Regulation of Coenzyme A Biosynthesis by Glucagon and Glucocorticoid in Adult Rat Liver Parenchymal Cells

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We studied the effects of glucagon, dibutyryl cyclic AMP and dexamethasone on the rate of [^{14}C]pantothenate conversion to CoA in adult rat liver parenchymal cells in primary culture. The presence of 30 nM-glucagon increased the rate by about 1.5-fold relative to control cultures (range 1.4–2.3) and 2.4-fold relative to cultures containing 1–3 m.i.u. of insulin/ml. The half-maximal effect was obtained at 3 nM-glucagon. Dibutyryl cyclic AMP plus theophylline also enhanced the rate by about 1.5-fold. Dexamethasone acted synergistically with glucagon; glucagon at 0.3 nM had no effect when added alone, but resulted in a 1.7-fold enhancement when added in the presence of dexamethasone (maximum effect at 50 nM). The 1.4-fold enhancement caused by the addition of saturating glucagon concentrations was increased to a 3-fold overall enhancement by the addition of dexamethasone. However, dexamethasone added alone over the range 5 nM to 5 μM had no effect on the rate of [^{14}C]pantothenate conversion to CoA. The stimulatory effect of dibutyryl cyclic AMP plus theophylline was also enhanced by the addition of dexamethasone. Changes in intracellular pantothenate concentration or radioactivity could not account for the stimulatory effects of glucagon, dibutyryl cyclic AMP or dexamethasone. Addition of 18 μM -cycloheximide, an inhibitor of protein synthesis, decreased the rate of incorporation of [^{14}C]pantothenate into CoA and the enhancement of this rate by glucagon and dibutyryl cyclic AMP plus theophylline in a reversible manner. These results demonstrate an influence of glucagon, dibutyryl cyclic AMP and glucocorticoids on the intracellular mechanism regulating total CoA concentrations in the liver.

Mammalian tissues synthesize CoA from pantothenate, cysteine and ATP according to the following scheme:

→ CoA pathway is influenced by the hormones of metabolic homeostasis (insulin and glucagon) *in vivo*

[for reviews, see Plaut *et al.* (1974) and Abiko (1975)]. Degradation of CoA occurs via a phosphatase specific for CoA (Bremer, *et al.*, 1972) and a pyrophosphatase specific for dephospho-CoA (Skrede, 1973). An amidohydrolase that catalyses the hydrolysis of pantetheine to pantothenate has also been identified (Dupre *et al.*, 1973; Abiko, 1975).

Evidence that the metabolism of the pantothenate

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in vivo has come from comparisons of CoA concentrations in tissues of control rats with those in alloxan-diabetic rats (Smith *et al.*, 1978) and from studies of rats subjected to various feeding regimens (Smith *et al.*, 1978; Tubbs & Garland, 1964; Kondrup & Grunnet, 1973). In general, livers of rats deprived of food for 21–48 h have total CoA (CoASH plus acylated CoA) concentrations 1.6–2-fold higher than those in fed or re-fed rats, and the livers of alloxan-diabetic rats have total CoA concentrations 2-fold higher than in the normal

controls (Smith *et al.*, 1978). Neither of these conditions produced increases in total CoA concentrations in the heart (Smith *et al.*, 1978). Starved rats force-fed with glucose also had a 2–3-fold lower rate of incorporation of [^{14}C]pantothenate into CoA in the liver *in vivo* than did starved rats (Smith, 1978). The tissue cysteine and pantothenate contents, the specific radioactivity of the tissue pantothenate and the influx of pantothenate into the tissue were all unable to account for the differences in rate of incorporation of [^{14}C]pantothenate into CoA (Smith, 1978). This suggested that the locus of hormonal action involved an enzyme(s) in the metabolic pathway between intracellular pantothenate and CoA.

An involvement of glucocorticoids in the regulation of CoA concentration in the liver has also been suggested from experiments *in vivo*. Nakamura *et al.* (1970) reported that there was a marked decrease in the amount of CoA in the livers of adrenalectomized rats (relative to sham-operated controls), and that daily administration of prednisolone resulted in an initial increase in CoA followed by a decrease during longer periods of administration.

The above studies relied on manipulation of hormonal concentrations *in vivo* to demonstrate hormonal regulation of CoA concentration or pantothenate $\rightarrow$ CoA metabolism. However, the intra-tissue flux of many other hormones and metabolites is associated with changing concentrations of insulin, glucagon or glucocorticoids, and thus the hormonal effects could occur through an indirect route. In order to determine if these hormones act on liver pantothenate $\rightarrow$ CoA metabolism by direct interaction at the cellular level, we have used rat liver parenchymal cells maintained in primary culture. This technique employs a chemically defined growth medium which permits the addition of hormones and other agents at known concentrations. The experiments established a direct effect of glucagon and glucocorticoids on the rate of conversion of pantothenate to CoA. A preliminary report of this work has been presented (Smith & Savage, 1979).

Materials

All rats were Sprague–Dawley males weighing 180–250 g maintained on a schedule of 12 h light, 12 h dark and fed on stock diet *ad libitum*. Medium M-199, Hanks balanced salt solution (calcium- and magnesium-free), penicillin/streptomycin and foetal calf serum were purchased from Grand Island Biological Co. Sodium heparin was from Riker Laboratories, and insulin was from E. R. Squibb and Sons. Collagenase (Type II), cycloheximide and Hepes [4-(2-hydroxyethyl)-1-piperazine-ethane-

sulphonic acid] were from Sigma Chemical Co. Optilux culture flasks (25 cm²) were obtained from Falcon Plastics. [^{14}C]Pantothenate (54 mCi/mmol) and [^3H]CoA were purchased from New England Nuclear. Difco bactopantothenate assay medium was purchased from Difco Laboratories.

Methods

Isolation and cultivation of rat hepatocytes

Rat liver parenchymal cells were isolated in essentially pure form by using the collagenase perfusion technique described previously (Savage & Green, 1976; Savage & Bonney, 1978) as modified by Seglen (1973) in that 2.5 mM- Ca^{2+} was added to the collagenase perfusion medium. The procedure permits the isolation of 2×10^8 – 4×10^8 hepatocytes from one liver, with a viability of greater than 85%, as judged by exclusion of 1.15% Trypan Blue.

About 3×10^6 – 4×10^6 cells were plated in 25 cm² Falcon flasks in 5 ml of medium M-199 containing 15% (w/v) foetal calf serum, 10 m-i.u. of insulin/ml, 0.37 mg of penicillin/ml and 100 μg of streptomycin/ml. Medium M-199 was used because it has a low pantothenate concentration (46 nM). Hepes was added to a final concentration of 25 mM, and the medium was adjusted to pH 7.3–7.4 at the time of cell plating. The cells were allowed to attach to the flask for 2–4 h at 37°C, and the medium was replaced with 5 ml of fresh M-199 medium containing foetal calf serum and insulin. Thereafter, M-199 medium without serum was used and the medium was changed daily. Unless otherwise indicated, the cultures were maintained for 2 days and experiments were initiated during the third day of culture. Internal ATP concentrations in the range 10.5–27.0 nmol/mg of protein was measured by luciferase assay (Bergmeyer, 1973). This is comparable with the values measured in freeze-clamped livers (Woods *et al.*, 1970).

Incubation of hepatocytes with [^{14}C]pantothenate

At 2 h before an experiment, cells were washed *in situ* at 37°C with 5 ml of M-199 medium which lacked both serum and insulin. The medium was removed by aspiration, replaced with 3 ml of the same medium, and the cultures were incubated for 2 h at 37°C. Then [^{14}C]pantothenate was added to the medium at an external concentration between 7.1 and 44 μM (the contribution of pantothenate in the medium was negligible), and the indicated amounts of hormones or other compounds were added. In M-199 medium, at 37°C, external pantothenate was equilibrated with internal pantothenate by 1 h. After an appropriate time of incubation at 37°C, the contents of the flask were removed and the cells were washed three times *in situ* with 5 ml of 0.9% NaCl at room temperature and frozen on solid CO_2 .

Since our measurements of the intracellular content of pantothenate could be influenced by inadequate removal of the medium, or excessive leakage of pantothenate during rinsing, a determination of the efficiency of the rinsing procedure was made. A comparison of the radioactivity appearing in the cellular extracts was made after incubating cells for 1 h in the presence of [^{14}C]pantothenate, at which time the incorporation of radioactivity into other compounds was negligible. After three washes with 0.9% NaCl, each successive washing did not decrease the amount of intracellular pantothenate by more than 3%, thus providing assurance that pantothenate does not readily leak from the cells. Control experiments using [^{14}C]sucrose as an extracellular marker indicated that three washes were sufficient to rid the cells of more than 99.9% of the added radioactivity.

Preparation of cell extracts and assays

The cells were extracted within 24 h. Freezing cells for longer periods resulted in a significant loss of CoA. The culture flasks were thawed on ice, and 0.5 ml of cold 3% (w/v) HClO_4 and 0.02 ml of 1.0M-dithiothreitol were added to each flask. The cells were scraped from the flask with a rubber 'policeman' and the mixture was transferred to a centrifuge tube. In order to convert acyl-CoA derivatives to CoASH, a basic hydrolysis was carried out as follows. The mixture in each centrifuge tube was adjusted to pH 11.0–11.5 by the addition of an appropriate volume (about 0.1 ml) of 3M-KOH. This was heated to 55°C for 10 min with shaking, followed by rapid cooling on ice. The pH was adjusted to 5–6 by the addition of approx. 0.05 ml of 0.5M-triethanolamine containing 6% (w/v) HClO_4 . This was centrifuged at 8000g for 10 min at 4°C. Samples of this supernatant were then taken for pantothenate determinations and for DEAE-Sephadex chromatography (as described below). The pantothenate was assayed with *Lactobacillus plantarum* (Skeggs & Wright, 1944). Turbidity of the cultures after 18–24 h of bacterial growth in pantothenate assay medium was measured spectrophotometrically at 600 nm. The turbidity was a linear function of pantothenate concentration in the range 0–0.4 μg of pantothenate. The HClO_4 precipitate was washed out by the addition of 1 ml of cold 3% HClO_4 , followed by resuspension and centrifugation at 8000g for 10 min at 4°C. The washed pellet was dissolved in 1 ml of 1.0M-NaOH overnight at room temperature. Samples were removed for protein determination by the method of Lowry *et al.* (1951) (with bovine serum albumin as standard) and for determination of protein-bound radioactivity. CoA was assayed by the fluorimetric recycling assay with phosphotransacetylase (Allred & Guy, 1969; Hansford, 1974).

Determination of radioactivity in CoA and intermediates

In order to determine the amount of ^{14}C radioactivity in CoA, pantothenate and other intermediates, ascending chromatography on Whatman DEAE-cellulose paper (DE-81) was performed as described by Smith (1978). Portions (50 or 100 μl) of the cell extracts were spotted on the paper and the chromatograms were developed in the ascending direction by using freshly distilled formic acid (0.5M) in aqueous solution (pH 3.1). The developing time was usually 3–4 h at room temperature. Standards consisting of CoA, dephospho-CoA pantetheine and phosphopantetheine were chromatographed under identical conditions, and the spots were identified by spraying for thiol groups with cyanide and nitroprusside (Smith, 1978). In addition, [^3H]CoA or [^{14}C]pantothenate were chromatographed alone or in the presence of non-radioactive cell extracts.

The air-dried chromatograms were cut into 0.63 or 1.27 cm segments and counted for radioactivity in 7 ml of New England Nuclear Formula 963 scintillation fluid. The specific radioactivity of pantothenate in the cells was calculated from the amount of radioactivity appearing in the area of the chromatogram corresponding to pantothenate and the total amount of pantothenate in the sample.

Results

Incorporation of [^{14}C]pantothenate into CoA in rat liver parenchymal cells

The amount of radioactivity incorporated into CoA was determined after incubating the hepatocytes with [^{14}C]pantothenate for time intervals ranging from 3 to 20 h. Cellular extracts were subjected to mild alkaline conditions to hydrolyse acyl-CoA derivatives to free CoASH, followed by addition of HClO_4 to reprecipitate protein. The incorporation of radioactivity into components of the acid-soluble fraction is shown in Fig. 1(a), and the incorporation into the final HClO_4 -insoluble fraction is shown in Fig. 1(b). Major peaks of radioactivity appeared only in the positions of pantothenate and CoA, which together accounted for 93.0–95.7% of all the cellular radioactivity. After 20 h of incubation, the HClO_4 -insoluble radioactivity accounted for approx. 2.7% of the total cellular radioactivity. Over the 20 h of incubation, total cellular protein decreased by 16%, 3.8% in the first 10 h. The radioactivity appearing in pantothenate rose sharply and remained fairly constant over the 3–20 h of incubation, whereas radioactivity appearing in CoA rose continuously over this time period. Incorporation of radioactivity before 2.5 h was difficult to measure at the concentration of cells employed, and experimental values are most often taken after 10 h of incubation.

Fig. 1. *Conversion of $[^{14}\text{C}]$ pantothenate to CoA*
Rat liver parenchymal cells were incubated for the designated time intervals with $1\mu\text{Ci}$ of $[^{14}\text{C}]$ pantothenate/ml in M-199 medium. HClO_4 extracts of the cultures were incubated under alkaline conditions to hydrolyse the acyl-CoA derivatives and adjusted to a neutral pH with triethanolamine/ HClO_4 . The solubilized portion of the extract was chromatographed and the radioactivity in the peaks corresponding to pantothenate and CoA were determined (a). The final HClO_4 precipitate was solubilized in NaOH and its radioactivity determined (b). O, Total radioactivity in the cells; ●, radioactivity in pantothenate; ▲, radioactivity in CoA; ■, radioactivity in all other peaks; □, radioactivity in final HClO_4 -insoluble fraction.

Fig. 2. *Composite chromatogram of extracts from cells incubated with insulin or glucagon*

Cells were incubated for 10h with $1\mu\text{Ci}$ of $[^{14}\text{C}]$ pantothenate/ml in M-199 medium. Each point is the average of duplicate cultures. ●, Incubations contained 30nM-glucagon; ○, incubations contained 1 m-i.u. of insulin/ml.

Effects of glucagon, dibutyryl cyclic AMP plus theophylline, and insulin on incorporation of $[^{14}\text{C}]$ pantothenate into CoA

Hepatocytes incubated with glucagon showed a higher incorporation of $[^{14}\text{C}]$ pantothenate into CoA than did controls or cells incubated with insulin. A composite chromatogram of cells extracted after 10h incubation with insulin or glucagon is shown in Fig. 2. The CoA peak was cleanly separated from the pantothenate peak and there was only a small accumulation of pathway intermediates. There was far more radioactivity in the CoA peak of those cells incubated with glucagon.

The amount of pantothenate converted to CoA was calculated by dividing the amount of radioactivity in CoA/mg of protein by the specific radioactivity of intracellular pantothenate. This

value eliminated differences between individual flasks by correcting for possible differences in protein content of the culture flasks or for differences in specific radioactivity of the precursor, pantothenate. This decreased the standard deviation for a given experimental condition. A graph of the incorporation of pantothenate into CoA as a function of glucagon concentration is shown in Fig. 3. Half-maximal activation occurred at about 3nM-glucagon and no activation was present at 0.3nM; 30nM was near saturation. This value shows the largest stimulation by glucagon (approx. 2-fold) observed in the course of these experiments. Two additional titrations, in which the maximum stimulation by glucagon was in the range 40–60% (relative to controls), also gave values of about 3nM-glucagon for the concentration required for half-maximum activation.

The effect of glucagon on pantothenate incorporation into CoA is compared with the effects of insulin and dibutyryl cyclic AMP plus theophylline

Fig. 3. Effect of glucagon on the rate of conversion of [¹⁴C] pantothenate to CoA

Cells were incubated for 10h in M-199 medium with 1μCi of [¹⁴C]pantothenate/ml and the indicated amounts of glucagon. Pantothenate incorporated into CoA was calculated from the radioactivity in CoA/mg of protein divided by the specific radioactivity of pantothenate. Each point is the mean for four cultures ± the sample s.d.

in Table 1. The addition of 1–3 m.i.u. of insulin/ml decreased pantothenate incorporation relative to the controls, but higher concentrations of insulin (up to 10 m.i.u./ml) did not result in a significant decrease in incorporation relative to controls. The addition of dibutyryl cyclic AMP also resulted in a significant increase (approx. 50%) in the incorporation of pantothenate into CoA. This increase was similar to that achieved with 30 nM-glucagon in the same experiments, and was a 130% increase over the rate of incorporation in cells incubated with insulin.

The rate of incorporation of pantothenate into CoA calculated from the ratio of the radioactivity in CoA/mg of protein to specific radioactivity of intracellular pantothenate should provide a measure of pantothenate incorporation independent of protein content in the flask or specific radioactivity of intracellular pantothenate. However, the data in Table 1 show that neither of these parameters (protein content or the specific radioactivity of pantothenate) was strongly affected by addition of insulin, glucagon or dibutyryl cyclic AMP plus theophylline. This was true of all experiments presented.

In contrast with the above two parameters, values expressed as nmol of pantothenate incorporated/mg of protein could reflect a regulatory influence of pantothenate concentration on enzymes in the pantothenate → CoA pathway. In these experiments (Table 1) there was no significant effect of glucagon, insulin or dibutyryl cyclic AMP on the intracellular concentration of pantothenate. These data suggest that a locus of hormone action exists in the metabolic pathway between pantothenate kinase and CoA which is not dependent upon mechanisms affecting either pantothenate transport or intracellular concentrations of pantothenate. However, in

Table 1. Pantothenate incorporated into CoA at 10 h

Hepatocytes were incubated in medium with [¹⁴C]pantothenate and the indicated additions as described in the Methods section. Values are given as means ± the sample s.d. The number of incubations is shown in parentheses; values are taken from three to eight separate experiments. Experiments were normalized from the ratio of the average values for the controls in that experiment to the overall average of the controls. *Values that differ significantly from the controls (*P* < 0.001) by Student's *t* test. No other values differ significantly from the controls at *P* < 0.05.

Conditions	Pantothenate incorporated into CoA (nmol/mg of protein)	Protein (mg)	Pantothenate (nmol/mg of protein)	10 ⁻³ × Radioactive pantothenate (c.p.m./mg of protein)	10 ⁻⁴ × Pantothenate specific radioactivity (c.p.m./nmol)
Control (8)	0.180	485	1.38	57.6	4.3
Insulin (8) (1–3 m.i.u./ml)	0.115 ± 0.011*	473 ± 64	1.37 ± 0.33	58.8 ± 13.9	4.3 ± 0.4
Glucagon (8) (30 nM)	0.272 ± 0.035*	452 ± 62	1.52 ± 0.14	60.8 ± 7.3	4.0 ± 0.4
Dibutyryl cyclic AMP (0.55 mM + theophylline (1.1 mM) (6)	0.265 ± 0.054*	446 ± 78	1.30 ± 0.26	54.6 ± 5.2	4.3 ± 1.0

some of the subsequent experiments, a significant effect of glucagon and dibutyl cyclic AMP plus theophylline on the intracellular pantothenate concentration was observed. Experiments were there-

fore undertaken to determine if a variation of pantothenate concentration with the range used in these experiments could affect the rate of incorporation [^{14}C]pantothenate into CoA.

Table 2. *Variation of pantothenate concentration*

Hepatocytes were incubated for 10h with medium and the indicated concentration of pantothenate. Values are given $\pm$ the sample s.d. Pantothenate incorporation was calculated from the radioactivity in CoA divided by the specific radioactivity of pantothenate.

	External [pantothenate] (μM)	Internal [pantothenate] (nmol/mg of protein)	$10^{-3} \times$ Specific radioactivity of intracellular pantothenate (c.p.m./nmol)	$10^{-3} \times$ Radioactivity in CoA (c.p.m./mg of protein)	Pantothenate incorporation (nmol/mg of protein)
Expt. A	7.1	0.36 ± 0.04	85.0 ± 4.9	25.2 ± 2.5	0.296 ± 0.029
	14.2	0.54 ± 0.05	89.2 ± 7.4	24.2 ± 3.9	0.271 ± 0.044
Expt. B	18.2	1.16 ± 0.06	78.3 ± 10.8	16.4 ± 2.2	0.210 ± 0.019
	44.2	2.06 ± 0.11	36.3 ± 4.9	7.2 ± 1.2	0.198 ± 0.034

Table 3. *Effect of dexamethasone on [^{14}C]pantothenate incorporation into CoA*

Hepatocytes were incubated with [^{14}C]pantothenate for 10h in the presence of the indicated additions. The pantothenate incorporation was calculated from the radioactivity appearing in the CoA peak/mg of protein divided by the specific radioactivity of pantothenate. Glucagon, where present, was at a concentration of 30 nM, dibutyl cyclic AMP at a concentration of 0.55 mM and theophylline at a concentration of 1.1 mM. Values are expressed as means $\pm$ s.d. of the sample. The number of cultures used is indicated in parentheses. Statistical significance was determined for the levels of $P < 0.05$ and $P < 0.01$ by using a two-tailed Student's *t* distribution. All values for pantothenate incorporation or pantothenate content in cells containing glucagon, glucagon + dexamethasone, dibutyl cyclic AMP + theophylline or dibutyl cyclic AMP + theophylline + dexamethasone differed significantly from controls at a level of $P < 0.01$. None of the values for the specific radioactivity of pantothenate differed significantly from control values at a level of $P < 0.05$. *P* values shown in the Table are determined for a significant difference between that condition and the same condition without dexamethasone. None of the values for pantothenate contents differed significantly from values for the same condition without dexamethasone at the level $P < 0.05$. Abbreviation: NS, not significant.

Conditions	Pantothenate incorporation (nmol/mg of protein)	<i>P</i>	Percentage of control	Pantothenate (nmol/mg of protein)	$10^{-3} \times$ Specific radioactivity of pantothenate (c.p.m./nmol)
Control	0.299 ± 0.033 (10)		100	0.42 ± 0.046	71 ± 6.9
Dexamethasone					
(5 nM)	0.250 ± 0.055 (4)	NS	84	0.37 ± 0.041	74 ± 16.5
(50 nM)	0.252 ± 0.048 (4)	NS	84	0.43 ± 0.041	71 ± 7.8
(0.5 μM)	0.292 ± 0.099 (4)	NS	98	0.37 ± 0.042	76 ± 21.6
(5 μM)	0.299 ± 0.111 (4)	NS	100	0.38 ± 0.065	71 ± 14.3
Glucagon	0.433 ± 0.058 (10)		145	0.72 ± 0.056	69 ± 9.0
Glucagon + dexamethasone (5 nM)	0.503 ± 0.092 (4)	NS	168	0.73 ± 0.041	69 ± 11.1
Glucagon + dexamethasone (50 nM)	0.574 ± 0.069 (6)	< 0.01	192	0.73 ± 0.068	75 ± 8.2
Glucagon + dexamethasone (0.5 μM)	0.584 ± 0.086 (10)	< 0.01	195	0.64 ± 0.068	72 ± 13.7
Glucagon + dexamethasone (5 μM)	0.565 ± 0.116 (4)	< 0.05	189	0.65 ± 0.121	66 ± 6.6
Dibutyl cyclic AMP + theophylline	0.488 ± 0.060 (4)		163	1.02 ± 0.080	70 ± 12.1
Dibutyl cyclic AMP + theophylline + dexamethasone (0.5 μM)	0.762 ± 0.083 (4)	< 0.01	254	1.09 ± 0.098	67 ± 13.4

Variation of intracellular pantothenate concentration

To establish that these experiments were conducted at intracellular pantothenate concentrations that were saturating for enzymes of the CoA-biosynthetic pathway, the rate of pantothenate incorporation into CoA was measured after incubation with different extracellular concentrations of pantothenate (Table 2). In Expt. A, the amount of [14 C]pantothenate added to the external medium resulted in an approx. 50% increase in internal concentration of pantothenate of nearly identical specific radioactivity. The radioactivity appearing in CoA differed by less than 6% for the two concentrations of pantothenate. Likewise, the calculated values for pantothenate incorporation into CoA differed by an insignificant amount between the two pantothenate concentrations. Similar results were obtained with higher concentrations of pantothenate. These experiments indicated that changes in intracellular pantothenate concentration within this range had no effect on the rate of conversion of pantothenate to CoA. This conclusion was tested again in Expt. B (Table 2). The external pantothenate concentration was varied by addition of an excess of non-radioactive pantothenate. The result was a doubling of the internal pantothenate concentration, and a 50% decrease in the specific radioactivity of the intracellular pantothenate. This resulted in a corresponding decrease of about 50% in the radioactivity in CoA/mg of protein. However, the values of pantothenate incorporation into CoA, calculated by dividing the radioactivity in CoA by the specific radioactivity of pantothenate, were within 5% of each other. This experiment demonstrates the validity of the calculation for pantothenate incorporation into CoA and demonstrates that variation of pantothenate concentration within this range does not affect the rate of incorporation of pantothenate into CoA. This is consistent with the relatively low K_m for pantothenate kinase of $11\mu\text{M}$ (Abiko *et al.*, 1972). Assuming a value of 20% protein/g wet wt. of liver tissue (Diem & Lentner, 1973), the lowest intracellular pantothenate concentration used (0.36 nmol/mg of protein) would be equivalent to approx. $86\mu\text{M}$.

Effect of dexamethasone on the incorporation of [14 C]pantothenate into CoA

In the absence of other stimulatory agents, dexamethasone in the concentration range 5 nM – $5\mu\text{M}$ did not have a significant effect on the rate of incorporation of [14 C]pantothenate into CoA; values for the rates of incorporation ranged from 100 to 84% of control (Table 3). However, in the presence of either glucagon or dibutyryl cyclic AMP plus theophylline, the addition of dexamethasone substantially increased the rate of [14 C]pantothenate incorporation into CoA. At $0.5\mu\text{M}$ -dexamethasone

and 30 nM -glucagon, the rate of incorporation was 35% higher than in the presence of glucagon alone, and 95% higher than in the controls. The maximum effect was achieved with 50 nM -dexamethasone. In the presence of dibutyryl cyclic AMP plus theophylline, $0.5\mu\text{M}$ -dexamethasone increased the rate of incorporation by 56%. Dibutyryl cyclic AMP plus theophylline stimulation was 63% of control in the absence of dexamethasone, and 154% of control in the presence of dexamethasone.

There was no effect of dexamethasone on the pantothenate content of the cells. In these experiments, the pantothenate content of incubations containing either glucagon or dibutyryl cyclic AMP plus theophylline was significantly elevated relative to controls ($P < 0.01$). However, the pantothenate content of the incubations containing only dexamethasone did not differ from controls. The presence of dexamethasone also had little effect on the pantothenate concentration of cells containing glucagon or dibutyryl cyclic AMP plus theophylline. The specific radioactivity of the intracellular pantothenate was approximately the same for all incubations (Table 3), and the protein content of the cells did not differ significantly between various conditions ($P > 0.05$).

Although there was no effect of dexamethasone in the absence of glucagon or dibutyryl cyclic AMP plus theophylline, an effect of dexamethasone could

Fig. 4. Effect of dexamethasone on the rate of [14 C]pantothenate conversion to CoA

Hepatocytes were incubated for 10h with $1\mu\text{Ci}$ of [14 C]pantothenate/ml and the indicated amounts of glucagon (●); O, $0.5\mu\text{M}$ -dexamethasone was added.

Table 4. *Effect of cycloheximide on activation of pantothenate incorporation into CoA*

Hepatocytes were incubated for 6 h in the presence of the added activator, [14 C]pantothenate and cycloheximide ($18\mu\text{M}$). The radioactivity in CoA per culture was calculated instead of the radioactivity in CoA/mg of protein to avoid effects of cycloheximide on general protein synthesis. The values for each condition (mean $\pm$ sample S.D.) were averaged from four to eight cultures obtained from two to four experiments.

Condition	Radioactivity in CoA/culture		
	Specific radioactivity of pantothenate		Preincubation with cycloheximide followed by rinse
	– Cycloheximide	+ Cycloheximide	
Control	0.150 ± 0.044	0.081 ± 0.028	0.243 ± 0.036
Dibutyryl cyclic AMP (0.55 mM) + theophylline (1.0 mM)	0.302 ± 0.068	0.135 ± 0.081	0.461 ± 0.017
Control	0.150 ± 0.012	0.112 ± 0.031	0.183 ± 0.008
Glucagon (30 nM)	0.259 ± 0.062	0.130 ± 0.017	0.286 ± 0.036

be measured at glucagon concentrations as low as 0.3 nM (Fig. 4). In the absence of dexamethasone, addition of 0.3 nM -glucagon had no effect, or resulted in a slight decrease in the rate of incorporation. In the presence of $0.5\mu\text{M}$ -dexamethasone, 0.3 nM -glucagon stimulated the rate of incorporation by about 50%. At $3\mu\text{M}$ -glucagon, which was well above the glucagon concentration required for saturating glucagon activation, the addition of dexamethasone caused a further 2-fold increase in the rate of incorporation. The presence of an effect of dexamethasone at saturating glucagon concentrations indicates that the two hormones activate at different loci in the biosynthetic and/or degradative pathway and/or are activating through different mechanisms.

Effect of cycloheximide on incorporation of pantothenate into CoA

Addition of cycloheximide ($18\mu\text{M}$) to the incubation medium substantially decreased the incorporation of [14 C]pantothenate into CoA. Values are given in Table 4 for the ratio of the radioactivity of CoA per culture flask to the specific radioactivity of pantothenate. There was no significant effect of cycloheximide addition on the pantothenate content of the cells or on the specific radioactivity of pantothenate. In the presence of cycloheximide, the rate of incorporation of pantothenate into CoA was decreased by 25–46% in the controls, by 55% in the cells incubated with dibutyryl cyclic AMP plus theophylline and by 50% in the cells incubated with glucagon. In the absence of cycloheximide, the activation of dibutyryl cyclic AMP plus theophylline was 100% above controls and the activation of glucagon was 73% above controls. In the presence of cycloheximide neither dibutyryl cyclic AMP plus theophylline nor glucagon resulted in a statistically significant increase over controls.

In order to determine that the cycloheximide was

not irreversibly damaging the cells, culture flasks were incubated for 6 h in the presence of cycloheximide with or without the addition of dibutyryl cyclic AMP plus theophylline or glucagon. After 6 h, cells were rinsed three times with medium and [14 C]pantothenate was added together with the appropriate activator. The high incorporation was recovered in the controls and the stimulation by dibutyryl cyclic AMP was 80% above the control values. The stimulation by glucagon was 56% above the control values.

After exposure to cycloheximide both dibutyryl cyclic AMP plus theophylline and glucagon were capable of increasing the rate of conversion of pantothenate into CoA, demonstrating that the effects of cycloheximide were reversible and that the cells were still able to respond to hormonal stimuli. The inhibition by the addition of cycloheximide could result from a requirement for protein synthesis for the stimulatory effect of glucagon and dibutyryl cyclic AMP. However, it could also occur if one of the enzymes were turning over so rapidly that in the presence of cycloheximide it would become rate-limiting for CoA biosynthesis.

Discussion

The stimulation of conversion of pantothenate into CoA by glucagon and by dibutyryl cyclic AMP plus theophylline occurred at a locus of the biosynthetic and/or degradative pathway between intracellular pantothenate and CoA. Dexamethasone, in the presence of glucagon or dibutyryl cyclic AMP plus theophylline also increased the incorporation of pantothenate into CoA by a mechanism(s) that did not involve differences in intracellular pantothenate concentrations or pantothenate uptake (Table 3). The enhancement of the glucagon effect by dexamethasone resembles, in part, the pattern of hormonal induction of the liver

enzymes, tyrosine aminotransferase and phosphoenolpyruvate carboxykinase. Both glucagon and cyclic AMP are capable of inducing these two enzymes *in vivo* (Wicks *et al.*, 1969, 1972). Furthermore, glucocorticoids are capable of inducing tyrosine aminotransferase in liver and additive inductive effects are observed in the presence of cyclic AMP or glucagon (Wicks *et al.*, 1969). The observed increase in activity of tyrosine aminotransferase by cyclic AMP and glucocorticoids are a result of enzyme synthesis *de novo* (Nickol *et al.*, 1978; Ernst & Feigelson, 1978). A relationship between cyclic AMP and glucocorticoid action was suggested by the fact that HTC hepatoma cells did not induce tyrosine aminotransferase upon exposure to cyclic AMP unless the cells were first exposed to dexamethasone (Granner *et al.*, 1977). In contrast, the Reuber H35 hepatoma cells were sensitive to the cyclic nucleotides in the absence of glucocorticoids (Barnett & Wicks, 1971). In primary cultures of hepatocytes, phosphoenolpyruvate carboxykinase was induced with cyclic AMP (Savage & Bonney, 1978; Oliver *et al.*, 1978) and tyrosine aminotransferase with either cyclic AMP (Bonney & Savage, 1979) or dexamethasone (Savage & Bonney, 1978). Furthermore, the presence of both cyclic AMP and dexamethasone caused additive increases in tyrosine aminotransferase activity (Bonney & Savage, 1979). In contrast with the above experiments with tyrosine aminotransferase and phosphoenolpyruvate carboxykinase, increases in the rate of pantothenate conversion to CoA were not achieved by the addition of dexamethasone alone, and dexamethasone appeared to act synergistically with either glucagon or cyclic AMP. The difference between the dexamethasone effect on pantothenate incorporation and the induction of tyrosine aminotransferase could be in the mechanism of dexamethasone activation itself, or in the fact that our parameter for activation is flux through a multi-enzyme pathway. Any of the stimulatory agents might affect more than one locus of the biosynthetic and/or degradative pathway, so that there was no net effect on the incorporation of labelled precursor into final product.

Addition of glucagon or insulin to the hepatocytes in culture produced changes in the rates of pantothenate incorporation into CoA that were quantitatively consistent with changes in rates observed *in vivo* caused by starving and feeding rats (Smith, 1978). *In vivo*, the rate of pantothenate conversion to CoA measured in the starved rat 2 h after intravenous injection of [^{14}C]pantothenate was about 4.5 nmol/h per g wet wt. of liver and about 1.9 nmol/h per g wet wt. of liver in rats given glucose at the time of injection (Smith, 1978). With the hepatocytes, rates varied from 14.0 nmol/h per g wet wt. [assuming 20% protein for conversion (Diem &

Letner, 1973)] to 2.3 nmol/h per g wet wt. The rate increased from 2.3 nmol/h per g wet wt. to 5.3 when 1 m-i.u. of insulin was replaced with 30 nm-glucagon. The increase in total CoA during food deprivation overnight was 229 nmol of CoA/g wet wt. of liver in 20 h, or an increase of at least 11.0 nmol/h per g wet wt. (Smith *et al.*, 1978).

Although addition of glucagon and dexamethasone produced changes in pantothenate incorporation into CoA in hepatocytes that were quantitatively similar to the changes observed during starvation and feeding *in vivo*, it is difficult to assess the contribution of changes in the circulating concentration of glucagon or glucocorticoid to the physiological alterations in total CoA concentrations in the liver. The concentration of dexamethasone required for the maximum response in the cultured hepatocytes was 50 nM, which is comparable with the physiological glucocorticoid concentrations ranging from 10 to 100 nM (Wicks *et al.*, 1974). Although glucagon concentration required for half-maximal effect (approx. 3 nM) is considerably greater than the concentration of pancreatic glucagon thought to be in the circulation (Fisher *et al.*), it is comparable with the concentrations used by Birnbaum & Fain (1977) for protein kinase-related activations in isolated liver cells. Sherwin & Felig (1974) have suggested that changes in circulating concentrations of pancreatic glucagon are too small to account for most physiological responses to food ingestion. However, in a situation such as this where the stimulatory effects of glucagon and dexamethasone are synergistic, small changes of one or the other are accentuated. In the physiological situation, an increase in glucagon and/or glucocorticoid might also be accompanied by regulatory effects from a decrease in insulin or a change in metabolite concentrations. An influx of fatty acids, for instance, could stimulate an increase in total CoA by decreasing the free CoASH concentration through acylation and relieving the feedback inhibition of pantothenate kinase (Abiko *et al.*, 1972; Smith *et al.*, 1978) or protecting CoA from degradation. Thus other hormonally directed factors could contribute to the regulation of total CoA concentration *in vivo*. However, the stimulation of [^{14}C]pantothenate incorporation into CoA by glucagon, cyclic AMP and dexamethasone shown by these data demonstrate a mechanism whereby the biosynthesis of CoA could be regulated in the liver.

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