Phenethylbiguanide and the Inhibition of Hepatic Gluconeogenesis in the Guinea Pig

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1. Phenethylbiguanide inhibits the synthesis of phosphoenolpyruvate from malate or 2-oxoglutarate by isolated guinea-pig liver mitochondria. This inhibition is time- and concentration-dependent, with the maximum decrease in the rate of phosphoenolpyruvate synthesis (80%) evident after 10 min of incubation with 1 mM-phenethylbiguanide. 2. The phosphorylation of ADP by these mitochondria is also inhibited at increasing concentrations of phenethylbiguanide and there is a progressive increase in AMP formation. Guinea-pig liver mitochondria are more sensitive to this inhibition in oxidative phosphorylation caused by phenethylbiguanide than are rat liver mitochondria. 3. Simultaneous measurements of O₂ consumption and ADP phosphorylation with guinea-pig liver mitochondria oxidizing malate plus glutamate in State 3 indicated that phenethylbiguanide at low concentrations (0.1 mM) inhibits respiration at Site 1. At higher phenethylbiguanide concentrations Site 2 is also inhibited. 4. Gluconeogenesis from lactate, pyruvate, alanine and glycerol by isolated perfused guinea-pig liver is inhibited to various degrees by phenethylbiguanide. Alanine is the most sensitive to inhibition (60% inhibition of the maximum rate by 0.1 mM-phenethylbiguanide), whereas glycerol is relatively insensitive (25% inhibition at 4 mM). 5. Gluconeogenesis from lactate and pyruvate by perfused rat liver was also inhibited by phenethylbiguanide, but only at high concentrations (8 mM). Unlike guinea-pig liver, the inhibitory effect of phenethylbiguanide on rat liver was reversible after the termination of phenethylbiguanide infusion. 6. The time-course of inhibition of gluconeogenesis from the various substrates used in this study indicated a time-dependency which was related in part to the concentration of infused phenethylbiguanidine. This time-course closely paralleled that noted for the inhibition by phenethylbiguanide of phosphoenolpyruvate synthesis in isolated guinea-pig liver mitochondria.

The synthesis of glucose in mammalian liver involves a series of integrated reactions occurring in both the mitochondria and the cytosol. Gluconeogenesis requires the generation of NADH for a specific reduction step at the triose level of glycolysis as well as the synthesis of ATP within the mitochondria. Of particular importance in gluconeogenesis has been the use of inhibitors that specifically alter key reactions in the process. Biebuyck *et al.* (1972) have reported that halothane, a general anaesthetic, inhibits gluconeogenesis in rat liver, presumably by a direct action on the mitochondrial NADH dehydrogenase, as suggested by Cohen & Marshall (1968). Studies in our laboratory (Jomain-Baum *et al.*, 1973) using both the perfused rat and guinea-pig liver indicated that cycloheximide blocks gluconeogenesis from lactate, pyruvate and glycerol, when infused at concentrations (about 5 mM) several orders of magnitude higher than that required to inhibit protein synthesis. This compound was also shown to block energy

transfer at NADH dehydrogenase (Site 1) of the electron-transport chain (Garber *et al.*, 1973). Further, its action was rapid (2-4 min for 60% inhibition) and completely reversible. Another compound which inhibits hepatic glucose synthesis and is also hypoglycaemic is diphenyleneiodonium (Holland *et al.*, 1973). It was shown to block the oxidation of NAD⁺-linked substrates at Site I of the respiratory chain in a manner similar to rotenone.

This relationship between inhibitors of energy transfer and hepatic glucose synthesis is perhaps best exemplified by the widely used hypoglycaemic agent phenethylbiguanide. This compound acts most specifically in guinea-pig liver [see Cook *et al.* (1973a) for a discussion of species differences in response to phenethylbiguanide]. The mechanism by which this compound acts has been investigated and its hypoglycaemic effect may be related to its role as an inhibitor of energy transfer (Haeckel & Haeckel, 1972; Jangaard *et al.*, 1967; Davidoff, 1968a,b). In the present paper we have further investigated the

mechanism of phenethylbiguanide action on isolated guinea-pig liver mitochondria and have related our findings to the regulation of gluconeogenesis in the isolated perfused guinea-pig liver.

Experimental

Materials

Adenylate kinase (EC 2.7.4.3), β -hydroxybutyrate dehydrogenase (EC 1.1.1.30), glucose 6-phosphate dehydrogenase (EC 1.1.1.49), hexokinase (EC 2.7.1.1), lactate dehydrogenase (EC 1.1.1.27), pyruvate kinase (EC 2.7.1.40), malate dehydrogenase (EC 1.1.1.37), glutamate dehydrogenase (EC 1.4.1.3), phosphoenolpyruvate, NAD^+ , NADH and NADP^+ were obtained from Boehringer (Mannheim) Corp., New York, N.Y., U.S.A. Phenethylbiguanide was from K & K Laboratories Inc., Plainview, N.Y., U.S.A. and L-malate, 2-oxoglutarate, L-lactate, L-alanine and pyruvate were from Sigma Chemical Co. Ltd., St. Louis, Mo., U.S.A. The Fermcozyme used for the analysis of glucose was from Fermcolab, Chicago, Ill., U.S.A.

Isolation and incubation of mitochondria

Liver mitochondria from rats and guinea pigs were isolated by differential centrifugation in 0.32M-sucrose and 4mM-Hepes [2-(N-2-hydroxyethyl)piperazin- N' -yl]ethanesulphonic acid] buffer, pH 7.35. After sedimentation of the nuclei (700g for 10min) the mitochondria were separated by centrifugation at 12000g for 20min, then washed twice with 12vol. of the same buffer/g of liver used. Mitochondria (approx. 12mg of protein) were added to an incubation medium which contained, in a final volume of 3ml, 160mM-sucrose, 6.6mM- MgCl_2 , 20mM-potassium phosphate, 2mM-Hepes (pH 7.5), 3mM-ADP and the appropriate substrates at the concentrations indicated in the Figures. The incubation was stopped with HClO_4 (final concn. 6%, w/v), the tubes were chilled in ice and the extracts neutralized with KHCO_3 . The resultant precipitate was removed by centrifugation and the concentrations of the various metabolites were measured enzymically. Mitochondrial protein was determined by its u.v. absorption, with bovine serum albumin as a standard, by the method of Layne (1957).

For simultaneous studies of O_2 consumption and ADP phosphorylation, a cuvette based on a model described by Pressman (1967) and Chance & Hagihara (1963) was adapted for use in a dual-wavelength spectrophotometer. O_2 consumption was measured with a Clark-type oxygen electrode, and changes in pH were monitored with a Radiometer combination glass electrode with amplification of the resulting signal. These determinations were

performed simultaneously in a thermostatically controlled cuvette equipped with a stirring motor in which mitochondria were incubated at 25°C in 4.8ml of 0.25mM-sucrose buffer containing 5mM-potassium phosphate, 5mM- MgCl_2 and 3.2mM-Hepes, pH 7.5.

Determination of metabolites

The analysis of ATP, ADP, AMP, phosphoenolpyruvate, β -hydroxybutyrate, acetoacetate, lactate and pyruvate was performed by standard enzymic methods (Bergmeyer, 1965). Glucose was measured by using a Technicon Autoanalyzer with the glucose oxidase-peroxidase system (Hill & Kessler, 1961).

Liver perfusion

The animals used in this study were male Sprague-Dawley rats maintained on Wayne Lab Chow or guinea pigs of the Hartley strain fed on Wayne Guinea-Pig Chow. They were normally starved for 24 or 48h as indicated in the appropriate Figure legends. The liver-perfusion technique has been described in detail by Jomain-Baum *et al.* (1973). We used the liver-perfusion apparatus designed by Scholz (Scholz *et al.*, 1966) with a non-recycling perfusion system. During the perfusion we continually monitored the O_2 consumption of the liver with a Clark-type oxygen electrode and, as indicated in the various Figures, measured the hepatic output of glucose, acetoacetate and β -hydroxybutyrate. The concentration of the various substrates was 2mM and phenethylbiguanide was introduced into the liver with a constant-infusion pump to give the final concentration indicated in the Figure legends. For the measurement of adenine nucleotides the livers were freeze-clamped by using tongs pre-cooled in liquid N_2 . The tissue was powdered in liquid N_2 , then homogenized in 6% (v/v) HClO_4 and neutralized with KHCO_3 for the measurement of ATP, ADP and AMP by standard enzymic methods (Bergmeyer, 1965).

Results

The synthesis of phosphoenolpyruvate and ATP by isolated guinea-pig liver mitochondria metabolizing malate or 2-oxoglutarate was progressively inhibited by increasing concentrations of phenethylbiguanide over a range of 0.01–1mM (Fig. 1). The concentrations of AMP also increased, indicating that a portion of the ADP added was being converted into AMP by adenylate kinase. This lowered even further the net formation of ATP by oxidative phosphorylation by guinea-pig liver mitochondria in the presence of phenethylbiguanide. Mitochondria from

Fig. 1. Effect of phenethylbiguanide on the metabolism of guinea-pig liver mitochondria

Mitochondria (about 4 mg of protein/ml) were incubated at 30°C in 3 ml of 160 mM-sucrose, 2 mM-Hepes, 6.6 mM-MgCl₂ and 20 mM-potassium phosphate, pH 7.5, with either 5 mM-malate (●), or 5 mM-2-oxoglutarate (○). Phenethylbiguanide was used at different concentrations from 0 to 1 mM. The mitochondria were maintained for 20 min in State 4 (Chance & Williams, 1956), then placed in State 3 for 10 min by the addition of 3 mM-ADP. The incubations were stopped by addition of ice-cold HClO₄ and the chilled extracts were centrifuged and then neutralized before the enzymic determinations of metabolites. Bars denote $\pm$ S.E.M. for five determinations.

both guinea-pig and rat liver mitochondria were sensitive to phenethylbiguanide. At lower inhibitor concentrations (0.05 mM) guinea-pig liver mitochondria were more sensitive (23 versus 8% inhibition), whereas at higher concentrations mitochondria from both species appeared to be equally affected (60% inhibition). This point was noteworthy in view of the report by Cook *et al.* (1973b), indicating that phenethylbiguanide was rapidly metabolized

Fig. 2. Time-course of the inhibitory effect of phenethylbiguanide on oxidative phosphorylation by guinea-pig mitochondria

Mitochondria were incubated at 25°C in 5 ml of 0.25 M-sucrose containing 5 mM-MgCl₂, 3.2 mM-Hepes and 5 mM-potassium phosphate, pH 7.5, with either 5 mM-malate plus 5 mM-glutamate (----) or 5 mM-succinate (—). Phenethylbiguanide (0.2 mM) plus ADP (0.125 mM) were added at zero time and ADP alone at 10 and 20 min as shown by the arrows. Control additions of ADP with no phenethylbiguanide added were carried out after 0, 10 and 20 min of mitochondrial incubation. This was to ensure that the mitochondria maintained normal respiratory-control ratios of about 7 during the entire 20 min incubation period. The numbers on the slopes of the lines are: (a) rates of H⁺ uptake (nmol/min per mg of protein per ml); (b) O₂ consumption (ng atoms/min per mg of protein per ml).

by microsomal enzymes in rat but not guinea-pig liver. This difference therefore might only partly explain the reason for a greater sensitivity of guinea-pig liver to phenethylbiguanide, since isolated guinea-pig liver mitochondria were more sensitive to lower concentrations of this inhibitor. Phenethylbiguanide, at concentrations (0.1–0.2 mM) used in this study to block hepatic gluconeogenesis from lactate, acted specifically to inhibit the respiratory chain at Site 1. The time-course of inhibition is shown in Fig. 2 for experiments in which ADP phosphorylation (as measured by H⁺ uptake into the mitochondria and subsequent alkalization of the incubation medium) and O₂ consumption were measured simultaneously. Initially 0.2 mM-phenethylbiguanide did not inhibit either phosphorylation or O₂ consumption by guinea-pig liver mitochondria (zero time) metabolizing malate plus glutamate (dashed lines, Fig. 2), since the results obtained were the same as for control experiments without phenethylbiguanide (results not shown). With increasing time of preincubation in State 4

(Chance & Williams, 1956), the rates of both phosphorylation and O_2 consumption were markedly inhibited. Succinate oxidation (continuous line, Fig. 2), which is flavin-linked at Site 2, was unaffected by phenethylbiguanide. We noted, however, that increasing the concentration of the inhibitor to 0.5 mM decreased the time required for the inhibition with Site 1-linked substrates and also caused a 30% inhibition of O_2 consumption and phosphorylation of ADP with succinate as substrate (results not shown).

The effect of time and phenethylbiguanide concentration on mitochondrial metabolism is shown in Fig. 3, where we present the time-course of phenethylbiguanide inhibition of phosphoenolpyruvate formation by isolated guinea-pig liver mitochondria. At a phenethylbiguanide concentration of 0.05 mM, phosphoenolpyruvate formation from malate was decreased by 25% over a 20 min period of preincubation in State 4. Higher concentrations of the inhibitor caused a more rapid decline in the rate of phosphoenolpyruvate synthesis, so that at 0.5 mM-phenethylbiguanide there was an 80% inhibition.

Gluconeogenesis from lactate by guinea-pig liver was very sensitive to phenethylbiguanide at low concentrations. Fig. 4 shows the result of a typical non-recycling perfusion in which phenethylbiguanide was introduced into the liver with a constant-infusion pump to give a final concentration of 1 mM. The phenethylbiguanide was infused for 10 min and it took an additional 10 min before the inhibition of gluconeogenesis became evident. The depressive effect of phenethylbiguanide on glucose synthesis from lactate was irreversible, with the rate of gluconeogenesis not returning to normal values. O_2 consumption was also decreased by phenethylbiguanide. As noted by Haeckel & Haeckel (1972), phenethylbiguanide increased in the β -hydroxybutyrate/acetoacetate ratio as measured by the concentration of these ketone bodies in the perfusate coming from the liver, but this effect was noted immediately after the termination of phenethylbiguanide infusion. Table 1 shows that 10 min after the infusion of 0.1 mM-phenethylbiguanide the ATP/ADP ratio was lowered from 5.47 to 2.83 in the livers of guinea pigs perfused with 2 mM-lactate. Associated with this was a decrease in the total pool of adenine nucleotides. The reason for this decrease of 16% at 10 min and 31% at 25 min after phenethylbiguanide administration was not clear, but a similar finding had been reported by Holland *et al.* (1973), for the hypoglycaemic agent diphenyleneiodonium. Both gluconeogenesis and O_2 consumption by guinea-pig liver perfused with glycerol were relatively unaffected by 1 mM-phenethylbiguanide. However, the shift toward reduction in the mitochondrial oxidation-reduction state occurred with the same kinetics as found for lactate.

The inhibitory effect of phenethylbiguanide on hepatic gluconeogenesis by the guinea pig varied with the substrate infused. As shown in Fig. 5, a 10 min infusion of phenethylbiguanide at the concentrations indicated had various effects on gluconeogenesis from the major gluconeogenic substrates. Gluconeogenesis from alanine was the most sensitive and that from glycerol the least. The reason for the extreme sensitivity of gluconeogenesis from alanine by isolated guinea-pig liver was not clear, but at 1 mM-phenethylbiguanide, a concentration that caused a 40% inhibition of glucose synthesis from lactate, there was a virtual cessation of synthesis from alanine within 20 min after the termination of infusion of the inhibitor. In general, both the extent and time-course of inhibition of gluconeogenesis by phenethylbiguanide depended on the concentration of inhibitor used. This is shown in Fig. 6, where glucose synthesis from both lactate and alanine was measured 30 min after the start of the infusion of 1 mM-phenethylbiguanide for durations of infusion indicated in the Figure. The decline in hepatic gluconeogenesis from alanine was negligible after 1 min of phenethylbiguanide infusion but inhibition was almost total by 10 min. Gluconeogenesis from lactate is less sensitive to phenethylbiguanide, but the inhibition of glucose synthesis showed the same time-dependence. In experiments not presented in this paper, we noted that the continued infusion of 0.1 mM-phenethylbiguanide

Fig. 3. Effect of concentration and preincubation time with phenethylbiguanide on phosphoenolpyruvate formation by guinea-pig liver mitochondria

Mitochondria isolated from guinea-pig liver were incubated in the medium described in Fig. 1 with malate as substrate. Incubations were carried out as follows: preincubations in State 4 of different durations as indicated in the Figure followed by 10 min of incubation in State 3. Phenethylbiguanide was added at 0.05 mM (▲), 0.1 mM (■) and 0.5 mM (●).

Fig. 4. Effect of phenethylbiguanide on gluconeogenesis O₂ consumption and the ratio of β-hydroxybutyrate to acetoacetate in guinea-pig liver

Livers from 48h-starved guinea pigs were perfused with Krebs-Ringer bicarbonate buffer, pH 7.4, and at 30min either 2mM-lactate or -glycerol was infused. At 60min phenethylbiguanide was introduced with a constant-infusion pump to give a final concentration of 1 mM. The infusion of phenethylbiguanide was terminated after 10min (represented by vertical black blocks); lactate (a, c, e) or glycerol (b, d, f) was present for the entire 50min of perfusion. Glucose and ketone bodies were determined on samples of the perfusate as outlined in the Experimental section. The values for O₂ consumption and glucose synthesis were expressed as μmol/min per g of liver. Bars denote the S.E.M. for five animals.

will cause an almost total inhibition of gluconeogenesis from lactate within 40min. Thus both the time of exposure of the guinea-pig liver to phenethylbiguanide, as well as the concentration of the inhibitor

used, are important factors in determining the extent of the decrease in gluconeogenesis from the four substrates tested.

Rat liver is also sensitive to phenethylbiguanide.

Table 1. *Effect of phenethylbiguanide on the concentration of adenine nucleotides in perfused guinea-pig liver*

Isolated guinea-pig livers (four) were perfused with Krebs-Ringer bicarbonate buffer (Umbreit *et al.*, 1959), pH 7.4, with 2 mM-lactate as substrate by the procedure described in the Experimental section. Phenethylbiguanide (0.1 mM) was introduced into the liver for 10 min by a constant-infusion pump and the liver was freeze-clamped after phenethylbiguanide infusion at the times indicated in the Table. The procedure for extracting the frozen tissue is described in the Experimental section. Values are means $\pm$ S.E.M. for the four livers.

Time after phenethylbiguanide infusion (min)	Adenine nucleotide concn. (μ mol/g of liver)			ATP/ADP
	ATP	ADP	AMP	
0	2.38 ± 0.20	0.435 ± 0.003	0.104 ± 0.02	5.47
10	1.73 ± 0.05	0.612 ± 0.02	0.170 ± 0.02	2.83
25	1.34 ± 0.10	0.530 ± 0.07	0.153 ± 0.01	2.53

Fig. 5. Time-course of the inhibition of gluconeogenesis in perfused guinea-pig livers by various concentrations of phenethylbiguanide

The values are expressed as a percentage of the rate of gluconeogenesis from the various substrates immediately before the infusion of phenethylbiguanide. The concentration of the substrates was 2 mM and the experimental conditions are as outlined in Fig. 4. Each point is the mean of four or five separate liver perfusions. Substrates: (a) pyruvate; (b) alanine; (c) lactate; (d) glycerol. The average rate of glucose synthesis (μ mol/min per g of liver) by these livers before phenethylbiguanide infusion was 0.375 for lactate, 0.400 for pyruvate, 0.300 for alanine and 0.200 for glycerol.

Fig. 6. Time-course of the inhibitory effect of 1 mM-phenethylbiguanide on gluconeogenesis from lactate by perfused guinea-pig livers

Phenethylbiguanide (1 mM) was infused into the livers for the times indicated in the Figure and the rate of glucose formation from 2 mM-lactate (●) or -alanine (■) was measured 30 min later. This value is expressed as a percentage of the rate of glucose synthesis immediately before the infusion of phenethylbiguanide. Each point is the mean value from four separate livers. Experimental conditions are similar to those outlined in Fig. 4. The average control rate of glucose synthesis before the addition of phenethylbiguanide was $0.420 \mu\text{mol/min per g}$ of liver for lactate and $0.334 \mu\text{mol/min per g}$ of liver for alanine.

Gluconeogenesis from pyruvate and lactate was inhibited, but only at relatively higher phenethylbiguanide concentrations (8 mM), and as shown in Fig. 7, the effect was at least partly reversible after the infusion of the inhibitor was terminated. We noted that the constant infusion of high concentrations of this compound would maintain the lower rate of glucose synthesis from both substrates in perfused rat liver (results not shown).

Discussion

Phenethylbiguanide is a widely used hypoglycaemic agent and its mechanism of action at the cellular level has been intensively studied since Ungar *et al.* (1957) first reported its effectiveness in diabetes. There is general agreement that phenethylbiguanide shares with the guanidines the ability to interfere with oxidative metabolism. Early work by Wick *et al.* (1958) indicated an inhibition of glucose and acetate oxidation by adipose tissue, and this effect was attributed to a block in the electron-transport chain between succinate dehydrogenase and a reduced cytochrome *c*. Since then a number of other demonstrations of phenethylbiguanide inhibition of respiration have been published [see Harsant (1969)

Fig. 7. Effect of phenethylbiguanide on gluconeogenesis from lactate or pyruvate by perfused rat liver

Animals were starved for 24 h and the livers isolated and perfused with 2 mM-lactate (a) or -pyruvate (b) as outlined in Fig. 4. Phenethylbiguanide at the concentrations indicated in the Figure was infused for 10 min and the rates of glucose synthesis are expressed as a percentage of the rate immediately before phenethylbiguanide infusion. Each point is the mean of four separate liver perfusions. The average control rate of glucose synthesis before the infusion of phenethylbiguanide was $0.556 \mu\text{mol/min per g}$ of liver for lactate and $0.368 \mu\text{mol/min per g}$ of liver for pyruvate.

and Jangaard *et al.* (1967) for reviews of these studies], but the exact site of action of phenethylbiguanide still remains uncertain. For example, Cook *et al.* (1973b) suggested, from studies with perfused rat liver, that phenethylbiguanide blocks hepatic gluconeogenesis by a mechanism not directly related to energy requirements for this process; this, despite the fact that phenethylbiguanide, at the concentrations which inhibited gluconeogenesis, also lowered the ATP/ADP ratio in the perfused liver. On the other hand, Jangaard *et al.* (1967) concluded that a 'strong case can be made that the biguanides act by blocking energy transfer, presumably at the cytochrome *b* site of the electron-transport chain, and that their effects on metabolism are a secondary consequence of this primary action'.

An important question that remains to be answered is whether the action of phenethylbiguanide on energy metabolism in isolated mitochondria reflects the site

of inhibition of this compound *in vivo*. Davidoff (1968*a,b*, 1971, 1972) has extensively studied the mechanism of phenethylbiguanide accumulation and its relationship to respiratory inhibition *in vitro*. He demonstrated that phenethylbiguanide accumulates in guinea-pig heart mitochondria at a slow rate in part dependent on the affinity of the inhibitor for anionic binding sites (Davidoff, 1971). The uptake of ^3H -labelled phenethylbiguanide was also shown to be sensitive to pH changes in the mitochondrial incubation medium and to correlate well with the extent of respiratory inhibition. Also small amounts of long-chain fatty acids caused the transport of phenethylbiguanide in the absence of any effect on energy coupling or intramitochondrial pH. Schäfer & Bojanowski (1972) reported that biguanides bind to the mitochondrial membrane in a rapid reaction, which was ten times faster than the observed inhibition of oxidative phosphorylation. During this initial binding phase, protons were released from the mitochondrial membrane. The affinity for binding was determined by the degree of hydrophobic interaction of the biguanides and the mitochondrial membrane, presumably with membrane phospholipids. These observations demonstrate the complex regulatory factors that control the rate of phenethylbiguanide uptake by mitochondria *in vitro*.

The association between the direct effect of phenethylbiguanide on isolated mitochondria and the inhibitory effect of this compound on gluconeogenesis in the intact liver is suggested by the almost identical time-course of inhibition noted in the two systems. There is also a concentration-dependent lag in the effective inhibition of phosphoenolpyruvate formation by guinea-pig liver mitochondria which corresponds to a lag in phenethylbiguanide inhibition of gluconeogenesis by the perfused liver. A similar delayed inhibition of glucose synthesis by rat liver perfused with 0.5 mM-phenethylbiguanide was reported by Toews *et al.* (1970), who noted a 15–20 min lag period with lactate, pyruvate or alanine as substrates. We also found a difference in the degree of sensitivity of these substrates to phenethylbiguanide (see Figs. 5 and 7) and the lag time in the inhibition of gluconeogenesis is also different. This is clearly demonstrated in Fig. 6, where the infusion of 1 mM-phenethylbiguanide for 5 min is twice as effective in inhibiting glucose synthesis from alanine as compared with lactate.

To simplify our considerations of the hypoglycaemic effect of phenethylbiguanide we have assumed that its action is caused by a single primary effect on the liver. This does not rule out other possible effects of phenethylbiguanide on other tissues such as intestinal mucosa (Kruger *et al.*, 1970; Agid & Marquié, 1972; Wingate & Hadley, 1973) or muscle (Butterfield, 1968). However, the liver is particularly sensitive to this inhibitor and from our studies we

can demonstrate a direct effect of phenethylbiguanide on hepatic mitochondria. Since phenethylbiguanide does block energy transfer at Site 1 of the electron-transport chain, can we link this effect to its more general inhibition of gluconeogenesis? One argument in favour of this is the fact that other specific Site 1 inhibitors, such as cycloheximide (Jomain-Baum *et al.*, 1973), diphenyleneiodonium (Holland *et al.*, 1973) or halothane (Biebuyck *et al.*, 1972), all inhibit gluconeogenesis, lower ATP formation and cause a shift in the mitochondrial NAD^+/NADH ratio toward reduction. Metabolic alterations of this type can have far-reaching effects, especially in the guinea pig. A shift in mitochondrial oxidation-reduction state toward reduction is known to lower the rate of phosphoenolpyruvate formation by isolated guinea-pig liver mitochondria (Garber & Hanson, 1971) and to be associated with a decreased rate of gluconeogenesis in perfused guinea-pig liver (Arinze *et al.*, 1973). Haeckel & Haeckel (1972) were the first to demonstrate that phenethylbiguanide can markedly shift the mitochondrial oxidation-reduction state of guinea-pig liver toward reduction. Glucose formation from alanine would be especially sensitive to alterations in the mitochondrial NAD^+/NADH ratio, since the formation of 2-oxoglutarate from glutamate by glutamate dehydrogenase would be unfavourable for net 2-oxoglutarate synthesis. This may explain the greater sensitivity of alanine to the action of low concentrations of phenethylbiguanide, and its effect on conversion of pyruvate into glucose further supports this point. However, concentrations (0.1 mM) of phenethylbiguanide that cause a 60% inhibition of gluconeogenesis from alanine by 30 min of infusion also cause a small but significant stimulation of gluconeogenesis from pyruvate (see Fig. 5). Other compounds such as glycerol, which are less clearly influenced by changes in the mitochondrial oxidation-reduction state, are also less sensitive to the infusion of phenethylbiguanide.

It has been known for some time that phenethylbiguanide is relatively ineffective as a hypoglycaemic agent in the rat (Altschuld & Kruger, 1968). This contrasts with the situation in the human (Pomeranyc *et al.*, 1957) and in the guinea pig. Cook *et al.* (1973*a*) have reported that phenethylbiguanide is metabolized by rat liver microsomal preparations to *p*-hydroxyphenethylbiguanide, a metabolically inactive compound, to a greater extent than was observed with guinea-pig liver. If microsomal hydroxylation was inhibited by the infusion of 2,4-dichloro-6-phenylphenoxyethyl-diethylamine hydrobromide (Lilly 18947), gluconeogenesis by rat liver was inhibited by phenethylbiguanide. Although this finding undoubtedly accounts for the major differences in species reactivity to phenethylbiguanide, it is also evident that mitochondria isolated from

guinea-pig liver are more sensitive to the inhibitor than are rat liver mitochondria. The rate of ATP formation by guinea-pig liver mitochondria is inhibited at a lower concentration of phenethylbiguanide than was found for rat liver mitochondria respiring with malate plus glutamate. We have demonstrated (Fig. 7) that gluconeogenesis in rat liver perfused with lactate or pyruvate can be inhibited by phenethylbiguanide, if the concentration of the inhibitor is raised to a high value (8mM). In these studies a single 10min infusion of phenethylbiguanide was used and the effects on conversion of both pyruvate and lactate into glucose was reversible. It then seems likely that phenethylbiguanide will effectively inhibit gluconeogenesis from most substrates in both rat and guinea-pig liver, if the concentration of the inhibitor is raised to values that effectively overcome the mechanisms that remove it, presumably its hydroxylation by the hepatic microsomal preparations. It is also clear that Site 1 inhibitors such as phenethylbiguanide, cycloheximide and diphenyleneiodonium are effective gluconeogenic inhibitors in both rat and guinea-pig liver. The shift in oxidation-reduction state of the mitochondria toward reduction noted in both species is probably not responsible for the dramatic fall in glucose synthesis noted in guinea-pig liver, but rather some, as yet not clearly understood, relationship between ATP formation at Site 1 and hepatic gluconeogenesis.

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