

Modifications of Citric Acid Cycle Activity and Gluconeogenesis in Streptozotocin-Induced Diabetes and Effects of Metformin

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To better define the modifications of liver gluconeogenesis and citric acid cycle, or Krebs' cycle, activity induced by insulin deficiency and the effects of metformin on these abnormalities, we infused livers isolated from postabsorptive or starved normal and streptozotocin-induced diabetic rats with pyruvate and lactate (labeled with [$3\text{-}^{13}\text{C}$]lactate) with or without the simultaneous infusion of metformin. Lactate and pyruvate uptake and glucose production were calculated. The ^{13}C -labeling pattern of liver glutamate was used to calculate, according to Magnusson's model, the relative fluxes through Krebs' cycle and gluconeogenesis. These relative fluxes were converted into absolute values using substrate balances. In normal rats, starvation increased gluconeogenesis, the flux through pyruvate carboxylase-phosphoenolpyruvate carboxykinase (PC-PEPCK), and the ratio of PC to pyruvate dehydrogenase (PDH) flux ($P < 0.05$); metformin induced only a moderate decrease in the PC:PDH ratio. Livers from postabsorptive diabetic rats had increased lactate and pyruvate uptakes ($P < 0.05$); their metabolic fluxes resembled those of starved control livers, with increased gluconeogenesis and flux through PC-PEPCK. Starvation induced no further modifications in the diabetic group. Metformin decreased glucose output from the liver of starved diabetic rats ($P < 0.05$). The flux through PC-PEPCK and also pyruvate kinase were decreased ($P < 0.05$) by metformin in both groups of diabetic rats. In conclusion, insulin deficiency increased in this model of diabetes gluconeogenesis through enhanced uptake of substrate and increased flux through PC-PEPCK; metformin decreased glucose production by reducing the flux through PC-PEPCK. *Diabetes* 48:1251–1257, 1999

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ANOVA, analysis of variance; CAC, citric acid cycle; CS, citrate synthesis; *F*, dilution factor; FAO, fatty acid oxidation; GCMS, gas chromatography-mass spectrometry; IE, isotopic enrichment; MPE, mole percent excess; PC, pyruvate carboxylase; PDH, pyruvate dehydrogenase; PEP, phosphoenolpyruvate; PK, pyruvate kinase; STZ, streptozotocin.

The antihyperglycemic agent metformin (dimethylbiguanide) has been used for over 25 years in the treatment of type 2 diabetes (1,2). It does not act through stimulation of insulin secretion (3). Increases in the sensitivity to insulin of glucose utilization have sometimes (4–7) but not always (8,9) been reported. The main action of metformin is considered to be a reduction of endogenous glucose production through suppression of gluconeogenesis (9–14), both independently and in interaction with insulin. This decrease in gluconeogenesis has been found in vivo (9,13) and in vitro, in both kidney (10) and liver (11,12,14). The exact mechanism is still unclear, and various explanations, such as modifications of lactate uptake (14) or pyruvate kinase (PK) activity (12), have been reported.

We examined in the present study the effects of metformin on the gluconeogenic flux from lactate and pyruvate in perfused livers isolated from postabsorptive and starved normal and streptozotocin (STZ)-injected diabetic rats. This allowed us to study metformin action in the absence of any modification of hormonal environment and in the presence of a controlled delivery of gluconeogenic substrates. The addition of [$3\text{-}^{13}\text{C}$]lactate in the infusate and the determination of the enrichment of the glucose released and of the labeling pattern of liver glutamate gave us information (14–16) on the alterations of intrahepatic metabolic fluxes induced by diabetes and on the possible sites of action of metformin.

RESEARCH DESIGN AND METHODS

Materials. Chemicals were from Sigma (St. Louis, MO) and enzymes were from Boehringer Mannheim (Mannheim, Germany). L- [$3\text{-}^{13}\text{C}$]lactate, [$3\text{-}^{13}\text{C}$]pyruvate, [$1\text{-}^{13}\text{C}$]glucose, [$1\text{-}^{13}\text{C}$]glutamate, [$5\text{-}^{13}\text{C}$]glutamate, and [$1,2\text{-}^{13}\text{C}_2$]glutamate were from Eurisotop (Saint-Aubin, France). STZ was from Sigma.

Experiments. Male Sprague-Dawley rats (IFFA-Credo, L'Arbresle, France), housed under controlled temperature (22°C) and lighting (lights on: 0800–2000), were divided into control and diabetic groups. Diabetes was induced by intraperitoneal injection of STZ (70 mg/kg freshly dissolved in 150 mmol/l citrate buffer, pH 4.5); only rats with glycemia > 15 mmol/l were selected. Normal rats were studied in the postabsorptive state ($n = 6$) (6 h after food removal, i.e., food was removed at 0800 and experiments started at 1400) and after 48 h of starvation ($n = 5$). Diabetic rats were studied in the postabsorptive state ($n = 5$) or after 24 h of starvation ($n = 5$). Livers were perfused (open circuit) (18) with Krebs-Ringer bicarbonate buffer (pH 7.40, equilibrated with 95% O_2 + 5% CO_2) alone during 60 min. This allowed us to obtain stable baseline values for glucose output in all groups. Then, pyruvate (0.2 mmol/l) and lactate (1.5 mmol/l, 9–14% mole percent excess [MPE]-enriched with [$3\text{-}^{13}\text{C}$]lactate) were added to the infusate (from 60 to 120 min) without metformin (60–80 min), without (control experiments) or with (metformin experiments) a simultaneous infusion of metformin (final concentration: 0.70 mmol/l) (80–120 min). Samples of infusates and effluents from livers were collected for the various determinations throughout the experiments (end of basal state, 70–80 min, and 110–120 min). At the end of the experiments, the livers were quick-frozen in liquid nitrogen and stored until analysis.

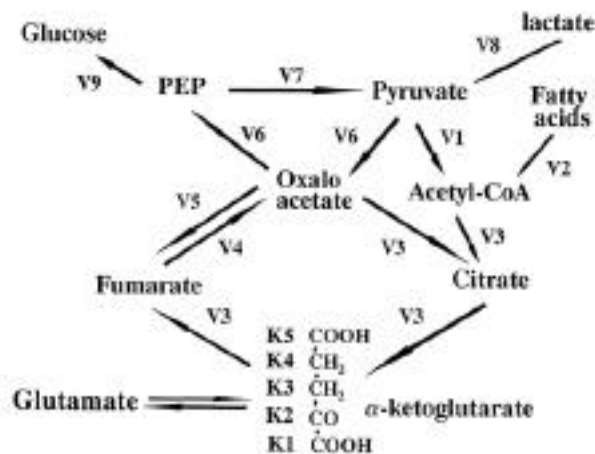


FIG. 1. Model of Krebs' cycle and gluconeogenesis. Rates are designated V1, V2, etc., in keeping with previous usage (15).

Analytical procedures. Glucose, lactate, and pyruvate concentrations in the infusates and effluents were assayed with enzymatic methods (19). The hepatic concentrations of lactate, pyruvate, ATP, phosphoenolpyruvate (PEP), and glycogen (19) were measured on neutralized perchloric acid extracts of liver. The total isotopic enrichment (IE) of glucose produced by livers was determined by gas chromatography-mass spectrometry (GCMS) using the aldonitrile pentaacetate (20) derivative after purification by ion-exchange chromatography. Lactate and pyruvate IE were measured using the *t*-butyldimethylsilyl derivative of lactate and the quinoxalinol *t*-butyldimethylsilyl derivative of pyruvate (17). Glutamate was purified from neutralized perchloric acid extract of livers by anion-exchange chromatography (16), and its ¹³C-labeling pattern was determined by GCMS as previously described (21). Standard curves prepared by mixing weighed amounts of natural and labeled metabolites (i.e., [1-¹³C]glucose, [3-¹³C]lactate, [3-¹³C]pyruvate, [1-¹³C]glutamate, [5-¹³C]glutamate, and [1,2-¹³C₂]glutamate) were run before and after the corresponding biological samples. Samples and standard were run in triplicate. All GCMS procedures have been described in detail previously (16,17,20,21).

Calculations. Net glucose output and lactate and pyruvate uptakes were calculated from the concentrations of these metabolites in the buffer infused and in the effluents from livers and from liver infusion rates (20–26 ml/min). In experiments with starved rats, the percentages of lactate and pyruvate taken up and used for gluconeogenesis were calculated from the net uptakes of lactate and pyruvate and the increases above basal values of glucose outputs. In each experiment, Krebs' cycle parameters as well as the correction factor *F* for dilution of the labeling at the oxaloacetate crossroad during gluconeogenesis were calculated from the absolute labeling pattern of liver glutamate using the equations of Magnusson et al. (15). The model developed by these authors is shown in Fig. 1. The main assumptions and possible limitations of this model for *in vitro* studies have been discussed previously (15–17). These equations yield rates relative to citrate synthesis (CS) (or Krebs' cycle activity). They were solved by computer using an Excel spreadsheet program (release 4.0). These relative fluxes were converted into

absolute fluxes using, for experiments in starved rats, either twice the total glucose output at the end of the protocol (110–120 min) or twice the increase above the basal value of this total glucose output as an absolute measure of rate V9 (PEP to glucose). These calculations give, respectively, the upper and lower limits for absolute fluxes. For experiments in postabsorptive rats, V9 was estimated from twice the total glucose output at the end of the protocols and the ratio of effluent glucose to liver pyruvate enrichment corrected by *F*.

All results are shown as averages and SEs. For liver metabolite concentrations, IEs, PEP:pyruvate enrichment ratios calculated according to Magnusson's model, and substrate balances, within- and between-group comparisons were performed using Student's *t* test for paired or nonpaired values as appropriate, except for the intragroup variations in glucose output, which were analyzed by two-way analysis of variance (ANOVA). For metabolic fluxes (relative and absolute), comparisons were performed by one-way ANOVA.

RESULTS

Liver metabolite concentrations. The addition of metformin to the infusate had no effect on the concentration of lactate, pyruvate, PEP, or glycogen (Table 1). The PEP:pyruvate and lactate:pyruvate concentration ratios were not modified despite a trend toward higher values of the former ratio in both diabetic groups and of the latter ratio in the starved diabetic group in the presence of metformin. ATP concentrations were not modified by metformin (postabsorptive control rats: 1.94 ± 0.15 vs. 2.10 ± 0.20; starved control rats: 1.40 ± 0.21 vs. 1.36 ± 0.05; postabsorptive diabetic rats: 2.10 ± 0.21 vs. 1.72 ± 0.16; and starved diabetic rats: 2.10 ± 0.25 vs. 2.32 ± 0.19 mol/g wet liver). As expected, glycogen was undetectable in livers from starved control rats. Its concentrations were low in the postabsorptive diabetic group compared with the normal postabsorptive group (*P* < 0.001) but were not further decreased in the starved diabetic group. Glycogen levels were higher in this last group than in the starved control rats (*P* < 0.01).

Substrate balances. Postabsorptive normal and diabetic rats released large amounts of glucose in the basal state; glucose output decreased then progressively despite the addition of lactate and pyruvate (Table 2). The net uptake of these gluconeogenic substrates increased slightly between the 70- to 80- and the 110- to 120-min periods. It was lower in normal than in postabsorptive diabetic rats (*P* < 0.05). These evolutions were not modified by the addition of metformin. Livers from starved control rats released small amounts of glucose in the basal state. This release increased, as expected, when lactate and pyruvate were added to the infusates (70–80 min); the addition of metformin (110–120 min) had no effect compared with either the 70- to 80-min period of the experi-

TABLE 1
Concentrations of liver metabolites

| | Lactate (μmol/g) | Pyruvate (nmol/g) | PEP (nmol/g) | Glycogen (mg/g) | PEP/pyruvate | Lactate/pyruvate |
|-----------------------------|------------------|-------------------|--------------|------------------------|--------------|------------------|
| Control rats | | | | | | |
| Postabsorptive ⁻ | 1.44 ± 0.15 | 143 ± 14 | 90 ± 10 | 23 ± 1 | 0.66 ± 0.05 | 10.6 ± 0.3 |
| Postabsorptive ⁺ | 1.12 ± 0.08 | 160 ± 10 | 100 ± 7 | 29 ± 2 | 0.63 ± 0.06 | 7.0 ± 0.6 |
| Starved ⁻ | 0.95 ± 0.05 | 180 ± 10 | 157 ± 17 | <0.1 | 0.88 ± 0.11 | 5.1 ± 0.2 |
| Starved ⁺ | 0.97 ± 0.03 | 149 ± 13 | 105 ± 10 | <0.1 | 0.71 ± 0.08 | 6.7 ± 0.6 |
| Diabetic rats | | | | | | |
| Postabsorptive ⁻ | 1.00 ± 0.07 | 95 ± 11 | 99 ± 18 | 4.1 ± 1.0 [†] | 1.06 ± 0.15 | 11.1 ± 1.1 |
| Postabsorptive ⁺ | 1.11 ± 0.11 | 99 ± 12 | 119 ± 8 | 3.0 ± 0.9 [†] | 1.35 ± 0.26 | 11.6 ± 1.1 |
| Starved ⁻ | 0.74 ± 0.08 | 106 ± 13 | 139 ± 14 | 2.4 ± 0.1 [*] | 1.34 ± 0.22 | 7.2 ± 0.6 |
| Starved ⁺ | 0.87 ± 0.04 | 83 ± 10 | 152 ± 10 | 2.5 ± 0.5 [*] | 1.84 ± 0.30 | 11.3 ± 0.9 |

Data are averages ± SE. **P* < 0.01, [†]*P* < 0.001 vs. the corresponding control group. -, livers infused without metformin; +, livers infused with metformin.

TABLE 2
Substrate balance across the perfused livers

| | Glucose output (nmol · min ⁻¹ · g ⁻¹) | | | L+P load (nmol · min ⁻¹ · g ⁻¹ wet liver) | L+P uptake (nmol · min ⁻¹ · g ⁻¹) | | % of L+P taken up and used as glucose | |
|-----------------------------|---|----------------|------------------|--|---|---------------------------|--|------------------|
| | Basal | 70–80 (min) | 110–120 (min) | | 70–80 (min) | 110–120 (min) | 70–80 (min) | 110–120 (min) |
| Control rats | | | | | | | | |
| Postabsorptive ⁻ | 1,275 ± 159 | 1,096 ± 107 | 979 ± 88 | 3,650 ± 95 | -66 ± 101 | -8 ± 125 | | |
| Postabsorptive ⁺ | 1,452 ± 424 | 1,214 ± 213 | 1,284 ± 363 | 4,334 ± 344 | 181 ± 59 | 348 ± 115 | | |
| Starved ⁻ | 106 ± 8 | 336 ± 21 | 368 ± 24 | 5,037 ± 494 | 908 ± 45 (18 ± 3%) | 972 ± 119 (20 ± 2%) | 50 ± 3 | 53 ± 4 |
| Starved ⁺ | 116 ± 11 | 435 ± 52 | 429 ± 86 | 5,240 ± 321 | 970 ± 62 (19 ± 1%) | 978 ± 153 (19 ± 2%) | 64 ± 5 | 63 ± 5 |
| Diabetic rats | | | | | | | | |
| Postabsorptive ⁻ | 1,506 ± 126 | 1,437 ± 210 | 1,249 ± 199 | 4,677 ± 73 | 592 ± 28† (13 ± 1%) | 754 ± 153† (16 ± 1%) | | |
| Postabsorptive ⁺ | 1,040 ± 224 | 1,048 ± 195 | 905 ± 207 | 6,125 ± 480 | 697 ± 107† (10 ± 1%) | 1,147 ± 219† (17 ± 2%) | | |
| Starved ⁻ | 413 ± 38† | 684 ± 76† | 704 ± 49† | 5,361 ± 332 | 1,418 ± 75† (27 ± 2%) | 1,373 ± 71† (26 ± 2%) | 39 ± 2 | 43 ± 4 |
| Starved ⁺ | 367 ± 123† | 637 ± 140† | 550 ± 97* | 5,937 ± 494 | 1,308 ± 51† (22 ± 1%) | 1,302 ± 124† (22 ± 3%) | 41 ± 4 | 31 ± 7*† |

Data are means ± SE. Glucose outputs were calculated in the basal state after the addition of lactate (L) and pyruvate (P) before (70–80 min) and after the addition (+) or not (-) of metformin (110–120 min). In experiments with starved rats, the percentages of lactate and pyruvate taken up and used for gluconeogenesis were calculated from the net uptake of lactate and pyruvate and the increase above basal values of glucose output. **P* < 0.05 vs. the corresponding value before metformin infusion. †*P* < 0.05 vs. the corresponding control group.

ments with metformin or the 110- to 120-min period of the control tests. Metformin also had no effect in starved control rats on either the uptake of lactate and pyruvate or the percentage of lactate and pyruvate taken up and converted into glucose. The 24-h starved diabetic rats had a greater (*P* < 0.05) glucose output in the basal state than did the starved control rats; the increases above initial values after the addition of lactate and pyruvate were comparable. The absolute net uptake of lactate and pyruvate was higher in starved diabetic rats than in starved normal rats (*P* < 0.05), but since the load of lactate and pyruvate was slightly higher, the percentage of lactate and pyruvate taken up were not different. Metformin addition decreased glucose output in the starved diabetic group during the 70- to 80-min period (*P* < 0.05), whereas no modifications were observed in the group without metformin. This decrease in glucose production occurred despite an unchanged uptake of gluconeogenic precursors. Therefore, the percentage of lactate and pyruvate taken up and used for gluconeogenesis decreased after the addition of metformin (*P* < 0.05) (Table 2). In addition, in the group with metformin, there was an increase in the release of alanine by livers (284 ± 34 vs. 166 ± 17 nmol · min⁻¹ · g⁻¹ of liver, *P* < 0.05), which accounted for most of the decrease in the use for gluconeogenesis of the lactate and pyruvate that was taken up. There were no modifications of alanine release with metformin in the other groups of perfused livers (postabsorptive diabetic rats: 94 ± 8 vs. 105 ± 12; postabsorptive control rats: 89 ± 13 vs. 72 ± 8; and starved control rats: 220 ± 18 vs. 192 ± 21 nmol · min⁻¹ · g⁻¹ of liver in the presence and absence of metformin, respectively).

Glutamate labeling and Krebs' cycle parameters. The ¹³C-labeling patterns of liver glutamate in the experiments without and with metformin are shown in Fig. 2. The corresponding rates calculated according to Magnusson et al. (15)

and expressed relative to V₃ (CS) are in Table 3. In normal rats, starvation increased the fluxes related to gluconeogenesis (V₈ [lactate and pyruvate utilization], V₉, and V₆ [PC-PEPCK]) and decreased the one through PDH, increasing the PC:PDH flux ratio. There were no modifications induced by metformin in normal rats, except for a small increase in the flux through PDH, resulting in decreased PC:PDH activity ratios (*P* < 0.05 for both groups). The differences between the starved and postabsorptive groups persisted. Livers from postabsorptive diabetic rats had increased gluconeogenic fluxes (V₈, V₉, and V₆) compared with the corresponding control group, but they

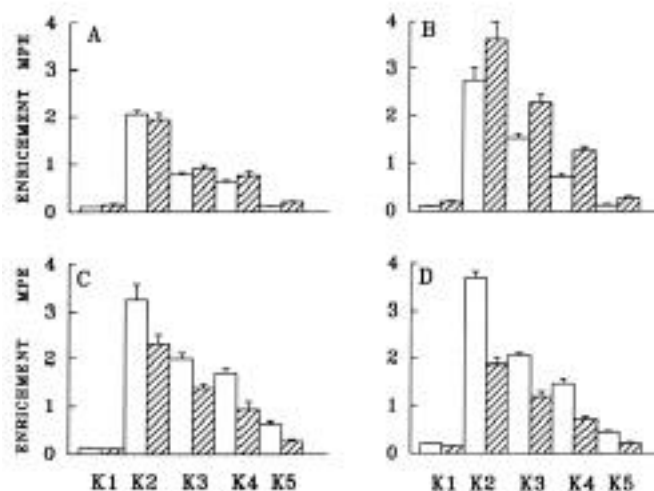


FIG. 2. Labeling patterns of liver glutamate. K1, K2, etc., refer to the five carbon atoms of α -keto-glutarate in keeping with the designation by Magnusson et al. (15). A: Postabsorptive control rats; B: starved control rats; C: postabsorptive diabetic rats; D: starved diabetic rats. □, experiments without metformin; ▨, experiments with metformin.

TABLE 3
Metabolic fluxes expressed relative to CS set to 10

| | Diabetic rats | | | | Control rats | | | |
|---------------------|----------------|-------------|--------------|-----------|----------------|-----------|--------------|-------------|
| | Postabsorptive | | 24-h starved | | Postabsorptive | | 48-h starved | |
| | - | + | - | + | - | + | - | + |
| V8: L+P utilization | 52 ± 5 | 46 ± 5 | 33 ± 3 | 31 ± 2 | 30 ± 6 | 23 ± 1 | 82 ± 12‡ | 59 ± 6§ |
| V1: PDH | 4.5 ± 0.3 | 3.0 ± 0.4 * | 3.3 ± 0.3‡ | 2.9 ± 0.1 | 2.6 ± 0.1 | 3.2 ± 0.1 | 1.9 ± 0.1§ | 2.6 ± 0.2*§ |
| V6: PC | 203 ± 30 | 117 ± 13* | 111 ± 7‡ | 80 ± 6†‡ | 104 ± 11 | 80 ± 5 | 140 ± 9‡ | 116 ± 14‡ |
| V2: FAO | 5.5 ± 0.2 | 7.0 ± 0.4* | 6.7 ± 0.2 | 7.1 ± 0.1 | 7.4 ± 0.1 | 6.8 ± 0.1 | 6.9 ± 0.1 | 7.4 ± 0.2 |
| V3: CS | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |
| V5: OAA to F | 203 ± 51 | 181 ± 17 | 85 ± 9§ | 110 ± 12§ | 28 ± 8 | 41 ± 3 | 231 ± 36§ | 253 ± 52§ |
| V4: F to OAA | 213 ± 51 | 191 ± 17 | 95 ± 9§ | 120 ± 12§ | 38 ± 8 | 51 ± 3 | 241 ± 36§ | 263 ± 52§ |
| V6: PEPCK | 203 ± 30 | 117 ± 13* | 111 ± 7‡ | 80 ± 6†‡ | 104 ± 11 | 80 ± 5 | 140 ± 9‡ | 116 ± 14‡ |
| V9: PEP to glucose | 48 ± 5 | 43 ± 5 | 30 ± 3§ | 28 ± 2‡ | 27 ± 6 | 20 ± 1 | 78 ± 12‡ | 57 ± 6§ |
| V7: PK | 159 ± 37 | 75 ± 8* | 81 ± 8 | 52 ± 5* | 77 ± 16 | 61 ± 5 | 61 ± 4 | 59 ± 12 |
| PC: PDH | 48±11 | 40 ± 4 | 35±5 | 28 ± 3‡ | 40 ± 5 | 25 ± 2* | 73 ± 5§ | 45 ± 6*‡ |

Data are means ± SE. * $P < 0.05$, † $P < 0.01$ vs. the corresponding value without metformin. ‡ $P < 0.05$, § $P < 0.01$ vs. the corresponding postabsorptive group. || $P < 0.05$ vs. the corresponding control group. L, lactate; OAA, oxaloacetate; P, pyruvate; +, with metformin; -, without metformin.

also had increased fluxes through PK and PDH. Starvation in diabetic rats decreased the flux through PDH and also through the gluconeogenic pathway. The PC:PDH ratio was not modified. In both groups of diabetic rats, metformin decreased the flux through PC-PEPCK and PK. In addition, there were moderate decreases in the flux through PDH and an increase in fatty acid oxidation (FAO) in the postabsorptive group.

The calculated intrahepatic dilution factors (F) were close to one in all groups (Table 4). They were slightly but significantly lower in normal postabsorptive rats ($P < 0.01$) compared with other groups. They were unchanged by metformin. Table 5 shows the IEs of lactate and pyruvate in the infusates and livers and the enrichment of glucose released by liver. As expected, glucose enrichment was higher in experiments with livers from starved rather than postabsorptive rats. There were no differences between the diabetic rats and the corresponding control groups. Metformin infusion induced no significant modifications.

Table 6 shows the absolute fluxes as estimated from the relative fluxes of Table 3 and either from the glucose balances, as increases of glucose output above basal values (starved rats), or from total glucose output and the ratio of glucose in effluent to liver pyruvate enrichment corrected by F (postabsorptive rats). Table 7 shows these fluxes calculated in starved rats using the total glucose output at 110–120 min instead of the rise above basal values. Whichever way calculations were performed, starvation decreased CS, FAO, and PDH and PK flux in control rats; PC-PEPCK flux was also decreased, but only when this flux was calculated from the rise above basal values of glucose output (Table 6). V8 and V9 were moderately increased by starvation when fluxes were calculated using total glucose output (Table 7). Postabsorptive diabetic rats had an increased PC-PEPCK flux compared with postabsorptive control rats; V7 (PK), V8, and V9 were also increased. Contrary to control rats, in starved diabetic rats, starvation induced modifications of fluxes only when calculated with total glucose output (Table 7). All these fluxes in starved diabetic rats were higher than those in starved control rats, since total glucose output (Table 2), and therefore V9, was much higher (Table 7). With the exception of V8,

these fluxes remained higher than those in starved control rats when calculated as in Table 6.

In normal rats, the only modification observed with metformin was again an increase in PDH flux. Absolute fluxes through PC-PEPCK and PK were largely decreased (roughly a 50% decrease, $P < 0.05$) in both diabetic groups. In addition, the starved diabetic group infused with metformin had, of course, a lower value for rate V9, since this rate is twice the increase in glucose output above the basal value or the total glucose output, which was decreased by metformin (Table 2). V8 was also slightly decreased by metformin in this group. Lastly, with metformin in starved diabetic rats, there was a trend toward a decrease in rates V1 (PDH), V2 (FAO), and V3 (CS), but these decreases did not reach significance.

DISCUSSION

In the present study we used perfused livers isolated from control and insulinopenic diabetic rats to test 1) the effect of insulin deficiency on gluconeogenesis from lactate and pyruvate and on the main parameters of Krebs' cycle activity and

TABLE 4
Hepatic PEP:pyruvate IE ratios calculated according to Magnusson's equations in control and diabetic rats

| | PEP:pyruvate ratio |
|-----------------------------|--------------------|
| Control rats | |
| Postabsorptive ⁻ | 0.90 ± 0.02 |
| Postabsorptive ⁺ | 0.92 ± 0.01 |
| 48-h starved ⁻ | 0.97 ± 0.01* |
| 48-h starved ⁺ | 0.97 ± 0.01* |
| Diabetic rats | |
| Postabsorptive ⁻ | 0.98 ± 0.02† |
| Postabsorptive ⁺ | 0.96 ± 0.01† |
| 24-h starved ⁻ | 0.95 ± 0.04 |
| 24-h starved ⁺ | 0.94 ± 0.01 |

Data are means ± SE. +, with metformin; -, without metformin. * $P < 0.01$ vs. postabsorptive control rats; † $P < 0.01$ vs. corresponding control rats.

TABLE 5
IEs (MPE) of the lactate infused, of liver lactate and pyruvate, and of glucose and alanine in the effluent

| | Lactate infused | Liver lactate | Liver pyruvate | Glucose effluent | Alanine effluent |
|-----------------------------|-----------------|---------------|----------------|------------------|------------------|
| Control rats | | | | | |
| Postabsorptive ⁻ | 10.21 ± 0.15 | 7.10 ± 0.25 | 4.70 ± 0.22 | 1.92 ± 0.32 | 4.45 ± 0.25 |
| Postabsorptive ⁺ | 9.89 ± 0.21 | 6.81 ± 0.31 | 4.88 ± 0.26 | 2.51 ± 0.48 | 4.71 ± 0.18 |
| 48-h starved ⁻ | 10.70 ± 0.20 | 7.40 ± 0.20 | 5.10 ± 0.29 | 9.90 ± 0.20* | 5.14 ± 0.14 |
| 48-h starved ⁺ | 12.28 ± 0.66 | 7.95 ± 0.25 | 5.15 ± 0.32 | 10.77 ± 0.66* | 4.98 ± 0.19 |
| Diabetic rats | | | | | |
| Postabsorptive ⁻ | 14.06 ± 0.28 | 8.61 ± 0.30 | 5.80 ± 0.14 | 3.71 ± 0.53 | 5.71 ± 0.18 |
| Postabsorptive ⁺ | 9.60 ± 0.91 | 6.51 ± 0.27 | 3.92 ± 0.18 | 2.69 ± 0.26 | 4.01 ± 0.20 |
| 24-h starved ⁻ | 11.88 ± 0.10 | 8.53 ± 0.15 | 5.19 ± 0.17 | 10.47 ± 0.24* | 4.94 ± 0.12 |
| 24-h starved ⁺ | 9.10 ± 0.16 | 7.51 ± 0.31 | 4.59 ± 0.21 | 9.53 ± 0.60* | 4.44 ± 0.19 |

Data are means ± SE. +, with metformin; -, without metformin. **P* < 0.01 vs. the corresponding postabsorptive group.

2) the action of metformin on these parameters. This was achieved by calculating substrate balances through the open circuit liver perfusion apparatus and by using the labeling pattern of liver glutamate during infusion of [³⁻¹³C]lactate (16) and the model developed by Magnusson et al. (15) to obtain information on intrahepatic metabolic fluxes. We showed that this approach is able to detect expected modifications of these metabolic fluxes (17). However, some potential limitations should be kept in mind. In particular, it is assumed that the fluxes through PC and PEPCK are equal and that there are no significant carbon outputs from Krebs' cycle other than CO₂ production and gluconeogenesis. Actually, there is some release of glutamate, citrate, malate, and aspartate by livers in this open circuit perfusion system, but this is a minor contributor to the substrate balances (17). Insulin deficiency clearly induced modifications of hepatic metabolism. Livers isolated from postabsorptive diabetic rats had, in contrast to livers from control postabsorptive rats, a large net uptake of lactate and pyruvate. They also had increases in intrahepatic gluconeogenic fluxes (V6, V8, and V9), expressed relative to

CS (V3) or as absolute values. Actually, the relative fluxes were closer to those of starved rather than postabsorptive control rats. However, since PDH activity also appeared increased in postabsorptive diabetic livers, the ratio of PC to PDH activity was not significantly modified. The fact that starvation induced no increases in either the relative gluconeogenic fluxes or the PC:PDH activity ratio in diabetic rats compared with control rats also suggests that insulin deficiency resulted in a hepatic metabolic status in postabsorptive diabetic rats resembling, in some aspects, that of starvation. This is similar to results obtained in human subjects by Landau et al. (22). These authors observed similar relative rates in insulin-deprived type 1 diabetic patients and in 60-h fasted normal subjects. This seems related to insulin deficiency and not to the existence of a diabetic state itself, since relative fluxes measured in postabsorptive type 2 diabetic patients were, on the contrary, similar to those of postabsorptive control subjects (23). Livers from starved diabetic rats released large amounts of glucose before addition of any gluconeogenic substrate in the infusate. The source of this basal glu-

TABLE 6
Absolute fluxes calculated from the relative fluxes and the increases above basal values of glucose output at the end (110–120 min) of the experiments taken as a measure of V9 (PEP to glucose) for experiments in starved rats.

| | Diabetic rats | | | | Control rats | | | |
|---------------------|----------------|--------------|--------------|--------------|----------------|-------------|--------------|--------------|
| | Postabsorptive | | 24-h starved | | Postabsorptive | | 48-h starved | |
| | - | + | - | + | - | + | - | + |
| V8: L+P utilization | 785 ± 86 | 599 ± 104 | 645 ± 47 | 405 ± 94* | 494 ± 46 | 731 ± 84 | 512 ± 40 | 665 ± 129 |
| V1: PDH | 72 ± 11 | 42 ± 11 | 64 ± 5 | 40 ± 8 | 50 ± 10 | 106 ± 12* | 13 ± 3§ | 28 ± 3†§ |
| V6: PC | 3,224 ± 718 | 1,575 ± 302* | 2,196 ± 189 | 1,091 ± 200* | 1,894 ± 312 | 2,696 ± 418 | 947 ± 150‡ | 1,289 ± 241‡ |
| V2: FAO | 89 ± 16 | 103 ± 25 | 133 ± 10 | 97 ± 17 | 144 ± 32 | 224 ± 26 | 57 ± 13‡ | 80 ± 7‡ |
| V3: CS | 161 ± 27 | 145 ± 35 | 197 ± 12 | 136 ± 34 | 194 ± 42 | 331 ± 37 | 71 ± 13‡ | 108 ± 9‡ |
| V5: OAA to <i>F</i> | 2,798 ± 460 | 2,196 ± 414 | 1,685 ± 194 | 1,559 ± 271 | 443 ± 41 | 1,328 ± 135 | 1,730 ± 490 | 2,749 ± 540 |
| V4: <i>F</i> to OAA | 2,959 ± 460 | 2,342 ± 448 | 1,882 ± 199 | 1,695 ± 304 | 637 ± 51 | 1,659 ± 418 | 1,798 ± 491 | 2,856 ± 545 |
| V6: PEPCK | 3,324 ± 718 | 1,575 ± 302* | 2,196 ± 189 | 1,091 ± 200* | 1,894 ± 312 | 2,696 ± 418 | 947 ± 150‡ | 1,289 ± 251‡ |
| V9: PEP to glucose | 714 ± 78 | 557 ± 94 | 581 ± 47 | 366 ± 82* | 444 ± 42 | 638 ± 65 | 505 ± 37 | 636 ± 127 |
| V7: PK | 2,510 ± 708 | 1,017 ± 209* | 1,615 ± 194 | 725 ± 158* | 1,450 ± 288 | 2,058 ± 357 | 448 ± 115§ | 653 ± 157§ |

Data are means ± SE in nanomoles per minute per gram of liver. For experiments in postabsorptive rats, V9 was estimated from the total glucose output and the ratios of glucose to liver pyruvate IEs. **P* < 0.05, †*P* < 0.01 vs. the corresponding value without metformin. ‡*P* < 0.05, §*P* < 0.01 vs. the corresponding postabsorptive group, ||*P* < 0.05 vs. the corresponding control group. L, lactate; OAA, oxaloacetate; P, pyruvate; +, with metformin; -, without metformin.

TABLE 7

Absolute fluxes calculated from the relative fluxes of Table 3 and the total glucose outputs at the end (110–120 min) of the experiments taken as a measure of V9 (PEP to glucose) for experiments in starved rats

| | Starved diabetic rats | | Starved control rats | |
|---------------------|-----------------------|----------------|----------------------|-------------|
| | - | + | - | + |
| V8: L+P utilization | 1,564 ± 116§‡ | 1,214 ± 204§ | 742 ± 57† | 897 ± 186 |
| V1: PDH | 156 ± 13§‡ | 119 ± 24§‡ | 19 ± 5† | 38 ± 5*† |
| V6: PC | 5,390 ± 564§ | 3,164 ± 508*§† | 1,373 ± 210 | 1,739 ± 340 |
| V2: FAO | 327 ± 35 §‡ | 284 ± 45§‡ | 83 ± 19† | 108 ± 10† |
| V3: CS | 483 ± 35 §‡ | 403 ± 69§† | 103 ± 18† | 146 ± 12† |
| V5: OAA to F | 4,056 ± 432 | 4,614 ± 933 | 2,508 ± 688 | 3,708 ± 730 |
| V4: F to OAA | 4,539 ± 457 | 5,017 ± 997 | 2,607 ± 675 | 3,853 ± 743 |
| V6: PEPCK | 5,390 ± 564§ | 3,164 ± 508*§† | 1,373 ± 675 | 1,739 ± 340 |
| V9: PEP to glucose | 1,408 ± 106§‡ | 1,096 ± 183*‡ | 736 ± 50† | 858 ± 112 |
| V7: PK | 3,983 ± 589§ | 2,068 ± 340*§† | 650 ± 165† | 881 ± 210† |

Data are means ± SE in nanomoles per minute per gram of liver. * $P < 0.05$ vs. the corresponding value without metformin. † $P < 0.05$, ‡ $P < 0.01$ vs. the corresponding postabsorptive group. § $P < 0.05$ vs. the corresponding control group. L, lactate; OAA, oxaloacetate; P, pyruvate; +, with metformin; -, without metformin.

cose production is unclear. It could represent gluconeogenesis from endogenously released substrates or the persistence of some glycogenolysis. The presence of significant amounts of glycogen in these livers at the end of the experiments, contrary to that observed in livers from fasted control rats, would support the last hypothesis. This persistence of liver glycogen stores in 24-h starved diabetic rats has already been reported (24,25), and the exact mechanism remains to be established. Total glucose output after addition of lactate and pyruvate was also higher in experiments with starved diabetic than with starved control rats, but the increase above basal values was comparable, suggesting that gluconeogenesis from exogenous substrates was comparable. Actually, the absolute flux through PC and PEPCK (V6) was increased in the diabetic group (Table 6), but recycling through PK was also largely enhanced. There were also no significant differences between the starved diabetic and normal groups with respect to the percentage of lactate and pyruvate taken up and to the percentage of these lactate and pyruvate molecules taken up and used for glucose production (as calculated from the increase of glucose production above basal values).

Metformin had no effect on livers from normal rats except for an increase in the calculated flux through PDH. A significant decrease in glucose output was observed only in livers from starved diabetic rats, while calculated fluxes through PC and PEPCK were reduced in both diabetic groups. Our results differ from those of Radziuk et al. (14) and Argaud et al. (12) who found that metformin decreased gluconeogenesis from lactate by perfused rat liver (14) and hepatocytes isolated from 24-h fasted normal rats (12). Argaud et al. observed this effect only for metformin concentrations higher than the one we used (10 instead of 0.7 mmol/l). However, they found, in perfused hepatocytes isolated from starved normal rats, an effect of 0.5 mmol/l metformin on gluconeogenesis from dihydroxyacetone. Radziuk et al. used metformin concentrations similar to ours. Their experiments lasted longer than ours, but a decrease in glucose accumulation was already apparent after only 40 min of metformin infusion (14). The experimental design was also different, since they used a recirculating perfusion system, higher lactate concentrations (4 mmol/l), and also infused glucose

(5.2 mmol/l in the initial perfusion medium). This glucose level increased throughout the experiment (up to 12–15 mmol/l), and there was a reflux from glucose to lactate; therefore, it is probable that the metabolic status of the livers was different. The mechanism of the decreased gluconeogenic flux we observed in livers from diabetic rats is also different from the explanations proposed by Radziuk (14) and Argaud (12). Radziuk et al. found a decrease in lactate extraction and suggested that the primary action of metformin is to inhibit hepatic lactate uptake. We have no evidence for this effect in the present study. Again, a main difference in the experimental design is the absence of glucose in the infusate in our experiments, whereas glucose was present and accumulated throughout the infusions in Radziuk's experiments. The reflux of carbon from glucose to lactate was increased by metformin and this could have contributed to the modification of lactate balance. If we had no modification of lactate and pyruvate net uptake, we observed an increased release of alanine in the only group with a significant decrease in glucose output in the presence of metformin (starved diabetic rats). The identity of alanine and pyruvate enrichment suggests that most of the alanine released came from pyruvate or that there was a rapid isotopic equilibration between pyruvate and alanine. Therefore, one action of metformin could be to divert in part the metabolism of liver pyruvate toward alanine; however, this could also result only from the decreased flux through PC. Argaud et al. (12) had evidence for an increase in pyruvate kinase flux, since gluconeogenesis from dihydroxyacetone was decreased by metformin, while lactate and pyruvate productions were increased. In addition, these effects were opposed by glucagon, which inhibits PK (26). Because PK activity itself was not modified, the authors suggested that the increase in PK flux was due to the decrease of ATP, a known allosteric inhibitor of PK (26), induced by metformin. We observed no decrease of ATP in the presence of metformin, and therefore this mechanism could not be operative in the conditions of the present study. Metformin has also been suggested to decrease hepatic FAO (13). The resulting decrease in acetyl CoA levels would enhance PDH and decrease PC activities (13). However, our experiments bring out no data in support of this hypothesis, at least in vitro,

since FAO, as estimated by the model, was unchanged or slightly increased.

In conclusion, we found, in the experimental conditions used in this study, that insulin deficiency increased gluconeogenesis through enhanced lactate and pyruvate uptake and flux through PC-PEPCK. Metformin decreased gluconeogenesis in livers of diabetic but not normal rats. We have evidence that this action is related to an inhibition of the flux through PC and PEPCK and perhaps also to an increased conversion of liver pyruvate into alanine. Neither the substrate balance nor the calculations of flux from the labeling pattern of glutamate showed evidence of modification of lactate and pyruvate uptake.

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