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INFLUENCE OF EXTRACELLULAR HYDROGEN IONS ON THE INHIBITION OF GLUCONEOGENESIS BY BUTYL-BIGUANIDE IN THE PERFUSED GUINEA PIG LIVER

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Abstract—Small changes of the pH value in the medium, such as can be expected to occur *in vivo*, had some effects on the metabolism of the isolated, perfused guinea pig liver. The rate of glucose formation did not alter as the extracellular pH values were changed. However, the inhibition of gluconeogenesis by *N*-butylbiguanide was very sensitive to alterations of the pH value. The intrahepatic ATP–ADP ratio decreased and the uptake of potassium ions by the liver was enhanced with increasing pH value. These findings led to the conclusion that the liver metabolism was partly influenced by the extracellular hydrogen ion concentration.

Severe cell damage by changing the pH value in the medium could be excluded, since the release of several enzymes from different compartments of the liver cell during the perfusion was not accelerated under the various experimental conditions.

FEW REPORTS deal with the influence of hydrogen ions on the metabolism of the isolated perfused liver. Ross *et al.*[1] found that the glucose formation from glutamate occurred at the same rate between pH 7·2 and 7·7. Exton and Parks[2] confirmed the original finding of Hems *et al.*[3] that the hepatic gluconeogenesis from lactate is not altered by varying the pH value of the medium in rat liver perfusion experiments. In contrast, renal gluconeogenesis from glutamate and 2-oxoglutarate was found to be accelerated in acidosis.[4]

To our knowledge, no clear-cut information is available, whether the metabolism of the isolated liver responds to small changes of the extracellular pH, which can be expected to occur *in vivo* under pathological conditions.

During studies on the inhibitory effect of biguanides on gluconeogenesis in the perfused guinea pig liver,[4–6] we noticed that the uptake of potassium ions by the liver and the action of *N*-butylbiguanide varied when the hydrogen ion concentration of the perfusate was altered.

METHODS

Albino male guinea pigs (250–350 g, random breed) fasted 48 hr prior to the experiments. Our modified perfusion procedure was that of Miller *et al.*[7] as described elsewhere.[5] The perfusion medium contained 3 g per cent bovine albumin (Behring Werke, Marburg), 1 mg sodium ampicillin (Farbwerke Hoechst), bovine erythrocytes, washed three times and taken up in Krebs–Ringer bicarbonate solution. The pH value was checked every 10 min with an Astrup apparatus (Type PHA 928, Radiometer Copenhagen).

The hemoglobin concentration was kept at 5 g per cent through all experiments. To determine the hemolysis (approximately 0·5 per cent) the erythrocytes were spun down

in perfusate samples. The hemoglobin concentration in the supernatant from this procedure increased from almost 0 to 23·0 ± 7·8 mg per cent (15 observations) during the perfusion experiments.

All perfusions lasted only 120 min, since Kaschnitz *et al.*[8] had reported that the isolated rat liver began to swell and to release alkaline phosphatase into the medium after this period. Liver metabolites were determined enzymatically[5] in samples taken at the end of the perfusion using Wollenberger's method.[9] Potassium ions were measured with an Eppendorf flame photometer (Eppendorf Gerätebau, Hamburg) and hemoglobin[10] with Drabkin's solution.

Enzyme activities* were determined in hemolysates or in perfusate samples after centrifugation of the erythrocytes with Boehringer test combinations (kinetic pro-

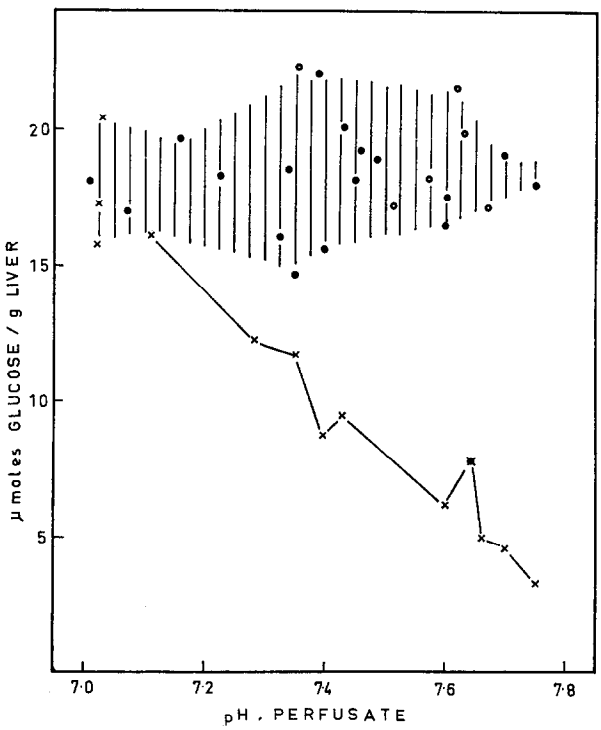

FIG. 1. The influence of hydrogen ions and of butylbiguanide on the glucose formation from alanine between the first and the second hour of the experiment. Butylbiguanide ($5·2 \times 10^{-5}$ M) was added 45 min and alanine (10 mM) 60 min after the beginning of the perfusion.

● the pH value was regulated by alteration of the CO_2 concentration in the aerating gas mixture.
○ alkalinization was achieved by the addition of sodium bicarbonate at constant CO_2 percentage of the gas mixture (5%).
× as ● in the presence of butylbiguanide.
⊗ as ○ in the presence of butylbiguanide. The hatched area covers all values from perfusion experiments which have been performed in the absence of biguanide.

* Lactate dehydrogenase, L-lactate: NAD oxidoreductase, EC 1.1.1.27 Glutamate dehydrogenase, L-glutamate: NAD oxidoreductase, EC 1.4.1.2. Glutamate pyruvate transaminase, L-alanine:2-oxoglutarate aminotransferase, EC 2.6.1.2. Glutamate oxalacetate transaminase, L-asparate:2-oxoglutarate aminotransferase, EC 2.6.1.1. Alkaline phosphatase, orthophosphoric monoester phosphohydrolase, EC 3.1.3.1. Acid phosphatase, orthophosphoric monoester phosphorylase 3.1.3.2 Arylamidase, L-leucyl-peptide hydrolase, 3.4.1.1.

cedure). The glutamyltranspeptidase was measured according to Szasz.[11] Hemolysates were obtained by mixing 1 ml of washed bovine erythrocytes with 10 ml distilled water and centrifugation after 20 min. The supernatant was used directly for the determination of enzyme activities.

N-butylbiguanide was a gift from Dr. Michael (Grünenthal GmbH, Stolberg).

RESULTS

The rate of glucose formation from alanine in perfused livers of guinea pigs fasted 48 hr was not dependent upon the hydrogen ion concentration of the medium between

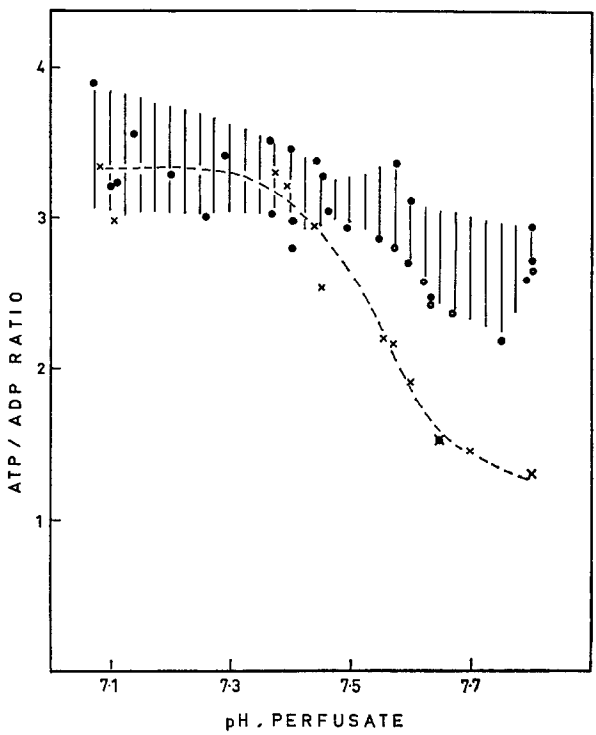

FIG. 2. The influence of the extracellular pH value on the intrahepatic ATP–ADP ratio. For further explanation of symbols see legend to Fig. 1.

pH 7·1 and 7·7 (Fig. 1). In the presence of butylbiguanide however, gluconeogenesis was more and more reduced with increasing pH value. In most experiments the pH value was regulated by changing the CO_2 concentration in the aerating gas mixture from 0 to 7 per cent. In some experiments the alkalinization was achieved by the addition of sodium bicarbonate. Both treatments gave similar results.

The hepatic ATP–ADP ratio tended to decrease with rising pH value. This effect was more distinct if butylbiguanide was added (Fig. 2). The influence of the biguanide on the adenosine phosphate concentrations probably reflected an inhibition of cell respiration since the 3-hydroxybutyrate–acetoacetate ratio (Table 1), an indicator of the intramitochondrial redox state, was increased. In the absence of butylbiguanide, the 3-hydroxybutyrate–acetoacetate ratio showed a slight downward trend with rising

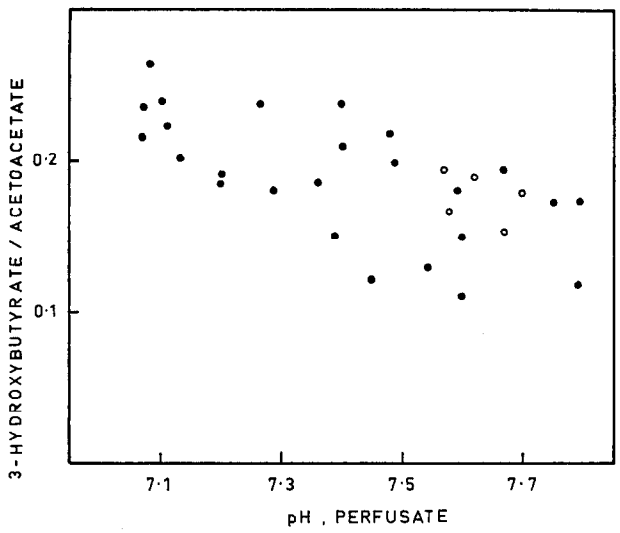

FIG. 3. The 3-hydroxybutyrate–acetoacetate ratio in the perfused guinea pig liver at various pH values. The symbols are explained in the legend to Fig. 1.

TABLE 1. THE INFLUENCE OF BUTYLBIGUANIDE ON THE HEPATIC 3-HYDROXYBUTYRATE–ACETOACETATE RATIO AT pH VALUES BETWEEN 7·6 AND 7·8

Butylbiguanide		$5{\cdot}2 \times 10^{-5}$ M
3-OH-butyrate	200 ± 60 (9)*	500 ± 30 (5)
Acetoacetate	1080 ± 170 (9)	1350 ± 230 (5)
Ketone bodies†	1280 ± 180 (9)	1850 ± 240 (5)
3-OH-butyrate/Acetoacetate	0·18 ± 0·03 (9)	0·37 − 0·05 (5)

* Means nmoles/g liver with standard deviations and the number of contributing values.

† Means the sum of the hepatic concentrations of 3-hydroxybutyrate and acetoacetate.

pH value (Fig. 3). The sum of the 3-hydroxybutyrate and the acetoacetate concentrations did not significantly change with alterations of the pH value.

Recently, we reported that the isolated guinea pig liver took up potassium ions during the perfusion experiments.[6] Figure 4 shows that this effect increased with rising pH value. Butylbiguanide ($5{\cdot}2 \times 10^{-4}$ M) inhibited this uptake of potassium ions by the liver (Table 2).

The bile production decreased when the hydrogen ion concentration was lowered (Fig. 5). This effect was not due to cholostasis since enzymes usually considered to indicate cholostasis (glutamyltranspeptidase, alkaline phosphatase and arylamidase) did not accumulate in the perfusate nor were less released into the bile.[12] Butylbiguanide had no effect on the bile production.

As sensitive indicators for liver cell damage some enzyme activities were measured

TABLE 2. THE INFLUENCE OF BUTYLBIGUANIDE ON THE UPTAKE OF POTASSIUM IONS BY THE PERFUSED LIVER

Butylbiguanide	Control	$2{\cdot}6 \times 10^{-5}$ M	$5{\cdot}2 \times 10^{-5}$ M
Potassium	$16{\cdot}3 \pm 4{\cdot}0$*	$14{\cdot}5 \pm 3{\cdot}0$	$9{\cdot}0 \pm 3{\cdot}0$
Number of experiments	34	12	8
Significance		not significant	significant ($P < 0{\cdot}05$)

* μmoles K^+/g liver (wet wt.), calculated from the difference of the potassium concentration at the beginning and at the end of the perfusion. The results were taken together from experiments at pH values between 7·35 and 7·70.

TABLE 3. ACTIVITIES OF SOME ENZYMES IN THE MEDIUM AFTER 120 min OF LIVER PERFUSION

pH range	7·10–7·35	7·35–7·45	7·45–7·80
AP*	$21{\cdot}9 \pm 18{\cdot}1$ (4)†	$7{\cdot}0 \pm 5{\cdot}8$ (5)	$9{\cdot}8 \pm 8{\cdot}2$ (13)
	14·1	4·5	6·3
Arylamidase	$25{\cdot}0 \pm 9{\cdot}8$ (4)	$26{\cdot}2 \pm 0{\cdot}8$ (4)	$19{\cdot}9 \pm 11{\cdot}8$ (8)
	65·0	68·3	51·0
GLDH	$3{\cdot}6 \pm 2{\cdot}1$ (4)	$9{\cdot}9 \pm 6{\cdot}6$ (4)	$7{\cdot}1 \pm 4{\cdot}2$ (8)
	4·9	13·6	9·7
GOT	$177{\cdot}1 \pm 33{\cdot}7$ (4)	$232{\cdot}8 \pm 55{\cdot}7$(4)	$171{\cdot}6 \pm 75{\cdot}2$ (12)
	151·0	208·0	146·0
GPT	$15{\cdot}3 \pm 9{\cdot}5$ (4)	$19{\cdot}4 \pm 4{\cdot}4$ (4)	$16{\cdot}4 \pm 15{\cdot}7$ (14)
	1·7	4·8	2·5
LDH	$433{\cdot}7 \pm 210{\cdot}5$ (4)	$302{\cdot}3 \pm 84{\cdot}0$ (4)	$229{\cdot}4 \pm 52{\cdot}1$ (9)
	195·0	91·5	33·6
SP	$14{\cdot}0 \pm 9{\cdot}9$ (4)	$15{\cdot}3 \pm 2{\cdot}1$ (4)	$8{\cdot}9 \pm 5{\cdot}8$ (15)
	15·9	17·4	10·1

* AP means alkaline phosphatase, GLDH glutamate dehydrogenase, GOT glutamate-oxalacetate-transaminase, GPT glutamate-pyruvate-transaminase, LDH lactate dehydrogenase, SP acid phosphatase.

† mU/g liver with standard deviations and the number of contributing values. The determination of the enzymatic activities were performed 8–10 hr after the perfusion experiments had been finished.

All values were corrected for the estimated activity of enzyme released from the erythrocytes and for the alteration of the activity caused by the ageing process.

in the perfusate. Acidification and alkalinization of the medium did not influence the release of enzymes from the liver significantly (Table 3). Only the activity of the lactate dehydrogenase appeared to be enhanced in the perfusate with increasing hydrogen ion concentration. To determine the error of the enzyme determinations which was introduced by the hemolysis we measured the enzyme activities in the erythrocytes per milligram hemoglobin and related them to the hemoglobin concentration found in the perfusate after centrifugation of the red cells at the end of the experiment. From this calculation the error appeared to be highest for the lactate dehydrogenase (approximately 5 per cent) and almost negligible for most of the other enzymes.

For comparison all enzyme activities were corrected for hemolysis and ageing (Table 3). However, these values can only serve as estimates, since the release of enzymes from the erythrocytes cannot be expected to parallel exactly the delivery of hemoglobin from the red blood cells.

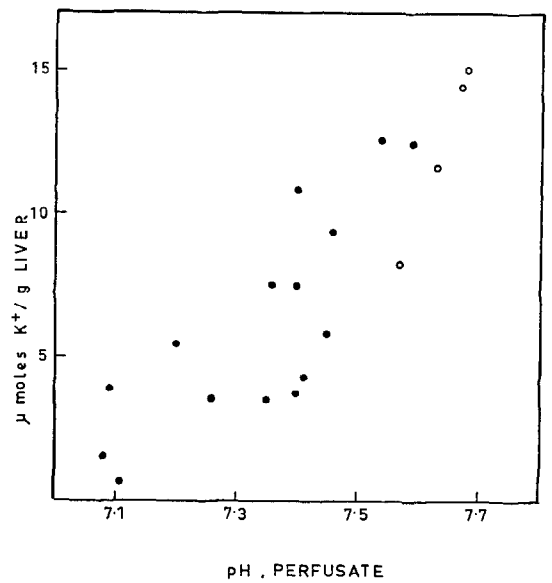

FIG. 4. The influence of the pH value on the uptake of potassium ions by the guinea pig liver between the 60th and the 120th min of the perfusion experiment. For further explanation see legend to Fig. 1.

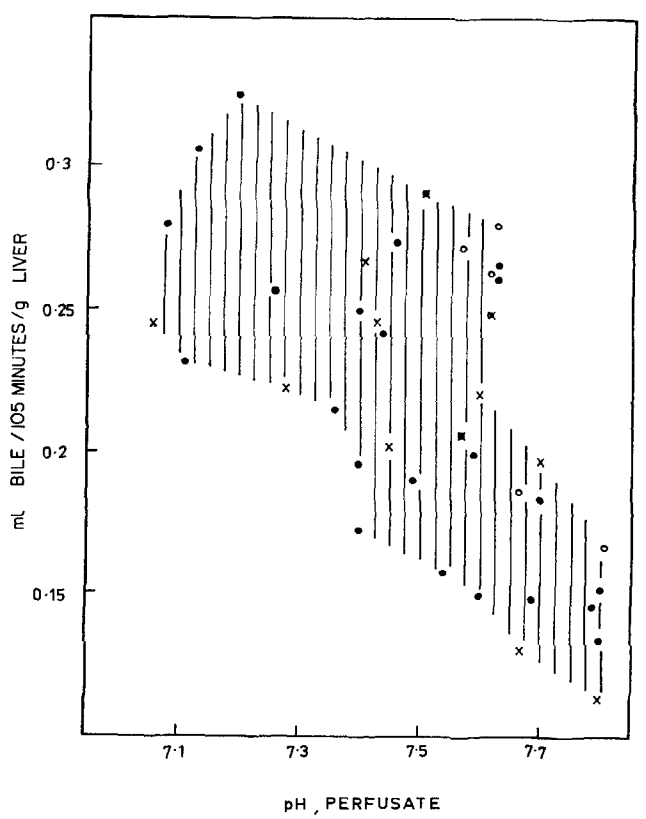

FIG. 5. The bile production at different extracellular hydrogen ion concentration between the 15th and the 120th min of the perfusion. For further explanation see legend to Fig. 1.

DISCUSSION

The ATP–ADP ratio was altered by the extracellular hydrogen ion concentration indicating that the energy potential of the liver cell might have been influenced. Rossi and Azzone[13] have demonstrated that in isolated mitochondria from rat liver the rate of ATP synthesis was markedly affected by the hydrogen ion concentration of the medium. The ATP formation was stimulated at pH 6·5 and inhibited at pH values above 7·5.

Although the ATP–ADP ratio was slightly reduced with increasing pH value, the glucose formation from alanine, which needs 6 μmoles ATP per 1 μmole glucose, was not influenced. However, the inhibition of gluconeogenesis by butylbiguanide strongly depended on the extracellular hydrogen ion concentration. So far, we have no explanation for this effect. An increasing pH value favours the monobasic ring formation[14] and reduces the dissociation of butylbiguanide ($pK_a = 11{\cdot}3$)[24] which both could facilitate the penetration of this compound into the liver cell. However, we do not know whether the hydrogen ion concentration influenced the penetration of biguanides into the liver cell or affected the cellular site of action of these compounds.

The inhibition of gluconeogenesis and the decrease of the ATP–ADP ratio with increasing pH value appeared to correlate in the presence of butylbiguanide. But so far, we cannot decide conclusively whether this correlation means a causal interrelationship as claimed by Altschuld and Kruger[15] according to Steiner's and Williams' original theory,[16] that the biguanides exert their hypoglycemic effect by reducing mitochondrial respiration. Recently, we reported experimental conditions under which the hepatic gluconeogenesis from lactate was inhibited by phenylethylbiguanide and the ATP–ADP ratio was not altered significantly.[6]

The perfused guinea pig liver took up potassium ions from the medium. According to Cashwell[17] and Graven *et al.*[18] isolated liver mitochondria accumulate potassium ions during oxidation of substrates and during aerobiosis. In analogy to these findings, the guinea pig liver could have lost intracellular potassium ions during the anoxic phase of the preparation. With the regeneration of the energy potential the cells might have restored their potassium pool.

The hepatic uptake of potassium ions increased with rising pH value. Similar effects were observed in isolated mitochondria and explained by the theory of Mitchell[19] with a proton exchange mechanism. A reduction of extramitochondrial hydrogen ions could facilitate the inflow of potassium ions into the mitochondria. Aldridge and Rose[20] assumed a coupling of oxidative phosphorylation with a potassium–hydrogen ion exchange. Also Cashwell[17] pointed out the possibility that the K^+ movement is associated with a H^+ countermovement and can "take place against the chemical gradient through utilization of energy derived from oxidizyable substrates or ATP". Rossi and Azzone[13] found the synthesis of ATP strongly dependent on the K^+_i–K^+_o ratio in isolated rat liver mitochondria. Above an external concentration of 3 mM K^+ the ATP formation was abolished. Addition of inhibitors of oxidative phosphorylation causes K^+ extrusion from isolated mitochondria.[17] Assuming that these conclusions are also applicable to the perfused organ, the driving force for the potassium uptake might be located in the mitochondria. This view could be supported by our finding that the uptake of potassium ions was inhibited by butylbiguanide under conditions which led to a decrease of the ATP–ADP ratio. However, so far

we cannot decide whether the uptake of potassium ions by the liver is regulated at the cellular or the mitochondrial membrane.

Since potassium ions usually are released from the erythrocytes during hemolysis, the true amount of these ions uptaken by the perfused liver can be expected to be even higher than has been calculated. Perfused livers from rats[21] and from pigs[22] release potassium ions into the medium according to several reports.

Acidification and alkalinization of the perfusate between pH 7·1 and 7·7 did not cause any severe cell damage, since the release of most enzymes examined was not accelerated. According to Schmidt *et al.*[23] the outflow of enzymes from the liver is a very sensitive sign for cellular alterations. We do not know whether the increased activity of the lactate dehydrogenase in the acidotic pH range had any significance. However, the ATP–ADP ratio and the low activity of the glutamate-pyruvate-transaminase and of the glutamate dehydrogenase in the perfusate are not consistent with severe alterations of the liver cell.

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