate into farnesyl pyrophosphate (which they hydrolyze rapidly to free farnesol) and into squalene. When leukocytes are incubated in a medium containing lipid-free serum, synthesis of sterols from acetate, but not from mevalonate, is much enhanced. It was shown that this increased synthesis resulted from increased levels of 3-hydroxy-3-methylglutaryl-CoA reductase activity in the cells.

A comparison was made of the activation of sterol synthesis from acetate in leukocytes of normal individuals and of heterozygous familial hypercholesterolemics. The latter group responded to incubation in lipid-free sera with a significantly higher activation than the cells of normocholesterolemics. This activation was shown to be well correlated with a higher induction of 3-hydroxy-3-methylglutaryl-CoA reductase in the heterozygous cells than in the normals. The leukocytes of a heterozygous familial hypercholesteremic individual were found to release, into a lipid-free incubation medium, more endogenously synthesized [3H]sterol (but not [3H]squalene) than the cells of a normal person. It is suggested that the genetic abnormality in heterozygous familial hypercholesterolemia could be accounted for by a mutation resulting in a weaker binding of a sterol repressor by heterozygous cells than by normal cells.

We have reported briefly on the use of leukocytes for studying sterol biosynthesis in man and on an abnormality of the control of sterol biosynthesis in cells of heterozygous familial hypercholesterolemics (1). We have observed that sterol biosynthesis from acetate in the leukocytes was stimulated when the cells were incubated for 6 hours in a lipid-free serum as compared to cells kept in a medium containing full serum. Utilization of mevalonate was not affected by the nature of the incubation medium. The stimulation of the conversion of [14C]acetate into sterols by cells kept in the lipid-free serum was inhibited completely by 10 μM cycloheximide which had no effect either on the utilization of [14C]acetate by cells kept in full serum or on the utilization of [2-14C]mevalonate by cells maintained in full or lipid-free serum. The data were interpreted to mean that in cells incubated in lipid-free sera an enzyme preceding those acting on mevalonate was induced and that the induction of this enzyme, presumed to be 3-hydroxy-3-methylglutaryl-CoA reductase, was repressed in media containing full serum. We have noted further that while the activation of acetate utilization by the lipid-free serum in the cells of normocholesterolemics was 2-fold in 6-hour incubations, in the cells of heterozygous familial hypercholesterolemics it was 3-fold. This was the first enzymic abnormality noted in heterozygous familial hypercholesterolemics.

The purpose of this paper is to document fully the sterol-synthesizing properties of human leukocytes and to present evidence that the activation phenomenon was indeed associated with the induction of HMG-CoA reductase and that the induction of this enzyme in leukocytes of heterozygous familial hypercholesterolemics was more rapid than in cells of normal individuals. Evidence will be presented also that the induction of HMG-CoA reductase may be a compensatory mechanism for the loss of sterols from the cells into the medium. We will discuss the correlation of our observations with those of Goldstein and Brown (2-6) made on cultured skin fibroblasts of normocholesterolemic, of homozygous and heterozygous familial hypercholesterolemic individuals.

MATERIALS AND METHODS

Human Subjects.—To date we have studied the leukocytes of 13 persons who were heterozygous for familial hypercholesterolemia by criteria previously defined (4, 6-8) and the cells of 17 age- and sex-matched normals.

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† Veterans Administration Research and Education Associate, 1972 to 1974.
The heterozygotes were a random sample of new referrals to the cardiac and lipid clinics of the UCLA Medical Center. The controls were new referrals to the cardiac clinic who proved to be normal in respect of the lipid and lipoprotein others were members of the Medical Center staff. No one in either group received drugs that might have affected serum cholesterol levels or lipid metabolism. All had normal hematocrits, reticuloocyte counts, white blood cell, and differential counts. Informed consent was obtained in writing from each person.

Collection, and Incubation of Leukocytes—Leukocytes were prepared by a modification of the method of Coulson and Chalmers (9). After a 12- to 14-hour fast, blood was drawn aseptically from a cubital vein through a 19-gauge scalp vein needle into 50 ml sterile plastic syringes. Their contents were emptied immediately into a 250-ml flask containing glass beads and the flask shaken at 140 rpm for 1 min in an Eberbach platform shaker. The cells were then centrifuged at 1000 X g for 15 min in order to defibrinate the blood. The defibrinated blood was then decanted from the clot into a wide mouthed test tube and 30 ml was drawn through an 18-gauge needle into a 50-ml plastic syringe containing 15 ml of Plasmagel (product of Laboratoire Roger Bellon, Neuilly, France; suppliers HTI Corp., Buffalo, N.Y.).

The contents were mixed and the syringes were rested on their plungers for 45 min in order to sediment the red cells. After the sedimentation was completed, a 19-gauge scalp vein needle was attached to the syringe and the barrel depressed over the plunger to express the supernatant. The first few drops and any subsequent particulate matter, e.g., small fibrin clots, seen through the plastic tubing of the needle were discarded. The supernatant leukocytes were washed once by sedimentation again after centrifuging at 250 X g for 12 min; the supernatant was discarded. The cells (about 0.5 ml) were then suspended in 1 ml of the Krebs-Ringer buffer and were transferred to 25-ml flasks packed in melting ice. The quantitative transfer of the leukocytes to the incubation flasks was made by rinsing of the centrifuge tubes with 3.3 ml of either full or lipid-free serum (see further on). The volume of the incubation was made up to 5 ml by the addition of 0.2 ml of a solution of sodium [2-14C]acetate (44.05 Ci/mol; 56 Ci/mol, or 18.67 Ci/mol) or of sodium [2-14C]mevalonate (9.46 Ci/mol; 10.3 Ci/mol). In some experiments [3-14C], [4-14C], and [5-14C]-mevalonate were also used; the amounts of these substrates and their specific activities will be specified at the description of the experiments. Before the start of the experiments the flasks were placed at 37°C, gassed with a 95 yo CO2 and 5 yo O2 stream, and sealed with Parafilm and were incubated at 37°C in a New Brunswick gyratory water bath shaker at 150 rpm.

The concentration of the serum (full or lipid-free) was 44% in the incubations. The cholesterol and triglyceride content of the incubations made with full serum was 0.81 and 0.59 mg per ml, respectively. Without the preliminary filtration through the Whatman No. 42 paper, the sterilization of sera by passage through Millipore filters is very difficult, as the filters rapidly become clogged.
standard scintillation solutions. Internal standards were used to determine counting efficiency.

Preparation of 3-Hydroxysterolacetate (3-14C)-Glycerol, CoA—HMG-CoA was synthesized by the method of Goldfarb and Pitot (17) from recrystallized 3-hydroxy-3-methyl[3-14C]glutaric anhydride (specific activity 11.4 Ci/mmol). A solution of 85.9 mg (~110 μmol) of CoA-SH in 3.2 ml of 0.2 mM KHC03 was added to 14.5 mg (100 μmol) of the anhydride packed in ice; the pH of the reaction mixture was 8.0. After 30 min the pH of the mixture was adjusted to 2.0 with 1 N HCl and the preparation was lyophilized. The dry, fluffy residue was dissolved in 2 ml of water.

4.0 ml samples of this solution were applied in a line onto four sheets, 20 × 55 cm, of Whatman No. 3MM paper which were developed overnight with butanol-acetic acid-water (5:2:3, by volume). The remaining 1.6 ml of the crude preparation was lyophilized and stored. After development, the dried sheets of paper were cut into 5 cm wide strips and were scanned for radioactivity and inspected for ultraviolet absorbing bands in a Chromato-Vue box (Ultra-Violet Products, Inc., San Gabriel, Calif.). The ultraviolet absorbing and radioactive band of HMG-CoA (Rf 0.22) was widely separated from HMG (Rf 0.79) and a little residual HMG-anhydride (Rf 0.90). The band of HMG-CoA was eluted from the paper with 103 ml 0.1 N HCl; the solution was lyophilized, leaving 15.0 mg of residue, which was then dissolved in 2 ml of water. The preparation contained no free CoA-SH as analyzed by the method of Hulcher and Oleson (18). By assay of its residual HMG-anhydride (RF 0.95), the band of HMG-CoA was confirmed by eluting from the paper with 103 ml of water.

The extracting buffer contained: 0.1 M sodium acetate, 7.5 mM dithiothreitol, and 154 μg/ml catalase (Boehringer and Company, Indianapolis, Ind.). The cells were sedimented by centrifuging at 250 g for 15 min at 4°C, and the supernatant was removed, and its protein content determined immediately spectrophotometrically (20). A sample was saved for a later determination of protein by the method of Lowry et al. (21).

Assay of HMG-CoA Reductase—The assay was based on the methods of Goldfarb and Pitot (17) and Shapiro et al. (22). The 0.2-ml reaction mixture contained 0.1 ml triethanolamine-HCl buffer, pH 7.5; 2 mM dithiothreitol; 5 mM EDTA; 0.2 μM KC1; and 0.26 μg K440 EOB detergent (Froster and Company, Cleveland, Ohio). The cell pellets were mixed with the extracting buffer with a glass stirring rod at 37°C for 15 min. The tubes were capped and centrifuged at 16,000 × g for 10 min at room temperature. The clear supernatant was removed, and its protein content determined immediately spectrophotometrically (20). A sample was saved for a later determination of protein by the method of Lowry et al. (21).

Here we present a new method for the preparation of human leukocytes for the assay of HMG-CoA reductase activity, which is more rapid and simpler than previous methods. The procedure is based on the method of Brown et al. (19), who were among the first to investigate the sterol-synthesizing ability of human leukocytes, have recorded an 80% loss of the enzyme activity, calculated from 14C content of mevalonolate, was corrected by subtracting the 14C content of a blank in which the enzyme was killed by heat at 80°C for 2 min before the addition of the substrate. Serum Cholesterol and Triglycerides—These were determined by methods cited previously (1).

Biochemicals, Radioactive Supplies, Reagents—Monosodium d-glucose 6-phosphate, NADP, NADPH, crystalline sodium ATP, glucose-6-phosphate dehydrogenase (from baker's yeast, 200 units/ml), dithiothreitol, coenzyme A, Trizma (2-amino-2-hydroxymethyl-1,3-propanediol) base, and Triton X-100 were supplied by Sigma, St. Louis, Mo. Sodium 3-[14C]acetate, RS-[14C]mevalonolactone, and 3Z-[4Z-4-3H]mevalonolactone were obtained from the Amersham-Searle Corp., Arlington Heights, Ill. The dibenzylethylenediamine salt of RS-15-3Hlmevalonic acid (0.74 Ci/mmol) and 3-hydroxy-3-methyl[3-14C]glutaric acid (15.8 and 8.5 Ci/mmol) were the products of New England Nuclear, Boston, Mass.

Chromatographic materials (precoated thin layer plates) were supplied by Applied Science Laboratories, Inc., Ingelwood, Calif.; scintillators (2,5-diphenyloxazole and 1,4-bis[2-(4-methyl)-5-phenyloxazolyl)]benzene) by the Packard Instrument Co., Chicago, Ill., and organic solvents (reagent grade and Nanograde) by Mallinkrodt, St. Louis, Mo., triethanolamine-HCl, by Calbiochem, San Diego, Calif.

RESULTS

General Properties of Isolated Human Leukocytes—Human leukocytes isolated both in the earth's field and from lymphocytes and neutrophils, as compared to those in whole blood.

We have compared sterol synthesis from [2-14C]acetate by leukocytes isolated by the Flaschel method with the synthesis by the leukocytes in whole blood. For the purpose of this experiment, we have incubated for 6 hours 50 ml of freshly drawn

Human erythrocytes do not synthesize sterols.
blood, kept liquid with 900 units of sodium heparin, under 95% O_2-5% CO_2 with concentrations of glucose and [2-^14C]acetate identical with those used in the standard 5-ml incubations of isolated leukocytes in full serum. The isolated leukocytes converted 300 pmol and the leukocytes in the whole blood converted 244 pmol of acetate per 10^6 cells into digitonin-precipitable sterols (to be referred to subsequently as sterols for short). Thus the isolated leukocytes retained fully their synthetic power, which may even have been stimulated slightly by the isolation or incubation in the serum.

The incorporation of [^14C]acetate into sterols was linearly proportional to the number of leukocytes incubated (Fig. 1). Thus leukocyte numbers provide a standard measure for intra- and interexperimental comparisons.

As will be shown later (cf. Fig. 2), the incorporation of [^14C]acetate into sterols was linearly proportional in respect of time also, up to 18 hours, but only in incubations made in full serum.

The system is highly reproducible, as the results of duplicate incubations differed by less than 5%. Repeated studies of the cells of the same individual gave identical results.

Nature of Sterols and Sterol Intermediates Synthesized by Leukocytes—Depending on the starting substrate, various intermediates of cholesterol biosynthesis or substances derived from these can be recognized in the incubations of leukocytes.

The data of Table I show that leukocytes incubated for 6 hours in a lipid-free serum synthesized from acetate substantially more sterol than in full serum, but the nature of the incubation medium had no effect on sterol synthesis from mevalonate. Since both acetate and mevalonate were always in excess in these experiments, this is the first demonstration that, as in other species, the rate-limiting step (or steps) of cholesterol synthesis in man precedes the formation of mevalonate. Moreover, this is also the first demonstration of limiting reactions even beyond mevalonate in fresh, intact human cells. The data of Table I show that in contrast to the great powers of the cells to synthesize farnesyl pyrophosphate (the presumed source of the free farnesol) and squalene, the ability of the cells to convert squalene into sterols is limited.

In the standard incubation the concentration of RS-[2-^14C]mevalonate was 0.92/5 = 0.184 μmol/ml or 92 μM in respect of the utilizable R-enantiomer. This was clearly in excess of the amount of mevalonate 10^6 cells could utilize, as only about 6 nmol of products accumulated of which only 71 to 80 pmol were accounted for in sterols (cf. Table I). In order to get an indication of the relative activities of the enzymes acting on mevalonate,
Effects of increasing concentrations of mevalonate on products formed by leukocytes

The isolated leukocytes of a 27-year-old normal male were incubated for 6 hours in full serum with increasing concentrations of R-S-[3H]mevalonate (11.7 Ci per mol). The concentrations shown refer to that of the utilizable R-enantiomer.

| Product      | Concentration of R-mevalonate (pmol product/10^8 cells) |
|--------------|--------------------------------------------------------|
|              | 2.7 μM | 5.4 μM | 27 μM | 54 μM |
| Farnesol     | 142    | 230    | 2100  | 4200  |
| Squalene     | 109    | 186    | 1100  | 1200  |
| Sterols      | 3      | 6      | 28    | 42    |

**Table III**

Products formed by 10,000 × g supernatant of disrupted leukocytes

Leukocytes (3.3 × 10^8) isolated from the blood of a 32-year-old normal male and suspended in 4 ml of 0.1 M Tris-HCl buffer, pH 7.5, containing 5 mM MgCl₂ and 30 mM nicotinamide, were disrupted by nine 15-s bursts of 20 KHz ultrasound in an MSE 100-watt ultrasonic disintegrator. The 10,000 × g supernatant (13), 4 ml, was incubated in air with [4R-4-3H]mevalonate, 43 μM (11.7 Ci/mol), ATP 5 mM, and NADPH 1 mM, for 3 hours at 37°C.

| Product      | Picomoles of product/10^8 cells |
|--------------|---------------------------------|
| Farnesol     | 6900                            |
| Squalene     | 475                             |
| Sterols      | 12                              |

and intermediates derived from it, we have incubated the leukocytes of a 27-year-old normal male with concentrations of mevalonate ranging from 2.7 to 54 μM (the concentration referring to that of the utilizable R-enantiomer). The results are set out in Table II which show that even at the lowest concentration of substrate, free farnesol, and squalene were the main products, and that the squalene-synthesizing capacity of the cells became saturated at 27 μM mevalonate (when a total of 4.36 nmol of farnesyl pyrophosphate had to be made), but synthesis of excess farnesyl pyrophosphate had to be made), but synthesis of excess farnesyl pyrophosphate had to be made, but synthesis of excess farnesyl pyrophosphate had to be made, but synthesis of excess farnesyl pyrophosphate had to be made.

Since farnesol was not detected when acetate was the substrate, isolated leukocytes were incubated for 6 hours with 44.05 μCi of [2-14C]acetate (18.67 Ci/mol) for the times indicated in media containing lipid-free serum.

**Table IV**

Distribution of sterols synthesized by leukocytes from [2-14C]acetate or [2-3H]mevalonate in 6 hours

Equal portions of the sterol digitonides from the five experiments shown in Table I were pooled; the digitonides were decomposed and analyzed by thin layer chromatography as described under "Materials and Methods."

| Sterol                      | [2-14C]Acetate | [2-14C]Mevalonate |
|-----------------------------|----------------|-------------------|
|                             | Full serum medium | Lipid-free serum medium | Full serum medium | Lipid-free serum medium |
| Lanosterol                  | 0.39           | 0.89              | 0.61              | 5.09                 |
| Dihydroxylanosterol         | 0.06           | 0.21              | 0.55              | 0.34                 |
| Unknown*                    | 3.91           | 5.57              | 23.39             | 20.92                |
| Desmosterol                 | 3.23           | 10.86             | 19.91             | 28.89                |
| Cholesterol                 | 5.86           | 12.14             | 20.15             | 24.61                |
| Total                       | 13.45          | 30.67             | 70.77             | 79.85                |

* This is probably either 14-nor-lanosterol or 4α-methyl cholesterol.

**Table V**

Amounts of squalene and sterols synthesized by leukocytes incubated with [2-14C]acetate in lipid-free sera for various times

Isolated leukocytes of a 39-year-old normal male were incubated with 44.05 μCi of [2-14C]acetate (18.67 Ci/mol) for the times indicated in media containing lipid-free serum.

| Product      | Time of incubation (hrs) | Picomoles of product/10^8 cells |
|--------------|--------------------------|---------------------------------|
| Squalene     | 3                        | 4                               |
|              | 6                        | 6                               |
| Sterols      | 14                       | 36                              |
|              | 15                       | 144                             |
|              | 18                       | 189                             |

in the two media are that cholesterol formed a higher proportion of sterols synthesized at the lower synthetic rate observed when [14C]acetate was the substrate as compared to the rates seen with [2-14C]mevalonate (cf. Table I) and that in the lipid-free medium more desmosterol was synthesized irrespective of the starting substrate.

Time Course of Sterol Synthesis in Leukocytes from Acetate. Comparison of Leukocytes of Normocholesterolemic and Heterozygous Familial Hypercholesterolemic Individuals—In all preceding experiments and in those reported by us previously (1), the cells were incubated for 6 hours. We became particularly interested to find out the time course of the apparent activation of sterol synthesis from acetate in leukocytes incubated in the lipid-free sera. The data of Table V show that the synthesis of sterols in cells incubated in lipid-free sera was not linearly proportional to time, but that a rapid rise ensued beyond the 6th hour of incubation. This is in contrast to the data obtained from incubation in full serum (cf. Fig. 2). The data of Table V also show that squalene did not accumulate in the cells proportionately to the increased synthesis of sterols. As will be shown later, HMG-CoA reductase levels rise markedly in cells incubated for more than 6 hours in lipid-free sera.

Subsequent to the observations reported by us previously (1), we have taken leukocytes from further five heterozygous
familial hypercholesterolemic individuals and their age- and sex-matched controls, and incubated the cells with 44.05 µCi of [2-14C]acetate (18.67 Ci/mol) in full and lipid-free sera for 6, 15, and 18 hours. In these experiments, as in the one shown in Table V, the concentration of acetate was increased 3-fold over that used in the earlier experiments in order to exclude the possibility of acetate becoming limiting in the long incubations. Also, in these long incubations, penicillin, 100 units/ml, and streptomycin, 100 µg/ml, were added to the media.

The data of the individual experiments are set out in Table VI. All were made with the same batch of full serum from which the lipid-free serum was subsequently prepared. The blood for the isolation of the leukocytes was taken from the familial hypercholesterolemics and from their controls in the morning after an overnight fast. There was no significant difference in full serum between the cells of normals and heterozygotes, even though the values for the heterozygotes were slightly higher than for the controls (Table VI and Fig. 2). In full serum, the incorporation of [14C]acetate into sterols was linear with time up to 18 hours. At 6 hours the cells of these five hypercholesterolemics behaved exactly as was found for the previous six heterozygotes (1), i.e., they incorporated 3 times more acetate into sterols in the lipid-free serum than in the full serum, whereas the control cells showed only a 2-fold rise. The amounts (picomoles) of [2-14C]acetate incorporated into sterols were also in the same range in this set of experiments at 6 hours as in the earlier ones (1) even though the concentration of acetate was 3 times higher, showing that acetate was not limiting, nor could the differences noted between normal and heterozygous cells be ascribed to differences in endogenous pools of acetyl-CoA. Moreover, we found that the results were identical when the same batch of cells were incubated in lipid-free sera for 18 hours with either 0.44 or 0.88 mM acetate.

Since acetate was not limiting in any of the experiments, and since we have shown that the effect of lipid-free serum was to induce an enzyme, or enzymes, preceding the formation of mevalonate (1), we can consider that the acetate incorporated into sterols in lipid-free serum minus that incorporated into sterols in full serum was the amount attributable to the activity of the "induced" enzyme. The calculated values for the cells of normals and heterozygous hypercholesterolemics are set out in Table VII and show a nearly twice as large activation of acetate utilization in the hypercholesterolemic cells as in the normal cells both in the early and in the later period of incubation. Our
The results from the experiments previously reported (1) were pooled with those described in Table VI and Fig. 2 to give the data calculated as described in the text.

| Time period | 0-6 hrs | 6-15 hrs |
|-------------|---------|----------|
| Normals     |         |          |
| Heterozygotes |       |          |
| pmoles acetate/(hr × 10⁸ cells) |         |          |
| Mean ± S.E. | 41 ± 4  | 76 ± 6   |
| Range       | (15-59) | (42-113) |
| No. of cases | 13     | 11       |
| Significance | p < 0.001 | p < 0.01 |

HMG-CoA Reductase in Human Leukocytes—We have suggested previously that the enhanced incorporation of [14C]-acetate in leukocytes incubated in lipid-free sera resulted probably from an induction of HMG-CoA reductase (1). To our knowledge, this enzyme has not been assayed previously in extracts of leukocytes. We have tested the method of Hulecher and Olecron (18) for the assay of this enzyme in leukocyte extracts, but found it unsatisfactory on account of the very high nonspecific deacylase activities of the extracts. The methods of Goldfarb and Pitot (17) and of Shapiro et al. (22), depending on the extraction from the reaction mixture of [14C]mevalonolactone, formed from [3H]HMG-CoA, with [3H]mevalonolactone added as internal standard followed by purification of [14C, 3H]mevalonolactone by thin layer chromatography, and determination of the 14C:3H ratio in the mevalonolactone could, however, be readily adopted in the assay of HMG-CoA reductase in extracts of leukocytes.

We have experimented with sonically disrupted cells and with cells lysed with detergent. We have abandoned the use of sonically disrupted preparations of leukocytes for assay for HMG-CoA reductase because such preparation converted, in the presence of even traces of ATP and NADH and NADPH, the minute amounts of mevalonate formed from HMG-CoA into farnesyl pyrophosphate, squalene, and sterols, thus making the assays difficult. In cell extracts made by the lysis of cells with the KYRO E09 detergent (19) we have never seen the conversion of mevalonate into other products under our assay conditions.

We have found that extracts of leukocytes, prepared as described under “Materials and Methods,” converted HMG-CoA to mevalonate at a rate that was linear with respect to protein concentration up to 2.4 mg/ml and up to 60 min of incubation. Duplicate analyses differed by less than 5%.

It remained to be established whether the higher utilization of [14C]acetate by cells incubated in lipid-free sera than in full sera was indeed associated with the induction of HMG-CoA reductase or not.

The experiment shown in Table VIII shows that there was a strict proportionality between HMG-CoA reductase levels in the leukocytes and the amount of [2, 14C]acetate incorporated into sterols.

In Table VIII we compare the amounts of mevalonate formed by the HMG-CoA reductase from HMG-CoA and the amounts of mevalonate that must have been formed in the whole cells from [14C]acetate to account for the incorporation of 14C into sterols. The data show unequivocally a parallel between HMG-CoA reductase activity and acetate incorporation into sterols, the squalene accumulating in the cells not being taken into account. The correlation between HMG-CoA reductase activity and acetate incorporation into sterols in good in the leukocytes that the synthesis of [14C]sterols from [14C]acetate may be taken as a measure of HMG-CoA reductase activity.

Comparison of HMG-CoA reductase activity in extracts of leukocytes incubated for 9 hours in full serum and in lipid-free serum

Endogenous mevalonate synthesis in whole cells was calculated from the incorporation of [14C]acetate into sterols in cells preincubated for 8 hours in full serum and lipid-free serum in the presence of 0.29 mM unlabeled acetate (cf. also text). A batch of leukocytes from a 37-year-old heterozygous familial hypercholesteremic man (serum cholesterol 360 mg/100 ml; serum triglycerides 60 mg/100 ml; corneal arcus and tendon xanthomata present; no coronary disease) was divided into two lots. One lot, divided into two flasks, was preincubated in full and lipid-free serum, respectively, for 8 hours with 0.29 mM unlabeled acetate. After 8 hours, [14C]acetate was added to the flasks, raising the concentration of acetate to 0.44 mM. The incubation with the [14C]acetate was continued for exactly 2 hours, hours 8 to 10 of the incubation. The flasks were then analyzed for [14C]sterols. The second lot of the leukocytes was also divided into two flasks and incubated with 0.44 mM unlabeled acetate in full and lipid-free serum, respectively, for 9 hours when the HMG-CoA reductase activity in these cells was determined.
was 40 years old; his serum cholesterol and triglyceride concentrations were 182 mg/100 ml and 134 mg/100 ml, respectively. The leukocytes were incubated, their extracts prepared, and assayed for HMG-CoA reductase as described in the text.

The similar reductase activities in the leukocytes of the heterozygote and normal took freshly from the blood (zero time, before incubation) confirm the data obtained with [14C]acetate in full serum.

The time course for the induction of the reductase in normal and heterozygous cells in lipid-free sera corresponds to the time course for the increased incorporation of acetate into sterols in normal and heterozygous cells in lipid-free sera (cf. Fig. 2). This is further proof of the excellent correlation between the incorporation of acetate into sterols and HMG-CoA reductase activity, and thus supports the validity of the conclusions drawn from the acetate data.

Release of Sterols from Leukocytes to Medium—The induction of HMG-CoA reductase in leukocytes incubated in lipid-free sera raised the question, what was the underlying cause of the induction, and why was a higher activity induced in the heterozygous (normal or heterozygous) associated with squalene in the first labeling of cellular squalene, there were only 2080 dpm/10^6 cells heterozygous cells in sterols (cf. Fig. 4). In spite of the heavy incorporation of acetate into sterols and HMG-CoA reductase activity, cell pellets contained much more 3Hsqualene than 3Hsterol at the end of the preliminary 3-hour incubation: 144,000 dpm/10^6 normal cells and 112,000 dpm/10^6 heterozygous cells in squalene, as compared to 4200 dpm/10^6 normal cells and 4000 dpm/10^6 heterozygous cells in sterols (cf. Fig. 4). In spite of the heavy labeling of cellular squalene, there were only 2080 dpm/10^6 cells (normal or heterozygous) associated with squalene in the first medium. After transfer of the cells to lipid-free sera (without exogenous substrate), both normal and heterozygous cells continued to accumulate [3H]sterols, presumably by the conversion of endogenous labeled substrates (e.g. of [3H]squalene) for 3 hours (Fig. 4). The heterozygous cells accumulated, however, less [3H]sterol than the normal cells. After the first 3 hours in full serum, the normal cells had lost 13% of the total [3H]sterols into the medium, while the heterozygous cells had lost 21%. Then upon transfer to the lipid-free serum, both normal and heterozygous cells rapidly lost sterol into the medium, but by 2 hours the normal cells had nearly reached a new steady state, while the heterozygous cells continued to lose an ever increasing

1 Unlabeled cholesterol (1 mg/ml) was added for the isolation of [3H]sterols, from the lipid-free media, as the digitonides.
per cent of their cellular sterol to the medium, reaching 40% of the total [3H]sterol after 6 hours as compared to only 26% loss by the normal cells (Fig. 5). The continued transfer of sterols from the heterozygous cells to the medium over 6 hours is contrasted by a very small loss of [3H]squalene to the medium after 2 hours (Fig. 4). Thus it appears, (a) that there is a differential and specific loss of [3H]sterol from leukocytes to the medium, and (b) that this loss is much larger from the heterozygous cells than from the normal ones.

**DISCUSSION**

The data presented confirm and extend our observations reported earlier (1) on the usefulness of leukocytes for the study of sterol biosynthesis in man. We have provided evidence that the most severely limiting reaction of sterol biosynthesis in man, as in other species, is that catalyzed by HMG-CoA reductase. Since in cells incubated in a medium containing full serum, sterol synthesis was linearly proportional to time (cf. Fig. 2), we may calculate from the data of Table I that the mean rate of sterol synthesis from acetate was 2.25 ± 0.3 pmol/10⁶ cells per hour corresponding to the generation of 13.5 ± 0.6 pmol of mevalonate. In addition, 3.6 ± 0.6 pmol of mevalonate are accountable in squalene giving an hourly total of 17.2 pmol of mevalonate formed from acetate per 10⁶ leukocytes. This value is in very close agreement with the HMG-CoA reductase activity measured in "uninduced" cells, 10 to 20 pmol/10⁶ cells per hour. From the incorporation of mevalonate into sterols, one might conclude that the maximum sterol-synthesizing capacity of the leukocytes per 10⁶ cells was 12 to 16 pmol per hour (cf. Table I). However, from the slopes of the curves on Fig. 2 beyond the 6th hour of incubation, we calculate that the rate of sterol synthesis in the normal cells increased to 17 to 20 pmol/hour and in the heterozygous cells to 32 to 38 pmol per hour per 10⁶ cells upon prolonged incubation in a lipid-free medium. The lower maximum rate of synthesis calculated from mevalonate utilization may be attributed to possible inhibitory effects of intermediates accumulating in the cells (cf. Tables I and II) when mevalonate is freely available. The capacity of the leukocytes to synthesize farnesyl pyrophosphate and squalene is very large as compared to their ability to generate mevalonate or to convert squalene into sterols. Our results in respect of the synthesis of squalene relative to the conversion of squalene to sterols are very similar to those of Edgren and Hellstrom (25) obtained by in vivo experiments on rats.

The evidence we have presented here proves firmly that the increased sterol synthesis from acetate seen in cells incubated in a lipid-free medium was indeed associated with the induction of HMG-CoA reductase as we have inferred previously (1) and that complete parallelism existed between the degree of enhanced acetate incorporation into sterols and the degree of induction of the reductase (cf. Table VIII, Figs. 2 and 3). We have also confirmed our earlier data (1) that the cells of heterozygous familial hypercholesterolemic responded to incubation in a lipid-free medium not only with a greater utilization of acetate, but also with a greater induction of HMG-CoA reductase than seen in normal cells. We suggest that this abnormally high induction results from a more rapid dissociation of a repressor of the system coding for HMG-CoA reductase from the heterozygous leukocytes than from normals into the medium. In contrast to the continued loss of [3H]sterol from the heterozygous cells into the lipid-free medium, there was no loss of [3H]squalene from the cells beyond the 2nd hour after their transfer into the lipid-free medium, and moreover this loss was only about 2.5% of the [3H]squalene synthesized in both normal and heterozygous cells, in contrast to the 26 and 40% loss of labeled sterols from the normal and heterozygous cells, respectively. Since we measured only radioactive squalene and sterol in these experiments, we cannot tell exactly how much sterol is represented by a certain number of disintegrations per min. But as the defect in familial hypercholesterolemia has been shown in both fibroblasts and leukocytes to affect HMG-CoA reductase, the rate-controlling enzyme in sterol biosynthesis, there is no reason to suspect that the heterozygous cells have a greater complement of enzymes in the biosynthetic pathway to cholesterol after mevalonate; that is why we chose mevalonate rather than acetate for these experiments. Proof that the sterol loss is associated with the primary defect in the leukocyte must await further study including the demonstration that the non-isotopic sterol content of the heterozygous cells is equal to or lower than the normal at a time when the heterozygous cells are producing more sterol from acetate. Khachadurian and Kawanahara have observed precisely such a situation in homozygous fibroblasts (26).

It is pertinent to discuss the observations of Goldstein and Brown and their associates (2–6) who have studied the repression of HMG-CoA reductase in human fibroblasts cultured from the skin of normal, heterozygous, and homozygous familial hypercholesteremic individuals by serum lipoproteins. They have found that the high levels of HMG-CoA reductase, induced in normal fibroblasts and in fibroblasts of heterozygous familial hypercholesterolemics by a serumless medium, or by a lipoprotein-deficient serum, could be repressed by low density lipoproteins. However, higher concentrations of LDL-cholesterol were
needed for the repression of the enzyme in the heterozygous than in the normal fibroblasts. The fibroblasts of homozygous familial hypercholesterolemies grown in 10% fetal calf serum had HMG-CoA reductase levels that were 40 to 60 times higher than those of normal fibroblasts similarly grown and, in contrast to the behavior of the normal cells, the HMG-CoA reductase levels in the homozygous cells did not increase upon the change to a lipoprotein-deficient medium, neither could the enzyme be repressed in the homozygous cells by as much as 2 mg of LDL-cholesterol per ml, whereas 25 to 40 µg per ml were sufficient to repress the enzyme in normal cells. Goldstein and Brown attribute the cellular defect in familial hypercholesterolemia to a defect of LDL binding to the cells and of LDL degradation, which they have demonstrated with the aid of 3H-labeled LDL (6). They attribute the abnormality to a genetic mutation that results in a failure of the synthesis of specific LDL receptors on the cell surface and a failure of degradation of LDL and hence transport of cholesterol into the cell.

It is not possible to decide at present whether the phenomena we have observed in leukocytes, the abnormally high rate of induction of HMG-CoA reductase in heterozygous cells and the high rate of loss of sterols from such cells to a lipid-free serum, and those observed by Goldstein and Brown, the impaired binding and degradation of LDL by heterozygous and homozygous fibroblasts, are related or not, as we have not studied LDL binding to leukocytes. Whatever might be the correlation of the recorded phenomena, the observations on cultured fibroblasts and on freshly isolated leukocytes indicate a genetic abnormality in the control of the synthesis of HMG-CoA reductase. Our interpretation of the observations differs from that given by Goldstein and Brown for the origin of the familial hypercholesterolemia abnormality in a fundamental way in that we propose that the abnormality results from a mutation with a defective binding for a sterol repressor synthesized within the cell and with a consequently greater loss of sterol from the cell. Once a sufficiently high level of extracellular cholesterol has been built up, rates of cholesterol synthesis and rates of loss of sterol from the cells, are depressed in normal values. Goldstein and Brown imply that the primary control of the phenotypic expression of HMG-CoA reductase lies not within the cell, but comes from without through the transfer of cholesterol from extracellular LDL to the cell. Only further work can decide between these contrary views.

In spite of the evidence for the existence of a genetic abnormality in the control of the phenotypic expression of HMG-CoA reductase in fibroblasts and leukocytes, a full understanding of the origin of familial hypercholesterolemias is still not at hand. The limited information available from in vivo studies does not suggest the existence of a derepressed or partially derepressed state for the synthesis of HMG-CoA reductase, as has been suggested from the study of J.P. For the first time her serum cholesterol fell by 300 mg/100 ml. Because of these encouraging results, an end-to-end portacaval shunt was made on the child. Subsequently, her serum cholesterol fell to the 200 to 300 mg/100 ml range, her xanthomas disappeared, she returned to school, and grew at a normal rate. It is difficult to reconcile these results with a structural gene mutation for LDL binding and degradation as has been suggested from the study of J.P.'s fibroblasts in culture.

The fibroblasts are studied under conditions which do not exist in vivo. So are the leukocytes in lipid-free sera. However, the situation in vivo, i.e. a high concentration of LDL, is mirrored by the leukocytes before incubation or after incubation in full serum, and our results under these conditions are in perfect agreement with the in vivo studies in man (27, 28).

We believe the transfer to lipid-free serum may also reflect an in vivo phenomenon, derepression. Because fibroblasts grown in 10% fetal calf serum have adapted to a cholesterol concentration one-tenth of that seen even in the interstitial fluid drained from the foot (32), and because they must be grown in culture for at least five generations before being studied, one cannot assess derepression as it must happen in vivo, namely, in the presence of a high LDL concentration. That is probably why the fibroblasts were taken routinely to a completely derepressed state and then repression studied. This cannot occur in vivo. What may occur in vivo is periodic derepression. The diurnal variations of HMG-CoA reductase levels in the liver documented for the rat (e.g. 33-35) may also exist in man, and in familial hypercholesterolemia the HMG-CoA reductase levels may rise higher to the postprandial stimulus, whatever that stimulus might be, than in normal individuals.

Malamos et al. have shown (36) that when human leukocytes were separated from whole blood and then incubated in their own plasma with [1-14C]-acetate, they released [14C]-sterols into the medium within 6 hours. We have shown that homozygous cells lost [3H]-cholesterol but not [3H]-squalene in the lipid-free sera more readily than did normal cells. Under steady state conditions in vivo, the heterozygotes do not synthesize cholesterol more rapidly (27, 28), but they have a higher extracellular concentration of cholesterol. We feel that this may be a homeostatic mechanism to compensate for a defect which leads to a higher rate of loss of cholesterol from cell membranes. If this hypothesis is correct, one would expect that the cellular content of cholesterol in heterozygotes is no higher than in normals. Indeed, except for specific sites of connective tissue and blood vessels, there is no evidence that cells of heterozygous hypercholesterolemics have a higher cholesterol content than normal. The cholesterol concentration in a liver biopsy from a person with type II hyperlipoproteinemia was no higher than the cholesterol concentration in liver biopsies from persons operated on for cholelithiasis without biliary obstruction and with normal serum concentrations of cholesterol (30). Moreover, Maurizi et al. (37) did not find any correlation between liver cholesterol content and atherosclerosis in a large autopsy series. The cholesterol concentration in fibroblasts from two individuals with homozygous familial hypercholesterolemia was no higher than that in fibroblasts from six normals, despite the fact that the homozygotes incorporated 10 times more acetate into cholesterol than the normals (29). Hence, we must assume that the homozygotes were losing the synthesized cholesterol from their cells into their environment faster than the normals.

The defect in LDL binding that Brown and Goldstein (6) have observed in familial hypercholesterolemic cells may in fact be a
consequence of the mass action effect of cholesterol loss from the
loss from the surface of those cells.

The leukocytes, parallel with the cultured fibroblast, is, we
believe, a most useful and readily accessible human cell for prob-
ing the many unanswered questions as to the control of sterol
biosynthesis in man.

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