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Regulation of 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase Activity in Human Fibroblasts by Lipoproteins

(cholesterol biosynthesis/enzyme suppression and stimulation/low-density and high-density lipoproteins)

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ABSTRACT **The activity of 3-hydroxy-3-methylglutaryl coenzyme A reductase (EC 1.1.1.34), the rate-limiting enzyme of hepatic cholesterol biosynthesis, is suppressed in human fibroblasts cultured in the presence of serum. This enzyme activity increases by more than 10-fold after the removal of serum from the medium. The rise in enzyme activity requires *de novo* protein synthesis and is not accompanied by changes in the activities of several other cellular enzymes. The factor responsible for the suppression of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity in cultured fibroblasts is present in the sera of at least four mammalian species, and in human serum it is found in the low-density lipoproteins. Human high-density lipoproteins, very low-density lipoproteins from chicken egg yolk, and the fraction of human serum containing no lipoproteins do not suppress the activity of 3-hydroxy-3-methylglutaryl coenzyme A reductase.**

Mammalian fibroblasts cultured *in vitro* synthesize cholesterol at a rate inversely related to the cholesterol content of the growth medium (1, 2). Similarly, in mammalian liver the rate of cholesterol biosynthesis is controlled by the cholesterol content of the diet (3). In liver this regulation is effected through alterations in the activity of the rate-limiting enzyme in the cholesterol biosynthetic pathway, 3-hydroxy-3-methylglutaryl coenzyme A reductase (reductase; EC 1.1.1.34) (3). This enzyme activity has not been previously demonstrated in fibroblasts.

The present study demonstrates that the reductase activity is detectable in cultured human fibroblasts. In addition, the results indicate that in fibroblasts the activity of this enzyme is regulated by a process that is coupled to the amount of specific extracellular lipoproteins.

MATERIALS AND METHODS

Cells. Human fibroblasts, established from normal newborn foreskin, were cultured by standard methods in a humidified CO_2 incubator at 37° in 75-cm² flasks (Falcon) containing 10 ml of Eagle's minimum essential medium (MEM) (Gibco), supplemented with penicillin (100 units/ml), streptomycin sulfate (100 μg/ml), 0.05 M tricine (pH 7.4) (Sigma), 0.05 g of $NaHCO_3$/100 ml, 1% (v/v) nonessential amino acids (Gibco), and 10% (v/v) fetal-calf serum (Flow Laboratories). The medium was changed every third day. Cells in stationary phase growth were dissociated for subculture by washing with 10 ml of Puck's saline A (Gibco) and then incubating with 0.05% trypsin–0.02% EDTA solution (Gibco) for 5–10 min at 37°. All cells used in the experiments had been grown in monolayer for 5–15 generations. In experiments in which cells were incubated without serum, growth medium was removed and the cellular monolayer was washed with 10 ml of Puck's saline A, after which 10 ml of MEM supplemented as above, but without fetal-calf serum, was added to each flask. All materials, including medium, serum, and lipoprotein fractions, were sterilized by Millipore filtration (HAWP 0.45 μm) before addition to culture flasks. Cycloheximide and actinomycin D were obtained from Sigma and Merck, Sharpe, and Dohm, respectively.

Extracts. The medium from each flask was discarded and the cells were scraped with a rubber policeman into 1 ml of buffer containing 0.05 M Tris·HCl (pH 7.4)–0.15 M NaCl (buffer A). All further operations were performed at room temperature (24°), except as indicated. After centrifugation (900 × *g*, 3 min, 24°), the cell pellet was suspended in 1 ml of buffer A and washed once more in the same manner. Each pellet was frozen once in liquid nitrogen and kept at −196° until use. Cell extracts were prepared by dissolving the thawed pellet of fibroblasts in 0.2 ml of buffer containing 50 mM K_2HPO_4 (pH 7.4)–5 mM dithiothreitol–1 mM EDTA–0.25% Kyro EOB (buffer B). Kyro EOB (Procter and Gamble Co.), a synthetic nonionic detergent that solubilizes plasma membranes but not endoplasmic reticulum of cultured cells (4), was a gift to Dr. Robert Dowben from Dr. D. H. Hughes, Miami Valley Research Laboratories, Procter and Gamble Co. This detergent was used because it yielded higher activities of reductase than were obtained by sonication, freeze-thawing, or Dounce homogenization. Moreover, this detergent had no effect on the activity of enzyme prepared by these three latter methods.

Assays. Reductase activity was assayed in human fibroblasts by a minor modification of the method described for rat liver in which the rate of formation of [3-^{14}C]mevalonate from [3-^{14}C]hydroxymethylglutaryl-CoA is determined (5). 50 μl of cell extract containing 100–250 μg of protein was incubated 10 min at 37° in a total volume of 0.2 ml containing 0.1 M K_2HPO_4, (pH 7.5); 20 mM glucose-6-phosphate; 2.5 mM TPN; 0.7 unit of glucose-6-phosphate dehydrogenase; and 5 mM dithiothreitol. The reaction was then started by addition of DL-[3-^{14}C]hydroxymethylglutaryl CoA (5.26 Ci/mol) to a final concentration of 30 μM. After 120 min at 37° the reaction was stopped by addition of 20 μl of 5 N HCl; 3 μmol of [5-^{3}H]mevalonolactone (21.8 mCi/mol) was added as an internal standard, and the mixture was extracted with diethyl ether. The mevalonolactone was isolated by thin-layer chromatography and counted as described, the efficiency of

Abbreviations: Reductase, 3-hydroxy-3-methylglutaryl-coenzyme A reductase; LDL, low-density lipoproteins; HDL, high-density lipoproteins; VLDL very low-density lipoproteins; MEM, Eagle's minimum essential medium.

the counter being 26% for ^{3}H and 68% for ^{14}C (4). Recovery of mevalonolactone averaged about 50%.

The activities of lactate dehydrogenase (6), alkaline phosphatase (7), and acid phosphatase (7) and the content of protein (8) and DNA (9) in fibroblasts were assayed by minor modifications of the cited methods. The activity of dehydroepiandrosterone sulfate sulfatase was assayed by measurement of the rate of conversion of [7-^{3}H]dehydroepiandrosterone sulfate (21 Ci/mmol, New England Nuclear Corp.) to dehydroepiandrosterone, as quantitated by diethyl ether extraction and liquid scintillation counting (efficiency 50%). The identity of the product was confirmed by celite chromatography.

Lipoproteins. Human lipoproteins were prepared from 350 ml of plasma collected in 0.1% EDTA from a healthy man who had not eaten for 15 hr. Lipoproteins from human and fetal-calf sera were fractionated by sequential flotation in a Beckman preparative ultracentrifuge at 214,000 × *g* (average) and 4–10° for 16–24 hr, according to standard techniques (10) using solid KBr for density adjustment (11). Isolated fractions were dialyzed at least 36 hr at 4° against three changes of at least 50 volumes of buffer containing 0.15 M NaCl–0.3 mM EDTA (pH 7.4) (buffer C). Each isolated fraction migrated as a homogeneous peak on lipoprotein electrophoresis (12). The cholesterol (13) and triglyceride (14) content of sera and lipoprotein fractions were measured by modifications of standard methods. Lipid-deficient fetal-calf serum was prepared by the method of Albutt (15). An emulsion of chicken egg yolk was prepared by sonification (Bronwill Biosonik II) for 5 min of the yolk of 1 egg suspended in 100 ml of 0.15 M NaCl. All animal sera except fetal-calf serum were obtained from Gibco and were dialyzed against buffer C.

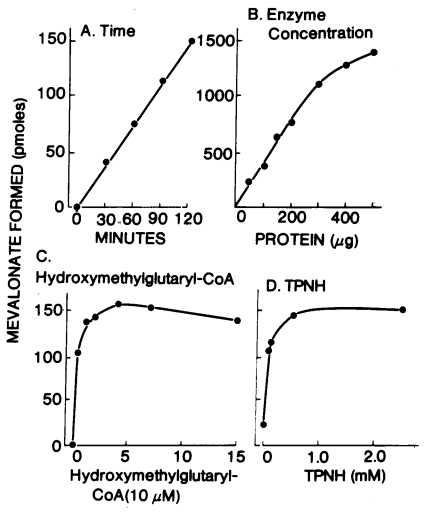

FIG. 1. Assay of reductase activity in cultured fibroblasts. Experiments *A*, *C*, *D:* cells grown to confluence were incubated 20 hr in MEM without serum, and extracts from four flasks were prepared and pooled in a final volume of 0.5 ml of buffer B. 25-μl Aliquots (60 μg of protein) were incubated 120 min at 37°, as described in *Methods* (exceptions indicated). Experiment *B:* cells were incubated and extracts were prepared as in Exp. *A*, except that extracts from three flasks were pooled in a final volume of 0.2 ml of buffer B and aliquots of 5–50 μl containing 50–500 μg of protein were incubated 120 min at 37°.

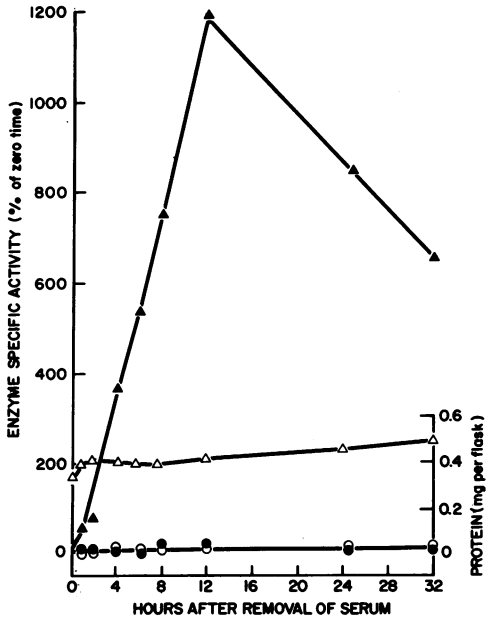

FIG. 2. Increase in reductase activity after removal of fetal-calf serum. 6 days before the experiment, 2 × 10^{5} trypsinized cells were added to each of 10 flasks and grown to confluence in growth medium containing 10% fetal-calf serum. At 0 time growth medium was replaced with MEM without serum. After the indicated interval, extracts were prepared from each flask, and enzyme activities and protein content (△——△) were measured. Enzyme specific activities determined in extracts from duplicate flasks at 0 time averaged: reductase (▲——▲), 4.5 pmol/min per mg; lactate dehydrogenase (●——●), 183 nmol/min per mg; acid phosphatase (○——○), 14.8 nmol/min per mg. Results are expressed as a percentage of these initial values.

RESULTS

Under standard assay conditions extracts of cultured human fibroblasts converted hydroxymethylglutaryl-CoA to mevalonate at a rate that was linear with time and protein concentration (Fig. 1*A* and *B*). In a 2-hr incubation the rate of the reaction remained linear up to the formation of 1000 pmol of mevalonate (Fig. 1*B*). The concentrations of DL-hydroxymethylglutaryl-CoA (Fig. 1*C*) and TPNH (Fig. 1*D*) giving half-maximal velocities (5 μM and 50 μM, respectively) were both about 5-fold lower than the corresponding concentrations for rat-liver reductase (5). When the crude extract of detergent-solubilized fibroblasts was fractionated by differential centrifugation, 44% of the total enzyme activity was found in the 100,000 × *g* pellet and 55% was contained in the supernatant (data not shown).

In fibroblasts grown to confluence in media containing 10% fetal-calf serum, the reductase activity was relatively low (Fig. 2). However, when the serum was removed from the medium, both the specific activity of the reductase and the total activity per flask progressively increased by more than 10-fold, reaching a peak between 12–16 hr (Fig. 2). During this interval there was little change in the total content of protein per flask, and there was a change of less than 50% in the specific activities of two control enzymes, acid phosphatase and lactate dehydrogenase. The increase in reductase activity was not merely the result of addition of fresh medium (Table 1). Although there was a slight increase in reductase activity when the growth medium was replaced with

TABLE 1. *Response of cultured human fibroblasts to removal of serum*

Modifications of growth medium	Time of cell harvest (hr)	Enzyme specific activities: Reductase (pmol/min per mg)	DHEA sulfate sulfatase (pmol/min per mg)	Alkaline phosphatase (nmol/min per mg)	Acid phosphatase (nmol/min per mg)	Protein content (mg per flask)	DNA content (μg per flask)
None	0	3.3	—	—	—	1.32	—
Replaced with:							
Fresh medium plus FCS	19	4.8	2.1	2.0	8.6	0.72	17.4
Fresh medium minus FCS	19	15.6	1.8	1.3	10.1	0.66	20.8
Fresh medium minus FCS plus 0.1 mM cycloheximide	19	0.6	2.9	1.8	9.1	0.46	16.2
Fresh medium minus FCS plus 0.1 mM actinomycin D	19	0.7	—	—	—	0.80	—

6 Days before the experiment, 2×10^5 trypsinized cells were added to each flask and grown to confluence in growth medium containing 10% fetal-calf serum. At 0 time the growth medium was modified as indicated. At either 0 time or 19 hr after modification of medium, the cells from each flask were harvested as described in *Methods*, except that a 0.5-ml aliquot of cells suspended in 1 ml of buffer A was removed at the time of the final wash. The cells in this 0.5-ml aliquot were centrifuged (900 × *g*, 3 min, 24°), suspended in 0.2 ml of buffer containing 50 mM Tris·HCl (pH 7.5), frozen-thawed thrice in liquid nitrogen, and then used for measurement of the content of DNA and protein and the activities of dehydroepiandrosterone (*DHEA*) sulfate sulfatase, alkaline phosphatase, and acid phosphatase. The remaining 0.5 ml of cells suspended in buffer A were washed, centrifuged, frozen-thawed once, suspended in 0.1 ml of buffer B, and assayed for protein content and reductase activity. All values are the means of measurements from duplicate flasks. *FCS*, fetal-calf serum.

fresh medium containing 10% fetal-calf serum (from 3.3 to 4.8 pmol/min per mg), a much greater increase (to 15.6) occurred when the added medium was free of fetal-calf serum (Table 1). However, in the same experiment removal of serum had no effect on the activities of the microsomal enzyme dehydroepiandrosterone sulfate sulfatase (16), the plasma membrane enzyme alkaline phosphatase, or the lysosomal enzyme acid phosphatase (Table 1). Moreover, removal of serum from the medium produced little change in the amount of protein or DNA per flask, indicating that cell division was not responsible for the stimulation of reductase activity. Both cycloheximide and actinomycin D prevented the increase in reductase activity after removal of fetal-calf serum, suggesting that both protein synthesis and RNA synthesis were required (Table 1). The maximal specific activity of reductase detected in extracts of serum-deprived human fibroblasts is about equal to that observed in crude extracts of rat liver obtained from an animal at the mid-point of the diurnal cycle (data not shown).

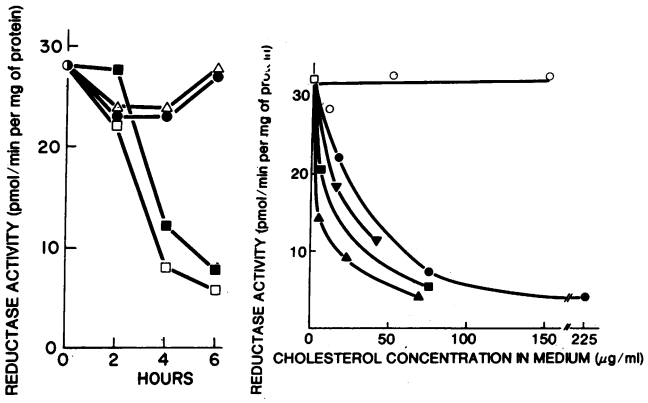

FIG. 3 (*left*). Decrease in reductase activity after addition of fetal-calf serum. Confluent cells were incubated in MEM without serum as described in legend to Fig. 2. After 14 hr in MEM without serum, the medium was replaced with 10 ml of fresh MEM containing the indicated concentration of fetal-calf serum (v/v): ●, none; ■, 10%; □, 20%; or △, 20% delipidated fetal-calf serum. Extracts from single flasks were assayed at the indicated time for reductase activity.

FIG. 4 (*right*). Effect of animal sera and egg yolk on reductase activity. Confluent cells were incubated in MEM without serum as described in the legend to Fig. 2. After 19 hr in MEM without serum, medium in each flask was replaced with 8.5 ml of fresh MEM and 1.5 ml of buffer C containing sera or egg yolk (□, buffer C; ○, chicken egg yolk; ▲, fetal-calf serum; ■, fetal-pig serum; ▼, guinea-pig serum; ●, calf serum) in an amount that gave the indicated cholesterol concentration in 10 ml. The cholesterol concentration of the sonicated egg yolk and of the fetal-calf, fetal-pig, guinea-pig, and calf sera before dilution with medium were 266, 50, 28, and 148 mg/dl, respectively.

The addition of fetal-calf serum to fibroblasts in which reductase activity had been increased by the prior removal of serum caused a prompt decrease in the activity of the enzyme (Fig. 3). Delipidation of the fetal-calf serum prevented this effect (Fig. 3). The depression of reductase activity by fetal-calf serum was observed only when the serum was added to the intact fibroblasts. Addition of serum directly to the cell extract caused no inhibition of reductase activity. Moreover, when extracts from serum-deprived cells with high reductase activity were mixed with extracts of serum-treated cells with low reductase activity, an additive amount of mevalonate was produced, suggesting that the reduced activity of the serum-treated cells was not the result of an intracellular enzyme inhibitor (data not shown).

Sera obtained from various mammalian species possessed the ability to lower reductase activity of cultured human fibroblasts (Fig. 4). The ability of each serum to inhibit enzyme activity was not strictly proportional to its cholesterol content. That cholesterol was not the sole factor required to

reduce reductase activity was further demonstrated by the failure of large amounts of egg-yolk cholesterol to affect the enzyme activity (Fig. 4). Evidence that reductase inhibitory activity was contained in a macromolecule in serum was provided by the fact that inhibitory activity was nondialyzable and that it failed to pass through a membrane ultrafilter with a nominal exclusion limit for globular proteins of 10,000 molecular weight (Table 2). Ultracentrifugation of fetal-calf serum localized the inhibitory factor to the lipoprotein fraction ($\rho <$ 1.21 g/cm^3), and by this technique the inhibitory factor was separated from the bulk of serum proteins and purified more than 100-fold (Fig. 5).

To examine in greater detail the nature of the factor that reduced reductase activity of human fibroblasts, lipoprotein fractions of human plasma were isolated and tested. Addition to fibroblasts of unfractionated whole plasma, very low-density lipoproteins (VLDL) ($\rho <$ 1.006 g/cm^3), and two fractions of low-density lipoproteins (LDL) ($\rho <$ 1.006–1.019 and ρ 1.019–1.063 g/cm^3) all led to a reduction of reductase activity (Table 3). On the other hand, high-density lipoprotein (HDL) (ρ 1.063–1.21 g/cm^3) inhibited only at much higher concentrations of protein and cholesterol. Whereas LDL produced a 50% reduction in reductase activity at a protein concentration in the medium of 0.7 μg/ml, a 500-fold greater concentration of HDL protein (360 μg/ml) was required to produce the the same effect. Moreover, when the data are compared on the basis of cholesterol concentration, LDL was 40-times more potent as an inhibitor than HDL. In contrast to the effects of unfractionated whole plasma, the addition to the cultured fibroblasts of human plasma devoid of lipoproteins ($\rho >$ 1.21 g/cm^3) stimulated reductase activity at all concentrations tested (Table 3).

DISCUSSION

This paper describes a method for the measurement of reductase activity in human fibroblasts cultured *in vitro* and demonstrates that the activity of this enzyme in fibroblasts is suppressed when certain serum lipoproteins are added to the culture medium and increased when these lipoproteins are removed. These results suggest that the previously reported increase in the rate of cholesterol synthesis that occurs when lipids are removed from the medium of cultured fibroblasts (1, 2) is due to an increase in the activity of the reductase. In our experiments the increase in reductase activity that occurred when cells were deprived of lipid was prevented by the addition of both cycloheximide and actinomycin D, suggesting that the rise in enzyme activity required *de novo* synthesis of protein and was not due to activation of preformed enzyme molecules. The increase in enzyme activity could not be explained by a stimulation of cell growth since (*1*) deprivation of serum is reported to inhibit cell division *in vitro* (17), (*2*) the cells used for these experiments were in stationary phase, and (*3*) there was no increase in DNA or protein content. In other experiments we observed that cells in log-phase growth not

TABLE 2. *Ultrafiltration of inhibitory factor in fetal-calf serum*

Addition to medium	Reductase activity (pmol/min per mg)	Percentage of control
None	40	100
Fetal-calf serum, 10% (v/v)		
Unfractionated	11	28
Ultrafiltrate	44	110
Retained fraction	15	38
Ultrafiltrate + retained fraction	12	30

Confluent cells were incubated in MEM without serum as described in the legend to Fig. 2. After 24 hr, the medium of each flask was replaced with fresh MEM containing 10% (v/v) of the indicated material. 4 Hr after these additions extracts from single flasks were assayed for reductase activity. The filtrate and retained fraction were prepared by ultrafiltration (35 lb/in^2) of 50 ml of fetal-calf serum through a UM-10 diaflo membrane (Amicon Corp.). After ultrafiltration, the volume of the retained fraction (10 ml) was adjusted with 0.15 M NaCl to that of the original serum.

TABLE 3. *Effect of human lipoproteins on reductase activity*

Fraction (density)	Concentration in medium (μg/ml)			Reductase activity (pmol/min per mg)
	Protein	Cholesterol	Triglyceride	
Buffer (−)	—	—	—	18.5
Whole plasma (−)	95	2	0.78	10.7
	280	6	2.3	10.3
	950	20	7.8	6.4
	1800	40	16	5.3
	3600	80	32	4.3
VLDL (<1.006)	—	6	32	15.5
	—	80	430	5.5
LDL (1.006–1.019)	0.7	2	6.1	9.6
	2.1	6	18	5.6
	7	20	61	3.6
	14	40	120	4.0
	28	80	240	1.9
LDL (1.019–1.063)	1.2	2	0.43	14.1
	3.5	6	1.3	7.3
	12	20	4.3	5.5
	23	40	8.6	2.2
	46	80	17	2.2
HDL (1.063–1.210)	9	2	0.75	18.8
	27	6	2.3	14.0
	90	20	7.5	17.2
	180	40	15	16.1
	360	80	30	8.7
Nonlipoprotein plasma (>1.210)	590	—	—	36.9
	1780	—	—	30.7
	5930	—	—	30.4
	11800	—	—	29.4
	23600	—	—	29.3

Confluent cells were incubated in MEM without serum as described in the legend to Fig. 2. After 18 hr, the medium of each flask was replaced with 7.5 ml of fresh MEM and 2.5 ml of buffer C containing the indicated fraction. 6 hr after additions, extracts from single flasks were assayed for reductase activity. Lipoproteins in human plasma were fractionated and their contents of protein, cholesterol, and triglyceride were measured. The content of protein in VLDL and the content of cholesterol and triglyceride in the nonlipoprotein fraction of plasma were too low for accurate measurement.

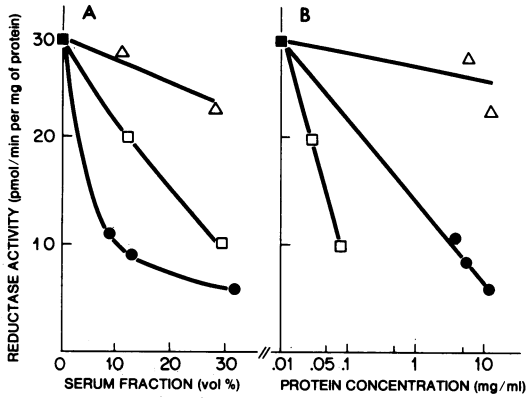

FIG 5. Decrease in reductase activity after addition of fetal-calf serum lipoproteins. Confluent cells were incubated in MEM without serum as described in legend to Fig. 2. After 14 hr in MEM without serum, the medium in each flask was replaced with fresh MEM containing the indicated concentration of fractions diluted in buffer C: ●, unfractionated fetal-calf serum; □, lipoprotein fraction of fetal-calf serum ($\rho < 1.21$ g/cm^3); △, nonlipoprotein fraction of fetal calf serum ($\rho > 1.21$ g/cm^3); ■, MEM alone. 4 hr after the additions were made, extracts from single flasks were assayed for reductase activity. To prepare the lipoprotein fractions, 200 ml of fetal-calf serum was spun in the ultracentrifuge at a density of 1.21, and the volumes of both the supernatant and infranatant fractions were adjusted to the original 200 ml with buffer C.

only had levels of reductase similar to those of cells in stationary phase but they also developed a similar increase in enzyme activity when serum was removed.

When reductase activity had been stimulated by prior incubation of fibroblasts in lipid-free medium, the addition of serum caused an inhibition of enzyme activity that was both time-dependent and proportional to the amount of serum added. Using this response to assay the activity of the inhibitory factor in serum, we obtained the following evidence that the inhibitory factor was a lipoprotein: first, it appeared to have a molecular weight greater than 10,000 as judged by ultrafiltration; second, its activity was destroyed by lipid extraction; and third, it floated when the serum was fractionated by ultracentrifugation at a density of 1.21 g/cm^3.

The inhibitory factor in the lipoprotein fraction of serum could be cholesterol itself, cholesterol bound to a specific apolipoprotein, or some other constituent of the lipoprotein fraction. The observation that human LDL was more effective than HDL in reducing reductase activity when added at an equal cholesterol concentration suggests that if cholesterol were the inhibitory factor, then the lipoprotein to which it was bound also participated in the inhibitory action. Since LDL and VLDL share a common apolipoprotein, apo B peptide, which is absent from HDL (18), and since LDL and VLDL both appeared to be more potent than HDL in their ability to lower reductase activity, it is possible that the apo B peptide is specifically involved in this regulatory process. An antibody to human LDL crossreacts with lipoproteins in 10 other mammalian sera, but fails to crossreact with chicken lipoproteins (19). Consistent with this observation is our finding that sera from four mammalian species reduced reductase activity in human fibroblasts, but chicken egg yolk, despite its high VLDL cholesterol content (20), did not.

In these experiments purified lipoproteins produced an almost complete inhibition of reductase activity in cultured cells at concentrations considerably lower than those existing physiologically in circulating plasma. If the enzyme activity in fibroblasts of skin and other nonhepatic tissues is as sensitive *in vivo* to the inhibitory effect of endogenous circulating lipoproteins as it appears to be *in vitro*, then cholesterol synthesis in these tissues must be under constant suppression. Such a conclusion is consistent with the observation that low rates of cholesterol synthesis can be observed in such tissues (21). In liver, reductase activity must be relatively more resistant to suppression by endogenous lipoproteins since this organ normally synthesizes much larger amounts of cholesterol (20). Synthesis in liver may be preferentially suppressed by cholesterol carried in exogenous lipoproteins (3, 21).

The *in vitro* system described in this report should be useful as a model for further study of the biochemical events involved in the regulation of cholesterol metabolism by extracellular lipoproteins in human fibroblasts. Hopefully, this system may allow a dissection of any derangements in this regulatory process that may be responsible for certain of the inherited hyperlipidemic states in man.

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