SUMMARY

The regulation of lipid synthesis from acetate in cultures of L cells in response to changes in exogenous lipid supply has been studied, and the enzyme acetyl-CoA synthetase has been investigated in reference to its possible role in the regulation of lipid biosynthesis from acetate.

When serum lipid was removed from monolayers of L cells, a relatively rapid stimulation of [14C]acetate incorporation into lipid was observed within 2 hours. This stimulation of incorporation was observed in both sterol and fatty acid fractions. Conversely, an inhibition of [14C]acetate incorporation was observed within 1 to 2 hours when cells cultured in lipid-free medium were transferred to serum-supplemented medium. As with the stimulation, this inhibition was observed both in sterol and fatty acid fractions of the cell lipid. The data indicated a coordination of fatty acid and cholesterol metabolism in the cells. Inhibition of [14C]acetate incorporation was observed in sterol as well as free fatty acid and glycerolipid fractions in cultures grown in lipid-free medium and transferred to fatty acid-supplemented medium; and similarly, cultures transferred to medium supplemented with cholesterol showed inhibition not only of acetate incorporation into sterol but also into fatty acid and glycerolipid fractions. Cycloheximide, actinomycin D and mitomycin C seemed to have no influence on the early stimulation or inhibition of [14C]acetate incorporation into total lipid.

Acetyl-CoA synthetase activity was assayed in homogenates of L cells cultured in the presence of serum-supplemented or lipid-free medium and a 5-fold decrease in enzyme activity was observed in homogenates of cultures grown in the presence of exogenous lipid. When cells grown in serum-supplemented medium were transferred to serum-free medium, a stimulation of enzyme activity occurred within 2 to 3 hours which reached a maximum by 6 hours. Conversely, when cells were cultured in lipid-free medium and transferred to serum-supplemented medium, inhibition of enzyme activity occurred within the same time course.

Cycloheximide, actinomycin D, and mitomycin C had no influence on the stimulation or inhibition of enzyme activity observed in response to changes in exogenous lipids. Inhibition of enzyme activity also was observed in cells cultured in lipid-free medium which were transferred to medium containing either fatty acid or cholesterol, indicating fatty acid and cholesterol might be part of the components of serum lipid that can influence the activity of acetyl-CoA synthetase. However, when various concentrations of serum lipid were added to the reaction mixture in vitro, there was no influence on enzyme activity. The data suggest that the enzyme acetyl-CoA synthetase may play a role in the regulation of lipid biosynthesis from acetate in cultured cells.

Cells in culture have provided an easily manipulatable system for the study of the regulation of lipid metabolism at the cellular level. It was observed that cells cultured in the presence of serum derived most of their lipid from the serum in the growth medium (1), while cells cultured in the absence of exogenous lipid synthesized cell lipids from carbon sources such as acetate and glucose (2). More recent studies have centered around attempts to localize the enzymatic sites of regulation of lipid biosynthesis. In investigations of the regulation of fatty acid synthesis in skin fibroblasts, Jacobs et al. (3) showed that there was induction and repression of the enzyme acetyl-CoA carboxylase over a 2-day time course. Recently, Alberts et al. (4) have demonstrated a similar regulation of the synthesis of the enzyme fatty acid synthetase in cultures of L and HeLa cells. In the case of sterol metabolism, the work of Williams and Avigan (5) and Rothblat et al. (6) suggested that there is a regulatory point in the pathway of cholesterol synthesis at the site of mevalonic acid. It was demonstrated by Watson (7) in hepatoma cells and Brown et al. (8) in skin fibroblast cultures that there were alterations in the activity of 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase within 4 hours after changes in exogenous cholesterol and that the rise in enzyme activity required de novo synthesis.

In considering lipid biosynthesis from acetate, however, several lines of evidence indicate that there might be other
In their study of the regulation of fatty acid biosynthesis in cultured fibroblasts, Jacob et al. (9) noticed a rapid inhibition of synthesis of [14C]acetate incorporation into fatty acid, sterol, and amino acids, suggesting a regulation at the level of 2-carbon metabolism. Coordinate inhibition of both fatty acid and sterol synthesis by exogenous cholesterol also was observed in some of the studies of Kandutsch and Chen (12).

In vitro studies have suggested that one possible site for coordination of fatty acid and sterol biosynthesis from acetate is at the level of the enzyme acetyl-CoA synthetase (acetate:CoA ligase, EC 6.2.1.1). A recent suggestion for alternative control mechanisms comes from previous studies (2) which indicated a coordinate inhibition of [14C]acetate incorporation into fatty acid, sterol, and amino acids, suggesting a regulation at the level of 2-carbon metabolism. Coordinate inhibition of both fatty acid and sterol synthesis by exogenous cholesterol also was observed in some of the studies of Kandutsch and Chen (12).

In vitro inhibition of enzyme activity in mitochondrial supernatants upon addition of oleate. A regulatory role for this enzyme also is supported by observations of changes in cellular acetate levels in relation to altered lipid metabolism (16, 17).

In this study, we present data on the kinetics of regulation of lipid metabolism from acetate in cultures of L cells in response to changes in exogenous lipid supply. The enzyme acetyl-CoA synthetase in these cultures also is investigated in reference to its possible role in the regulation of lipid biosynthesis from acetate. A preliminary report of these studies has been presented previously (18).

**METHODS**

Cells—L-929 cells were obtained from the American Type Culture Collection and from Dr. George Rothblat, Wistar Institute. L-2071 cells were obtained from Dr. Virginia Evans, National Cancer Institute. L-929 cells were grown as monolayer cultures at 36° in Eagle’s minimal essential medium (MEM) supplemented with nonessential amino acids, 10% fetal calf serum, penicillin (50 units per ml), and streptomycin (50 µg per ml). The pH was maintained at 7.2 to 7.4 by flushing with CO₂. For subcultivation, the monolayer was removed with a 0.25% trypsin solution in 40% trichloroacetic acid (0.03 ml) and the mixture was centrifuged for 5 min at 1000 g. The precipitated protein was collected by filtration and washed as described above. After thorough drying to remove all traces of ether, the protein was weighed, dissolved in MEM, and sterilized by filtration through a Millipore filter with a pore size of 0.22 µm. Free fatty acid (FFA) was dissolved in the medium on an albumin carrier (molar ratio of free fatty acid to albumin of 4:1) by heating a solution containing 1.7 mg per ml of fatty acid-poor albumin (Schwarz-Mann) in MEM to 60° and adding, with stirring, the salt of the free fatty acid (NuChek Prep, Inc.) dissolved in ethanol. Cholesterol (NuChek) was added to the medium using DLSP in MEM for a final concentration of 30 µg per ml, and stirred at 37° for 1 hour.

**Assay of [14C]Acetate Incorporation into Cell Lipid—Replicate cultures of cells were established in 25-cm² Falcon flasks or 60-mm Petri dishes which were maintained at 37° in a humidified atmosphere of 6% CO₂ in air. Lipid biosynthesis from acetate was assayed in confluent monolayers. At zero time the monolayers were washed three times with CBSS and the medium was replaced with experimental medium. At the designated time, sodium [1-14C]acetate (Amersham-Searle) was added for a 1-hour pulse. After 1 hour, the monolayer was rinsed three times with cold CBSS and the cells were lysed by freezing and thawing in 1 vol of distilled water. The cell lysate then was filtered through a 0.22-µm filter and an aliquot was taken for determination of cell protein. Cell lipids were extracted with a 2:1 solution of chloroform-methanol and the extract was washed according to the method of Folch et al. (25). The extract then was evaporated to dryness in a stream of nitrogen and redissolved in ethyl ether. For separation of fatty acid from sterol fractions, the ether extract was evaporated under a stream of nitrogen and saponified with 10% potassium hydroxide in 70% ethanol (3 ml) and heating at 70° for 4 hours. After adding 6 ml of water, nonsaponifiables were extracted from three 3 ml aliquots of ether extract. Thin layer chromatography of this fraction indicated the majority of the radioactivity migrated with an RF value of 0.8 similar to sterols such as cholesterol, and in therefore referred to as the sterol fraction. Fatty acids were isolated by acidifying the saponification mixture with concentrated hydrochloric acid and extracting with 3 volumes of ethyl ether. It was confirmed by TLC that the radioactivity in this fraction migrated with an RF value equal to free fatty acid.

For thin layer chromatography, aliquots of the ether extract were spotted on plates of Silica Gel G, 250 µm, which had been prepared from a generous supply and activated for 30 min at 100°. Plates were developed in a solvent system of petroleum ether-ethyl ether-acetic acid (65:25:4). After drying, the lipids were visualized in iodine vapor and identified by comparison with authentic standards. For determination of radioactivity, sections of the chromatograms were scraped into vials and radioactivity was determined by liquid scintillation spectrometry.

**Enzyme Assays—Acetyl-CoA synthetase assay was assayed by the method of Tove et al. (24). Monolayers were rinsed three times in CBSS and harvested by mopping with perforated cellophane. Cells were homogenized in 0.88 m sucrose containing 0.2% Triton X-100 by 20 strokes of a Donnate Type B hand homogenizer. The reaction mixture contained cell homogenate (0.2 ml), 0.1 m Tris (pH 8.5), 5 mm magnesium chloride, 10 mm sodium fluoride, 1 mm dithioerythritol, 0.6 nm CoA, 60 nm ATP, 0.6 nm [1-14C]acetate (1.78 µCi per µmol) in a total volume of 0.4 ml. After incubation for 20 min at 37°, the reaction was stopped by the addition of 40% trichloroacetic acid (0.03 ml) and the mixture was centrifuged at 12,000 × g for 10 min. The supernatant (0.05 ml) of the reaction mixture was spotted on filter paper, and the excess acid was removed by steaming for 10 min. After drying, the radioactivity was determined directly on the filter disc and corrected for nonenzymatic binding of acetate using controls containing the reaction mixture minus CoA.

**Radioactivity—Radioactivity was determined in a Packard liquid scintillation spectrometer. Quenching was determined by the channels ratio method. Lipid extracts and thin layer chromatography samples were counted in a scintillation solution containing toluene, 2,5-diphenyloxazole (6 g per liter) and 1,4-bis-2-

---

1 The abbreviations used are MEM, minimal essential medium; HMG, 3-hydroxy-3-methylglutaryl-CoA; CBSS, buffered balanced salt solution; DLSP, delipidized serum protein; TLC, thin layer chromatography; Tₘ, zero time.
Inhibitors of Macromolecule Synthesis—Cycloheximide, actinomycin D, and mitomycin C were obtained from Sigma Chemical Co. Inhibition of protein synthesis by cycloheximide was assessed by incorporating it into the medium at 2 to 10 μg per ml and pulsing for 30 min at hourly intervals thereafter with [3H]leucine (5 μCi per ml; 10 mCi per mmol). Monolayers were rinsed three times and harvested into 5% trichloroacetic acid. The precipitate was collected on filter paper and radioactivity was determined as described above. For inhibition of RNA synthesis by actinomycin D, a similar assay was carried out using actinomycin D at 1 to 5 μg per ml and [3H]uridine (10 μCi per ml; 6.3 Ci per mmol). For assay of inhibition of DNA synthesis by mitomycin C, [3H]thymidine (5 μCi per ml; 0.25 mCi per μg) was employed using 10 to 100 μg per ml of mitomycin C.

RESULTS

[14C]Acetate Incorporation into Cell Lipids—Studies were conducted to determine the time course of stimulation or inhibition of lipid synthesis from acetate in cells in culture upon changes in exogenous lipid. These experiments were carried out with the 2071 strain of L cells cultured in serum-free or serum-supplemented medium and with the L-929 cells which were cultured in the absence of exogenous lipid by supplementing the medium with DLSP. Essentially similar results were obtained with the two strains of L cells. Therefore, the results described here are mainly from the L-929 cells since their ease of handling allowed a greater number of experiments to be conducted. For the determination of kinetics of stimulation of [14C]acetate incorporation into cell lipids upon removal of serum lipids, replicate cultures were grown to confluency in serum-supplemented medium. At zero time (T0) the monolayers were rinsed and lipid-free medium added. Control experiments established that acetate incorporation into lipid was linear for up to 6 hours under the culture and assay conditions utilized (Fig. 1). Lipid synthesis was evaluated at various time points with 1-hour pulses of [14C]acetate. The results of these experiments are shown in Fig. 2. The data are expressed as per cent of control so that direct comparison can be made between total lipid and sterol and fatty acid subfractions. When the specific activity of [14C]acetate was 20 μCi per mg, the absolute values for acetate incorporation per hour per μg of cell protein averaged 1 to 5 dpm per μg of protein for cells cultured in the presence of serum lipid and 10 to 20 dpm per μg for cells cultured in lipid-free medium. The data show that when exogenous lipid was removed from monolayers of L cells, there was a relatively rapid stimulation of acetate incorporation into lipid which was observed within 2 hours. Data are not given for time points greater than 12 hours because of the variables introduced by random cell proliferation. It should be mentioned that stimulation of [14C]acetate incorporation into lipid occurred whether or not the medium contained DLSP; the magnitude of this stimulation was no greater in DLSP containing medium than in MEM. The stimulation of incorporation was observed in both sterol and fatty acid fractions (Fig. 2) of the cell lipid and the time courses for these two fractions were relatively similar. Sterol and fatty acid represented greater than 90% of the total radioactivity from acetate incorporated into lipid under those conditions, and the ratio of fatty acid to sterol fractions averaged approximately 8:1. Incorporation rate of [14C]acetate into lipid remained constant in controls where the medium was replaced with serum-supplemented medium.

Fig. 3 shows the data obtained from inverse experiments where cells were cultured in serum-free medium or medium supplemented with DLSP and transferred to serum-supplemented medium at T0. An inhibition of [14C]acetate incorporation into lipid was observed within 1 to 2 hours which plateaued at about 6 hours. As with the stimulation, this inhibition was observed in both the sterol and fatty acid fractions of the cell.
lipid. It should be noted that an inhibition which was more rapid and of greater magnitude was observed when \[^{14}C\]acetate of specific activity greater than 100 \(\mu\)Ci per mg was utilized. This suggests the presence of significant amounts of acetate in fetal calf serum. It also should be noted that in control cultures, which received DLSP-supplemented medium at \(T_0\), there was usually a temporary stimulation of \[^{14}C\]acetate incorporation into cell lipid which occurred around 2 to 3 hours and returned to normal by 6 hours. The data are not corrected for this stimulation, however, since the constant values for controls in Fig. 2 suggest this stimulation is due to the DLSP, which was not present when inhibition of lipid synthesis was being evaluated.

Since a coordinate time course for sterol and fatty acid stimulation and inhibition was observed, experiments were conducted to determine if there was a coordination of fatty acid and cholesterol metabolism. Cultures were grown in lipid-free medium and at \(T_0\) received medium supplemented with either serum, fatty acid, or cholesterol. Lipid biosynthesis after 24 hours was assayed by a 1-hour pulse of \[^{14}C\]acetate, and incorporation of radioactivity into total lipid and lipid subfractions was determined. The results, shown in Table I, indicate that there was a coordinate inhibition of acetate incorporation into lipid by exogenous fatty acid and cholesterol in these cells. When cultures were supplemented with free fatty acids at \(T_0\), the expected inhibition of acetate into the free fatty acid and glycerolipid fraction was observed, and in addition the incorporation of acetate into sterol was inhibited. Similarly, cultures which received cholesterol showed an inhibition of acetate not only into sterol, but also into fatty acid and glycerolipid fractions. The time course of the inhibitory effect of cholesterol and fatty acid was determined in experiments similar to those described for whole serum (Fig. 2 and 3). The data (Fig. 4)
indicated that when either cholesterol or fatty acid was added to the medium [14C]acetate incorporation into both fatty acid and cholesterol was inhibited and the patterns of inhibition were similar and resembled that of whole serum.

In order to begin an investigation of the possible mechanism of regulation of acetate incorporation into lipid in these cells, and in order to coordinate the data on acetate incorporation with that of enzyme activity to be presented below, stimulation and inhibition of acetate incorporation into lipid was evaluated in the presence of certain inhibitors of macromolecule synthesis: cycloheximide was utilized at a concentration of 10 µg per ml which yielded a 95% inhibition of protein synthesis in these cultures within 30 min; actinomycin D was utilized at 5 µg per ml which yielded an inhibition of RNA synthesis of 90% in these cultures within 30 min. Mitomycin C was utilized at a concentration of 100 µg per ml which resulted in a 60% inhibition of DNA synthesis in these cells within 2 hours. For evaluation of their influence on the stimulation of acetate incorporation into lipid observed upon removal of serum lipid, cells were cultured in serum-supplemented medium and replicate cultures received at T₀ either lipid-free medium or lipid-free medium supplemented with one of the indicated inhibitors. Lipid biosynthesis was evaluated after 6 hours with a 1-hour pulse of [14C]acetate. The results, shown in Fig. 5, indicate that none of the inhibitors of macromolecule synthesis had any influence on the stimulation of [14C]acetate incorporation observed after removal of serum lipid. A similar lack of effect of the inhibitors on inhibition of [14C]acetate incorporation into total lipid was demonstrated in converse experiments (Fig. 5). In this situation, cells were cultured in the presence of delipidized serum protein and at T₀ received serum-supplemented medium with or without the indicated inhibitor. The data indicate the inhibitors had no effect on the inhibition of acetate incorporation into total lipid in response to addition of serum lipid. The differences between the magnitude of inhibition observed in the presence of the various inhibitors are not significant since considerable variability was observed in the acetate incorporation in the presence of these inhibitors from experiment to experiment, as indicated by the range of values in Fig. 5.

**Effect of Alterations in Exogenous Lipid on Acetyl-CoA Synthetase Activity in Cell Cultures**—The data from the studies on [14C]acetate incorporation into cell lipid indicated a rapid time course of stimulation and inhibition of lipid biosynthesis from acetate and a coordination of the effects of cholesterol and fatty acid. Since one possible mechanism for this coordinated control could be a regulation at the 2-carbon level of metabolism, attention was focused on studies of the enzyme acetyl-CoA synthetase in these cultures. Experiments were conducted in parallel fashion to those described above which assessed acetate incorporation. Fig. 6 shows the results of experiments to standardize the assay conditions. Homogenates of L cells converted acetate to acetyl-CoA at a rate that was linear with time and protein concentration (Fig. 6, A and B). The concentrations of coenzyme A (Fig. 6C) and ATP (Fig. 6D) that gave half-maximal velocities were 0.08 and 18 mM, respectively, for enzyme activity in cell homogenates. The kinetics of enzyme activity versus ATP concentrations yielded a sigmoidal curve, suggesting either a possible autocatalytic effect of ATP, or significant ATPase activity despite the presence of fluoride. Although enzyme activity was assayed by steaming unreacted acetate from the reaction mixture, it was confirmed that the acetate was indeed converted to acetyl-CoA by paper chromatography of the reaction mixture according to the method of Huang (25).

Acetyl-CoA synthetase activity was assayed in homogenates of L-929 cells cultured in the presence of serum or delipidized serum protein, and in homogenates of L-2071 cells cultured in serum-supplemented or serum-free medium. The data shown in Table II indicated a 5-fold decrease in enzyme activity in cells cultured in the presence of exogenous lipids. The data thus suggested that there is a regulation of this enzyme in the L cell

![Diagram](http://www.jbc.org/)
Table II

Acetyl-CoA synthetase levels in cells grown in lipid-free versus serum-supplemented medium

L-929 cells were subcultivated at a 1:4 ratio in MEM supplemented with 10% fetal bovine serum or 2 mg per ml of DLSP. L-2071 cells were subcultivated at 1:4 ratios in the modified NCTC-135 described under “Methods,” or NCTC-135 supplemented with 10% fetal bovine serum. When cultures reached confluency, the monolayers were rinsed, cells were harvested and homogenized, and the enzyme was assayed as described under “Methods.” Values are expressed ± the standard error of the mean; they are the average of determinations on four to eight separate cultures.

| Cell type | Culture medium | Synthetase activity (nmol/hr/mg cell protein) |
|-----------|----------------|-----------------------------------------------|
| L-929     | MEM + delipidized serum protein | 200 ± 17 |
| L-929     | MEM + fetal calf serum | 68.4 ± 6.9 |
| L-2071    | Lipid-free NCTC-135 | 296 ± 24 |
| L-2071    | NCTC-135 + fetal calf serum | 54.9 ± 11 |

To investigate the mechanism of this regulation, and to compare the activity of the enzyme to the rate of lipid synthesis, the kinetics of stimulation and inhibition of the enzyme activity in response to changes of exogenous lipids were studied in experiments similar to those described in Figs. 2 and 3. The results are shown in Fig. 7. The data indicate that when cells grown in serum-supplemented medium were transferred to serum-free medium at zero time, a stimulation of enzyme activity was observed within 2 to 3 hours and reached a maximum by 6 hours. Conversely, when cells cultured in lipid-free medium were transferred to serum-supplemented medium, an inhibition of enzyme activity occurred within 3 to 6 hours. The time course of stimulation and inhibition of enzyme activity was quite similar to that observed for stimulation and inhibition of acetate incorporation into total lipid. On the other hand, the relative magnitudes of stimulation or inhibition were somewhat less in the case of enzyme activity than they were for lipid biosynthesis from acetate.

In order to evaluate the mechanism of action of serum or individual lipids on enzyme activity, inhibition and stimulation of enzyme was assessed in the presence of inhibitors of macromolecule synthesis described in Fig. 5. Experiments were...
Fig. 8. Failure of inhibitors of macromolecule synthesis to influence stimulation and inhibition of acetyl-CoA synthetase activity in cell cultures. The effect of inhibitors of macromolecule synthesis on the stimulation and inhibition of enzyme activity in response to changes in exogenous lipids was determined in a manner similar to that described in Fig. 5. LF, lipid-free medium; CYCLOHEX, cycloheximide; ACTIN.D, actinomycin D; MITO.C, mitomycin C.

Fig. 9. Decay of the activity of acetyl-CoA synthetase in the presence of cycloheximide. Cultures were grown to confluency in medium supplemented with 2 mg per ml of DLSP and the medium was changed at T0 to similar fresh medium including 10 µg per ml of cycloheximide. At the indicated times enzyme activity was assayed in homogenates of harvested cells as described under "Methods."

Conducted in a fashion similar to that described for acetate incorporation. Cells at T0 received experimental medium in the presence and absence of the indicated inhibitor. Enzyme activity was determined after 1 to 6 hours. The data, shown in Fig. 8 indicate that the inhibitors had no influence on stimulation or inhibition of enzyme activity in response to changes in exogenous lipid. The decay rate of the enzyme under these culture conditions was assessed by adding 10 µg per ml of cycloheximide to monolayer cultures of L-929 cells grown in medium supplemented with DLSP. Enzyme activity was assayed over a 22-hour period in the presence of this inhibitor. The data (Fig. 9) showed a slow decay of enzyme activity over the time period evaluated, with an apparent half-life of approximately 20 hours under these conditions.

The results of the inhibitor experiments and the long half-life suggested that acetyl-CoA synthetase might be regulated at the level of enzyme activity. It was thus of interest to determine the effects of cholesterol and fatty acid on the enzyme. This was first evaluated by adding these compounds to the medium over monolayers of cells and then assaying enzyme activity in cell homogenates. Cells were cultured in medium supplemented with DLSP and, at T0, were transferred to medium supplemented with either fetal calf serum, fatty acid, or cholesterol. After 6 hours, enzyme activity was determined in homogenates of each culture. The data (Table III) indicate that the cultures supplemented with calf serum displayed an inhibition of enzyme activity to about 30% of the control value. Cultures receiving either fatty acid or cholesterol also showed inhibition, but the extent of inhibition was less than that produced by total serum. The results suggested that fatty acid and cholesterol might be part of the components of serum that can influence the activity of acetyl-CoA synthetase. However, in cell homogenates, when various concentrations of serum lipid from 5 to 40% were added to the reaction mixture, there was no influence on enzyme activity (Table IV). Preliminary experiments using isolated serum lipids added to the reaction mixture also showed no inhibitory effect.

Table III

| Culture medium | Enzyme activity (nmol/mg/20 min) |
|----------------|---------------------------------|
| MEM + delipidized serum protein | 134–195 |
| MEM + 20% fetal calf serum | 35.8–39.4 |
| MEM + fatty acid | 71–72 |
| MEM + cholesterol | 73–86 |

DISCUSSION

Acetate Incorporation into Lipids—Alterations of exogenous lipid levels produced rapid compensatory changes in the rate of [14C]acetate incorporation into cellular lipid. Total cell lipid was measured in these experiments to investigate possible coordinate regulation of sterol and fatty acid synthesis. When incorporation of [14C]acetate into sterol and fatty acids were measured separately, similar changes were observed which paralleled those of total lipid under the culture conditions employed. The inhibition and stimulation of sterol synthesis...
pressed f standard error.

tion of fatty acid synthesis from acetate observed upon changes
monolayers were harvested and homogenized in 0.88 M sucrose
was assayed as described under "Methods." For determination
Brown et al. (8), and Kandutsch and Chen (12). The stimula-
inhibition of [r4C]acetate incorporation into fatty acid which
in serum lipid supply was similar to that observed by Jacobs
added to the reaction mixture at a concentration of 20% (equiva-
lent to 22 μg per ml of free fatty acid and 20 μg per ml of choles-
terol), and DLSP was added at 8 mg per ml. Values are expressed
experiments is probably a reflection of the complex action of these
compounds. However, the fact that their presence seemed to
have no effect on the time course of the early stimulation and
inhibition of enzyme levels is an indication that the regulation is
occurring at the level of enzyme activity; in cases of control
where changes in absolute amounts of enzyme protein levels
have been confirmed, these inhibitors did influence the course
of events. For example, Brown et al. (8) report that cyclo-
heximide inhibits the stimulation of HMG-CoA reductase in
cultured fibroblasts, and the data of Raff (28) indicate that,
when glucose is used as a precursor of fatty acid, cycloheximide
prevents stimulation of fatty acid synthesis, reflecting the long
term regulatory steps at the level of acetyl-CoA carboxylase (3)
and fatty acid synthetase (4).

Acetyl-CoA Synthetase Activity—No other studies have been
reported on the activity of this enzyme in cultured cells. Acetyl-
CoA synthetase has been studied, however, in whole animals
(29, 30). The Km observed for the enzyme present in cell
homogenates corresponds quite well with that obtained for more
purified preparations. Londesborough et al. (31) report a Km
of 30 mM for ATP and 1 mM for CoA in a purified enzyme from
rat liver mitochondria, and Klein and Jahnke (32) report satu-
ration of the yeast acetyl-CoA synthetase by 0.2 mM CoA and
8 mM ATP.

Several lines of evidence suggest that the enzyme may have a
role in the regulation of acetate incorporation into lipid in
cultured cells. In the first place, the time course of stimulation
and inhibition of enzyme activity parallels that of acetate
incorporation into lipid. Second, both responses are insensitive
to inhibitors of macromolecule synthesis. Third, both acetate
incorporation and enzyme activity are inhibited by addition of
fatty acid and cholesterol to the medium. Finally, calculations
of the amount of carbon converted to lipid based on the observed
values for enzyme activity and lipid synthesis from acetate indi-
cate that the enzyme in cells cultured in the presence of serum
could be the rate-limiting step of conversion of acetate into cell
lipid.

Several studies conducted on preparations from whole animals
support a possible regulatory role for this enzyme (13, 17, 22).
Steiner and Cahill (13) found that, upon feeding high fat diet,
lipogenesis from acetate was decreased in brown adipose tissue
homogenates, but lipogenesis from acetyl-CoA remained constant.
Murthy and Steiner (15) found that activity of acetyl-CoA
synthetase decreased in livers of starved or alloxan diabetic rats.
Furthermore, they correlated changes in the enzyme with
alterations in free acetate concentration in livers of 72 hour
fasted and fed rats (17). Briefly et al. (16) had previously
observed alterations in acetyl-CoA concentrations in tissues of
the developing lamb, which also suggested that there was a
regulation of the use of acetate as a substrate for lipogenesis.

The mechanism of regulation of this enzyme is intriguing.

from acetate observed here agrees in magnitude and duration with the data reported by Bates and Rothblat (26), Watson (7),
Brown et al. (8), and Kandutsch and Chen (12). The stimulation
of fatty acid synthesis from acetate observed upon changes in
serum lipid supply was similar to that observed by Jacobs
et al. (3). These workers found, however, an even more rapid
inhibition of [14C]acetate incorporation into fatty acid which
occurred within 10 min of addition of serum or fatty acid to the
medium.

It is important to note that the present study deals with the
early control events which occur within 12 hours after change
in external lipid source. Additional changes in rates of acetate
incorporation, especially into fatty acids, occur during the next
several days; these could be due to induction or repression of
several enzymes (3, 4) and also to multiple effects related to
induction of cell division in the culture.

A coordinate effect between fatty acid and sterol metabolism
has been demonstrated in these studies. That is, fatty acids
added to the culture medium resulted in inhibition of sterol
synthesis from acetate and, conversely, cholesterol in the me-
dium inhibited incorporation of [14C]acetate into both fatty
acids and glycerolipids. Although the inhibition of sterol
synthesis from acetate by fatty acid could result partly from
dilution of intracellular acetyl-CoA pools through oxidation of
fatty acid, this dilution effect could not be applicable to the
converse observation of inhibition of acetate incorporation into
fatty acid by cholesterol since cholesterol is not converted to
acetate in animal cells. Similar coordination has been observed
previously in long term growth experiments (2). An inhibition
of fatty acid synthesis by cholesterol was observed in some of
the experiments of Kandutsch and Chen (12), who investigated
the effect of various sterol analogs on sterol synthesis in L cells
and liver cell cultures. In our hands, inhibition of fatty acid
synthesis by sterol was quite reproducible, but always of lesser
magnitude (around 40 to 60%) as compared to the magnitude
of inhibition (85 to 90%) when fatty acid or total serum are
added (Table 1 and Figs. 4 and 5). This suggests that the
effects of sterol on fatty acid synthesis may be influenced by
other variables such as growth conditions or be mediated by
only one of the possible control points in Fig. 10.

It is interesting that the stimulation of [14C]acetate incorpora-
tion into lipid observed in L cell cultures occurred when cells
were transferred both to serum-free medium and to medium
supplemented with delipidized serum proteins and that no
greater magnitude of stimulation was observed in the presence
of DLSP. Williams and Avigan (6) have investigated the
effect of DLSP on the stimulation of sterol and fatty acid syn-
thesis, and their data indicate a stimulatory effect of DLSP
which is more pronounced in fibroblast cultures.

The results of the studies of stimulation and inhibition of
acetate incorporation into total lipid in the presence of inhibitors
of macromolecule synthesis must be interpreted with great
cautionsince it is well established that these inhibitors do not
have single clearly defined actions in animal cells (27). In fact,
the increased variability observed in the data in these experi-
ments is probably a reflection of the complex action of these
compounds. However, the fact that their presence seemed to
have no effect on the time course of the early stimulation and
inhibition of enzyme levels is an indication that the regulation is
occurring at the level of enzyme activity; in cases of control
where changes in absolute amounts of enzyme protein levels
have been confirmed, these inhibitors did influence the course
of events. For example, Brown et al. (8) report that cyclo-
heximide inhibits the stimulation of HMG-CoA reductase in
cultured fibroblasts, and the data of Raff (28) indicate that,
when glucose is used as a precursor of fatty acid, cycloheximide
prevents stimulation of fatty acid synthesis, reflecting the long
term regulatory steps at the level of acetyl-CoA carboxylase (3)
and fatty acid synthetase (4).

| Assay mixture | Enzyme activity (nmol/hr/mg protein) |
|--------------|--------------------------------------|
| Control      | 24 ± 3.6                             |
| + Serum      | 23 ± 0.71                            |
| + DLSP       | 24 ± 3.5                             |
Fig. 10. Possible sites of control and types of regulation in the over-all process of fatty acid and sterol synthesis in cultured cells. 1, acetyl-CoA carboxylase; 2, HMG-CoA reductase; 3, fatty acid synthetase; 4, acetyl-CoA synthetase; 5, one or more enzymes in the terminal stages of sterol synthesis. Open bars across paths represent enzyme regulated at the level of activity, solid bars represent enzymes controlled at the level of synthesis, and dotted bar indicates undetermined mechanisms.

cell cultures and the lack of effect of inhibitors on macromolecule synthesis suggested that lipids might be acting as allosteric effectors of this enzyme. The sigmoidal shape of the response of the activity to changing ATP concentration also would be consistent with it being allosterically controlled. However, no effect was observed in these studies when serum lipid was added directly to the homogenates during assay of enzyme activity. Preliminary attempts were made in this study to add individual lipids to the reaction mixture with negative results. It is interesting that Murthy and Steiner (15) report inhibitory effect of sodium oleate (1 to 4 mm) on enzyme activity in vitro. It is very difficult, however, to separate this observation from a detergent effect of the lipid. Further studies are planned to elucidate whether isolated lipids added in vitro are allosteric effectors of the enzyme, or whether a secondary regulator such as a protein kinase might be involved.

Mechanisms of Regulation of Acetate Incorporation into Lipid—Although it disguises the undoubtedly greater complexity of the regulation of cell lipid biosynthesis, Fig. 10 illustrates schematically the control points for lipid synthesis that thus far have been identified and partially characterized in cultured cells. From the available evidence it seems that two major control points are at the first committed steps in fatty acid and sterol biosynthesis (Fig. 10, 1 and 2). It appears that these steps are regulated via the induction and repression of the appropriate enzymes acetyl-CoA carboxylase (3) and HMG-CoA reductase (7, 8). Another control point in fatty acid synthesis via enzyme induction and repression occurs at the level of fatty acid synthetase (Fig. 10, 3) (4). From the evidence presented here a fourth point of control which would influence both pathways is via regulation of the acetate activating enzyme (Fig. 10, 4) the mechanism, which is so far unidentified, appears to be at the level of enzyme activity rather than to involve induction and repression of synthesis. It is also indicated in Fig. 10 that other control points may exist between mevalonial and cholesterol (Fig. 10, 5) (33).

It is difficult, however, to determine which control point is rate-limiting and most important physiologically. It is possible that, although a coordinate effect between fatty acid and sterol has been observed, separate points of regulation in fatty acid and sterol pathways might be the rate-limiting ones. It is clear in the case of fatty acid synthesis that the time course of regulation of lipid synthesis from acetate observed in our hands and by Jacobs et al. (3, 9) could not be accounted for by the kinetics of regulation of acetyl-CoA carboxylase or fatty acid synthetase. It also has been observed in in vivo experiments that acetate incorporation into fatty acid is greatly inhibited in a rapid fashion after a short period of fasting (10, 11). The definition of the relative role of various control points in sterol synthesis is more difficult since the induction and repression of HMG-CoA reductase occurs within 4 to 6 hours of change in exogenous lipid (8). Therefore, a stimulation of [14C]acetate incorporation into sterol would reflect either changes in HMG-CoA reductase or regulation at the level of acetate or both. Similarly, inhibition of [14C]acetate incorporation into sterol could reflect changes in either HMG-CoA reductase or acetate activation and in addition be subjected to dilution effects in the oxidation of fatty acid to acetyl-CoA. Some indications of the relative importance of acetyl-CoA synthetase and HMG-CoA reductase in regulation of sterol synthesis come from data of Bates and Rothblat (26). They compared inhibition of sterol synthesis from [14C]acetate and H2O and observed a greater per cent inhibition of synthesis in L cells using acetate as a precursor, the difference presumably reflecting controls at the level of 2-carbon metabolism.

The significance of various cellular control points may be related to the importance of acetate as a substrate for lipid synthesis. In cultured cells grown in medium containing 0.1 mg per ml of acetate, it serves as approximately 20% of the carbon source for lipid synthesis. Acetate is also a substrate for acetate metabolism in vitro at times of ethanol ingestion, since ethanol is converted to acetate by the liver (34). It is also possible that regulation of acetyl-CoA synthetase is involved in vivo in the metabolism of acetoacetate, which also can serve as its substrate (29). It thus would be important in situations of ketoadidosis when ketone bodies are significant carbon sources to peripheral cells.

Finally, there are several points in addition to acetyl-CoA synthetase at which control of acetate metabolism could occur. One possible site is acetate transport into and out of the cell. It also has been established recently that three pools of acetyl-CoA exist in certain cells (35, 36) and that tolbutamide can effect the intracellular flow of acetyl-CoA between the three pools. The final possibility is that there could be a competition intracellularly for coenzyme A so that availability of CoA and its intracellular transport may be a site of coordination and control. Studies are currently in progress using cells in culture to determine which mechanisms are active in control of 2-carbon metabolism and to elucidate the physiological significance of acetyl-CoA synthetase in the regulation of lipogenesis.

Acknowledgments. We wish to thank Ms. Marion Dillard and Mr. John Findley for providing excellent technical assistance for this project, and Dr. George Rothblat for aid in the preparation of this manuscript.

REFERENCES
1. Howard, B. V., and Kritchevsky, D. (1969) Biochim. Biophys. Acta 187, 293-301
2. Bailey, J. M. (1966) Biochim. Biophys. Acta 125, 226-236
3. Jacobs, R. A., Sly, W. S., and Majerus, P. W. (1973) J. Biol. Chem. 248, 1268-1276
4. Alberts, A. W., Ferguson, K., Hennessy, S., and Vagelos, P. R. (1974) J. Biol. Chem. 249, 5241-5249
5. Williams, C. D., and Atigian, J. (1973) Biochim. Biophys. Acta 260, 413-423
6. Rothblat, G. H., Boyd, R., and Deal, C. (1971) Exp. Cell Res. 57, 430-440

* Unpublished data.
7. WATSON, J. A. (1973) in Tumor Lipids, Biochemistry and Metabolism (Wood, R. ed) pp. 34–53, American Oil Chemical Society Press, Champaign, Ill.
8. BROWN, M. S., DANA, S. E., AND GOLDSTEIN, J. L. (1973) Proc. Nat. Acad. Sci. 70, 2162–2166
9. JACOBS, R. A., AND MAJERUS, P. W. (1973) J. Biol. Chem. 248, 8392–8401
10. LION, I., MARRI, M. G., AND CAHILL, C. L. (1953) J. Biol. Chem. 196, 25–32
11. BAKER, N., AND HUEBOTTER, R. J. (1973) J. Lípid Res. 14, 87–94
12. KANDUTSCH, A. A., AND MEIER, H. W. (1973) J. Biol. Chem. 248, 8392–8401
13. STEINER, G., AND CAHILL, G. F. (1966) Can. J. Biochem. 44, 1587–1596
14. RAFF, C., SLADEK, M., AND DECKER, K. (1972) Biochim. Biophys. Acta 300, 1–9
15. MURPHY, V. K., AND STEINER, G. (1972) Metabolism 21, 213–221
16. BRIERLEY, R. V., JEACOCK, M. K., AND SHEPHERD, D. A. L. (1969) Biochem. J. 114, 70P
17. MURPHY, V. K., AND STEINER, G. (1973) Metabolism 22, 115–125
18. HOWARD, B. V., AND BAILEY, J. M. (1973) Fed. Proc. 32, 602A
19. CRISTOFALO, V. J., AND KRITCHEVSKY, D. (1966) J. Cell Physiol. 67, 125–132
20. EVANS, V. J., BRYANT, J. C., KERR, H. A., AND SCHILLING, E. L. (1964) Exp. Cell Res. 36, 439–474
21. ALBUTT, E. C. (1966) J. Med. Lab. Tech. 23, 61–82
22. LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L., AND RANDALL, R. J. (1951) J. Biol. Chem. 193, 265–275
23. FOLCH, J. M., LEERS, M., AND SLOANE-STANLEY, G. H. A. (1957) J. Biol. Chem. 226, 497–509
24. TOVEY, S. R., STACKY, R. E., AND LATIMER, S. B. (1964) Biochim. Biophys. Acta 44, 192–195
25. HUANG, K. P. (1970) Anal. Biochem. 37, 98–104
26. BAYED, S. R., AND ROTHBLAT, G. R. (1974) Biochim. Biophys. Acta 360, 38–55
27. CRAIG, N. (1973) J. Cell. Physiol. 82, 133–150
28. RAFF, R. A. (1970) J. Cell. Physiol. 75, 341–352
29. AAS, M., AND BREMER, J. (1968) Biochim. Biophys. Acta 164, 157–166
30. AAS, M. (1971) Biochim. Biophys. Acta 231, 32–47
31. LONDESBOURGH, J. C., YUAN, S. L., AND WEBSTER, L. T. (1973) Biochem. J. 133, 23–36
32. Kлейн, И. П., и Янкне, Л. (1968) J. Bacteriol. 96, 1632–1639
33. HOWARD, B. V., BUTLER, J. D., AND RAYLE, J. M. (1973) in Tumor Lipids, Biochemistry and Metabolism (Wood, R. ed) pp. 200–214, American Oil Chemical Society Press, Champaign, Ill.
34. LUNDBERG, F., SEXTOPF, L., DAMGAARD, S. E., CLAUSEN, J. P., AND TRAP-JENSEN, J. (1973) J. Clin. Invest. 52, 3231–3235
35. RAOI, G. J., LIANG, T., AND BLUM, J. J. (1973) J. Biol. Chem. 245, 8064–8072
36. LIANG, T., RAOI, G. J., AND BLUM, J. J. (1973) J. Biol. Chem. 245, 8075–8078
Acetyl Coenzyme A Synthetase and the Regulation of Lipid Synthesis from Acetate in Cultured Cells
Barbara V. Howard, William J. Howard and J. Martyn Bailey

*J. Biol. Chem. 1974, 249:7912-7921.*

Access the most updated version of this article at http://www.jbc.org/content/249/24/7912

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/249/24/7912.full.html#ref-list-1