Pantethine inhibits cholesterol and fatty acid syntheses and stimulates carbon dioxide formation in isolated rat hepatocytes

Giuliana Cighetti, Marina Del Puppo, Rita Paroni, Elena Fiorica, and Marzia Galli Kienle

Department of Medical Chemistry and Biochemistry, University of Milan, Via Saldini 50, 20133 Milano, Italy

Abstract The effects of pantethine on cholesterol and fatty acid metabolism were investigated in isolated rat hepatocytes. Preincubation of the cells with pantethine induced a concentration-dependent decrease of the radioactivity incorporated into carbon dioxide and lipids in incubations with [2-^{14}C]acetate. When pantethine and the labeled substrate were simultaneously added to the cell suspension, there was an enhancement of carbon dioxide radioactivity at short incubation time (5 min) whereas, at longer incubation time, values were comparable to those of controls; lipid radioactivity, instead, was dramatically reduced by pantethine even at short incubation time and decreased further during the incubation, being 23% of that of controls at 60 min. Analysis of the incubation medium showed that pantethine induced a concentration- and time-dependent release of acetate into the medium. Results of the effect of the acetate concentration on the incorporation of [2-^{14}C]acetate radioactivity into CO_2 and lipids in control hepatocytes allowed the conclusion that the above-described modifications induced by pantethine are only partially attributable to the dilution of the labeled substrate, and that catabolism of acetate to carbon dioxide is stimulated by the disulphide pantethine, whereas cholesterol and fatty acid syntheses are inhibited. — **Cighetti, G., M. Del Puppo, R. Paroni, E. Fiorica, and M. Galli Kienle.** Pantethine inhibits cholesterol and fatty acid syntheses and stimulates carbon dioxide formation in isolated rat hepatocytes. *J. Lipid Res.* 1987. **28:** 152–161.

Supplementary key word acetate

Pantetheine is a naturally occurring thiol and its dual biological functions in mammalian cells, as a coenzyme A precursor and as the prosthetic group of acyl carrier protein in fatty acid synthetase, are well known (1, 2). It has also been reported (3) that, in vivo, an equilibrium exists between the thiol and the corresponding disulphide, namely pantethine (D-*bis*-(N-pantothenyl-β-aminoethyl)-disulphide). The latter compound was shown to act as a hypolipidemic agent in clinical hyperlipidemias (4–7). In order to investigate the biochemical mechanism of this action, we have previously determined the effects of pantethine on the incorporation of labeled mevalonate into cholesterol in liver cells and found that the disulphide causes a reduction of the cholesterol synthetic rate when this is stimulated by high mevalonate concentrations in the incubation medium of isolated hepatocytes (8).

The present work deals with the effects of pantethine on cholesterol biosynthesis at level(s) preceding the formation of mevalonate as well as on fatty acid synthesis and catabolism. The reported results demonstrate that in isolated hepatocytes pantethine inhibits both cholesterol and fatty acid syntheses. Carbon dioxide production from acetate is instead enhanced in pantethine-treated cells. This mode of action of the disulphide suggests that pantetheine/pantethine might function as physiological regulators of enzymes involved in lipid metabolism.

EXPERIMENTAL PROCEDURES

Materials

[2-^{14}C]Acetate (55–58 mCi/mmol), [1-^{14}C]palmitate (58 mCi/mmol), [1-^{14}C]stearic acid (55 mCi/mmol), and [2-^{14}C]cholesterol (56 mCi/mmol) were supplied by Amersham International (Amersham, Bucks, U.K.). Cofactors, enzymes, and bovine serum albumin (lipid-free, fraction V) were obtained from Boehringer Mannheim GmbH (Mannheim, FRG). Solvents and reagents were of analytical grade (Merck, Darmstadt, FRG). Pantethine, was from Maggioni Farmaceutici S.p.A. (Milan, Italy).

Preparation and incubation of isolated hepatocytes

Sprague-Dawley male rats (150–180 g) fed ad libitum were used. The preparation of the hepatocytes was usually started at 9AM, 2 hr after the onset of the 12-hr light cycle, and was carried out as described previously (9). Preparations showing a viability lower than 90% by

the Trypan blue exclusion test were discarded. The isolated hepatocytes were suspended in a medium containing 137 mM NaCl, 5 mM KCl, 0.12 mM $CaCl_2$, 0.9 mM $MgSO_4$, 10 mM sodium phosphate, 25 mM $NaHCO_3$, 55 mM glucose, and 2% (w/v) bovine serum albumin, pH 7.4, equilibrated with an O_2–CO_2 95:5 atmosphere. The incubations were started by the addition of the labeled precursor and were carried out at 37°C in a Dubnoff incubator in stoppered polycarbonate flasks containing the hepatocyte suspension (0.9–1.2 × 10^7 cells, 3 ml final volume). When indicated, the cells were preincubated in the presence or absence of pantethine. During both the preincubation and the incubation the cells were maintained under O_2–CO_2 95:5. $^{14}CO_2$ was collected in 0.2 ml of N hyamine in methanol absorbed on filter paper and placed in a center-well cup attached to the rubber stopper of the incubation flask.

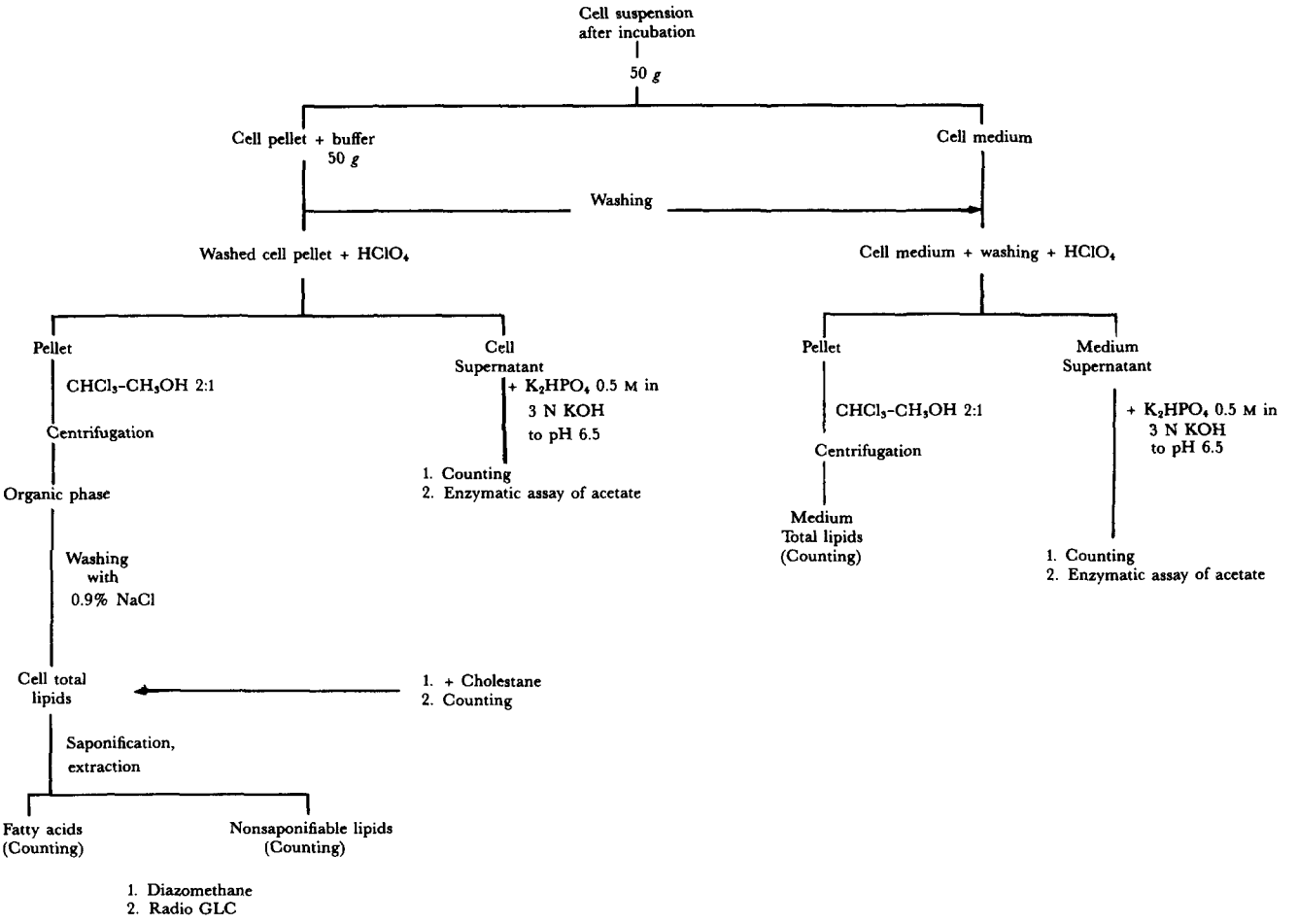
At the end of the incubation, the samples were generally treated as shown in **Scheme 1** and the various fractions were analyzed as described below.

Counting of radioactive samples was carried out in a Packard Tricarb 460 CD liquid scintillation spectrometer using Lipoluma and Aqualuma (Lumac, Schalsberg, Netherlands) as the scintillation fluids for organic and aqueous samples, respectively. For the determination of radioactivity associated with CO_2, the filter paper was transferred from the center well of the incubation flask to the counting vial with methanol (1 ml). Lipoluma (10 ml) was then added to the scintillation vials.

Incubation of liver homogenates

The livers obtained from three Sprague-Dawley male rats were homogenized (1:2, w/v) in the incubation buffer described above. Aliquots of the homogenate (0.5 ml, 20 mg of protein) were incubated for various times with [2-^{14}C]acetate (1.5 × 10^6 dpm; 22 μM) in the presence of 1 mM pantethine or in its absence (controls); final volume was 0.62 ml. The triplicate incubations were started by the addition of the labeled substrate and were carried out at 37°C in stoppered vials. Radioactive CO_2 was collected and counted as described above for isolated hepatocytes.

Scheme 1.



Determination of acetate levels

The concentration of acetate in the medium and cell supernatant was evaluated by enzymatic assay (Boehringer, Mannheim, FRG) (10). For this purpose, aliquots (0.1 ml) of the supernatants taken to pH 6.5 (Scheme 1) were mixed with a solution (3.1 ml) of L-malic acid in triethanolamine buffer, pH 8.4, containing $MgCl_2$, ATP, CoA, and NAD^+. After determining the absorption (Eo) at 340 nm, formation of oxaloacetate from malate was started by the addition of malate dehydrogenase (0.3 U), and citrate synthase (0.07 U) was also added. After 3 min the absorption was determined. Acetyl CoA synthetase (0.4 U) was added and formation of NADH was followed through the increase of absorption for 6 min. The concentration of acetate in the sample was computed on the basis of standard curves prepared with supernatants obtained from control hepatocytes to which known amounts of acetate were added.

Analysis of lipids

Lipids were recovered both from the cells and from the medium by chloroform–methanol 2:1, (v/v) extraction of the pellets obtained after $HClO_4$ treatment (Scheme 1). In all experiments about 90% of the lipid radioactivity was within the cells; moreover, the radioactivity in lipids found in the medium followed the same trend of variation observed in the cells. Therefore, data on total lipids in the Results section always refer to those of the cells.

The molar radioactivity of fatty acids and of cholesterol was determined by radiogas–liquid chromatography using a Carlo Erba model GV gas chromatograph with a flame ionization detector, connected with a Nuclear Chicago flow counter, model 4998. A glass silanized column, 2 m long, packed with 3% SE 30 on Gas Chrom Q (80–100 mesh) was used. Total lipids were saponified in 2 N KOH in 60% methanol, for 1 hr at 60°C. After acidification, extraction with diethyl ether, and treatment with an ethereal solution of diazomethane, they were analyzed under programmed temperature from 180 to 250°C (2°C/min); the temperature was then kept at the latter level value up to the retention time of cholesterol. Helium flow was 80 ml/min and methane flow was 60 ml/min. The retention times of methyl palmitate, stearate, and oleate, and of cholestane and cholesterol were 9.6, 12.7, 13.4, 21.8, and 24.0 min, respectively. The amounts of the various compounds and their molar radioactivities were calculated on the basis of standard curves prepared with ^{14}C-labeled palmitate, stearate, and cholesterol of known molar radioactivity and nonlabeled cholestane as the internal standard.

Statistical analysis

When not otherwise indicated, significance of the differences among samples was determined by the Duncan's new multiple range test.

RESULTS

The distribution of radioactivity among the fractions of cells, preincubated for 1 hr in the presence of various concentrations of pantethine and then incubated for 1 hr with [2-^{14}C]acetate is shown in **Fig. 1.** As compared to control cells, which were preincubated in the absence of the disulphide, a progressive decrease of radioactivity with the increase of pantethine concentration was observed in CO_2 and in total cell lipids, whereas the radioactivity in the cell medium increased. Mean values (± SEM) in experiments with various hepatocyte preparations preincubated for 1 hr without and with 1 mM pantethine were 1.7 ± 0.24 and 0.80 ± 0.12 nmol [2-^{14}C]acetate incorporated/10^7 cells (n = 6; $P < 0.001$ by the paired Student's t test), respectively, for CO_2, and 12.4 ± 3.1 and 2.0 ± 0.5 (n = 5; $P < 0.001$) for cell lipids.

At shorter incubation times with [2-^{14}C]acetate, preincubation with pantethine induced a decrease of the incorporation of radioactivity into lipids, whereas it increased the radioactivity associated with CO_2 as compared to controls (**Fig. 2**). Incorporation of [2-^{14}C]acetate radioactivity into CO_2 was also clearly stimulated in liver homogenates (**Fig. 3**), where its incorporation into lipid fractions was unaffected by the disulphide (data not shown).

The opposite effects of pantethine on the incorporation of radioactivity into CO_2 and into lipids were also found when preincubation was omitted. The results are reported in **Fig. 4;** at short incubation time, CO_2 radioactivity was higher with pantethine, even if not significantly, than in its absence, whereas the contrary was observed for lipids. At longer incubation times, the lipid-associated radioactivity remained very low, and that in CO_2 was also lower in the samples incubated with pantethine as compared to controls. Radioactivity in the medium supernatant was always higher in samples with pantethine than in controls, the difference being significant after 15 and 60 minutes.

Isolation of nonsaponifiable lipids and fatty acids from total lipids showed that the decrease of radioactivity was due to a decrease of the incorporation into both fractions. In order to understand whether this was attributable to a modification of the cellular pools of these compounds, their molar radioactivities were evaluated by radiogas–liquid chromatography. **Fig. 5** shows a typical chromatogram obtained in the analysis of the nonsaponifiable sterols and of fatty acids of control cells and of cells pretreated with 1 mM pantethine. From the ratio of the intensities of the radioactivity and mass peaks, the molar radioactivities were computed on the basis of standard curves. The results in **Fig. 6** show that the decrease of molar radioactivity of palmitate, stearate, and cholesterol induced by pantethine is dependent upon a decreased incorporation of radioactivity without modification of the cell levels of the compounds.

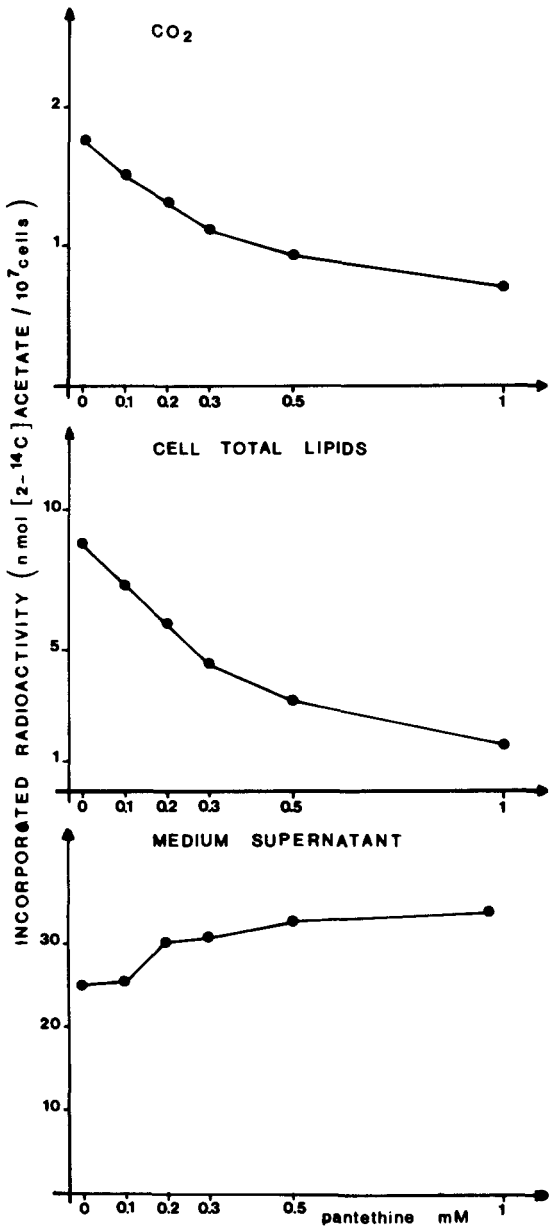


Fig. 1. Effect of pantethine on CO_2, total lipid, and medium radioactivity in incubations of isolated rat hepatocytes with [2-^{14}C]acetate. Points represent the nmol of the radioactive substrate incorporated and are means obtained in triplicate incubation samples in two experiments performed with hepatocytes prepared from two rats. Aliquots of the cell supsensions (10^7 cells/3 ml of buffer) were preincubated for 1 hr in the presence of the indicated concentrations of pantethine or in its absence. [2-^{14}C]Acetate (3.5 μCi/flask; 58 mCi/mmol) was then added and incubation was carried out for 1 hr. Radioactivity in medium total lipids and in cell supernatants (Scheme 1) is not reported because it never exceeded the 10% of that in cell total lipids and in the medium supernatant.

Since a decrease of the incorporation of radioactivity into lipids and CO_2 might be due to a modification of the cellular pool of a common biosynthetic precursor of these compounds, we evaluated the levels of acetate both in the medium and in the cells. In the medium of control cells,

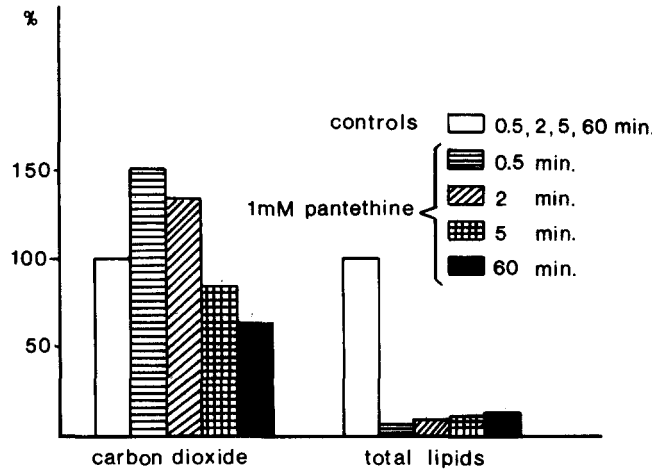


Fig. 2. Incorporation of radioactivity into CO_2 and cell total lipids in incubations of isolated rat hepatocytes with [2-^{14}C]acetate in the presence of 1 mM pantethine. Cells were preincubated for 1 hr as in experiments reported in Table 1. Incubations with [2-^{14}C]acetate were then carried out for various times. Results are expressed as % of controls and are means of two experiments in which each assay was performed in triplicate. Radioactivity in medium total lipids (Scheme 1) never exceeded 10% of that shown for cell lipids (data not shown).

the acetate concentration increased in 5 min of incubation from the basal value of 0.09 ± 0.02 (mean ± SEM in six hepatocyte preparations) to 0.47 ± 0.09 (determined in eight hepatocyte preparations) μmol/10^7 cells, then it remained constant. As shown in the right panel of **Fig. 7**, the presence of 1 mM pantethine in the incubation medium highly stimulated the increase; at 5 min of incu-

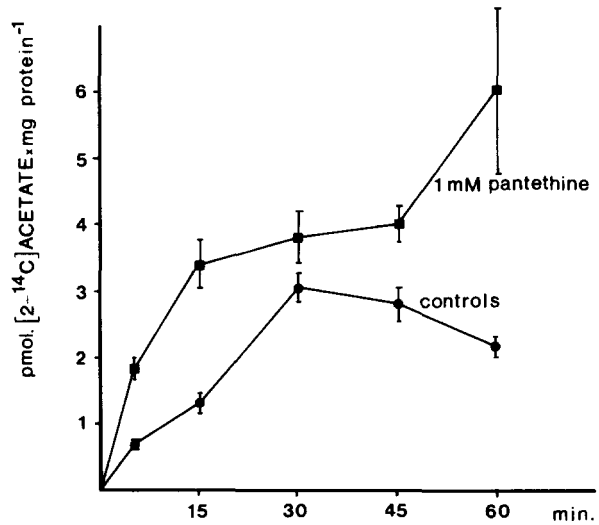


Fig. 3. Effect of pantethine on the incorporation of radioactivity into CO_2 in rat liver homogenate incubated with [2-^{14}C]acetate. Aliquots of liver homogenate obtained from three pooled rat livers were incubated for the shown times with [2-^{14}C]acetate in the presence of 1 mM pantethine or in its absence (controls). Results are means ± SEM of triplicate incubation samples. CO_2 radioactivity in cells preincubated with pantethine was significantly different from controls at all incubation times.

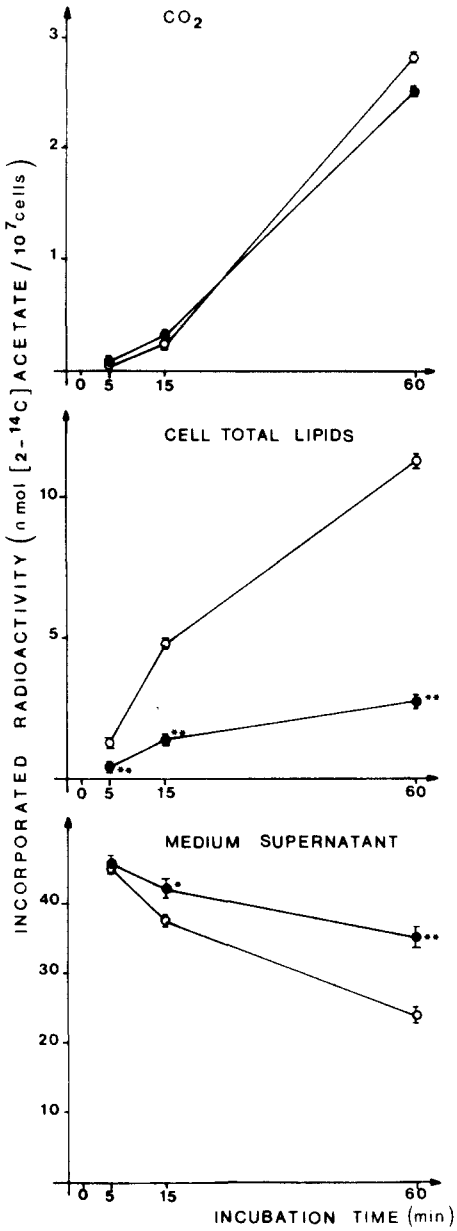


Fig. 4. CO_2, total cell lipid, and medium radioactivity in incubations of rat hepatocytes with [2-^{14}C]acetate in the presence of 1 mM pantethine. Points represent the nmol of labeled substrate incorporated into the various fractions and are means ± SEM obtained in triplicate incubations of aliquots (10^7 cells/3 ml) of a cell preparation. The cell suspensions were added with pantethine (●) or with the corresponding volume of buffer (controls) (○) followed immediately by [2-^{14}C]acetate (3.5 μCi/flask, 58 mCi/mmol) and were incubated for the indicated times. $^*P < 0.05$; $^{**}P < 0.01$ vs. control.

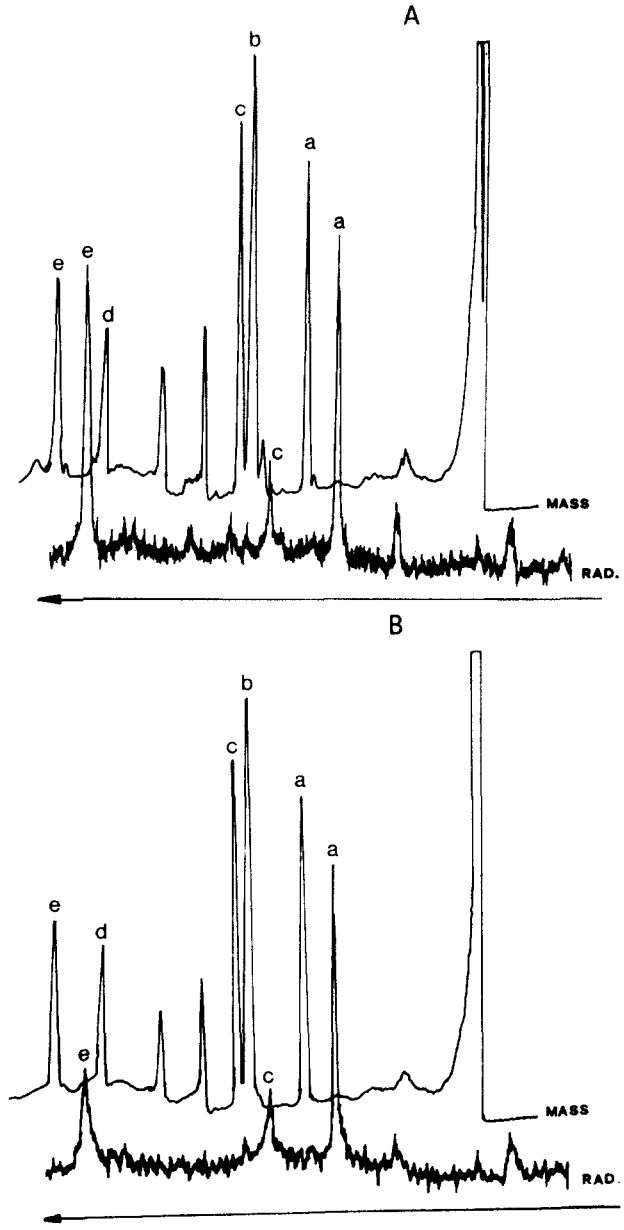


Fig. 5. Radiogas-liquid chromatogram of total lipids extracted from cells preincubated with or without pantethine. The analysis was carried out as described in the Experimental section on lipid extracts after esterification with diazomethane using cholestane as the internal standard. The reported chromatograms refer to control cells (A) and cells preincubated with 1 mM pantethine (B) in one of the experiments of Fig. 1. Sensitivity for mass detection in B was ¼ that in A; a) palmitate; b) oleate; c) stearate; d) cholestane; e) cholesterol.

bation, the acetate level was twice that in controls and became 5- to 10-fold higher after 1 hr of incubation. Curves representing the modification of medium and cell acetate levels with the concentration of pantethine are shown in the left panel of Fig. 7. Values reported in the figure are means obtained in two experiments with different hepatocyte preparations. In both experiments incubations with each pantethine concentration were carried out in triplicate, and the statistical evaluation of the data showed that in the medium, acetate levels were significantly higher than in controls (no pantethine added) even at 0.2 mM pantethine ($P < 0.05$). In the cells, acetate levels were much lower than in the medium and were not modified during incubation even with pantethine up to 1 mM. It was therefore established that when [2-^{14}C]acetate is added to

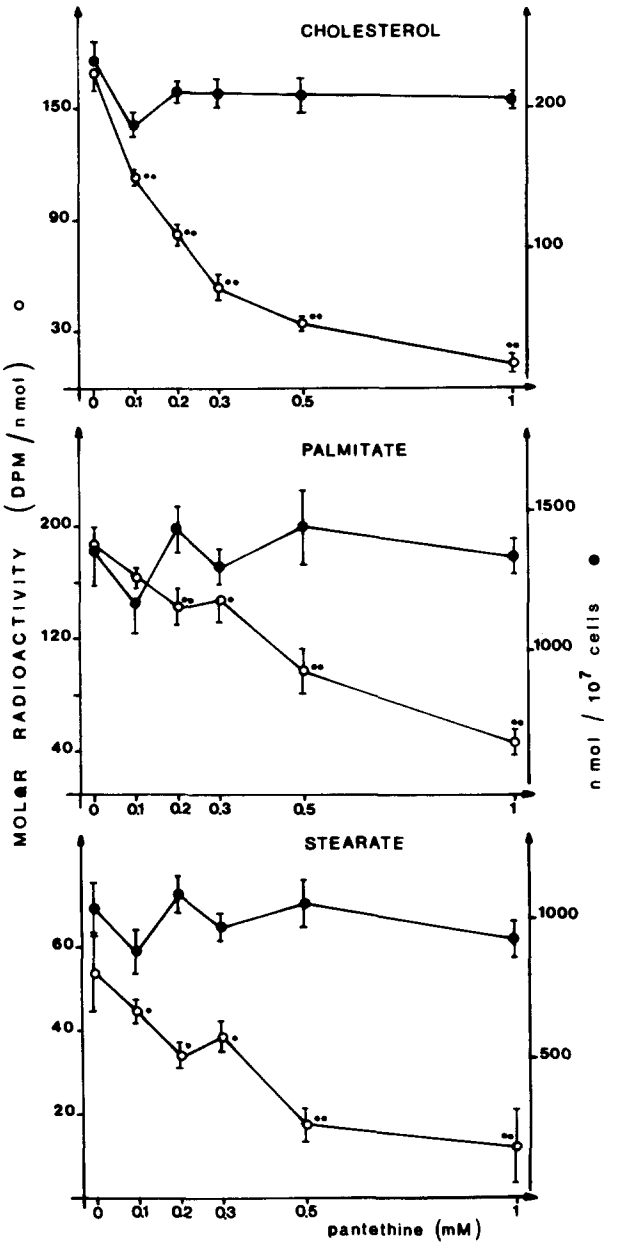


Fig. 6. Effect of pantethine on the molar radioactivity and the cell concentrations of cholesterol, palmitate, and stearate in incubations of rat hepatocytes with [2-^{14}C]acetate. Results are mean ± SEM of triplicate incubation samples with the same hepatocyte preparation. Analyses were carried out on the lipid extracts whose radioactivity is reported in Fig. 1 after saponification, acidification, extraction with diethyl ether, and esterification with diazomethane. Analytical conditions were as described in the Experimental section. $^{*}P < 0.05$; $^{**}P < 0.01$ vs. cells preincubated without pantethine.

cells preincubated with pantethine, a "dilution" of the substrate with nonlabeled acetate occurs which is higher than that in cells preincubated without pantethine. As a consequence, the molar radioactivity of the substrate becomes lower in pantethine-treated cells than in controls. This should result in a decreased incorporation of radioactivity when the amount of acetate catabolized by the cells is not modified by the substrate concentration. However, it was shown previously that the increase of the concentration of acetate added to the medium of hepatocyte incubations induces a stimulation of its incorporation into lipids (11). Therefore, we carried out an experiment to compare the radioactivity of [2-^{14}C]acetate incorporated into CO_2 and lipids in the presence of various amounts of unlabeled acetate with that found in samples preincubated with pantethine. For this purpose, cells (10^7/3 ml) were preincubated for 1 hr. Various amounts of unlabeled acetate (0.5, 1, 2, 5 μmol/10^7 cells) were then added, followed immediately by the addition of a constant amount of [2-^{14}C]acetate (0.06 μmol/10^7 cells; 3.5 μCi). In three samples preincubated with pantethine and three preincubated without it, only the radioactive substrate (0.06 μmol/10^7 cell, 3.5 μCi) was added. The results in **Table 1** show the radioactivity associated with CO_2, lipids, and the medium supernatant as a function of acetate concentration, including also the amount of acetate found in the medium after the preincubation without pantethine (0.09 ± 0.005 μmol/10^7 cell). Radioactivity in CO_2 of pantethine-treated cells (samples P in Table 1) was almost twice that obtained in control cells with the highest acetate concentration (samples E in Table 1) despite the fact that this concentration was higher than that present in the medium supernatant after the preincubation with pantethine and addition of the labeled substrate. Lipid radioactivity was instead almost half in pantethine-treated cells as compared to those incubated with the highest acetate concentration. From the radioactivity found in the various fractions and from the molar radioactivity of acetate in the medium, the amounts of acetate incorporated into sterols, fatty acids, and CO_2 were calculated. Plotting the reciprocals of these amounts versus the reciprocals of acetate concentration gave significant linear correlations (**Fig. 8**). From the obtained lines and from the results obtained in cells preincubated with pantethine, it was calculated that carbon dioxide production was 2.4-fold higher than that in controls, whereas sterol and fatty acid syntheses were inhibited by 52.7 and 27.1%, respectively.

In order to test whether pantethine also affects lipid catabolism, hepatocytes were preincubated with [2-^{14}C]acetate for 1 hr to label endogenous lipids. Cells were then separated from the medium, washed, and incubated for 1 hr with various concentrations of pantethine and without it. As shown in **Fig. 9,** radioactivity in CO_2 in pantethine-treated cells did differ from that in controls after 30 min of incubation. This effect may again depend upon the release of acetate by pantethine, resulting in a decreased molar radioactivity of that oxidized to CO_2 by the cells. The lack of effect of pantethine on lipid radioactivity suggests, however, that the overall catabolism of fatty acids is not affected by the pantethine.

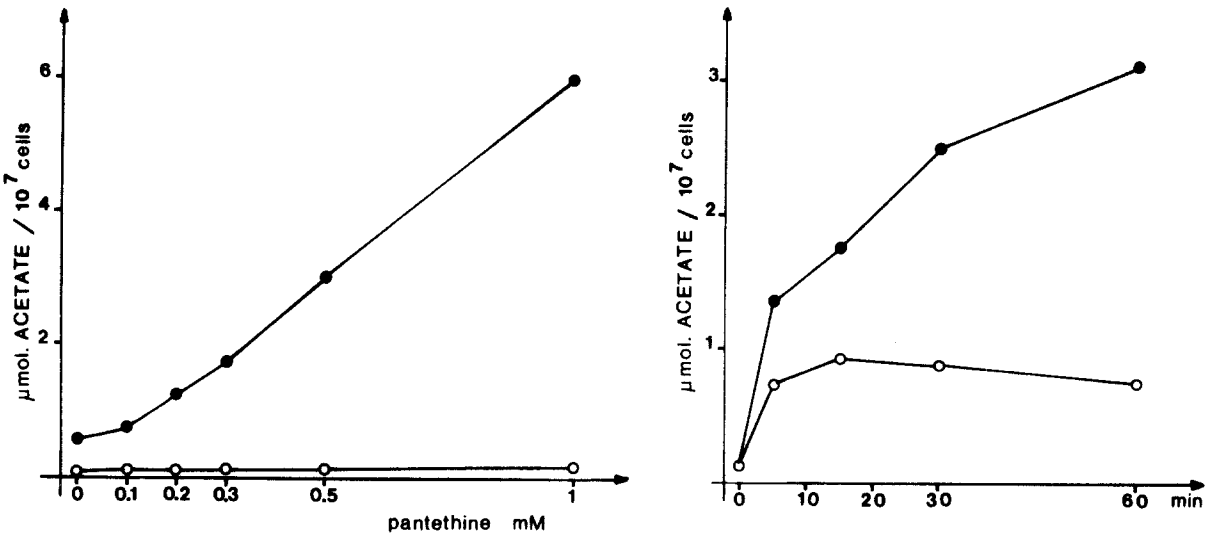


Fig. 7. Acetate levels after incubation of hepatocytes with pantethine. Acetate levels were determined in the medium and cell supernatants (Scheme 1) as described in the Experimental section. Results are means obtained in triplicate incubations with two different cell preparations. Right panel: points represent the acetate concentration in the medium supernatant after incubation of hepatocytes (10^7 cells/3 ml) with 1 mM pantethine (●) or without it (○) for the indicated time. Values in pantethine-treated cells differed significantly ($P < 0.01$) from those in controls at all incubation times. Left panel: points represent the acetate concentration in cell (○) and medium (●) supernatants after incubation of hepatocytes (10^7 cells/3 ml) with various concentrations of pantethine for 1 hr. One-hr incubations with 1 mM pantethine and without it were carried out with additional hepatocyte preparations; acetate concentration (mean ± SEM) was 4.56 ± 0.36 and 0.47 ± 0.09 µmol/10^7 cells (n = 8; $P < 0.001$ by the paired Student's *t* test), respectively, in the medium and 0.23 ± 0.02 and 0.19 ± 0.04 (n = 5; n.s.), respectively, in the cells.

DISCUSSION

Effects of pantethine on the metabolism of acetate have been described previously in human skin fibroblasts by Ranganathan, Jackson, and Harmony (12), similar to what we report here for hepatocytes. These authors found a decreased incorporation of the acetate radioactivity into cholesterol and fatty acids when fibroblasts were incubated with pantethine. In contrast with our present data, however, radioactivity associated with nonsaponifiable lipids was unaffected by the pantethine (12). Moreover, in fibroblasts pulsed with labeled acetate, incubation with pantethine induced an increase of the radioactivity associated with methyl sterols. A similar effect was previously observed by us in hepatocytes pretreated with pantethine and incubated with a high mevalonate concentration, but was absent in cells incubated with concentrations of mevalonate that do not alter the endogenous pool

TABLE 1. CO_2 and lipid radioactivity in rat hepatocytes incubated with various concentrations of [2-^{14}C]acetate

Sample	Acetate	CO_2	Total Lipids	Medium Supernatant	Nonsaponifiable Lipids	Fatty Acids
	µmol/10^7 cells			*dpm/10^4cells*		
A	0.15	12.1 ± 1.86	2170 ± 19	2874 ± 48	185 ± 1.0	1625 ± 73
B	0.65	4.63 ± 0.93	492 ± 27	5074 ± 122	52.5 ± 0.74	372 ± 38
C	1.15	6.21 ± 0.90	402 ± 52	5098 ± 60	38.0 ± 6.7	288 ± 42
D	2.15	3.72 ± 0.82	201 ± 54	5548 ± 92	20.6 ± 1.3	164 ± 15
E	5.15	2.28 ± 0.22	108 ± 5.4	5816 ± 56	10.2 ± 1.2	88.9 ± 4.8
P	4.37	4.62 ± 0.68**	67.6 ± 1.6**	5964 ± 34**	4.45 ± 0.13**	56.1 ± 1.7**

Hepatocytes (10^7 cells/3 ml) were preincubated for 1 hr without (samples A–E) or with 1 mM pantethine (samples P) and were then incubated for 5 min with labeled acetate. ^{14}C Radioactivity added to each sample corresponded to 3.5 µCi. Acetate levels (µmol/10^7 cells) in samples without pantethine (A–E) include labeled acetate (0.06 µmol/10^7 cells) and unlabeled acetate added after 1 hr of preincubation (0, 0.5, 1, 2, and 5 µmol/10^7 cells) plus the amount found in the medium supernatant after 1 hr preincubation of the cells, 0.09 ± 0.005 µmol/10^7 cells (mean ± SEM in three incubation samples). In samples with pantethine (P), the acetate concentration was that found after 1 hr incubation (4.31 ± 0.02) plus the radioactive substrate added before the incubation (0.060 µmol/10^7 cells). Results are means ± SEM in triplicate incubation samples. **$P < 0.01$ vs. cells preincubated without pantethine and incubated with 20 µM [2-^{14}C]acetate (acetate levels = 0.15 µmol/10^7 cells).

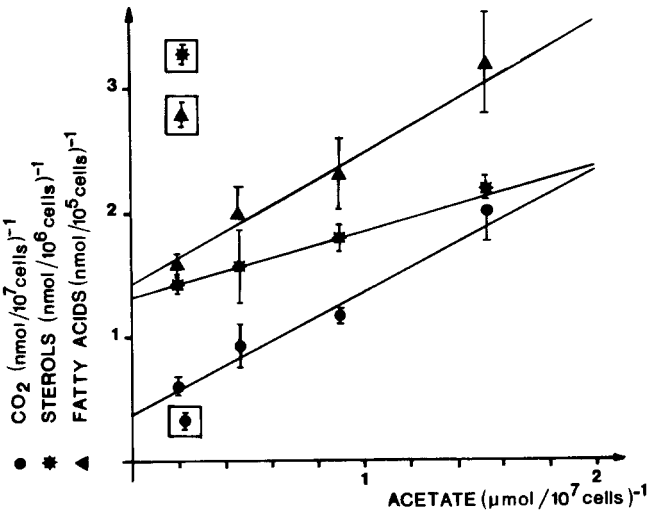


Fig. 8. Linear correlation between the reciprocals of the amounts of sterols, fatty acids, and CO_2 and the reciprocals of acetate concentration in the cell medium. The concentration of sterols, fatty acids, and CO_2 formed from acetate are expressed as the amount of acetate incorporated and were calculated from the radioactivity found in the corresponding fraction (see Table 1) and the molar radioactivity of acetate in the cell medium, computed on the basis of the added radioactivity and the amount of acetate in the medium supernatant after 1 hr preincubation. Results are means ± SEM of triplicate incubations with the same cell preparation. Equations of the lines obtained by regression analysis (Y = a(± SEM)X + b(± SEM)) were as follows: Y = 0.97(± 0.1)X + 0.39(± 0.1); r = 0.990 for CO_2; Y = 0.53(± 0.02)X + 1.3(± 0.02); r = 0.998 for sterols; Y = 1.1(± 0.07)X + 1.4(± 0.07); r = 0.995 for fatty acids. Points represented within a square are values found in pantethine-treated cells at the 4.37 μmol/10^7 cells concentration of acetate (acetate^{-1} = 0.227) found in the medium after preincubation with 1 mM pantethine.

(8). When cells were incubated with labeled acetate as in the experiments described here, no accumulation of radioactivity in sterols other than cholesterol was observed (data not shown). Ranganathan et al. (12) concluded that in fibroblasts the inhibition of fatty acid synthesis by pantethine occurs at a point beyond the steps common to fatty acid and cholesterol syntheses. However, our data suggested modifications in hepatocytes at early stages of the two pathways. For this reason, in the present experiments, we measured the levels of acetate in the medium of cells after their preincubation and found that the presence of pantethine induced an increase of acetate levels, dependent upon the concentration of pantethine and the preincubation time (Fig. 7). The lower radioactivity in CO_2 and lipids in cells pretreated with pantethine could therefore depend on the dilution of the radioactive substrate with the nonlabeled acetate present in the incubation medium, leading to a specific radioactivity of the substrate metabolized during the incubation, lower in cells pretreated with pantethine as compared to controls. However, the lesser decrease of radioactivity induced by pantethine in CO_2 than in lipids (Figs. 1 and 4) prompted us to check the effect of the modification of the concentration and molar radioactivity of acetate on the incorporation of radioactivity in control cells preincubated in the absence of pantethine. As expected, in hepatocytes incubated with the same [2-^{14}C]acetate amount but with increasing concentrations of unlabled acetate, a decrease of the radioactivity incorporated into CO_2 and lipids was observed (Table 1). The amount of incorporated acetate, as calculated from the molar radioactivity of the added substrate (labeled plus unlabeled) and from the incorporated radioactivity, increased with the increase of acetate concentration. On the other hand, the results obtained with hepatocytes pretreated with pantethine (Table 1) ensured that modifications of acetate radioactivity incorporated into the various fractions are not attributable only to modification of the molar radioactivity of the substrate; incorporation of acetate into CO_2 in pantethine-pretreated cells was much higher than that expected from the concentration of acetate found in the medium after pantethine treatment, whereas incorporation into sterols and fatty acids was much lower than that expected. The linear correlations obtained when the reciprocals of acetate incorporation into CO_2, sterols, and fatty acids were plotted against the reciprocal of acetate concentration (Fig. 8) allowed us to calculate that the substrate conversion into CO_2 in pantethine-treated cells is about twice that expected from the acetate concentration after 1 hr of preincubation with pantethine. For the conversion into sterols and fatty acids, an inhibition of 52.7% and 27.1%, respectively, was calculated. These results, together with the enhancement of CO_2 radioactivity induced by pantethine at short incubation times (Figs. 2 and 4) and in liver homogenate (Fig. 3), suggest that the compound stimulates acetate oxidation via the tricarboxylic acid cycle.

Inhibition of sterol and acid syntheses is demonstrated instead by the results of the above-described experiment (Table 1, Fig. 8) and by the low radioactivity found in these lipids even at a very short incubation time (Fig. 2), particularly in experiments in which incubation with labeled acetate was started immediately after the addition of pantethine (Fig. 4). In these samples, the low radioactivity in cell lipids cannot be accounted for by the dilution of the labeled substrate with unlabeled acetate released by pantethine because, after 5 min preincubation with pantethine, the acetate levels are only twice those at zero time (Fig. 7).

Accumulation of acetate has been described to occur under conditions implying increased fatty acid oxidation (13–15). We therefore tested the possibility that pantethine may induce such an effect. However, the lack of an increase of CO_2 radioactivity and decrease of lipid radioactivity by pantethine in cells prelabeled with radioactive acetate (Fig. 9) and the above-described stimulation of the Krebs cycle render this hypothesis quite improbable. This seems to be in contrast with previously reported data (16) that

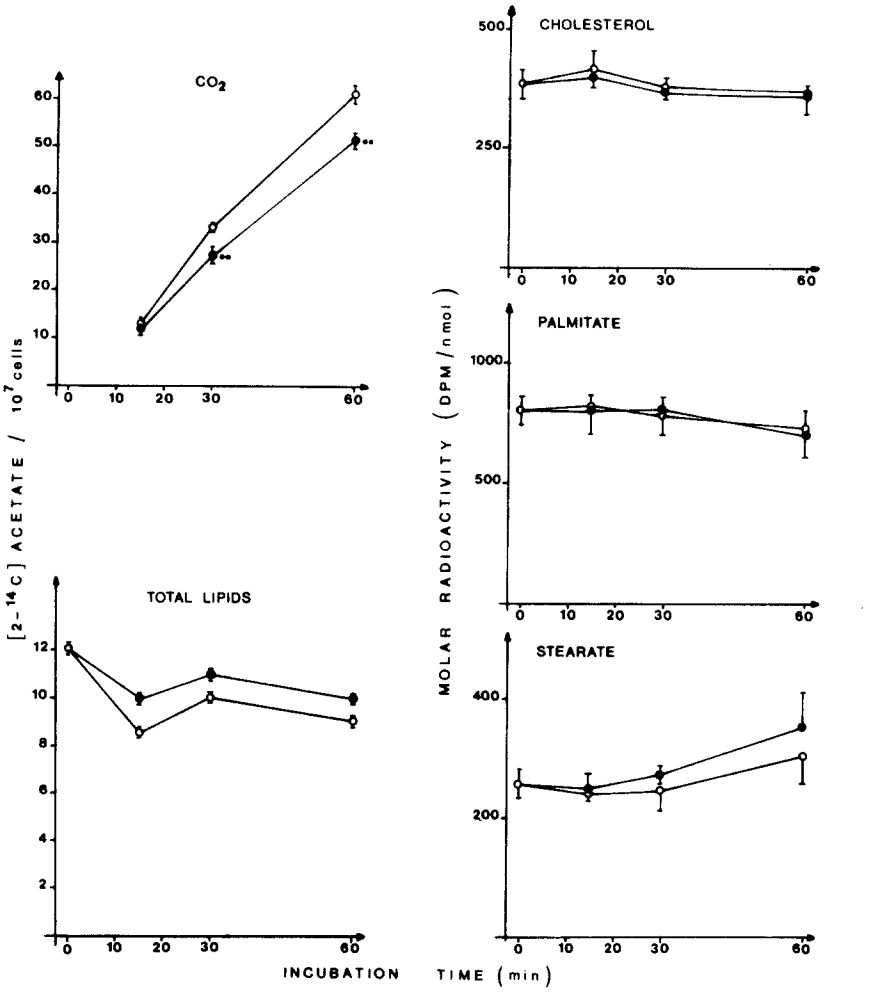


Fig. 9. Effect of pantethine on lipid catabolism. Hepatocytes (21×10^7 cells; 10^7 cells/3 ml) were incubated for 60 min in the presence of [2-^{14}C]acetate (57 μCi; 58.3 mCi/mmol). Cells were then spun down at 50 *g*, washed twice by centrifugation at the same speed, and resuspended in the incubation medium. Aliquots (2 ml, 10^7 cells) were added either with pantethine (●) dissolved in the incubation medium (1 ml, final concentration 1 mM) or with the medium (1 ml) (○). The suspensions were then incubated for the indicated times. Incorporated radioactivity is expressed as pmol of [2-^{14}C]acetate/10^7 cells for CO_2 and nmol of [2-^{14}C]acetate/10^7 cells for cell lipids. Results are means ± SEM obtained in triplicate incubation samples. $^{**}P < 0.01$ vs. samples incubated for the same time without pantethine.

indicate a stimulation of fatty acid oxidation by pantethine in in vitro experiments. However, the effect was observed only when CoA was also added to the in vitro system (16), whereas in our experiments, both with homogenates and isolated cells, no addition was made other than the radioactive substrate and, eventually, pantethine.

Inhibition of acetyl-CoA synthetase (15) as an effect of pantethine can also be excluded, due to the observed stimulation of CO_2 production by the compound, which implies the previous conversion of acetate to the thiol ester derivative. Accumulation of acetate by pantethine may therefore be due to the observed inhibition of sterol and fatty acid syntheses, with a consequent rise of acetyl-CoA levels, stimulating, in turn, the hydrolysis to acetate catalyzed by acetyl-CoA hydrolase (15).

The intimate mechanism of the inhibition of lipid synthesis induced by pantethine in hepatocytes is still difficult to understand. As far as cholesterol synthesis is concerned, the inhibition may occur at the level of HMG-CoA reductase, because the enzyme is known to be transformed into a latent inactive form by disulphides (12–20). Unfortunately, this mechanism cannot be evaluated by measuring the activity of the enzyme because its isolation in the absence of thiols from rat liver (17) and from isolated hepatocytes (personal observation) gives an almost completely inactive preparation.

Inhibition of fatty acid synthesis is also difficult to interpret. The 4-phosphopantetheine prosthetic group of acyl carrier protein (ACP) might be considered as the site of inhibition by pantethine. A fast turnover of the pros-

thetic group has been described (21) catalyzed by ACP phosphodiesterase (22), which hydrolyzes the prosthetic group from ACP, and by ACP synthase which transfers the 4-phosphopantetheine moiety from CoA to apoACP (22). The turnover rate was shown to be inversely correlated with CoA concentration (21) and this was considered as an indication that the turnover may be more related to the regulation of CoA than of fatty acid biosynthesis. As a hypothesis one may consider a double effect of pantethine. Pantethine may cause the inactivation of the prosthetic group via oxidation of its thiol function to a disulphide. In addition, turnover of the prosthetic group may be lowered, either due to its oxidation or to the increase of CoA levels induced by pantethine (3). Both effects would contribute to the decreased activity of fatty acid synthetase.

In conclusion, the present study demonstrates that, in isolated hepatocytes, pantethine stimulates the oxidation of acetate and inhibits sterol and fatty acid formation at early stages of the synthetic pathways. These findings are of potential interest in the elucidation of the complex mechanisms underlying the therapeutic efficacy of pantethine in hyperlipidemia. It is difficult at present to understand whether a regulation of lipid metabolism by pantetheine/pantethine also occurs under physiological conditions. Concentrations used by us in hepatocyte incubations appear to be quite high. However, experiments in human fibroblasts (12) have demonstrated that less than 0.5% of pantethine added at 0.5 mM concentration to the culture medium is taken up by the cells. If this also occurs in hepatocytes where pantethine effects were observed at a concentration of 0.2 mM, its activity in stimulating the Krebs cycle and inhibiting fatty acid syntheses may occur at physiological levels of pantethine/pantetheine.

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