Effect of Phenformin on Gluconeogenesis from Lactate and Intracellular pH in the Isolated Perfused Guinea Pig Liver

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SUMMARY

Gluconeogenesis from lactate and hepatic cell pH (pH_i) were measured in the isolated perfused livers of starved guinea pigs in the presence and absence of phenformin (phenethylbiguanide). The observed decrease in lactate consumption and glucose output in the presence of phenformin was associated with a fall in pH_i. The fall in glucose output observed was considerably greater than accountable for by the decrease in lactate consumption. A possible mechanism for the pathogenesis of clinical lactic acidosis due to phenformin therapy is suggested. DIABETES *24*:618-24, July, 1975.

It has been suggested that one of the ways by which phenformin therapy may give rise to lactic acidosis is by suppression of gluconeogenesis from lactate, an effect which has been demonstrated in the isolated perfused liver preparation[1-5] and in liver slices.[6,7]

In previous work, we have demonstrated a relationship between cell pH and lactate metabolism using the isolated perfused rat liver.[8] It was found that when lactate uptake by the liver was varied by changing the lactate concentration of the medium, hepatic cell pH changed in the same direction as lactate consumption. Furthermore, lactate uptake decreased when cell pH fell below about 7.1 during simulated metabolic acidosis.[9] When hepatic cell pH became markedly decreased (pH<7.0), lactate uptake ceased altogether and lactate was in some cases actually produced by the liver. We have suggested[9] that inhibition of gluconeogenesis from lactate by phenformin might thus be expected to lead to a fall in cell pH; if a severe degree of cellular acidosis developed, the liver might cease to take up lactate from the circulation and later produce lactate. Such a sequence of events might be of importance in the pathogenesis of the lactic acidosis which complicates phenformin therapy in some patients.

The present series of experiments were designed to investigate the effect of phenformin on lactate uptake, glucose production and intracellular pH in the isolated perfused guinea pig liver.

METHODS

Inbred guinea pigs (weight 130-200 gm.) which had been starved for forty to forty-eight hours were used. There were eighteen animals in the control group and thirty-three in the phenformin group. Isolated liver perfusions were set up as previously described.[8,10] The livers were perfused with a medium containing equine erythrocytes obtained from blood which had been taken into acid citrate dextrose six days before the perfusions. They were suspended in physiologic buffer (Krebs-Henseleit) containing 4 per cent w/v bovine albumin (fraction V, Armour Pharmaceuticals, Eastbourne, Sussex). The albumin had been previously dialyzed for forty-eight hours against Krebs buffer. The packed cell volume ranged between 0.15 and 0.20. L (+) lactic acid (Sigma) was added to the perfusate to obtain an initial concentration of about 14 mM. Control and phenformin perfusions were distributed in an even manner chronologically over the course of the study.

Approximately 150 ml. of medium was used for each experiment. This was equilibrated in the circuit at a PCO_2 of approximately 40 mm. Hg, and the pH was adjusted to be in the physiologic range by the addition of Krebs buffer modified in that chloride was replaced by bicarbonate. During each experiment the pH of the perfusate did not vary by more than 0.08 units or the PCO_2 by more than 8 mm. Hg. At the beginning of each experiment [2-^{14}C] 5,5-di-

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methyloxazolidine-2,4 dione ([^{14}C] DMO), $^{3}H_2O$ and hydroxy [^{14}C]-methyl inulin were added with non-radioactive carrier to the perfusate for measurement of cell pH (pH_i) as previously described.[8]

Each experiment lasted for one hour, and the medium was recirculated throughout that period. The perfusion rate was approximately 7 ml. min.$^{-1}$ (100 gm. animal)$^{-1}$ and was held constant during the experiment.

Twenty minutes after the perfusion was set up, samples of arterial and venous blood were taken for measurement of pH, PCO_2 and PO_2 and for the determination of lactate and glucose concentration. Phenformin was then added to the reservoir to produce a concentration of 0.05, 0.1 or 0.25 mM. An equivalent volume of 154 mM. sodium chloride was added to the reservoir in the control group. Further samples of perfusate were taken at 40, 50 and 60 min. Each experiment was terminated at sixty minutes and samples of medium and liver were taken so that the distribution of isotopes could be measured and pH_i calculated. In some experiments in the phenformin group, a second dose of phenformin, which doubled the existing nominal reservoir concentration of phenformin, was added at forty minutes.

At the conclusion of each experiment, the volume of medium remaining in the circuit and reservoir was measured. By adding to this final volume the quantity of medium removed for the purpose of estimations, the volume of the medium at 20, 40, 50 and 60 min. was calculated. From these values and the measured arterial lactate and glucose concentrations, the lactate and glucose content of the reservoir at the time of sampling was calculated. Glucose production and lactate uptake during the period 20-40, 40-50 and 50-60 min. could thus be calculated. Perfusate alanine concentrations were measured in some of these experiments.

The above experiments are referred to as series A.

A number of additional groups of experiments (series B-F) were carried out. To examine the effect of phenformin on glycogen content of the liver at the end of perfusion, this was determined in a further group (series B) of twelve perfusions of livers from starved animals. Lactate was present in the perfusate at an initial concentration of 15 mM. In six of these perfusions phenformin was added twenty minutes after the start of perfusion to produce a concentration of 0.1 mM. and a second similar addition was made at forty minutes. In the remaining perfusion, no addition was made. The livers were freeze-clamped at sixty minutes. To assess the relative contribution of gluconeogenesis and glycogenolysis to glucose production, the experiments were repeated in the absence of added lactate (series C). In a further group (series D) of similar perfusions, the effect of phenformin addition on perfusate ketone body, pyruvate and free fatty acid concentrations was studied. To investigate the extent of glucose consumption by erythrocytes in the presence and absence of phenformin, experiments were also carried out in the absence of a liver preparation and added lactate; for these studies (series E), glucose was added to the perfusate to produce a concentration of 4-6 mM.

To determine the rate of equilibration of DMO between the perfusate and the liver cell, four perfusions (series F) were set up, $^{3}H_2O$ and hydroxy-[^{14}C] methyl inulin were added and were allowed to equilibrate for forty minutes. A bolus of [^{14}C]-DMO (with carrier) was then added to the reservoir and liver lobes were excised at four, eleven and twenty minutes; these times allowed for the predetermined interval between addition of a substance to the reservoir and its reaching the liver. Intracellular [^{14}C]-DMO was then determined in each of the lobes as for intracellular pH measurements.

Chemical Methods

The methods for determination of perfusate pH, PCO_2, PO_2, lactate concentration and intracellular pH, and the calculations employed, were as previously described.[8] Glucose concentration of the perfusate plasma was measured using a glucose oxidase method on a Technicon AutoAnalyzer. Whole perfusate glucose content was calculated from the perfusate plasma glucose and the packed cell volume, assuming an erythrocyte water content of 65 per cent and erythrocyte water glucose concentration equal to that of perfusate plasma water. Free fatty acids were determined in perfusate plasma by the method of Chernick and Novak.[18] The following additional methods were employed, using the same perchloric acid supernatant of perfusate as for lactate: 3-hydroxybutyrate and acetoacetate;[19,20] pyruvate and alanine-AutoAnalyzer adaptations of the methods of Hohorst et al.[21] and Williamson, Lopes-Vieira, and Walker.[22] Rates of metabolite uptake and output are expressed per minute per 100 gm. guinea pig.

Frozen tissues for glycogen estimation were ground, 30 per cent potassium hydroxide solution was added (7 ml./gm. tissue), and the slurry was homogenized. Hepatic glycogen was then determined by the method of Schwartz and Rall.[11] The results were compared with hepatic glycogen content esti-

mated in the livers of similarly starved animals not subjected to perfusion.

The effect of incubation at 37° C. for one hour with phenformin (0.25 mM.) on the oxygen dissociation curve of the perfusate was determined in vitro by the mixing technic.[24] The five-point curves are defined in terms of the PO_2 at half-saturation (P_{50}) and the Hill constant (n).

The difference between the control and phenformin groups was analyzed by both parametric and non-parametric methods; these gave similar results. Means are expressed ± S.E.M. One-tailed tests are used unless otherwise stated. Standard parametric correlation methods were used.

RESULTS

1. Effect of Phenformin on Cell pH and Lactate Uptake (Series A)

(a) Lactate concentration of the perfusate. The mean lactate concentration in the perfusate at the start of each experiment was 14.37 ± 0.41 mM. for the control group and 13.59 ± 0.40 mM. for the phenformin group. The difference was not significant.

(b) pH, PCO_2 and PO_2 of the perfusate. The mean portal venous pH and PCO_2 at the time of pH_i measurement were 7.40 ± 0.007 and 42.5 ± 0.10 mm. Hg respectively in the control group and 7.38 ± 0.007 and 41.4 ± 0.73 mm. Hg in the phenformin group; these differences were not significant.

The corresponding mean hepatic venous pH was 7.40 ± 0.008 for the control group and 7.36 ± 0.006 for the phenformin group. This difference was significant ($P<0.01$). The hepatic venous PCO_2 measured at the same time was 42.7 ± 0.93 in the control group and 44.4 ± 0.95 in the phenformin group.

The reservoir PO_2 was always about 400 mm. Hg. In the control series, the mean hepatic venous PO_2 at twenty minutes was 32.2 ± 2.28 mm. Hg and at sixty minutes was 33.7 ± 2.27 mm. Hg, the difference being insignificant. In the phenformin series, the mean hepatic venous PO_2 at the same times were 32.6 ± 1.43 and 42.1 ± 2.23 mm. Hg, the difference (9.45 mm. Hg) being highly significant ($P<0.01$, Mann-Whitney U test).

When the oxygen-dissociation curve was determined in vitro on the same perfusate in the presence and absence of 0.25 mM. phenformin (see Methods), the curves were virtually identical (control: P_{50}, 20.2 mm. Hg, Hill constant (n), 2.33; with phenformin: P_{50}, 20.7 mm. Hg, n, 2.33).

(c) Lactate uptake. The response to phenformin was variable and no clear dose-response relationship was evident. For the purpose of analysis all phenformin dose groups have therefore been considered together.

The mean lactate uptake during the course of the perfusion in the two groups is shown in figure 1. No difference between the control and phenformin groups was seen until the forty-to-fifty-minute period. The mean lactate uptake during the final ten minutes of the perfusion was 10.51 ± 1.35 μmol. min.$^{-1}$ (100 gm. guinea pig)$^{-1}$ in the control group and 6.83 ± 1.39 μmol. min.$^{-1}$ (100 gm. guinea pig)$^{-1}$ in the phenformin groups. The difference is significant ($P = 0.033$).

(d) Relationship between lactate uptake (L) and cell pH (pH_i). This is shown in figure 2. The regression equation in the control group is

$$pH_i = 7.098 + 0.0116\ L;\ r = 0.492,\ 0.01<P<0.05$$

and in the phenformin-treated group is

$$pH_i = 7.072 + 0.0078\ L;\ r = 0.551;\ P<0.001$$

These two regression lines do not differ significantly in either slope or intercept.

In the phenformin series, lactate output occurred in some instances where pH was particularly low.

(e) Glucose production. During the perfusion, the mean glucose production fell in both groups but the fall was much greater in the phenformin group (figure 3). During the forty-to-fifty-minute perfusion period, the mean glucose production was 4.33 ± 1.22 μmol. min.$^{-1}$ (100 gm. guinea pig)$^{-1}$ in the control group and 0.73 ± 0.52 μmol. min.$^{-1}$ (100 gm. guinea pig)$^{-1}$ in the phenformin group; this difference is

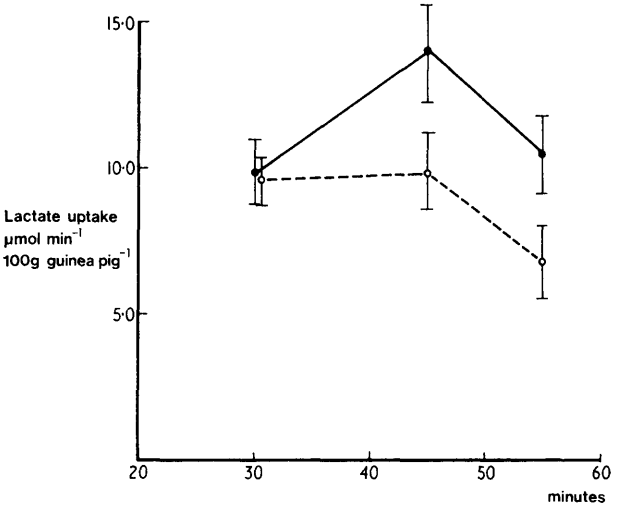

FIG. 1. Mean lactate uptake (± S.E.M.) during the 20-40 min. 40-50 and 50-60 min. perfusion periods in series A. • control animals o phenformin animals.

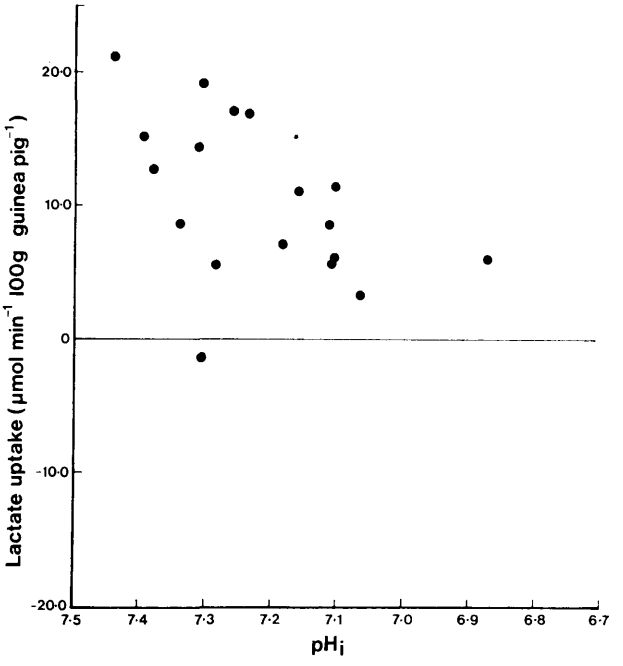

FIG. 2. (a) Relationship between lactate uptake in the fifty-to-sixty-minute period and hepatic intracellular pH (pH_i) in the control perfusions of series A. Points above the zero line represent lactate uptake and those below represent lactate output by the liver.

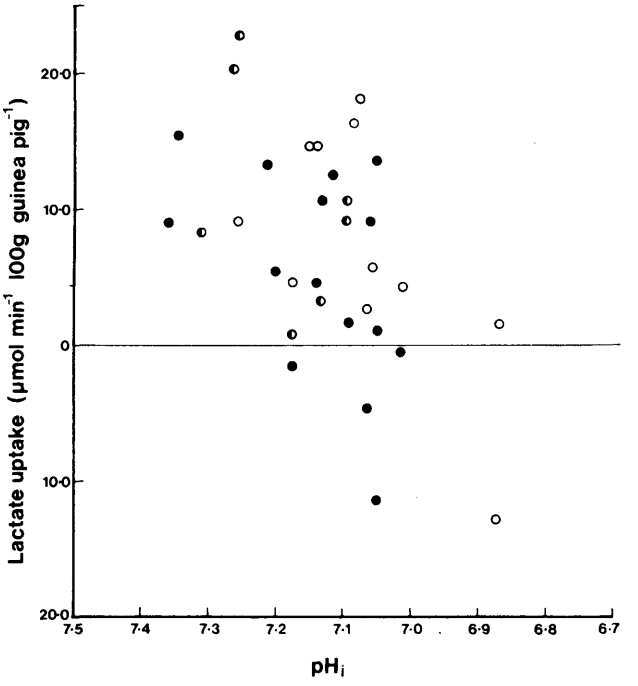

FIG. 2. (b) The same relationship in the phenformin perfusions of series A. The different symbols indicate the nominal concentrations of phenformin employed: • 0.05-0.1 mM. at zero time; ◦ 0.1 mM. at zero time and a similar increment at twenty minutes; o 0-0.025 mM. at zero time and a similar increment at twenty minutes (see Methods).

highly significant ($P<0.01$). During the fifty-to-sixty-minute period, the mean glucose production in the control was 1.38 ± 0.58 μmol. min.$^{-1}$ (100 gm.)$^{-1}$ while in the phenformin group, glucose production had actually ceased and the glucose content of the reservoir was falling at a rate of 1.69 ± 0.46 μmol. min.$^{-1}$ (100 gm.)$^{-1}$. The difference between the two groups is significant ($P<0.01$).

There is also a significant correlation ($P<0.01$) between cell pH and glucose production during the final ten-minute perfusion period.

(f) Changes in alanine production. Alanine production was very small, there being no significant difference between the control (-0.15 ± 0.29 μmol. min.$^{-1}$ (100 gm. guinea pig)$^{-1}$, n=4) and the phenformin series (0.041 ± 0.045, n=4).

2. Effect of Phenformin on Hepatic Glycogen Content After Perfusion (Series B)

The mean glycogen content in the nonperfused livers of forty-eight-hour starved guinea pigs was 0.039 ± 0.012 gm./100 gm. liver (n=4). In livers after perfusion in the absence of phenformin, the mean glycogen was 0.069 ± 0.022 gm./100 gm. liver (n=6). In perfusions to which phenformin had been added as described in Methods, mean glycogen was 0.032 ± 0.0039 gm./100 gm. (n=6). These levels are very low, and the difference between the groups is not significant.

3. Glucose Production in the Absence of Added Lactate (Series C)

In the absence of added lactate, mean glucose pro-

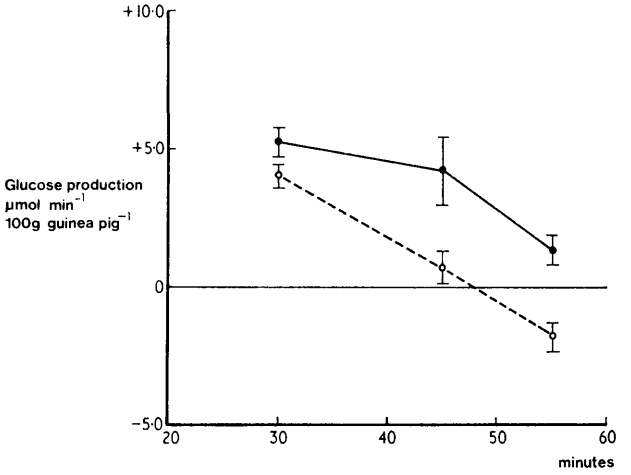

FIG. 3. Mean glucose production (± S.E.M.) during the 20-40 min., 40-50 min. and 50-60 min. perfusion periods in series A. Points above the zero line represent glucose output by the liver, and points below glucose uptake. • control animals o phenformin animals.

duction was 1.44 ± 0.084 μmol. min.$^{-1}$ (100 gm. guinea pig)$^{-1}$ in the control group (n=4) and 0.967 ± 0.507 μmol. min.$^{-1}$ (100 gm.)$^{-1}$ in the phenformin group (n=6) in the twenty to forty minute period. These values represent 27 per cent and 23 per cent, respectively, of the glucose produced during the same period when lactate was added to the perfusate. By fifty to sixty minutes, glucose production had apparently ceased.

4. Changes in Perfusate Ketone Bodies, Pyruvate, and FFA (Series D)

Table 1 shows the mean liver output (or uptake) of ketone bodies (3-hydroxybutyrate + acetoacetate), pyruvate and FFA in the final period. There is no significant difference between the control and phenformin series for these metabolites, but, as in series A, there were significant differences in glucose production and lactate output.

5. Perfusate Glucose Consumption and Lactate Production (Series E)

The mean consumption of glucose by the perfusate erythrocytes was 0.40 ± 0.08 μmol. min.$^{-1}$ 100 ml.$^{-1}$ (n=4) in the absence of phenformin, and the lactate production 0.67 ± 0.06 μmol. min.$^{-1}$ 100 ml.$^{-1}$. In the presence of 0.25 mM. phenformin, glucose consumption was 0.45 ± 0.18 μmol. min.$^{-1}$ 100 ml.$^{-1}$ and lactate production 0.71 ± 0.21 μmol. min.$^{-1}$ 100 ml.$^{-1}$ (n=4). Phenformin thus made no significant difference to glucose production and lactate consumption.

6. Rate of DMO Equilibration Between Perfusate and Liver (Series F)

There was very little change in intracellular concentration of [^{14}C]-DMO between eleven and twenty minutes after addition of DMO. Taking the twenty minute level as 100 per cent, the mean extent of equilibration in the four experiments at four and eleven minutes were 85.3 ± 3.50 and 95.4 ± 2.12 per cent respectively. An approximate half-time of equilibration of 1.9 min. may be calculated from these figures.

TABLE 1

Mean (± S.E.M.) hepatic output of metabolites at the end of perfusion in series D (μmol. min.$^{-1}$ (100 gm. guinea pig)$^{-1}$

	Control group	Phenformin group
Ketone bodies	1.14 ± 0.18 (4)	1.43 ± 0.26 (4)
Pyruvate	0.10 ± 0.091 (6)	−0.12 ± 0.079 (5)
FFA	−0.11 ± 0.10 (4)	−0.04 ± 0.08 (4)
Glucose	3.89 ± 0.87 (5)	−0.27 ± 0.70* (6)
Lactate	7.64 ± 1.40 (5)	2.82 ± 1.45† (6)

Number of experiments in each group in parentheses.
*P<0.01; †P<0.05, compared with control series.
Other comparisons are not significant.

DISCUSSION

The use of the DMO technic to measure cell pH is dependent on a number of assumptions which have been discussed elsewhere.[12-17] An important requirement is that a steady state with regard to intra- and extracellular pH should have been present for a sufficient period of time for full equilibration of DMO to have occurred; we have previously described in quantitative terms the effect of a nonsteady state.[25] In the present experiments, a steady state was not present since the mean lactate uptake in the fifty-to-sixty-minute period, at the end of which cell pH was measured, was less than that in the forty-to-fifty-minute period in both the control and phenformin experiments. The absolute decrease in lactate uptake over these two periods was, however, similar in the two groups; furthermore, although a highly significant correlation was demonstrable between pH_i and lactate uptake in the fifty to sixty minute period, no such relationship existed between the *change* in lactate consumption between the 40-50 and 50-60 min. periods and pH_i. These considerations make it unlikely that the significant correlation demonstrated between pH_i and lactate uptake (figure 2) was an artifact due to failure of DMO equilibration. The equilibration studies show that the half-time of DMO equilibration in the perfused guinea pig liver is approximately two minutes. This means that the DMO distribution at the time of measurement of cell pH is influenced by changes in pH_i which might have taken place over the previous eight to ten minutes (four to five half-times).

The positive correlation between lactate uptake and pH_i is comparable with the results of previous studies[8] in which lactate uptake was varied by changing perfusate lactate content rather than by administering an inhibitor of gluconeogenesis. It therefore may well be that the rate of lactate uptake and metabolism is in many circumstances a major factor in the control of hepatic pH_i. We suggest[8] that the relationship arises from at least part of the lactate entering the liver, so doing in the ionized form rather than entirely as the undissociated acid. Intracellular H^+ is then consumed according to the over-all equations

$$2CH_3CHOH\,COO^- + 2H^+ \rightarrow 2C_6H_{12}O_6$$

$$\text{or } CH_3CHOH\,COO^- + H^+ + 3O_2 \rightarrow 3CO_2 + 3H_2O$$

Cell pH will, therefore, rise as lactate consumption rises provided the cellular mechanisms for pH_i control are not completely effective. The regression lines between pH_i and lactate uptake were statistically similar in the control and phenformin groups, and this suggests that the low pH_i values observed in some of the phenformin group were due to phenformin inhibition of lactate uptake. Such inhibition could be due either to depression of lactate metabolism or to interference with lactate transport into the cell; the present data do not allow discrimination between these possibilities.

If lactate is not metabolized completely to glucose or glycogen, or carbon dioxide and water, but instead is converted, for instance, to ketone bodies, pyruvate or alanine, then intracellular H^+ would not be consumed, and the relationship between pH_i and lactate uptake would disappear. It was shown in series B and D that there was no difference in the production of these metabolites between the control and phenformin series; this is consistent with the similar pH_i/lactate uptake relationship in the control and phenformin series.

We have shown elsewhere[9] that, although above a hepatic pH_i of about 7.1 there is no obvious effect of pH_i on lactate uptake, at values below 7.1, lactate uptake rapidly diminishes. This effect of pH_i upon lactate uptake together with that of lactate uptake on pH_i described in this paper could form the two arms of a positive feedback system which might account for the rapid development of lactic acidosis due to phenformin and other causes.

Although at the time of determination of pH_i the mean portal venous pH in the two groups were similar, the corresponding hepatic venous pH in the phenformin groups was slightly but significantly lower than in the control group. This is presumably due to a smaller bicarbonate output of the liver in the phenformin group because of lowered lactate consumption. It should be noted that this small difference could contribute only very slightly to the changes in pH_i observed (see Lloyd et al.[9], figure 1).

A feature of the present studies is the lack of stoichiometry between lactate uptake and glucose production in series A. Though this is most evident in the phenformin series, it is also prominent in the control series, in which, allowing for glucose production in the absence of added lactate, the proportion of lactate uptake accountable for by glucose production fell during the course of the experiment from 80 per cent to about one third; nevertheless, oxygen consumption, as judged by the lack of change in hepatic venous PO_2, remained unaltered. Neither the discrepancy between lactate uptake and glucose production in the control series, nor the increased discrepancy in the phenformin series can be explained by diversion of lactate into ketone body, pyruvate, alanine, FFA or glycogen formation, nor by erythrocyte glycolysis nor by the sum of such effects. It remains possible that lactate has been partially diverted into the tricarboxylic acid cycle and has been completely oxidized. The rise in hepatic venous PO_2 in the phenformin series can be shown, using the oxygen dissociation curve of the perfusate and taking dissolved oxygen into account, to represent approximately a 20 per cent fall in oxygen consumption due to phenformin; despite this fall, lactate could have replaced other substrates for oxidation. Other studies in which lack of lactate/glucose stoichiometry has been observed have been described and discussed.[23,26] This feature of the present study does not affect the interpretations offered in the previous paragraphs.

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