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# Effect of Alloxan-Diabetes on Gluconeogenesis and Ureogenesis in Isolated Rabbit Liver Cells

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1. Neither alloxan-diabetes nor starvation affected the rate of glucose production in hepatocytes incubated with lactate, pyruvate, propionate or fructose as substrates. In contrast, glucose synthesis with either alanine or glutamine was increased nearly 3- and 12-fold respectively, in comparison with that in fed rabbits. 2. The addition of aminooxyacetate resulted in about a 50% decrease in glucose formation from lactate in hepatocytes isolated from fed, alloxan-diabetic and starved rats, suggesting that both mitochondrial and cytosolic forms of rabbit phosphoenolpyruvate carboxykinase function actively during gluconeogenesis. 3. Alloxan-diabetes resulted in about 2–3-fold stimulation of urea production from either amino acid studied or $NH_4Cl$ as $NH_3$ donor, whereas starvation caused a significant increase in the rate of ureogenesis only in the presence of alanine as the source of $NH_3$. 4. As concluded from changes in the [3-hydroxybutyrate]/[acetoacetate] ratio, in hepatocytes from diabetic animals the mitochondrial redox state was shifted toward oxidation in comparison with that observed in liver cells isolated from fed rabbits.

Much of our knowledge about the effect of diabetes on hepatic gluconeogenesis and ureogenesis has been derived from studies with rats, exhibiting essentially the cytosolic phosphoenolpyruvate carboxykinase activity (Nordlie & Lardy, 1963). Since this enzyme activity has been demonstrated in both mitochondrial and cytosolic compartments of human liver (Wieland *et al.*, 1968; Diesterhaft *et al.*, 1971), rabbit liver (Ilyin *et al.*, 1966; Usatenko, 1970), guinea-pig liver (Nordlie & Lardy, 1963) and some other mammals (see, e.g., Tilghman *et al.*, 1976), one might predict that the process of gluconeogenesis in species exhibiting phosphoenolpyruvate carboxykinase activity in both cytosol and mitochondria could deviate considerably from that expected in rats. In addition, there are different observations with respect to the ability of dietary and hormonal manipulations to influence gluconeogenesis in mammals with an equal distribution of intracellular phosphoenolpyruvate carboxykinase activity. First, starvation increases the cytosolic phosphoenolpyruvate carboxykinase activity in rabbit liver (Usatenko, 1970; Huibregtse *et al.*, 1976) and increases both cytosolic and mitochondrial activities in guinea-pig liver (Elliott & Pogson, 1977*a*), whereas no change in the enzyme activity was observed in both sheep (Filsell *et al.*, 1969) and ox (Ballard *et al.*, 1968) livers. Secondly, diabetes does not result in a significant change of phosphoenolpyruvate carboxykinase activity in guinea-pig liver (Elliott & Pogson, 1977*a*), but increases the cytosolic enzyme activity in rabbit liver (Usatenko, 1970; Huibregtse *et al.*, 1976). On the contrary, in the sheep liver (Filsell *et al.*, 1969; Taylor *et al.*, 1971) both mitochondrial and cytosolic activities are increased under conditions of experimental diabetes. Thirdly, glucagon enhances lactate-induced gluconeogenesis in rabbit liver (Huibregtse *et al.*, 1977), but not in starved guinea-pig liver (Söling *et al.*, 1970; Cook, 1977). These observations suggest that subtle differences in glucose formation also exist in species with an equal distribution of intracellular phosphoenolpyruvate carboxykinase activity. The present paper reports studies on the effect of alloxan-diabetes on both glucose and urea synthesis in rabbit hepatocytes.

## Materials and Methods

### *Animals*

Male young white rabbits (Popielno strain), weighing 500–700g, were used throughout. Fed rabbits had free access to laboratory chow and tap water *ad libitum*. Starved rabbits were deprived of laboratory chow 27h before the experiment. Starvation of rabbits for 48h or longer did not result in a stimulation of rates of synthesis of both glucose and urea compared with that determined in hepatocytes isolated from animals starved for 27h.

Alloxan-diabetes was induced by the single injection of alloxan (200mg/kg body wt.) dissolved in 10mM-citrate buffer (pH4.5) into the marginal vein of

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the ear of a rabbit starved for 48h. After injection animals were allowed a standard diet and water *ad libitum*. To avoid hypoglycaemic shock, animals were given 1% glucose solution to drink during the 24h after the alloxan treatment. Only those alloxan-treated animals that exhibited decreased or stabilized body weight and blood glucose concentrations in excess of 300mg/100ml [447 (S.E.M. ±36) for nine animals] 3 days after treatment were considered 'diabetic' and used for experiments. In alloxan-diabetic rabbits glucose in urine was above 2g/100ml, as detected with Ames (Stoke Poges, Bucks., U.K.) reagent strips. Alloxan-diabetes induced a decrease of liver glycogen content determined as glucose equivalent [from 16.0 (S.E.M. ± 5.4) mg/g of liver in fed rabbits to 5.4 (S.E.M. ± 2.3) mg/g of liver in alloxan-diabetic animals, as calculated for four measurements]. Starvation of alloxan-diabetic rabbits for 24h markedly decreased blood glucose. Hepatocytes were isolated on day 4 after the alloxan treatment.

### *Chemicals*

Collagenase, alloxan and all substrates were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Other enzymes were purchased from either Sigma or Boehringer (Mannheim, Germany). Amino-oxyacetate was from Eastman Kodak Co. (Rochester, NY, U.S.A.) and quinolinate from Fluka A.G. (Buchs, Switzerland). Other chemicals were of analytical grade.

### *Isolation and incubatinn of hepatocytes*

Liver parenchymal cells were obtained basically by the procedure of Berry & Friend (1969) with modifications by Krebs *et al.* (1974) and Seglen (1972, 1973*a*,*b*) as described previously (Zaleski & Bryła, 1977), except for changes described below. Livers were preperfused with 200ml of $Ca^{2+}$-free Krebs–Ringer bicarbonate buffer (Krebs & Henseleit, 1932)/100g body wt. before the perfusion with 0.03% collagenase. Isolated hepatocytes were washed with 3×200ml of Krebs–Ringer bicarbonate solution. Hepatocyte preparations obtained by this procedure had negligible glycogen content. Hepatocytes from starved rabbits isolated by this procedure show the same ATP content, $O_2$ uptake and rates of synthesis of both ketone bodies and glucose as those reported before (Zaleski & Bryła, 1977). Incubations of hepatocytes (10–15mg dry wt./ml) were carried out for either 30 or 60min at 37°C.

The dry weight of isolated cells was obtained by drying 1ml of cell suspension at 105°C to constant weight. At the same time 1ml of suspension medium without cells was treated in the same way. The difference gave the dry weight (Krebs *et al.*, 1974). Measurements of ratios of the fresh weight of liver (bled out)/dry weight of hepatocytes in starved, fed and diabetic rabbits were 3.69 (S.E.M. ±0.06) (four observations), 3.48±0.20 (five observations) and 3.87±0.10 (five observations) respectively.

### *Assays*

Glucose, urea, $NH_3$ and ketone bodies were assayed in supernatants after centrifugation of the neutralized $HClO_4$ extracts, as described elsewhere (Zaleski & Bryła, 1977). Ketone bodies were measured fluorimetrically or spectrophotometrically as described by Williamson & Corkey (1969). Glycogen was extracted from the liver and hydrolysed to glucose as described by Pfleiderer (1965). Glucose was measured with glucose oxidase and peroxidase (Huggett & Nixon, 1957). Urea after treatment with urease was determined as $NH_3$ by the method of Chaney & Marbach (1962).

### *Expression of results*

Glucose and urea synthesis and ketone-body concentrations in the cell suspensions are expressed as means±S.E.M., calculated as μmol/g dry wt. of cells. The statistical significance of results was calculated by Student's *t* test.

## Results and Discussion

Utilization of various gluconeogenic precursors in hepatocytes isolated from fed, starved or alloxan-diabetic rabbits is shown in Table 1. Without any substrate added to the reaction medium, the rates of glucose formation in hepatocytes isolated from all rabbits studied were very low, but linear with time. In liver cells obtained from either fed or alloxan-diabetic animals the glucose formation was linear with time in the presence of all substrates studied. In contrast, when isolated hepatocytes from starved animals were incubated with amino acids as substrates there were lags of some 15–30min before the rates of gluconeogenesis became linear. Urea production from either alanine or glutamine was linear in hepatocytes from starved rabbits for at least 90min and showed no significant lag after the addition of substrate. Thus the low initial rates of glucose formation in hepatocytes from starved rabbits incubated with amino acids are probably not due to the permeability barrier to those substrates. The reason for the lag in glucose production in hepatocytes from starved animals in the presence of amino acids is not known. A similar lag in gluconeogenesis was also reported for lactate as substrate in hepatocytes isolated from the rat (Cornell *et al.*, 1974) and guinea pig (Arinze & Rowley, 1975; Elliott & Pogson, 1977*b*).

Table 1. *Glucose formation in hepatocytes isolated from fed, alloxan-diabetic and starved rabbits*

Rabbit hepatocytes were isolated and incubated as described in the Materials and Methods section. Pyruvate was used at 5mM, and all other substrates were added at 10mM concentrations. Values for glucose formation ± S.E.M. (in μmol/30min per g dry wt.) are shown for the numbers of observations given in parentheses. With either pyruvate or propionate as substrates the values for glucose synthesis in hepatocytes from starved rabbits are significantly different from those in hepatocytes from alloxan-diabetic rabbits ($P<0.01$). With either alanine or glutamine the values for glucose formation after 30min of incubation of hepatocytes from starved rabbits are significantly different from those in cells from alloxan-diabetic rabbits ($P<0.001$). $P$ (against corresponding fed controls): *<0.05; **<0.01; ***<0.001; other differences are not significant.

| Substrate | Period of incubation (min) | Glucose formation | | |
|---|---|---|---|---|
| | | Fed | Alloxan-diabetic | Starved |
| None | 0–30 | 3.0± 1.1 (6) | 4.4±1.1 (6) | 3.0±0.4 (43) |
| L-Lactate | 0–30 | 45.5± 2.2 (6) | 53.3±3.7 (6) | 46.2±2.2 (37) |
| Pyruvate | 0–30 | 30.0± 5.2 (6) | 37.0±4.8 (6) | 24.0±1.1 (35) |
| Propionate | 0–30 | 48.5± 5.9 (3) | 58.5±3.3 (6) | 37.4±3.0 (5) |
| Fructose | 0–30 | 104.7±13.3 (5) | 119.9±5.9 (6) | 104.3±7.8 (10) |
| L-Alanine | 0–30 | 17.0± 2.6 (4) | 41.1±3.3 (6)** | 15.9±0.7 (26) |
| | 30–60 | | | 33.7±1.8 (26)** |
| L-Glutamine | 0–30 | 5.5± 2.6 (5) | 31.1±3.3 (5)*** | 11.5±1.1 (14)* |
| | 30–60 | | | 24.8±1.8 (14)*** |

Data in Table 1 show that the order of rates at which fructose, lactate and alanine are converted into glucose in isolated hepatocytes of starved rabbits is the same as in perfused livers (Huibregtse *et al.*, 1977), perfused rat livers (Exton & Park, 1967; Ross *et al.*, 1967) and guinea-pig hepatocytes (Arinze & Rowley, 1975; Elliott & Pogson, 1977*b*). In contrast, according to Söling *et al.* (1970) guinea-pig liver utilizes lactate for glucose synthesis at least as well as fructose. The rates of glucose formation obtained with hepatocytes of starved rabbit with fructose, lactate or alanine are similar to those reported by Huibregtse *et al.* (1977) for perfused livers; however, they are approximately half of those obtained with rat livers (Exton & Park, 1967; Ross *et al.*, 1967) and guinea-pig hepatocytes (Elliott & Pogson, 1977*b*). Propionate is utilized for glucose formation 2-fold faster in guinea-pig (Elliott & Pogson, 1977*b*) than in rabbit hepatocytes. Although pyruvate is as good a glucose precursor as lactate in both rat (Ross *et al.*, 1967) and guinea-pig (Elliott & Pogson, 1977*b*) livers, the rate of its utilization for glucose production in rabbit hepatocytes is half of that with lactate. Although alanine is a poor precursor for glucose in the perfused rabbit liver (Huibregtse *et al.*, 1977), it is a rather good substrate for glucose production in rabbit hepatocytes (as concluded from the maximal rate of glucose formation, i.e. measured in the period between 30 and 60min of incubation) and in perfused livers (Söling *et al.*, 1970) and hepatocytes (Elliott & Pogson, 1977*b*) isolated from guinea pig.

Although both starvation and alloxan-diabetes resulted in a marked increase of phosphoenolpyruvate carboxykinase activity (Huibregtse *et al.*, 1976; Ray, 1976), they did not induce an increase in glucose formation in rabbit hepatocytes with lactate, pyruvate, propionate or fructose as substrate (Table 1). However, from the data of Kim & Miller (1969) the induction of regulatory enzymes may not be associated with an increased flux through the overall pathway in which those enzymes are involved. Our observations are consistent with nearly identical rates of glucose synthesis in livers isolated from fed and starved rabbits (Arinze *et al.*, 1973) and rats (Story *et al.*, 1976), but differ from data of Huibregtse *et al.* (1977) and Elliott & Pogson (1977*a*) for perfused rabbit livers and guinea-pig hepatocytes respectively. When either alanine or glutamine was used as substrate in rabbit hepatocytes, both starvation and alloxan-diabetes resulted in a marked stimulation of maximal rates of glucose production (i.e. measured in the period between 30 and 60min of incubation), suggesting, in contrast with Huibregtse *et al.* (1977), that an alanine cycle may function actively in the rabbit.

The addition of amino-oxyacetate, an inhibitor of transamination (Hopper & Segal, 1964), resulted in about a 50% decrease in glucose formation from lactate in hepatocytes isolated from fed, alloxan-diabetic or starved rabbits, suggesting that both mitochondrial and cytosolic forms of rabbit phosphoenolpyruvate carboxykinase function actively in gluconeogenesis (Table 2). This finding confirms data of Arinze *et al.* (1973) and Huibregtse *et al.* (1977) for perfused livers from starved rabbits. The data obtained with amino-oxyacetate were confirmed with the use of quinolinate, an inhibitor of mainly cytosolic phosphoenolpyruvate carboxy-kinase (Ray *et al.*, 1966; Veneziale *et al.*, 1967; Söling *et al.*, 1971) and/or cytosolic aspartate amino-transferase (Hsu &

Table 2. *Inhibition of glucose formation by amino-oxyacetate and quinolinate in hepatocytes isolated from fed, alloxan-diabetic and starved rabbits*

Rabbit hepatocytes were incubated for 30min with either pyruvate (5mM) or L-lactate (10mM) as described in the Materials and Methods section. Amino-oxyacetate and quinolinate were used at 0.2 and 14mM concentrations respectively. Values (means±S.E.M., with numbers of observations in parentheses) express percentage of inhibition of glucose formation in the presence of either amino-oxyacetate or quinolinate. Values obtained in the absence of inhibitors are shown in Table 1. *P* (against corresponding fed controls): *<0.001; **<0.02; other differences are not significant.

| | Percentage inhibition | | | | | |
|---|---|---|---|---|---|---|
| | +Amino-oxyacetate | | | +Quinolinate | | |
| Substrate | Fed | Alloxan-diabetic | Starved | Fed | Alloxan-diabetic | Starved |
| L-Lactate | 53±4 (6) | 54±3 (6) | 59±2 (26) | 56±6 (6) | 35±3 (6)* | 51±3 (26) |
| Pyruvate | — | — | — | 52±3 (5) | 30±2 (5)** | 47±3 (19) |

Table 3. *Urea production and $NH_3$ release in hepatocytes isolated from fed, alloxan-diabetic and starved rabbits*

Hepatocytes were incubated for 30min as described in the Materials and Methods section. Ornithine was added at 2mM concentration, and other substrates were at 10mM concentrations. Values shown are means±S.E.M. (expressed in μmol/30min per g dry wt. of cells) with numbers of observations in parentheses. *P* (against corresponding fed controls): *<0.05; **<0.02; ***<0.001. With alanine as substrate the value for $NH_3$ release in alloxan-diabetic rabbits is significantly different from that in starved rabbits ($P<0.01$). With glutamine the values for both $NH_3$ release and urea formation in alloxan-diabetic animals are significantly different from those in starved ones ($P<0.001$ and $P<0.02$, respectively). Urea production in alloxan-diabetic rabbits is significantly different from that in starved rabbits in the presence of either $NH_4Cl$+lactate ($P<0.05$) or $NH_4Cl$+lactate+ornithine ($P<0.001$). Other differences are not significant.

| | Urea | | | $NH_3$ | | |
|---|---|---|---|---|---|---|
| Substrate | Fed | Alloxan-diabetic | Starved | Fed | Alloxan-diabetic | Starved |
| None | 5.2± 1.5 (6) | 12.9± 2.2 (7)** | 9.6± 0.7 (43)*** | 0 | 0 | 0 |
| Alanine | 25.5± 4.8 (6) | 52.2± 5.9 (8)*** | 46.6± 3.0 (36)** | 8.5±2.6 (6) | 56.2±15.9 (8)* | 16.6±3.7 (28) |
| Glutamine | 35.9± 4.4 (6) | 97.7±11.5 (5)*** | 54.0± 5.9 (34) | 18.5±2.2 (6) | 75.8±16.6 (7)*** | 16.3±3.7 (27) |
| L-Lactate, $NH_4Cl$ | 61.0± 7.4 (8) | 99.5±11.5 (8)** | 72.1± 5.9 (15) | — | — | — |
| L-Lactate, $NH_4Cl$, ornithine | 124.3±15.2 (8) | 321.6±10.7 (7)*** | 151.3±11.1 (17) | — | — | — |

Fahien, 1976). Quinolinate was used at relatively high concentration (14mM), owing to poor transport through the cell membrane of rabbit liver (Söling & Kleineke, 1976). A similar degree of inhibition of glucose formation by either amino-oxyacetate or quinolinate was obtained in hepatocytes from both fed and starved rabbits. A lower inhibitory effect of quinolinate in comparison with that of amino-oxyacetate in diabetic rabbits is consistent with the report by Alvares & Ray (1974) that the inhibitor is ineffective in diabetic rats, but is contrary to its strongly inhibitory effect on glucose production in rat hepatocytes (Elliott *et al.*, 1977).

Without any substrate added to the reaction medium, the rate of urea formation in hepatocytes isolated from fed rabbits was low (Table 3). Alanine and glutamine stimulated urea production about 5- and 7-fold respectively, and induced $NH_3$ release. The highest stimulation of urea production was observed when ornithine was added to hepatocytes incubated with $NH_4Cl$ and lactate. Starvation increased the urea production from endogenous substrate and alanine, whereas it did not affect the urea formation when either glutamine or $NH_4Cl$ was used as $NH_3$ donor. On the contrary, alloxan-diabetes significantly stimulated urea synthesis from each of the substrates studied. This might be due to an increase in both aminotransferase activities (Belfiore *et al.*, 1974; Johnson *et al.*, 1973) and those of enzymes of the urea cycle (McLean & Novello, 1965).

As shown in Table 4, the concentration of ketone bodies in suspensions of hepatocytes isolated from alloxan-diabetic rabbits was similar to that in cell suspensions obtained from starved rabbits (Zaleski & Bryła, 1977), but about 50% higher than in hepatocyte suspensions obtained from fed animals. Similarly to starvation (Garber & Hanson, 1971*b*; Zaleski & Bryła, 1977), alloxan-diabetes resulted in a decline of the [3-hydroxybutyrate]/[acetoacetate] ratio, suggesting a marked decrease in the mitochon-

Table 4. [3-*Hydroxybutyrate*]/[*acetoacetate*] *ratio in suspensions of hepatocytes isolated from alloxan-diabetic and fed rabbits*

Hepatocytes were incubated for 30min either without substrate, with 10mM-lactate or with 5mM-pyruvate. Values shown are means±S.E.M. for five separate experiments. *P* (against fed controls): *<0.05; **<0.02; ***<0.01; †<0.001; other differences are not significant.

| Substrate | Metabolite concentrations in cell suspensions (μmol/g dry wt.) 3-Hydroxybutyrate | Acetoacetate | Ratio [3-hydroxybutyrate]/[acetoacetate] |
|---|---|---|---|
| Fed | | | |
| None | 1.8±0.4 | 13.3±1.8 | 0.13±0.04 |
| Pyruvate | 2.6±0.4 | 2.1±0.7 | 0.32±0.04 |
| L-Lactate | 2.6±0.4 | 5.9±0.7 | 0.44±0.04 |
| Alloxan-diabetic | | | |
| None | 0.4±0*** | 22.2±0.7† | 0.02±0.01** |
| Pyruvate | 1.8±0.4 | 13.3±1.1*** | 0.13±0.03*** |
| L-Lactate | 1.5±0* | 10.4±1.5* | 0.14±0.03 |

drial [NADH]/[NAD$^+$] ratio. On starvation, the mitochondrial redox state is also shifted toward oxidation in guinea-pig liver (Garber & Hanson, 1971*a*), whereas in rat liver it is shifted toward reduction (Williamson *et al.*, 1967). In agreement with Exton *et al.* (1972), lactate markedly suppressed ketogenesis, whereas pyruvate was less efficient in inhibiting the ketone-body production in rabbit hepatocytes.

The present results indicate that hepatocytes from rabbit differ from those isolated from other mammals with respect to the rates of gluconeogenesis from major substrates as well as the metabolic responses to both starvation and diabetes. The mechanism for the control of gluconeogenesis in hepatocytes from diabetic rabbits requires investigation.

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