Increase of L-serine dehydratase activity under gluconeogenic conditions in adult-rat hepatocytes cultured on collagen gel/nylon mesh

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Hormonal regulation of L-serine dehydratase [L-serine hydro-lyase (deaminating), EC 4.2.1.13] was studied in primary cultures of adult-rat hepatocytes. The hepatocytes were isolated by collagenase perfusion and maintained in culture on collagen-gel/nylon-mesh substrata. L-Serine dehydratase activity was measured with [^{14}C]threonine as substrate. The enzyme activity in hepatocytes of normal adult rats was low and declined rapidly in culture in L-15 medium containing $0.1\text{ }\mu\text{M}$ -insulin and even more in the presence of glucose. L-Serine dehydratase activity in hepatocytes of rats with streptozotocin-induced diabetes was initially 20-fold higher than that of normal rats, but fell rapidly to a low value by 4 days in culture. Hormonal regulation of the enzyme activity was manifested by treatment of the cultured hepatocytes with insulin ($0.1\text{ }\mu\text{M}$), glucagon ($0.3\text{ }\mu\text{M}$), dexamethasone ($10\text{ }\mu\text{M}$) and combinations of these hormones. Either glucagon or dexamethasone in the absence of insulin enhanced the activity of L-serine dehydratase, but failed to do so in the presence of insulin. Treatment with both hormones resulted in a 2–3-fold increase in enzyme activity in culture on days 3 and 4. Under conditions in which the enzyme activity was enhanced, glucose production by the cultured hepatocytes was concomitantly increased. Glucose production resulted in part from gluconeogenesis from pyruvate and not entirely from glycogenolysis. The gluconeogenic conditions of culture resulted in a decrease in cellular lipids in the cultured hepatocytes, as evidenced by ultrastructural studies.

The activity of the rat liver enzyme L-serine dehydratase [L-serine hydro-lyase (deaminating), EC 4.2.1.13] can be dramatically increased by the forced feeding of tryptophan, an amino acid mixture, or casein hydrolysate to rats that have been fed on a protein-depleted diet for 5 days (Pitot & Peraino, 1964; Peraino & Pitot, 1964; Peraino *et al.*, 1965). The increase in enzyme activity has been shown to represent protein synthesis *de novo* (Pitot & Peraino, 1964; Jost *et al.*, 1968) and may be completely repressed by the administration of glucose or fructose (Peraino *et al.*, 1966; Jost *et al.*, 1968). Hormonal regulation of L-serine dehydratase by glucocorticoid and glucagon occurs in intact (Ishikawa *et al.*, 1965; Inoue *et al.*, 1971; Cihak *et al.*, 1975), adrenalectomized or pancreatectomized (Ishikawa & Nakajima, 1970; Nakajima & Ishikawa, 1971; Cihak *et al.*, 1975) rats. However, there have been virtually no studies on the regulation of this enzyme in cultured rat hepatocytes.

The development of techniques for the isolation and maintenance of primary cultures of adult-rat

hepatocytes on collagen gel (Michalopoulos & Pitot, 1975) or collagen gel/nylon mesh (Sirica *et al.*, 1979) substrata has provided a means to maintain hepatocytes *in vitro* in a viable and functional state for 2 weeks or more, with morphological and biochemical properties comparable with those of parenchymal liver cells *in vivo* (Michalopoulos *et al.*, 1978; Sattler *et al.*, 1978). The hormonal regulation of several enzymes (Michalopoulos *et al.*, 1978; Oliver *et al.*, 1978; Spence *et al.*, 1979; Spence & Pitot, 1979) has been studied in these systems. In the present work, L-serine dehydratase activity in adult-rat hepatocytes maintained on collagen-gel/nylon-mesh substratum was measured under gluconeogenic conditions, as evidenced by the criteria of glucose production and a diminution of intracellular lipids.

Experimental

Materials

Animals. Adult male albino rats were supplied by

Holtzman Co. (Madison, WI, U.S.A.). Animals were maintained in polycarbonate cages and fed on laboratory chow. Diabetes was induced in fed rats by a single injection of streptozotocin (60 mg/kg body wt.) in 0.1 M-sodium citrate buffer, pH 4.5, via the tail vein of the animals anaesthetized lightly with diethyl ether. The diabetic state in rats was confirmed 5–7 days after injection by their high blood glucose concentration (5–10 mg/ml).

Chemicals. Insulin, L-threonine, streptozotocin and calf thymus DNA were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Glucagon was the product of Eli Lilly and Co. (Indianapolis, IN, U.S.A.), and dexamethasone sodium phosphate was obtained from Organon Inc. (West Orange, NJ, U.S.A.). [^{14}C]Threonine (specific radioactivity 203 Ci/mol) and sodium [^{14}C]pyruvate (specific radioactivity 21 Ci/mol) were obtained from New England Nuclear.

Methods

Isolation of cells and culture conditions. Hepatocytes were isolated from normal adult male albino rats (180–220 g) or rats with streptozotocin-induced diabetes (160–180 g) by a collagenase perfusion method, as described by Berry & Friend (1969) and modified by Bonney *et al.* (1974). The cells were maintained in culture on collagen gel/nylon meshes (Sirica *et al.*, 1979) with Leibovitz (L-15) medium (Leibovitz, 1963) as described by Mak *et al.* (1980).

Preparation of cell extracts. The hepatocyte cultures were harvested by the use of collagenase as previously described (Mak *et al.*, 1980). The resulting cell pellets were resuspended in 1 ml of 0.1 M-potassium phosphate buffer, pH 8.0, containing 1 mM-EDTA, 0.05 mM-pyridoxal phosphate and 1 mM-dithiothreitol, and disrupted with a Polytron (model PT10, Brinkmann Instruments) for 15 s at half-maximal setting. The suspension was centrifuged at 105 000 g for 1 h. The resulting supernatant was assayed for L-serine dehydratase activity. The pellets were covered with 1 M-KOH and left overnight at room temperature. The content of DNA in the pellets was determined by the method of Burton (1956) as modified by Bonney *et al.* (1974), with calf thymus DNA used as the standard.

Enzyme assays. L-Serine dehydratase activity was measured by a modification of the method of Suda & Nakagawa (1971), with [^{14}C]threonine as substrate. Briefly, 0.9 ml of the cell homogenate in the reaction buffer was preincubated for 5 min at 37°C, and then 0.1 ml of [^{14}C]threonine (final concn. 0.1 M, 0.1 $\mu\text{Ci/ml}$) was added to start the enzyme reaction. The reaction was stopped after 15 min with trichloroacetic acid, and the supernatant was treated with 2,4-dinitrophenylhydrazine as described by Suda & Nakagawa (1971), but without the subse-

quent NaOH treatment. α -Oxobutyrate was added to a final concentration of 1 mM to give quantitative precipitation. The crystalline precipitate was collected on Millipore filters and counted for radioactivity in toluene-based scintillation fluid, containing 2,5-diphenyloxazole (PPO, 4 g/l) and 1,4-bis-(5-phenyloxazol-2-yl)benzene (POPOP, 0.1 g/l), in a Beckman liquid-scintillation counter (model LS 7000). The deep-yellow solution resulting from the dissolution of the phenylhydrazone derivatives in toluene had a very high degree of quenching, but could be counted for radioactivity in this Beckman counter, which was equipped with automatic quenching compensation utilizing the 'H Number' concept. A standard quenching curve prepared with different amounts of phenylhydrazone derivatives was established to correct for the quenching of samples. Specific activity of the enzyme was expressed as μmol of product/h per μg of DNA.

Glucose measurements. (a) Unlabelled glucose. The glucose concentration in the medium was determined by a fluorimetric method originally described for the measurement of ATP (Greengard, 1963). The medium was first centrifuged at 800 g for 10 min at 4°C to remove any cell debris, and then precipitated with an equal volume of 10% HClO_4 . The supernatant was neutralized with KOH, and the insoluble KClO_4 was removed by centrifugation at 800 g for 5 min at 4°C. A sample of the resultant supernatant was assayed in the hexokinase/glucose 6-phosphate dehydrogenase system with fluorimetric determination of the product, NADH. A standard curve of glucose concentration was used to determine the glucose concentration in the medium.

(b) [^{14}C]Glucose. Deproteinized medium was analysed for the content of [^{14}C]glucose by successive mixing with cation- and anion-exchange resins as described by Exton & Park (1967). The unabsorbed radioactivity was counted in a liquid-scintillation counter; this value was taken to be largely due to [^{14}C]glucose.

Lipid measurements. Total lipid contents in the medium and in the cell pellets were extracted with chloroform/methanol (2:1, v/v) as described by Folch *et al.* (1957). The extracted material was evaporated to dryness under a stream of N_2 and counted for radioactivity with toluene-based scintillation fluid in a liquid-scintillation counter.

Results

L-Serine dehydratase activity was measured in this study by an assay method, modified from the colorimetric assay described by Suda & Nakagawa (1971), employing a radioactive substrate. This assay method was developed for the cultured hepatocytes, which generally possessed low enzyme activity and contained indeterminate amounts of

pyruvate from either endogenous production or exogenous contamination from the medium. Although the interfering amounts of pyruvate could be subtracted as blank values in the colorimetric assay, low enzyme activity was difficult to measure in the presence of the high blank values. The radioactive assay method circumvented this problem. The method was tested with a rat liver L-serine dehydratase preparation purified as described by Inoue *et al.* (1971), and validated by the linearity of the enzyme reaction over a 30 min interval and the sensitivity of the reaction in the range 0.2–1 μg of protein (results not shown).

L-Serine dehydratase activity in hepatocytes cultured on collagen-gel/nylon-mesh substratum was measured with this assay method. Enzyme activity in hepatocytes of normal adult rats was low and declined rapidly with time in culture, more so when glucose was present in the culture medium (Fig. 1b). Enzyme activity in hepatocytes of the diabetic rats was at least 20-fold higher than that of normal rats at the beginning of the culture period, but fell rapidly to a low value by 4 days in culture (Fig. 1a). The rapid decline was not accelerated by the presence of glucose in the culture medium.

Fig. 1. Time course of L-serine dehydratase activity in normal and streptozotocin-induced diabetic-rat hepatocytes

The hepatocytes isolated from (a) streptozotocin-induced diabetic rats or (b) normal rats were cultured with L-15 medium supplemented either with insulin (0.1 μM) (●, ○) or with insulin (0.1 μM) plus glucose (8 mM) (▲, △) for 4 days. Specific activity of the enzyme, expressed as nmol of product/h per μg of DNA, was determined in cells cultured for the number of days indicated.

The responsiveness of the enzyme activity to hormonal regulation was studied in hepatocytes from normal rats that had been in culture for 2 days to allow for their recovery after the collagenase dissociation. Since the cells had to be maintained in culture medium containing insulin (0.1 μM) in order to preserve viability and functional integrity (Michalopoulos & Pitot, 1975; W. W.-N. Mak & H. C. Pitot, unpublished work), the enzyme activity measured in the presence of insulin as the only added hormone was taken as the base-line for comparison with other hormone-supplemented conditions. Fig. 2(a) shows that in a day-3 culture of hepatocytes, 24 h treatment with 0.3 μM -glucagon and 10 μM -dexamethasone resulted in more than a 2-fold increase in enzyme activity compared with the treatment with insulin (0.1 μM) alone. Either glucagon or dexamethasone in the absence of

Fig. 2. Hormonal regulation of L-serine dehydratase activity in cultured hepatocytes

The hepatocytes were maintained in culture with daily changes of L-15 medium supplemented with insulin (0.1 μM) for (a) 2 days or (b) 3 days. At the end of this period, the medium was replaced with fresh L-15 medium supplemented only with hormones specified as follows: (I) insulin (0.1 μM) plus glucose (8 mM); (II) insulin (0.1 μM); (III) insulin (0.1 μM) plus glucagon (0.3 μM); (IV) insulin (0.1 μM) plus dexamethasone (10 μM); (V) glucagon (0.3 μM); (VI) dexamethasone (10 μM); and (VII) glucagon (0.3 μM) plus dexamethasone (10 μM). At 24 h after the hormone treatment, the cultures were harvested and the specific activity of the enzyme, expressed as nmol of product/h per μg of DNA, was measured in each culture. Results represent means $\pm$ S.D. obtained from three to five separate experiments.

insulin also enhanced the enzyme activity; but in the presence of insulin, their effects were nearly eliminated. The presence of glucose appeared to lower the enzyme activity further in the insulin-treated culture. In the absence of insulin, the cells after 24 h showed signs of deterioration, and the enzyme activity was even lower than that measured in the insulin-treated culture (results not shown). When insulin was omitted from the medium at the beginning of culture, the hepatocytes could not be maintained until day 3 for experimentation. When the hepatocytes were kept 1 more day in culture, enzyme activity in the basal culture was significantly decreased compared with that in a day-3 culture (Fig. 2*b*). However, glucagon, dexamethasone and their combination were still able to increase the enzyme activity 2–3-fold. The absolute enzyme activities in day-4 cultures were generally lower than those of day-3 cultures.

The hormonal conditions under which L-serine dehydratase activity in the cultured hepatocytes was enhanced resulted in glucose production into the culture medium. In Table 1, the amounts of glucose produced by the cultured hepatocytes during 5 days in culture were found to be decreasing rapidly when the cells were maintained in culture medium with

insulin (0.1 μM) alone. In a day-3 culture, glucagon (0.3 μM) and dexamethasone (10 μM) each by itself increased the glucose production 2-fold, and both together resulted in a 3-fold increase in glucose formation. Similarly, in a day-4 and a day-5 culture, either of the hormones alone and their combination could still increase glucose production, although the absolute amounts of glucose produced by the hormonal stimulation declined with time in culture. Glucose production and its stimulation by hormones suggested that L-serine dehydratase activity was increased under gluconeogenic conditions. To ascertain that the glucose production arose at least partially from gluconeogenesis and not entirely from glycogenolysis, the following experiment was performed.

In L-15 medium, the pyruvate concentration is 5 mM (Leibovitz, 1963). By labelling the pyruvate pool with [3- ^{14}C]pyruvate, any production of radioactive glucose will require gluconeogenesis from the C_2 unit of pyruvate. Table 2 shows the contents of glucose and lipid in the cultures treated with insulin or glucagon and dexamethasone. The glucose content in the medium of the hepatocyte cultures declined from day 3 to day 4, as observed in Table 1. Dexamethasone and glucagon again were capable of

Table 1. *Glucose production into the culture medium*

The hepatocytes were maintained in culture with daily changes of L-15 medium supplemented with insulin (0.1 μM). At the beginning of the experiments, the culture medium was replaced with L-15 medium supplemented with different hormones specified in the first column of the Table. The culture medium was removed on the following day, at which time the cells had been in culture for the time denoted in the Table. Glucose concentration in the culture medium was determined by a fluorimetric method (see under 'Methods') and expressed as nmol/ μg of DNA. Results represent the means $\pm$ S.D. obtained from three separate experiments.

Supplements in medium	Glucose production (nmol/ μg of DNA)				
	Day 1	Day 2	Day 3	Day 4	Day 5
Insulin (0.1 μM)	136.5 $\pm$ 24.0	73.9 $\pm$ 9.4	13.2 $\pm$ 0.9	2.4 $\pm$ 0.2	2.0 $\pm$ 0.2
Glucagon (0.3 μM)	—	—	26.5 $\pm$ 0.7	14.8 $\pm$ 1.2	—
Dexamethasone (10 μM)	—	—	22.8 $\pm$ 0.5	13.7 $\pm$ 0.9	—
Glucagon (0.3 μM) + dexamethasone (10 μM)	—	—	34.3 $\pm$ 1.6	28.9 $\pm$ 1.5	24.5 $\pm$ 1.7

Table 2. *Glucose and lipid contents in cultured hepatocytes*

The hepatocytes were maintained in culture with daily changes of L-15 medium supplemented with insulin (0.1 μM). At the beginning of the experiments, the culture medium was replaced with L-15 medium containing [3- ^{14}C]pyruvate (1 $\mu\text{Ci}/\text{ml}$, 5 $\mu\text{mol}/\text{ml}$) and supplemented with either insulin or glucagon and dexamethasone. On the following day, at which time the cells had been in culture for the time denoted in the Table, the culture medium was removed and the cells were collected by collagenase treatment. Contents of glucose and lipids in the medium or in the cell pellet were determined and expressed as d.p.m./ μg of DNA (see under 'Methods'). Results represent the means $\pm$ S.D. obtained from three separate experiments.

Supplements in medium	$10^{-4} \times$ Glucose in medium		Lipid in medium		Cellular lipid	
	Day 3	Day 4	Day 3	Day 4	Day 3	Day 4
Insulin (0.1 μM)	3.2 $\pm$ 0.5	1.9 $\pm$ 0.4	42 $\pm$ 7	33 $\pm$ 6	80 $\pm$ 5	58 $\pm$ 5
Glucagon (0.3 μM) + dexamethasone (10 μM)	5.8 $\pm$ 0.8	3.0 $\pm$ 0.5	45 $\pm$ 5	37 $\pm$ 4	60 $\pm$ 3	38 $\pm$ 6

EXPLANATION OF PLATE 1

Ultrastructural differences in adult rat hepatocytes treated with various hormones in culture

(a) The hepatocytes were maintained in culture for 3 days with daily changes of L-15 medium supplemented with insulin ($0.1\mu\text{M}$) and glucose (8mM). (b) The hepatocytes were maintained in culture for the first 2 days with daily changes of L-15 medium supplemented with insulin ($0.1\mu\text{M}$) and for the third day with L-15 medium supplemented with glucagon ($0.3\mu\text{M}$) and dexamethasone ($10\mu\text{M}$) and no insulin. After 24 h of hormone treatment, a depletion of lipid droplets occurred, together with the appearance of small autophagic vacuoles as noted in (b). This is in contrast with the lipid droplets seen in cells cultured in the presence of insulin and glucose (a). Cellular organelles shown are mitochondria (M), nuclei (N), lipid droplets (L), bile canaliculi (BC) and autophagic vacuoles (AV).

increasing the glucose production into the medium compared with the insulin-treated cultures. When the contents of the total lipids in the medium and cells were measured, there was 25–35% less cellular lipid in the glucagon/dexamethasone-treated cultures than in those treated with insulin (Table 2), but there was not a significant difference in the output of lipids into the medium in both cultures. This difference in cellular lipid content in the two cultures was further confirmed qualitatively by ultrastructural analysis of the hormone-treated cells. Electron micrographs of the insulin-treated cells (Plate 1a) revealed large lipid droplets in the cytoplasm, whereas in the glucagon/dexamethasone-treated cells there were very few lipid droplets of significant size (Plate 1b). The overall morphology of other organelles was normal and similar in both cells. There were also more autophagic vacuoles in the glucagon/dexamethasone-treated cells than in the cells of insulin-treated cultures.

Discussion

Rat liver L-serine dehydratase (EC 4.2.1.13) utilizes as its substrate both L-serine and L-threonine, with K_m values of $5.7 \times 10^{-2} \text{ M}$ and $8.7 \times 10^{-2} \text{ M}$ respectively (Nakagawa & Kimura, 1969). The enzyme was shown to be identical with the previously described L-threonine dehydratase (EC 4.2.1.16) of rat liver by the studies of Nakagawa & Kimura (1969). In the present paper, the name L-serine dehydratase was used, although the substrate for the measurement of enzyme activity was L-threonine. Since there are other serine-metabolizing enzymes in the liver cytosol, such as serine aldolase (serine hydroxymethyltransferase, EC 2.1.2.1), cystathionine β -synthase [L-serine hydro-lyase (adding homocysteine), EC 4.2.1.22] and the mitochondrial L-serine:pyruvate aminotransferase (EC 2.6.1.51; Snell, 1975), the use of L-threonine as substrate and of 100 000 g supernatants as the enzyme source may alleviate possible interference by these enzymes with L-serine dehydratase activity measurements. The radioactive assay method described here was adequate and sensitive to detect enzyme activities in the hepatocyte cultures. L-Serine dehydratase activity declined rapidly in cultured hepatocytes, such a decline being consistent with the reported short half-life (6–8 h) of this enzyme in rat liver (Pitot, 1974). However, after 2 days in culture, the hepatocytes could still respond to the combined actions of dexamethasone and glucagon by an increase of L-serine dehydratase activity, enhanced production of glucose, and decreased cellular lipid production from pyruvate. The increase of L-serine dehydratase activity was 2–3-fold in hepatocytes cultured for 3–4 days, but

the basal as well as the increased activity of the enzyme appeared to decline with time in culture. Prolonged maintenance of the hepatocytes in glucagon-supplemented medium is not feasible, since stimulation of autophagic-vacuole accumulation by glucagon was observed in this study (Plate 1b) and has been reported to occur *in vivo* by others (Deter, 1971; Abe & Ogawa, 1978). Glucose production in these cultures, which was enhanced by dexamethasone and glucagon, arose partially from gluconeogenesis, as evidenced by the conversion of [^{14}C]pyruvate into [^{14}C]glucose. [^{14}C]Serine was not employed to demonstrate gluconeogenesis in conjunction with increased L-serine dehydratase activity, because of the large pool of pyruvate (5 mM) in the culture medium. Any conversion of [^{14}C]serine into [^{14}C]pyruvate would be diluted by the endogenous pool, and the dilution would render interpretations difficult. However, some degree of glucose production from glycogenolysis could not be ruled out, since the degree of stimulation of glucose production measured in terms of unlabelled glucose was greater than that measured in terms of [^{14}C]glucose. On the other hand, lipid accumulation in the cells and medium appeared to be diminished by the actions of dexamethasone and glucagon, in agreement with reports that glucagon and cyclic AMP could stimulate gluconeogenesis (Exton *et al.*, 1970) and inhibit glycolysis and lipogenesis (Geelen & Gibson, 1976; Ochs & Harris, 1978). An inhibition of lipogenesis was demonstrated by the depletion of lipid vesicles observed morphologically in the cultured hepatocytes. Glucagon and dibutyl cyclic AMP were found to block the formation of these triacylglycerol-rich vesicles in chicken liver cells *in vitro* rather than to accelerate their turnover (Watkins *et al.*, 1977).

The possible relationship between gluconeogenesis and increased L-serine dehydratase activity shown *in vitro* in the present study would further lend support to the belief that the L-serine dehydratase-dependent pathway for gluconeogenesis from serine is a significant one in rat liver (Rowell *et al.*, 1973; Sandoval & Sols, 1974; Bhatia *et al.*, 1975), compared with the serine:pyruvate aminotransferase-dependent pathway (Rowell *et al.*, 1973; Williamson & Ellington, 1975), which may be important in some carnivorous mammals (Rowell *et al.*, 1979).

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