Biochem. J. (1978) **172**, 91–96
Printed in Great Britain

The Effects of Pyruvate Concentration, Dichloroacetate and α-Cyano-4-hydroxycinnamate on Gluconeogenesis, Ketogenesis and [3-Hydroxybutyrate]/[3-Oxobutyrate] Ratios in Isolated Rat Hepatocytes*

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(*Received* 14 *June* 1977)

1. In isolated rat hepatocytes incubated with pyruvate, ketogenesis increased with increasing pyruvate concentrations and decreased under the influence of 1 mM-α-cyano-4-hydroxycinnamate, a known inhibitor of pyruvate transport. Ketogenesis from pyruvate was higher by 30% in hepatocytes prepared from starved than from fed rats. 2. With pyruvate as substrate, 2 mM-dichloroacetate had no effect on ketogenesis of starved-rat hepatocytes, but increased ketogenesis of fed-rat hepatocytes to the 'starved' value. Gluconeogenesis from pyruvate, lactate and alanine, but not from glycerol, was inhibited by dichloroacetate. Both increased ketogenesis and decreased gluconeogenesis may result from an inhibition of pyruvate carboxylase by dichloroacetate. 3. Mitochondria were rapidly isolated from incubated hepatocytes, and [3-hydroxybutyrate]/[3-oxobutyrate] ratios were measured in the mitochondrial pellet ('mitochondrial' ratios) and in whole-cell suspensions ('total' ratios). Increasing pyruvate concentrations increased mitochondrial and decreased total ratios. In the presence of pyruvate (2 to 10 mM), dichloroacetate decreased mitochondrial and increased total ratios.

Isolated liver mitochondria (Schaefer & Veneziale, 1973) and, as shown in the present study, isolated rat hepatocytes are able to form ketone bodies from unphysiologically high concentrations of pyruvate. Gluconeogenesis and ketogenesis from pyruvate share some common steps, such as pryuvate transport and oxidation, which provides NADH required for the cytosolic reductive step of gluconeogenesis (Zahlten *et al.*, 1973). The influence of known effectors of these steps, namely dichloroacetate and α-cyano-4-hydroxycinnamate, was thus investigated. Pyruvate mitochondrial transport is known to depend on exchange with counter anions (Mowbray, 1975; Paradies & Papa, 1975), such as 3-oxobutyrate via the antiporter described by Papa & Paradies (1974). α-Cyano-4-hydroxycinnamate inhibits pyruvate transport into erythrocytes and isolated mitochondria (Halestrap & Denton, 1974), as well as biosynthesis of fatty acid from glucose or fructose in fat-pads (Halestrap & Denton, 1975). Dichloroacetate interferes with pyruvate mitochondrial transport (Halestrap, 1975; Paradies & Papa, 1976); it also activates pyruvate dehydrogenase (Whitehouse & Randle, 1973; Whitehouse *et al.*, 1974). In intact animals, dichloroacetate increases blood ketone bodies and decreases blood sugar (Blackshear *et al.*, 1974). Thus it may be predicted that, in isolated hepatocytes, α-cyano-4-hydroxycinnamate and dichloroacetate are able to produce similar or opposite effects on the studied metabolic pathways, depending on their predominant site of action. Moreover, pyruvate itself and effectors of its mitochondrial transport may presumably impair [3-hydroxybutyrate]/[3-oxobutyrate] ratios through the action of the pyruvate–3-oxobutyrate antiporter. The whole-cell [3-hydroxybutyrate]/[3-oxobutyrate] ratio is generally assumed to reflect the mitochondrial [NADH]/[NAD^+] ratio (Williamson *et al.*, 1967), but discrepancies are foreseeable if the repartition of metabolites between mitochondrial and cytosolic compartments is no longer uniform. Thus [3-hydroxybutyrate]/[3-oxobutyrate] ratios were simultaneously measured in whole-cell suspensions and mitochondria rapidly prepared from hepatocytes incubated with pyruvate and dichloroacetate.

Abbreviation used: Hepes, 4-(2-hydroxethyl)-1-piperazine-ethanesulphonic acid.

* Part of this work was presented at the 9th FEBS Meeting (Budapest, 1974) and part by F. Demaugre & J. P. Leroux (1976) in *Use of Liver Cells and Kidney Tubules in Metabolic Studies* (Tager, J. M., Söling, M. D. & Williamson, J. R., eds.), pp. 430–433, North-Holland, Amsterdam.

Materials and Methods

Isolation of hepatocytes

Male Sprague–Dawley rats weighing about 200 g were fed with a standard diet or starved for 24 h and then anaesthetized by an intraperitoneal injection of Nembutal (10 mg). Hepatocytes were isolated by the method of Berry & Friend (1969) with some minor

modifications. Calcium-free Krebs–Ringer bicarbonate buffer (Krebs & Henseleit, 1932), continuously gassed with O_2/CO_2 (19:1), was used. The concentrations of collagenase (clostridiopeptidase A; EC 3.4.24.3) and hyaluronidase (hyaluronate 4-glycanohydrolase; EC 3.2.1.35) in the perfusion medium were 0.25 and 0.30mg/ml respectively. Isolated cells were suspended in a well-aerated medium (pH7.4; 304mosM) containing 100mM-Hepes buffer, 70.6mM-NaCl, 4.5mM-KCl, 2.5mM-$CaCl_2$, 1.2mM-KH_2PO_4 and 1.2mM-$MgSO_4$.

The cell suspension (between 3×10^6 and 4×10^6 hepatocytes/ml) was stored in ice before use, for a maximum period of 2h. Glass Labware (Prolabo, Paris, France) was used throughout the isolation procedure.

Incubations

In most experiments, 2 or 4ml of suspended cells were incubated at 37°C in 25 or 50ml Erlenmeyer flasks with a shaking rate of about 60 cycles/min. Before addition of the substrate, preincubation for 10min was performed with or without the tested effector.

In a first set of experiments, whole-suspension metabolites were measured either at the end of the incubation time or at regular time intervals on portions of the cell suspension.

In a second set of experiments, mitochondria were rapidly isolated from hepatocytes at the end of the incubation time, by using the method of Zuurendonk & Tager (1974). Suspended cells (1ml) were poured into 3.5ml of ice-cold 20mM-Hepes, pH7.0, containing 3mM-EDTA, 250mM-saccharose and 24mg of digitonin/100ml. After 10s of contact, the suspension was centrifuged for 5s at 4000*g* in a Martin Christ UJ 1 KS refrigerated centrifuge (3360 Osterode/Harz, Germany). Immediately after centrifugation, the supernatant was discarded and the pellet deproteinized by $HClO_4$. A period of 70s elapsed between the first contact of cells with digitonin and the final deproteinization. 3-Hydroxybutyrate and 3-oxobutyrate were assayed on neutralized extracts of both pellet and simultaneously run incubations of whole cells. [3-Hydroxybutyrate]/[3-oxobutyrate] ratios are referred to as 'mitochondrial' for pellet and 'total' for whole-cell suspensions. In all experiments, the validity of this procedure was assessed by enzyme measurements [90–95% of total lactate dehydrogenase (EC 1.1.1.27), <15% of total adenylate kinase (EC 2.7.4.3) and <5% of total glutamate dehydrogenase (EC 1.4.1.3) were found in the supernatant].

Analytical methods

Glycolytic intermediates and ATP (Cartier *et al.*, 1967) were assayed by enzyme spectrophotometric methods. 3-Hydroxybutyrate and 3-oxobutyrate were measured by spectrophotometric (Williamson *et al.*, 1962) or fluorimetric (Williamson & Corkey, 1969) methods. Lactate dehydrogenase, adenylate kinase and glutamate dehydrogenase activities were measured at 30°C as described by Bergmeyer (1974).

Chemicals

Hepes was from Calbiochem, Lucerne, Switzerland. Dichloroacetic acid was from Merck–Schuchardt, Darmstadt, Germany. α-Cyano-4-hydroxycinnamate was from Aldrich Chemical Co., Milwaukee, WI, U.S.A. Digitonin was from Fluka AG Buchs SG, Switzerland. Enzymes and substrates were from Boehringer (Mannheim) Corp., Paris, France.

Results

Ketogenesis and gluconeogenesis as a function of pyruvate concentration (Figs. 1 and 2)

In hepatocytes prepared from starved or fed rats, ketogenesis increased with pyruvate concentration and reached a maximum at about 3mM-pyruvate. Ketogenesis was always higher in starved-rat hepatocytes. Total ketone-body formation at 5mM-pyruvate was (μmol/min per 10^9 cells; mean values±S.D.): for starved rats, 9.31±0.36 (six determinations); for fed rats, 7.13±0.52 (three determinations), significantly different after Student's *t* test ($P<0.001$). Gluconeogenesis also increased with [pyruvate].

Influence of α-cyano-4-hydroxycinnamate on gluconeogenesis and ketogenesis from pyruvate

Concentrations of 0.3–1mM-α-cyano-4-hydroxycinnamate decreased gluconeogenesis in hepatocytes incubated with 2mM- or 10mM-pyruvate (Table 1). The compound also inhibited ketone-body production, which remained constant irrespective of pyruvate concentration (Fig. 1).

Table 1. *Effect of α-cyano-4-hydroxycinnamate on glucose production with pyruvate as exogenous substrate*

Hepatocytes isolated from starved rats were preincubated for 10min without or with α-cyano-4-hydroxycinnamate, then incubated for 40min after pyruvate addition. Results are expressed as means for three determinations±S.D.

α-Cyano-4-hydroxycinnamate added (mM)	Glucose formed (μmol/min per 10^9 cells)	
	2mM-Pyruvate present	10mM-Pyruvate present
—	3.06±0.25	4.43±0.32
0.3	1.39±0.07	1.41±0.04
0.5	1.08±0.07	1.16±0.05
1	0.99±0.02	1.09±0.03

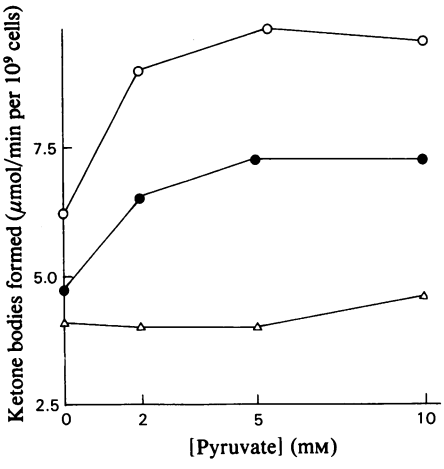

Fig. 1. *Ketone-body formation (3-hydroxybutyrate+3-oxobutyrate) in hepatocytes isolated from fed rats, as a function of pyruvate concentration*

Before pyruvate was added, hepatocytes were preincubated for 10min under standard conditions (●——●), with 2mM-dichloroacetate (○——○) or with 1mM-α-cyano-4-hydroxycinnamate (△——△). Then incubations (20min) were performed after pyruvate addition. Results of a representative experiment are shown. Experiments were repeated with 5mM-pyruvate. Ketone bodies formed (μmol/min per 10^9 cells; mean for three determinations±S.D.): controls, 7.13±0.52; with 2mM-dichloroacetate, 9.17±0.30; with 1mM-α-cyano-4-hydroxycinnamate, 3.89±0.10. Both values were significantly different from controls at $P<0.005$ by Student's t test.

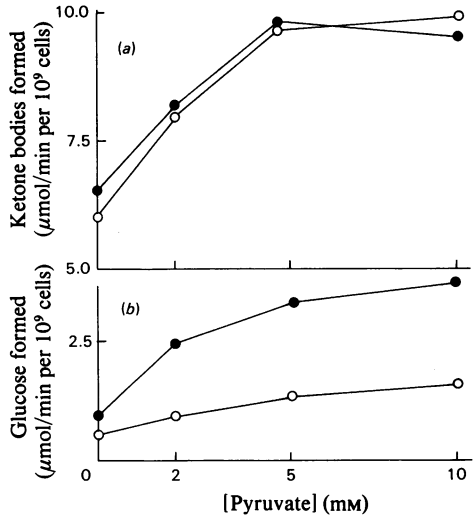

Fig. 2. *Effect of dichloracetate on glucose and total ketone-body production by starved-rat hepatocytes incubated with various concentrations of pyruvate*

Hepatocytes were preincubated for 10min without (●——●) or with (○——○) 2mM-dichloroacetate, then incubated for 20min after pyruvate addition. Results of a representative experiment are shown. Experiments were repeated at a pyruvate concentration of 5mM. Products formed (μmol/min per 10^9 cells; mean for six determinations±S.D.): ketone bodies (*a*), 9.31±0.36 without or with dichloroacetate; glucose (*b*), 3.37±0.27 without and 1.56±0.09 with 2mM-dichloroacetate (significantly different at $P<0.001$ by Student's t test).

Influence of dichloroacetate on gluconeogenesis and ketogenesis from pyruvate

In hepatocytes prepared from fed rats, 2mM-dichloroacetate enhanced ketone-body production at every concentration of pyruvate tested (Fig. 1). Dichloroacetate had almost no effect on ketogenesis of hepatocytes prepared from starved animals (Fig. 2). Thus the same production of ketone bodies was observed in the presence of dichloroacetate, irrespective of the nutrition status.

Dichloroacetate inhibited gluconeogenesis from pyruvate and this inhibition was not overcome by increasing pyruvate concentrations (Figs. 2 and 3). Other gluconeogenic precursors were tested. Dichloroacetate lowered glucose formation from lactate and alanine, but not from glycerol (Table 2).

Influence of pyruvate and dichloroacetate on [3-hydroxybutyrate]/[3-oxobutyrate] ratios (Table 3)

Increasing pyruvate concentrations raised mitochondrial and lowered total [3-hydroxybutyrate]/[3-oxobutyrate] ratios. At a given concentration of

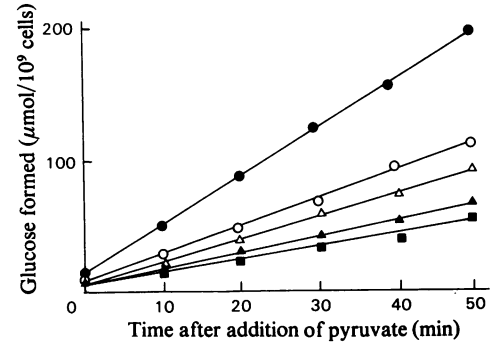

Fig. 3. *Effect of various dichloroacetate concentrations on glucose formation in starved-rat hepatocytes incubated with 10mM-pyruvate*

Before substrate was added, hepatocytes were preincubated for 10min without (●——●) or with 0.5mM-(○——○), 1mM-(△——△), 2mM-(▲——▲) or 3mM-dichloroacetate (■——■). Results of a representative experiment are shown.

Table 2. *Effect of dichloroacetate on glucose production from various substrates*

Hepatocytes prepared from starved rats were preincubated for 10min with or without dichloroacetate, then incubated for 1h after addition of the indicated substrate. Results are expressed as means±S.D. (numbers of determinations in parentheses).

Substrate	Dichloroacetate added (mM)	Glucose produced (μmol/min per 10^9 cells) Total	Corrected from endogenous production (incubation without substrate)
—	—	0.81±0.09 (5)	0
—	2	0.39±0.05 (5)	0
10mM-Pyruvate	—	4.43±0.32 (3)	3.62
10mM-Pyruvate	2	1.72±0.14 (3)	1.33
10mM-Alanine	—	2.92±0.13 (3)	2.11
10mM-Alanine	2	0.57 (2)	0.18
10mM-Lactate*	—	3.46±0.10 (3)	2.65
10mM-Lactate*	2	0.51±0.06 (3)	0.12
10mM-Glycerol	—	2.40±0.15 (3)	1.59
10mM-Glycerol	2	2.04±0.11 (3)	1.65

* Owing to a 20min lag period in gluconeogenesis from lactate, result refers to the last 30min of incubation for 1h, when glucose production is linear with time.

Table 3. *Effect of dichloroacetate and pyruvate concentration on [3-hydroxybutyrate]/[3-oxobutyrate] ratios in hepatocytes prepared from fasted rats*

Before pyruvate was added, hepatocytes were preincubated for 10min with or without dichloroacetate. Incubations (20min) were performed after pyruvate addition. Total and mitochondrial ratios were then measured as described in the Materials and Methods section. Results are the means for three experiments±S.D. *P* values (Student's paired *t* test) are shown in parentheses and express the significance of difference between the two adjacent values. * Difference between values significant at $P=0.05$. ** Difference between values significant at $P=0.05$. N.S., Not significant.

Substrate	Total [3-hydroxybutyrate]/[3-oxobutyrate] ratios: Without dichloroacetate	Total: With 2mM-dichloroacetate	Mitochondrial [3-hydroxybutyrate]/[3-oxobutyrate] ratios: Without dichloroacetate	Mitochondrial: With 2mM-dichloroacetate
1mM-Pyruvate	0.337±0.038* (<0.02)	0.390±0.026	1.06±0.10** N.S.	0.98±0.09
5mM-Pyruvate	0.310±0.035 (<0.02)	0.397±0.040	1.36±0.12 (0.05)	1.01±0.18
10mM-Pyruvate	0.290±0.010* (<0.01)	0.357±0.015	1.40±0.15** (0.05)	1.03±0.18

pyruvate, dichloroacetate decreased mitochondrial and increased total [3-hydroxybutyrate]/[3-oxobutyrate] ratios.

Discussion

Evaluation of experimental methods

Viability of hepatocytes was immediately assessed by optical microscopic examination and the Trypan Blue test. However, the best viability test appeared to be ATP content of cells. In all submitted experiments, ATP content of hepatocytes was about 19μmol/10^9 cells (fed animals) or 11μmol/10^9 cells (starved animals). Furthermore, ATP content of cells slightly increased during incubations. In hepatocytes prepared from starved rats, initial ATP content was 11.0±1.3μmol/10^9 cells; after 10min of preincubation followed by incubation for 20min with 5mM-pyruvate, final ATP content was 12.4±1.1μmol/10^9 cells (mean for five determinations±S.D.). Under the same conditions, ATP content of fed-rat hepatocytes increased from 18.9±1.3 to 21.0±1.3μmol/10^9 cells during the incubation (mean for seven determinations±S.D.). During similar incubations, lactate dehydrogenase activity of the supernatants increased from 7.7±2.2 to 10.7±3.3% of total activity (mean for four determinations±S.D.). Glucose synthesis from pyruvate per time unit remained constant up to 50–60min (Fig. 3) and, as ATP content, within published limits (Berry & Kun, 1972; Zahlten *et al.*, 1973; Krebs *et al.*, 1974). As an example, individual values of glucose synthesis reported by Zahlten *et al.* (1973) and Lardy *et al.* (1974) vary between 3.6 and 5.0μmol/min per 10^9 cells at 5mM-pyruvate and are slightly higher than our own results (3.37±0.27) because the authors added gelatin (Elliott, 1976) and incubated hepatocytes with Krebs–bicarbonate

medium. We observed that incubations in Krebs–bicarbonate buffer increased glucose synthesis up to 5.8–6.1 μmol/min per 10^9 cells (two experiments), but had no qualitative effect on the reported actions of dichloroacetate.

The validity of rapid mitochondrial separation was assessed by enzyme measurements, but a limited exchange of metabolites as well as trapping of supernatant metabolites in the pellet cannot be completely ruled out. However, such artifacts would tend to even 'supernatant' and 'pellet' metabolite ratios and their variations. Measured ratios are definitely different, and exhibit opposite variations under the influence of increasing pyruvate concentrations or dichloroacetate (Table 3). Thus, actual variations must be higher than the observed ones.

Ketogenesis from pyruvate

Ketone-body formation increased with pyruvate concentration and decreased under the action of α-cyano-4-hydroxycinnamate, an inhibitor of pyruvate transport (Figs. 1 and 2). Thus, under the described experimental conditions, pyruvate behaves like a ketone-body precursor. Quantitative evaluation of this process is difficult; however, if we assume a complete inhibition of pyruvate transport by 1 mM-α-cyano-4-hydroxycinnamate as is suggested by inhibition of gluconeogenesis from pyruvate (Table 1), the minimum ketogenesis from 5 mM-pyruvate may be calculated to be approx. 3.2 μmol/min per 10^9 cells in fed-rat hepatocytes (Fig. 1).

The observed ketogenesis is not an artifact caused by the absence of added bicarbonate from the incubation media. Ketogenesis was only slightly decreased (15%), and gluconeogenesis slightly increased (20%), when 5 mM-bicarbonate was added. Endogenous production presumably provided suboptimal quantities of bicarbonate to pyruvate carboxylase. The inhibitory action of α-cyano-4-hydroxycinnamate on gluconeogenesis and ketogenesis does not involve general energy inhibition, as shown by its lack of effect on ATP content. In hepatocytes prepared from fed rats, ATP concentrations measured after incubation for 20 min with 5 mM-pyruvate was 20.5 ± 0.7 without and 20.0 ± 0.4 μmol/10^9 cells with 1 mM-α-cyano-4-hydroxycinnamate (mean for three determinations ± S.D.).

The unphysiological process of ketogenesis from pyruvate certainly results from the high concentration of pyruvate used, which produces a maximum activation of pyruvate dehydrogenase without any concomitant increase of pyruvate carboxylase activity. Patzelt *et al.* (1973) showed that, in perfused liver, the active portion of total pyruvate dehydrogenase depended on pyruvate concentration. Half-maximal increase of the active form was obtained at about 1 mM-pyruvate. Modification of ketogenesis as a function of pyruvate concentration gives grossly similar curves (Fig. 1). It has already been shown (Garland *et al.*, 1968) that ketogenesis proceeds in a well-coupled system, i.e., a moderately active tricarboxylic acid cycle, and when oxaloacetate supply is limiting (Lopez Cardozo & Van den Berg, 1974). The higher ketogenesis from pyruvate in hepatocytes prepared from starved animals (Fig. 2) may then derive from a lower mitochondrial oxaloacetate concentration (Greenbaum *et al.*, 1971), owing to oxaloacetate overconsumption in the gluconeogenic pathway.

The effects of dichloroacetate on ketogenesis may also be related to variations of mitochondrial oxaloacetate concentration. In fed-rat hepatocytes, oxaloacetate concentration is high, and its lowering to the 'starved' value, possibly by pyruvate carboxylase inhibition, is able to activate ketogenesis; in fasted-rat hepatocytes, ketogenesis already proceeds at a high rate, and a further lowering of oxaloacetate concentration by dichloroacetate has no effect.

Effects of dichloroacetate on gluconeogenesis

Inhibition of gluconeogenesis by dichloroacetate may similarly be a consequence of pyruvate carboxylase inhibition, especially as the inhibition is observed with the substrates pyruvate (Figs. 2 and 3), lactate and alanine, but not glycerol (Table 2). Stacpoole & Berry (1971) and Stacpoole (1977), but not Crabb *et al.* (1976), found similar results.

As also reported by Stacpoole (1977), dichloroacetate did not significantly modify ATP content of starved-rat hepatocytes, which was 12.3 ± 1.2 without and 12.5 ± 1.3 μmol/10^9 cells with 2 mM-dichloroacetate after incubation for 20 min in the presence of 5 mM-pyruvate (mean for three determinations ± S.D.). Thus inhibition of gluconeogenesis is not a consequence of general energy inhibition.

[3-*Hydroxybutyrate*]/[3-*oxobutyrate*] *ratios*

The unexpected opposite variations of mitochondrial and total [3-hydroxybutyrate]/[3-oxobutyrate] ratios with increasing pyruvate concentrations and dichloroacetate (Table 3) may be the result of 3-oxobutyrate exchange with pyruvate (Papa & Paradies, 1974) and the antagonistic effects of dichloroacetate on the monocarboxylate carrier (Halestrap, 1975; Paradies & Papa, 1976).

Thus discrepancies are possible between mitochondrial and total [3-hydroxybutyrate]/[3-oxobutyrate] ratios, owing to an uneven distribution of metabolites in the cell compartments. Under the described conditions, the usually measured total ratio does not reliably reflect the mitochondrial redox state.

The technical assistance of Mrs. Arlette Moncion and Mrs. Claude Cepanec is gratefully acknowledged.

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