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Effects of Guanidine Derivatives on Mitochondrial Function

I. PHENETHYLBIGUANIDE INHIBITION OF RESPIRATION IN MITOCHONDRIA FROM GUINEA PIG AND RAT TISSUES

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ABSTRACT Derivatives of guanidine, such as phenethylbiguanide, are potent inhibitors of mitochondrial respiration in vitro, but the relevance of this inhibition to their in vivo blood sugar–lowering action is not clear. We have studied the metabolism of pyruvate and long chain fatty acids by mitochondria from several tissues of guinea pigs and rats and observed the effects of phenethylbiguanide on these processes. The rate of pyruvate decarboxylation and of β-oxidation of long chain fatty acyl-CoA derivatives by guinea pig heart mitochondria in vitro has been found to exceed the flux of substrate through the citric acid cycle, both in the presence and absence of phosphate acceptor.

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INTRODUCTION

Guanidine derivatives are potent inhibitors of coupled respiration in mitochondria in vitro (1). The therapeutically useful hypoglycemic properties of these agents have been ascribed to increased anaerobic glycolysis resulting from mitochondrial inhibition in vivo (2), analogous to the increase in glycolysis induced by other respiratory inhibitors or anaerobiosis itself (3). Support for this hypothesis has been lacking, however, since the hypoglycemia obtained with relatively low doses of phenethylbiguanide in animals (4, 5) and in humans (5, 6) is not necessarily accompanied by an increase in lactate : pyruvate ratio or of serum lactate, nor is there evidence that the tissues of biguanide-treated subjects are functionally hypoxic (7). A further objection to the respiratory inhibition hypothesis stems from the observation that many other pharmacologic agents which in vitro resemble guanidine derivatives in their ability to inhibit mitochondrial respiration have no hypoglycemic activity in the whole animal (8). In addition, the increase in sugar uptake by the intact diaphragm induced by phenethylbiguanide does not exhibit the same specificity with regard

to sugar structure as the increase induced by other respiratory inhibitors (9).

Several alternative hypotheses, such as specific inhibition of gluconeogenesis in liver (7, 10) and kidney (10) or of pyruvate decarboxylation in heart (11) and adipose tissue (12), rather than nonspecific tissue anoxia, have been proposed as primary metabolic events which may be relevant to the hypoglycemic action of guanidine derivatives in vivo. Although the alkyl monoguanidines are specific inhibitors of NAD-linked mitochondrial oxidations, phenethylbiguanide inhibits at the second mitochondrial energy conservation site, (13) and, therefore, at the mitochondrial level appears to be a relatively nonspecific inhibitor in terms of substrate (14). Furthermore, mitochondria from different tissues within the same species appear to be about equally sensitive to respiratory inhibition in vitro (8), suggesting that if tissue specificity of inhibition exists in vivo, it is due to metabolic factors other than intrinsic sensitivity.

Extrapolation from the in vitro effects of biguanides on mitochondria to in vivo mechanisms is further complicated by the discrepancy between the high levels of drug generally required to achieve inhibitory effects in vitro (2, 8) and the much lower tissue levels actually achieved in vivo (11, 15, 16). Widely varying species sensitivity to the hypoglycemic actions of the biguanides, although not widely commented upon, probably provides much of the explanation for this apparent discrepancy (4, 8, 17). The basis for this species variation has not been explored in depth, although variations in drug metabolism have been demonstrated in vivo (16), and variation among species in sensitivity to respiratory inhibition in vitro seems to parallel in vivo sensitivity to the hypoglycemic action of the same drug (8). The greater sensitivity of diabetics than of normals to the hypoglycemic action of biguanides (18) remains unexplained.

The current studies were carried out to explore in more detail the effects of guanidine derivatives on mitochondrial oxidation of the major metabolic fuels, pyruvate and fatty acids. Limited studies of biguanide inhibition of pyruvate oxidation in cell-free systems have been reported previously with respirometric techniques alone (8), but effects on fatty acid oxidation have not been examined. Guinea pig tissues were chosen for the bulk of these studies because of the exquisite sensitivity of this species to the hypoglycemic activity of guanidines (4). It was reasoned that in vitro effects obtained at drug levels approaching the very low concentrations known to be achieved in vivo might justifiably be implicated in the mechanisms of hypoglycemia in the intact animal.

These studies demonstrate that under the proper in vitro conditions phenethylbiguanide significantly inhibits mitochondrial respiration at concentrations very close to those found to accompany hypoglycemia in vivo. Furthermore, when pyruvate and long chain fatty acyl-CoA are the substrates, the inhibition of respiration and phosphorylation which is achieved appears to be nonspecific with regard to substrate or oxidative reaction. However, oxidation of long chain free fatty acids is resistant to inhibition by phenethylbiguanide. The reasons for this resistance to inhibition of long chain free fatty acid oxidation are explored in detail in the accompanying paper (19).

METHODS

Preparation of mitochondria. English strain guinea pigs and CD strain albino rats were obtained from local breeders. Animals were decapitated and exsanguinated under ether anesthesia and the tissues chilled in 0.25 M sucrose–0.5 mM Na_2 ethylenediaminetetraacetate (EDTA)–triethanolamine HCl buffer, pH 7.2. All subsequent procedures were carried out at 0–2°C. Tissues were put through the coarse seive of a stainless steel tissue press (Harvard Apparatus Co., Inc., Dover, Mass.). Heart and skeletal muscle were then homogenized in 5 volumes of buffer in a Dounce homogenizer with a loose-fitting pestle (Blaessig Glass Specialties, Rochester, N. Y.) in the presence of Nagarse bacterial proteinase (Enzyme Development Corporation, New York) (20). The homogenate was incubated for 10 min at 0°C, rehomogenized, diluted 5-fold with buffer, and centrifuged at 600 *g*. Mitochondria were then collected by centrifuging the supernatant at 5000 *g* and washing the pellet once by resuspension in the homogenizer and recentrifugation. Finally mitochondria were suspended until used in 0.1 M sucrose–0.5 mM Na_2EDTA–0.15 M KCl and the protein concentration adjusted to a known dilution of 5.5 mg of protein per ml. Liver was homogenized directly in 10 volumes of isolation medium, nuclei and cell debris were removed by low speed centrifugation, and the mitochondrial pellet was washed three times and suspended in sucrose-EDTA-KCl medium at a protein concentration of approximately 10 mg/ml. Mitochondrial protein was estimated by dissolving an aliquot of the suspension in 30% formic acid and measuring the optical density at 280 and 260 mμ (21).

Incubation techniques. Substrates, cofactors, and in-

hibitors were dissolved in sucrose-EDTA-KCl medium and pH adjusted to 7.2 with KOH. Sodium pyruvate and palmitylcarnitine solutions were prepared fresh daily. ^{14}C-labeled pyruvate solutions were stabilized according to the method of von Korff (22) and stored frozen in aliquots at $-10°C$. These solutions were not used after storage for longer than 4–6 wk.

Oxygen uptake was measured in a Warburg respirometer in 6-ml flasks, with 10% KOH in the center wells. For experiments not involving collections of $^{14}CO_2$, folded filter papers were placed in the center wells containing alkali. When $^{14}CO_2$ was to be collected, the filter paper was omitted, and after completion of the incubation, the alkali was transferred quantitatively to the main compartment of a small center well flask containing 1 ml of water. 0.2 ml of 1 M Hyamine hydroxide base [*p*-(diisobutylcresoxy-ethoxyethyl) dimethylbenzyl ammonium hydroxide] in methanol was then placed in the center well, the flask sealed with a rubber serum cap, and 0.4 ml of 4 N HCl injected through the rubber diaphragm into the main compartment of the flask. The flasks were then shaken at 37°C for 45 min, and the Hyamine-$^{14}CO_2$ counted in a scintillator solution consisting of 6% naphthalene, 0.4% 2,5-diphenyloxazole, and 0.02% 1,4-bis[2-(5-phenyloxazolyl)]benzene in methanol: ethylene glycol-*p*-dioxane (5:1:44) in a scintillation spectrometer; corrections were made for quenching. Incubations were also performed without enzyme, and corrections made for the small, nonenzymatic release of radioactivity. Control experiments indicated that with the respiratory rates and substrates used in these experiments, omission of filter paper from the center wells did not affect the apparent oxygen uptake rate.

For measurement of the water-soluble radioactive products of fatty acid oxidation, the reaction was stopped with 0.1 ml of 25% perchloric acid, the precipitated proteins and coprecipitated fatty acids centrifuged off, and an aliquot of the supernatant counted.

In most experiments with guanidine derivatives a preincubation was carried out which was timed from the immersion of the flasks in the 37°C bath until the tipping in of hexokinase from the side arms. Approximately 8 min was then required for oxygen uptake to become linear, at which point the first (zero time) reading was taken. In most experiments identical duplicate or triplicate flasks were incubated to determine each experimental value and oxygen uptake, or $^{14}CO_2$ evolution data were rejected if results from paired flasks did not agree within 10%.

Biochemicals. Substrates and cofactors were obtained from commercial sources and were of the highest purity available. Hexokinase (type VI, specific activity 1300 Kunitz-McDonald Units/mg) (23) was obtained from Sigma Chemical Co., St. Louis, Mo. Fatty acids were analyzed as the methyl esters by gas-liquid chromatography (GLC) before use and were at least 95% pure. Phenethylbiguanide hydrochloride was obtained from the Aldrich Chemical Co., Milwaukee, Wis. and was found to be chromatographically homogenous by thin-layer chromatography on silica gel in acid and alkaline solvent systems; it contained about 0.1% of an uncharacterized impurity by chromatography on Dow 50 H^+ (16) and analysis by paper chromatography. Respirometric studies using phenethylbiguanide freed of this impurity by ion-exchange chromatography on Dow 50 H^+ gave identical results with those using the unpurified material. Radiochemicals were obtained from New England Nuclear Corp., Boston, Mass.

Fatty acid–free bovine serum albumin was prepared by the method of Goodman (24), brought to pH 7.5 with KOH, and dialyzed exhaustively against distilled water before use. The molecular weight of albumin was assumed to be 66,000. Fatty acyl-CoA derivatives were prepared by the mixed anhydride method (25), and the products were assayed as described previously (26) by measurement of optical density at 262 and 232 mμ and by hydrolysis and quantitative analysis for fatty acid by GLC.

Palmitylcarnitine was synthesized by the method of Brendel and Bressler (26) and purified by repeated precipitation from *n*-butanol–diethyl ether. Quantitative analysis for fatty acid was carried out by hydrolysis and GLC of the methyl ester of palmitic acid. Thin-layer chromatography of the final product on alumina gave a single spot with an R_f of 0.9. (27).

Fatty acid albumin complexes were prepared by adding a warm, slightly alkaline solution of the sodium or potassium salt of the fatty acid to the requisite amount of fatty acid–free albumin in solution. The resulting solutions were optically clear.

RESULTS

Oxidation of pyruvate by guinea pig heart mitochondria. When respiration of guinea pig heart mitochondria isolated under the gentle conditions outlined in Methods was measured with pyruvate as substrate, oxygen uptake was linear for at least 1 hr and continued until inorganic phosphate or substrate was exhausted. Respiratory rate was a function of mitochondrial concentration and of amount of hexokinase present. In most of the experiments to be described hexokinase was present in excess; under these conditions, the respiratory control ratio with pyruvate was always in excess of 8 and frequently in the range of 14–18 or higher, as in the experiment included in Table I. (The respiratory control ratio represents the ratio of oxygen uptake rate in the presence of phosphate acceptor, in this case the hexokinase–glucose adenosine diphosphate (ADP)-generating system, or state 3, to the rate in the absence of phosphate acceptor, state 4 [28]).

When pyruvate-2-^{14}C was employed as substrate, the ratio of $^{14}CO_2$ evolved during state 3 respiration to that evolved in state 4 was higher

TABLE I

Oxidation of Pyruvate-1-^{14}C and -2-^{14}C to $^{14}CO_2$ by Guinea Pig Heart Mitochondria: Inhibition by Phenethylbiguanide and Malonate

Substrate	Metabolic state*	Inhibitor		Oxygen uptake		$^{14}CO_2$ produced	
		Phenethylbiguanide	Malonate				
		Concentration (M)		*μatoms*	*% of state 3 control*	*μmoles*	*% of state 3 control*
Pyruvate-1-^{14}C	State 4	0	—	0.3	4	0.19	9
Pyruvate-1-^{14}C	State 3	0	—	8.4	100	2.10	100
Pyruvate-1-^{14}C	State 3	4×10^{-5}	—	4.1	49	1.02	62
Pyruvate-2-^{14}C	State 4	0	—	0.3	4	0.013	1
Pyruvate-2-^{14}C	State 3	0	—	7.7	100	1.30	100
Pyruvate-2-^{14}C	State 3	4×10^{-5}	—	2.8	36	0.38	29
Pyruvate-1-^{14}C	State 3	—	0	7.9	100	2.2	100
	State 3	—	1.6×10^{-3}	2.3	29	1.2	53
Pyruvate-2-^{14}C	State 3	—	0	8.4	100	1.45	100
	State 3	—	1.6×10^{-3}	2.9	35	0.10	7

Each incubation flask contained 4×10^{-3} M adenosine triphosphate (ATP), 5×10^{-3} M $MgCl_2$, 0.1 M glucose, 8×10^{-3} M phosphate buffer, pH 7.2, 3.6×10^{-3} M pyruvate, 3×10^{-4} M fumarate, 0.45×10^{-4} M fatty acid–free bovine serum albumin, 0.1 M sucrose, 0.15 M KCl, 0.5×10^{-4} M Na_2 ethylenediaminetetraacetate (EDTA), either 36,500 cpm of pyruvate-1-^{14}C or 48,500 cpm of pyruvate-2-^{14}C, specific activities 3.49 and 3.37 mc/mmole respectively, and 0.27 mg of mitochondrial protein in a total volume of 1.15 ml. Phenethylbiguanide and malonate were present in the main compartment. After preincubation for 15 min, 0.1 ml of 0.35 M potassium phosphate buffer, pH 7.2, with or without 6 μg of hexokinase was added from the side arm, and measurements of $^{14}CO_2$ evolution and oxygen uptake were carried out for 40 min at 37°C. as described in Methods. Zero time control flasks were prepared in parallel and incubated in identical fashion up to the time of the first reading. The $^{14}CO_2$ contained in the center wells was then determined and this figure used to correct the experimental flasks in calculating the final results.

* Metabolic state as defined by Chance and Williams (28). State 4 represents respiration of a tightly coupled mitochrondrial system in the absence of phosphate acceptor (adenosine diphosphate (ADP) generating system); state 3, respiration in the presence of phosphate acceptor.

than the over-all respiratory control ratio (Table I). The ratio of $^{14}CO_2$ evolved from pyruvate-1-^{14}C in these two metabolic states was closer to the overall respiratory ratio, suggesting that pyruvate decarboxylation may be less tightly controlled than is the function of the tricarboxylic acid cycle. As a result, in both state 4 and in state 3, with the rate of ADP generation at a maximum, the rate of pyruvate decarboxylation exceeded that of oxidation of acetyl-CoA derived from pyruvate, as measured by production of $^{14}CO_2$ from pyruvate-2-^{14}C. In all of these experiments, the oxygen uptake as measured manometrically was accounted for nearly stoichiometrically by the total pyruvate oxidized to $^{14}CO_2$ and to acetate plus the oxidation of added fumarate via malate to oxaloacetate.

Effect of phenethylbiguanide on pyruvate oxidation. When phenethylbiguanide was added to the incubation mixture at zero time, i.e. at the time of initiation of state 3 respiration with hexokinase, no effect on oxygen uptake was discernible for about 20 min. At this point, inhibition of respiration became increasingly evident (Fig. 1). In contrast, phenethylbiguanide added during the state 4 preincubation produced maximal inhibition from the time of initiation of state 3

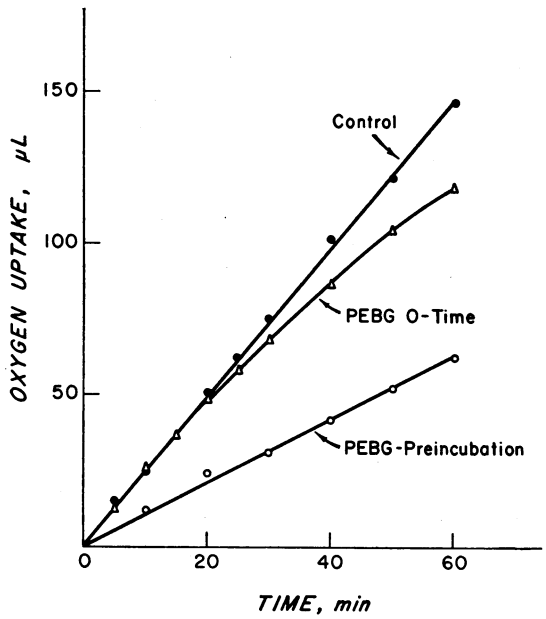

FIGURE 1 Inhibition of pyruvate oxidation by phenethylbiguanide (*PEBG*) added at different times. Conditions of incubation were as described in Table I except that in the experiment marked *PEBG 0-time*, the phenethylbiguanide was present in the side arm and was added with the phosphate buffer and hexokinase. Phenethylbiguanide was present in a concentration of 6×10^{-5} mole/liter.

respiration, and oxygen uptake rate was linear thereafter. It should be pointed out that the respiratory rate after the delayed inhibition finally approximated that of inhibition induced during state 4. The data in Fig. 1 also serve to illustrate that the over-all inhibition of respiration of mitochondria in hexokinase-glucose–induced state 3 is not due to the inhibition of the hexokinase reaction, since preincubation of hexokinase in the side arm with a relatively high concentration of phenethylbiguanide did not inactivate the enzyme.

It was further noted that the presence of fatty acid–free serum albumin markedly potentiated the inhibitory effects of phenethylbiguanide on oxidation of pyruvate, in contrast to the protective effects of albumin against the inhibitory and uncoupling effects of the halophenols (29). In the presence of as little as 1 mg/ml of fatty acid–free bovine serum albumin, 50% inhibition of respiration was consistently achieved at about $3–5 \times 10^{-5}$ M phenethylbiguanide (Fig. 2). In contrast, in the absence of albumin, the initial rate of oxygen uptake was less affected by phenethylbiguanide, a difference of approximately 5-fold (Figs. 2 and 3).

In addition, the rate of oxygen uptake in the absence of albumin was frequently not constant with time, a secondary increase in rate being observed after 15–30 min, the final slope then approximating the control rate. Control experiments with varying amounts of albumin indicated no significant inhibitory effects of albumin alone; increasing the concentration of albumin above about 1 mg/ml had no further potentiating effect on phenethylbiguanide inhibition. In the presence of serum albumin, phenethylbiguanide, in concentrations which inhibited oxygen uptake by 50–70%, inhibited both the decarboxylation of pyruvate to acetyl-CoA and the subsequent oxidation of the 2-carbon fragment in the citric acid cycle (Table I). Each of these processes was inhibited in proportion to its activity in the uninhibited state. Thus the rate of decarboxylation of pyruvate consistently exceeded that of oxidation of acetyl-CoA in the inhibited state 3 as it did in both uninhibited state 3 and state 4. This finding indicates that over-all inhibition of citric acid cycle activity by phenethylbiguanide in vivo or in whole tissues probably does not result from direct, selective

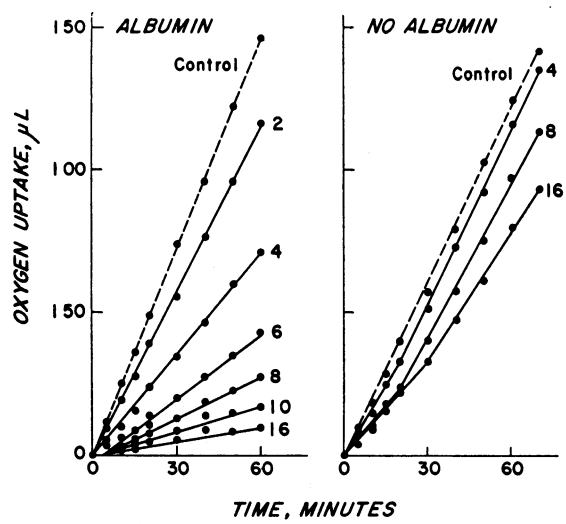

FIGURE 2 Inhibition of pyruvate oxidation by increasing concentrations of phenethylbiguanide in the presence and absence of fatty acid–free serum albumin. Conditions of incubation were identical with those described in Table I except that albumin was omitted where indicated; flasks with albumin contained 0.6×10^{-4} M fatty acid–free bovine serum albumin. Phenethylbiguanide was present in the main compartment during the 15 min preincubation; phenethylbiguanide concentration is indicated in the figure, expressed as concentration $\times 10^{5}$ moles/liter.

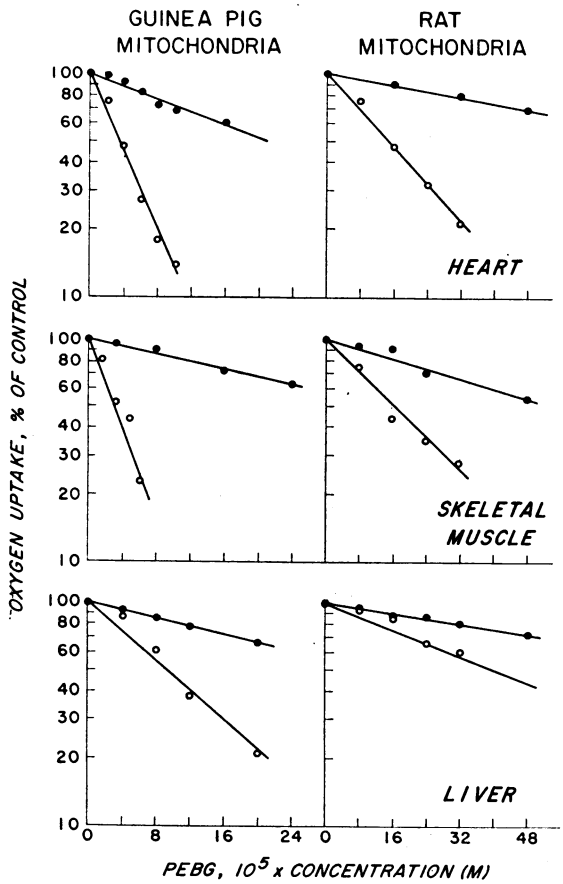

FIGURE 3 Inhibition of mitochondrial respiration by phenethylbiguanide in the presence and absence of albumin. Conditions of incubation were the same as in Table I except that flasks with albumin contained $0.6–0.7 \times 10^{-4}$ M fatty acid–free bovine serum albumin. Flasks with heart and skeletal muscle mitochondria contained 0.27 mg of mitochondrial protein and 3 μg of hexokinase per flask.

Data for guinea pig heart are replotted from Fig. 2; the values shown were calculated for the oxygen uptake during the first 30 min; some data is included from this experiment which was not presented in Fig. 2. Data for other mitochondria were calculated from oxygen uptake over either 40 or 50 min, during which time uptake was linear. Open circles represent flasks with albumin; closed circles represent flasks without albumin. Note the difference in scales of phenethylbiguanide concentration on the abscissa for guinea pig and rat mitochondria.

inhibition of pyruvate decarboxylation to the point at which it becomes rate limiting for over-all pyruvate oxidation.

However, the possibility still existed that the primary action of the drug might be to selectively inhibit the citric acid cycle; since coenzyme A is a necessary cofactor for pyruvate decarboxylation, continued decarboxylation of pyruvate in the absence of citric acid cycle activity could lead to increasing sequestration of coenzyme A in the pool of acetyl-CoA, and eventually to secondary inhibition of pyruvate decarboxylation (30). That this effect is probably not operative in the mechanism of state 3 phenethylbiguanide inhibition of pyruvate oxidation is demonstrated by the data in Table I. Thus when the tricarboxylic acid (TCA) cycle was inhibited by over 90% with malonate, a substrate-level inhibitor, the resultant secondary inhibition of pyruvate decarboxylation was only 45%. In contrast, the same degree of inhibition of pyruvate decarboxylation was achieved with phenethylbiguanide despite a much smaller degree of TCA cycle inhibition. Phenethylbiguanide inhibition appears, therefore, to affect directly all the coupled reactions of pyruvate in proportion to their relative rates in the uninhibited state, such that with increasing over-all inhibition, the rates and patterns of reactions approach those present in uninhibited state 4.

Effect of phenethylbiguanide on oxidation of pyruvate by other guinea pig tissues. Phenethylbiguanide inhibited pyruvate oxidation by guinea pig skeletal muscle mitochondria at concentrations very similar to those effective in heart muscle mitochondria, and the potentiating effect of albumin was fully as striking (Fig. 3). In contrast to heart muscle mitochondria, oxidation of pyruvate by guinea pig liver mitochondria was not entirely linear with time, and somewhat higher concentrations of phenethylbiguanide were needed to achieve inhibition than with muscle mitochondria. In the presence of serum albumin approximately 1×10^{-4} M phenethylbiguanide was required to inhibit respiration by 50%, while in the absence of serum albumin $3–4 \times 10^{-4}$ M phenethylbiguanide was needed. It should be noted that the Qo_2 of liver mitochondria for pyruvate oxidation in the presence of an excess of hexokinase was approximately one-tenth as great as the Qo_2 for muscle mitochondria. The smaller inhibitory effect of phenethylbiguanide for liver mitochondria may be related to the lower Qo_2, since the effectiveness of respiratory inhibition generally increases with the rate of electron flow (14).

Although respiratory control with liver mitochondria was not as great as with mitochondria from other tissues, the proportions of pyruvate

decarboxylated and oxidized fully to CO_2 by liver mintochondria resembled those with heart mitochondria. Phenethylbiguanide in a concentration of 1.6×10^{-4} mole/liter inhibited citric acid cycle activity and pyruvate decarboxylation approximately in proportion to their relative activities in the uninhibited state.

Effect of phenethylbiguanide on pyruvate oxidation by rat tissues. Rats are much less sensitive than are guinea pigs to the hypoglycemic action of biguanides (4). Previous studies have indicated that mitochondria from rat tissues are correspondingly about five times less sensitive to the in vitro inhibitory action of phenethylbiguanide (8), suggesting that differences in intrinsic tissue sensitivity, in addition to differences in pharmacologic disposition of the drug, may constitute the basis for the in vivo differences in species sensitivity. Similar conclusions have been reached from studies of perfusion of rat and guinea pig livers (31).

As may be seen in Fig. 3, mitochondria from rat tissues when incubated with pyruvate as substrate were significantly less sensitive to phenethylbiguanide inhibition than were the corresponding mitochondria from the guinea pig, even in the presence of fatty acid–free bovine serum albumin. The difference between the two species needed to achieve 50% inhibition was about 5-fold. It was of particular interest to note that rat tissue mitochondria demonstrated sensitization to phenethylbiguanide inhibition by serum albumin that was qualitatively quite analogous to that observed with guinea pig mitochondria.

These data confirm the previous studies and are compatible with the suggestion that intrinsic mitochondrial sensitivity constitutes a major source of species difference in sensitivity to phenethylbiguanide hypoglycemia. Since serum albumin can exert such a significant effect on the sensitivity of mitochondria to in vitro inhibition, it may be concluded that extramitochondrial factors functioning in vivo in a fashion analogous to albumin may also contribute to the differences in species sensitivity.

Effects of phenethylbiguanide on oxidation of long chain fatty acyl–CoA derivatives. Oxidation of palmityl- and oleyl-CoA was always carried out in the presence of fatty acid–free bovine serum albumin to prevent the damaging effects of these substrates on mitochondrial function. In the absence of carnitine, the rate of oxidation of palmityl-CoA by guinea pig heart mitochondria was negligible. In the presence of carnitine, control ratios, oxygen uptake rates, and oxidation of palmityl-CoA were identical with those for oxidation of pyruvate, for rates of ADP generation which were low to intermediate (Fig. 4), since state 3 and state 4 rates of respiration were identical for the two substrates. As the rate of ADP generation was further increased, respiratory rates with pyruvate continued to increase, while a limiting rate was quickly achieved with palmityl-CoA, resulting in a maximal control ratio which was smaller for fatty acyl-CoA than for pyruvate. Similar results were obtained with oleyl-CoA.

With palmityl-1-^{14}C–CoA as substrate, the proportion of substrate oxidized completely to $^{14}CO_2$ and incompletely to acetate, acetoacetate, β-hydroxybutyrate (22), and TCA cycle intermediates (soluble products) could be determined for each flask. The data from such an experiment, as shown in Table II, indicate that in state 3, β-oxidation only doubled, while TCA cycle activity increased by a maximum of about 15-fold. Thus, as with oxidation of pyruvate, the oxidative reactions pre-

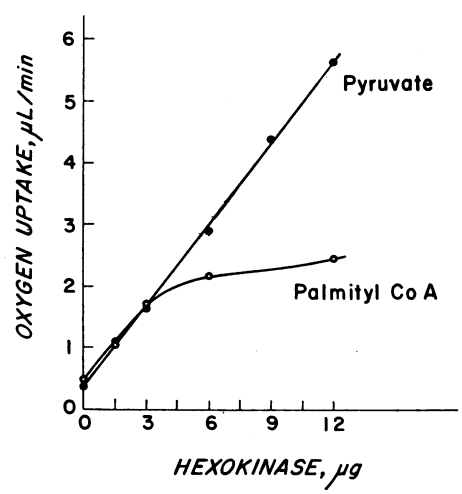

FIGURE 4 Respiratory rates for guinea pig heart mitochondria with pyruvate and palmityl-CoA substrates at increasing rates of adenosine diphosphate (ADP) generation. Conditions of incubation were as described in Table I with the following differences: fatty acid–free bovine serum albumin was present in all flasks in a concentration of 1.5×10^{-4} mole/liter; D,L-carnitine was present in all flasks at 5×10^{-3} mole/liter; flasks with palmityl-CoA contained 0.26×10^{-3} M palmityl-CoA; all flasks contained 0.54 mg of guinea pig heart mitochondrial protein. Incubation was for 25 min after induction of state 3.

ceding the TCA cycle within the mitochondrion appeared to be less tightly controlled than the TCA cycle itself.

Oxidation of oleyl-CoA by guinea pig heart mitochondria was inhibited 50% by 6×10^{-5} M phenethylbiguanide. As with pyruvate, the presence of the inhibitor suppressed the oxidative function of both the TCA cycle and the extra-TCA cycle reactions in parallel, such that the rates of these reactions in the inhibited state resembled those in state 4 (Table II).

Phenethylbiguanide inhibition of palmitylcarnitine oxidation. Studies with palmitylcarnitine were somewhat less satisfactory than those with palmityl-CoA, since the surface active properties of the long chain acylcarnitine substrate limited the maximal concentrations of substrate that could be employed, even in the presence of large quantities of serum albumin. Nonlinear respiratory rates also became apparent after relatively short time intervals with this substrate. Phenethylbiguanide was somewhat less effective as an inhibitor of oxidation of this substrate by guinea pig heart mitochondria than it was of palmityl-CoA and pyruvate oxidation; 50% inhibition was achieved at a concentration of about 1.2×10^{-4} M phenethylbiguanide.

Phenethylbiguanide inhibition of free palmitate oxidation. Oxidation of free palmitic acid by guinea pig heart mitochondria was observed in the presence of added CoA and of serum albumin and carnitine in amounts which were optimal for the oxidation of palmityl-CoA. As with palmityl-CoA the maximal Qo_2 values achieved with free palmitic acid as substrate were only about one-half as great as with pyruvate; addition of hexokinase in excess of the optimal amount led to some inhibition of respiration, as observed by others, presumably by removing the ATP necessary for activation of fatty acids to their CoA esters (32). Maximal respiratory control ratios of only 2.0–2.3 were achieved with 0.2 mM palmitate alone as substrate, even with a palmitate : albumin ratio of about 3 : 1, due to the combination of limited Qo_2 and a state 4 rate of oxygen uptake which was somewhat higher with palmitate than with pyruvate as substate (Table III).

Under these conditions, it is apparent that in state 4 more palmitate was oxidized to the level of acetate than was oxidized completely to CO_2 (Table III), as was the case for palmityl-CoA (Table II). Upon the addition of hexokinase the total amount of palmitate subjected to β-oxidation did not increase, but the activity of the TCA cycle

TABLE II

Oxidation of Palmityl-1-^{14}C-CoA to $^{14}CO_2$ and Soluble Radioactive Products by Guinea Pig Heart Mitochondria: Inhibition by Phenethylbiguanide

Metabolic state*	Phenethylbiguanide concentration	Hexokinase concentration	$^{14}CO_2$ produced	^{14}C-soluble products	Total palmitate oxidized (β-oxidation)	Calculated oxygen needed to burn palmityl-1-^{14}C-CoA to: $^{14}CO_2$	Soluble products	Total	Observed oxygen uptake
	M	*mg/flask*		*μmoles*			*μatoms*		*μatoms*
State 4	0	0	0.005	0.076	0.081	0.23	1.16	1.29	1.3
State 4	8×10^{-5}	0	0.008	0.067	0.075	0.37	0.94	1.31	1.1
State 3	0	6	0.068	0.067	0.135	3.12	0.94	4.06	4.4
State 3	0	24	0.089	0.046	0.135	4.09	0.64	4.73	5.6
State 3	8×10^{-5}	6	0.019	0.049	0.078	0.87	0.67	1.54	1.6
State 3	8×10^{-5}	24	0.018	0.070	0.088	0.83	0.98	1.81	2.0

Conditions of incubation were identical with those described in Fig. 4 except that the substrate consisted of 0.26×10^{-3} M palmityl-1-^{14}C-CoA (10.9×10^4 cpm/flask), and flasks were preincubated for 15 min. Collection and counting of $^{14}CO_2$ and of the soluble products were carried out as described in Methods. Oxygen uptake required to convert palmitate to soluble products was calculated by assuming the product was acetate. Incubations were for 40 min. Corrections for palmitate converted to $^{14}CO_2$ and soluble products during the preincubation were made by incubating a series of flasks simultaneously up to the point of the zero time reading, thus determining the radioactivity in these products and subtracting these values from these obtained in the experimental flasks.

* Metabolic state as defined in footnote to Table I.

TABLE III

Oxidation of Free Palmitate-1-^{14}C to $^{14}CO_2$ and Soluble Radioactive Products by Guinea Pig Heart Mitochondria: Inhibition by Phenethylbiguanide

Experiment	Metabolic state*	Phenethylbiguanide concentration	$^{14}CO_2$ produced	^{14}C-soluble products	Total palmitate oxidized	Calculated oxygen needed to burn palmitate-1-^{14}C to: $^{14}CO_2$	Soluble products	Total	Observed oxygen uptake
		M		*μmoles*			*μmoles*		*μatoms*
1	State 4	0	0.046	0.116	0.162	2.12	1.62	3.84	4.2
	State 3	0	0.009	0.042	0.141	4.56	0.59	5.15	6.6
	State 3	8×10^{-5}	0.039	0.046	0.149	4.28	0.65	4.93	6.6
2	State 4	0	—	—	—		—		2.5
	State 3	0	—	—	—		—		6.2
	State 3	1.6×10^{-4}	—	—	—		—		5.3

Conditions of incubation were the same as in Fig. 4, except that concentration of fatty acid–free albumin was 0.6×10^{-4} mole/liter, and all flasks contained 2×10^{-5} M coenzyme A. In the first experiment substrate concentration was 2×10^{-4} M sodium palmitate-1-^{14}C (6.03×10^4 cpm/flask); in experiment 2, substrate concentration (unlabeled) was 3×10^{-4} mole/liter. State 3 flasks contained 6 μg of hexokinase per flask, and duration of incubation after induction of state 3 was 60 min in the first experiment and 20 min in the second experiment. $^{14}CO_2$ and soluble radioactivity were determined as described in Methods; correction for radioactivity released into these products during incubation was calculated as described in the legend to Table II.

* Metabolic state as defined in footnote to Table I.

was stimulated, which accounted for the increase in respiratory rate.

Phenethylbiguanide produced a degree of respiratory inhibition which was strikingly less pronounced than that observed with the other substrates (Table III). In some experiments, no increase in respiration of guinea pig heart mitochondria was achieved over the state 4 rate upon addition of hexokinase. Under these circumstances respiratory control could be demonstrated by inclusion of guinea pig liver supernatant as a source of long chain fatty acylthiokinase to the system (33). Phenethylbiguanide exerted the same degree of inhibition in this system as in the absence of the requirement for thiokinase.

DISCUSSION

Control of mitochondrial oxidation reactions. Judging from the respiratory control ratios obtained with pyruvate as substrate, the functional integrity of the mitochondria used in these studies was well maintained. The limited oxidations occurring in state 4 probably represent both a lack of completely tight coupling between oxidation and energy conservation reactions, and the presence of some intrinsic ATPase activity, perhaps Mg^{++} stimulated (28). The data in these studies and from the work of others (21) indicate that the resultant oxidative activity in state 4 favors reactions outside the TCA cycle. Although the concentration of pyruvate used in these experiments was probably higher than that occurring in vivo, the relative preponderance of pyruvate decarboxylation over citric acid cycle activity in both states 3 and 4 has also been observed at lower, more physiologic concentrations of pyruvate (22). This phenomenon could possibly be artifact in which significant TCA cycle activity was masked by isotope dilution with unlabeled pools of endogenous citric acid cycle intermediates. Such an explanation seems most unlikely, since the total oxygen uptake in state 4 was almost quantitatively accounted for by $^{14}CO_2$ released from pyruvate-1-^{14}C. In liver mitochondria control of citric acid cycle activity is also relatively independent of non-TCA cycle oxidations (34). The oxidation of malate to oxaloacetate in liver mitochondria has been suggested as the rate-limiting step in determining the over-all rate of citrate synthesis, and hence of citric acid cycle activity; maintenance of malate reduction and (or) compartmentation of oxaloacetate away from citrate synthetase and the citric acid cycle appears to be an energy-requiring process. Under the state 4 conditions of the present studies with heart mitochondria the TCA cycle may have been suppressed by a similar mechanism,

since ATP was maintained at a high level, and electrons were available to the respiratory carriers from pyruvate decarboxylation.

β-oxidation of long chain fatty acyl substrates is even more effective than is pyruvate decarboxylation in suppressing oxaloacetate production and citrate synthesis in liver mitochondria, an observation which has been ascribed to the occurrence of two oxidations in β-oxidation as opposed to one in pyruvate decarboxylation (34). From the data in Fig. 4 and Table II, it appears that in the present experiments, β-oxidation occurred fully as rapidly in state 4 as did pyruvate decarboxylation, while citric acid cycle activity was very limited under these conditions.

The significance to the organism of the relative excess of acetyl-CoA–producing "extra-TCA cycle" reactions with both pyruvate and long chain fatty acyl-CoA may lie in the fact that muscle in the basal state probably maintains a metabolic equilibrium somewhat closer to state 4 than to state 3 (35), with relatively limited activity of the TCA cycle and of oxidative energy production. When a demand for extra energy is imposed, ADP is rapidly generated, and TCA cycle function is stimulated. It would seem advantageous, therefore, to maintain a constant supply of excess acetyl units, perhaps in storage form, in readiness for sudden demands for oxidatively generated energy. The formation of acetylcarnitine from pyruvate in insect muscle (36) and chain elongation of fatty acids in mammalian heart mitochondria (37) have been postulated to represent such storage forms of acetyl units.

Respiratory rate of tightly coupled mitochondria in state 4 is thought to be controlled by the unavailability of inorganic phosphate (P_i) or phosphate acceptor, or of energy-requiring processes, such as ion translocation, which make use of "high-energy intermediates." In the presence of maximal levels of ADP and P_i, however, substrate availability may become the rate-limiting process for respiration. Work by a number of investigators (38–40) has suggested that, at least in the presence of ATP and P_i, the rate of transfer of long chain fatty acyl derivatives across the outer (41) or inner (42) mitochondrial membrane may govern the over-all rate of β-oxidation. The long chain fatty acyl-CoA : carnitine fatty acyltransferase reaction has been proposed as the rate-limiting step in this process.

In the present studies, the reactions of β-oxidation, or some reaction before β-oxidation, appeared to become rate limiting for over-all respiration when the rate of ADP generation was maximal, since over-all respiration reached a limiting rate at a lower level of ADP generation with palmityl-CoA than with pyruvate as substrate (Fig. 4). This hypothesis was further confirmed with palmityl-1-^{14}C–CoA as substrate, since the rate of β-oxidation was found to reach a maximum before the TCA cycle was operative at a maximal rate (Table II).[1]

There is to date only minimal evidence that the rate of pyruvate transfer across mitochondrial membranes is rate limiting in either state 4 or 3 (45). However, it seems possible that, at least for some species, pyruvate transfer, particularly into heart and skeletal muscle mitochondria, may be a significant rate-determining step, as it may be for other carboxylic acids (22, 46), and that this process may be influenced by the presence of other factors such as Mg^{++}, ADP, or fatty acids.

Effects of phenethylbiguanide on respiration and specific oxidative reactions. These experiments indicate that inhibition by phenethylbiguanide is nonspecific, in the sense that citric acid cycle function is depressed as much as non-TCA cycle oxidations, resulting in a metabolic state resembling state 4 in terms of relative and absolute rates of individual oxidative reactions.

When compared with state 3, state 4 is characterized by relative reduction of the respiratory carriers (28) and their linked intramitochondrial oxidation-reduction couples. Similar reduction of the carriers through cytochrome *b* has been observed with isolated mitochondria in state 3 in the

[1] A finite pool of soluble radioactive products was found with palmityl-1-^{14}C-CoA as substrate even under conditions of maximal citric acid cycle activity (Table II), a finding which appears to be inconsistent with the hypothesis that β-oxidation was the rate-limiting process. However, some this soluble radioactivity represented amino acids and acetyl- or acetoacetylcarnitine groups which had escaped from the mitochondria (43, 44; also F. Davidoff, unpublished observation); although these compounds are measured as soluble radioactivity by the techniques employed, they are present at low concentrations in the extramitochondrial medium and are presumably not easily available to the TCA cycle enzymes as substrate.

presence of phenethylbiguanide (14). It has been suggested that in the presence of such a reducing environment the cytoplasmic lactate dehydrogenase system would divert pyruvate into lactate. Thus, in cells exposed to phenethylbiguanide, the availability of pyruvate to mitochondria, and hence the rate of pyruvate decarboxylation, could be decreased in the absence of a major degree of blockage to electron transfer (11); the resultant decrease in ATP, and increase in P_i, adenosine monophosphate (AMP), and fructose-1,6-diphosphate levels could secondarily lead to an increase in the rate of glycolysis (35). Consistent with this hypothesis, the rate of acetate oxidation by perfused rat heart was only slightly diminished by concentrations of phenethylbiguanide which greatly accelerated lactate production from glucose (11).

However, this hypothesis is not supported by the observation in vivo of normal lactate: pyruvate ratios after treatment with phenethylbiguanide (4–6) or of the depression in vitro of acetate oxidation by phenethylbiguanide to the same degree as pyruvate oxidation in adipose tissue (12). Furthermore, the oxidation-reduction state of mitochondria in liver and muscle frequently varies independently of that in the cytoplasmic compartment (35, 47).

Kinetic studies of glucose utilization have revealed that phenethylbiguanide causes a significant increase in glucose utilization by peripheral tissues (48) in the fasting state, when fatty acids are the major respiratory fuel. This observation further suggests that if phenethylbiguanide increases glycolytic rate by interference with mitochondrial function, this interference is probably not limited to specific inhibition of pyruvate oxidation, a conclusion further supported by the present finding that phenethylbiguanide limits respiration at essentially the same inhibitor concentrations when the substrate is long chain fatty acyl-CoA as when it is pyruvate (Table II).

The rate of glycolysis is normally diminished in the fasting state, probably in part through the allosteric inhibitory effects of citrate and ATP on phosphofructokinase activity (49). A fairly small decrease in citrate production, and (or) release from mitochondria rather than a drop in ATP levels due to limited respiration could represent the effect of phenethylbiguanide which was primarily responsible for the drug-induced increase in rate of peripheral glycolysis in the fasting state. This drop in cytoplasmic citrate level could result from either an over-all decrease in flux of substrate through the TCA cycle or specific inhibition of the transfer of citrate or α-ketoglutarate through the mitochondrial membrane to the cytoplasmic compartment. Changes in citrate level may also be important in regulating glycolytic flux when glucose is the major substrate (35, 49); hence, phenethylbiguanide might also increase glycolysis during oxidation of carbohydrate at least in part through regulation of cytoplasmic citrate levels. It is known that when citrate accumulates, as when the aconitase reaction of the TCA cycle is inhibited by fluoroacetate, glycolysis is diminished, and glucose tolerance is impaired (50).

Finally, the potentiation of phenethylbiguanide inhibition with fatty acid–free serum albumin (Figs. 2 and 3) suggests that free fatty acids endogenously present within the mitochondria may be antagonizing the in vitro effects of the drug, since albumin appears to affect several other mitochondrial functions by binding and removing free fatty acids (51). This conclusion is supported by the diminished inhibitory effects of phenethylbiguanide when the substrate is long chain free fatty acid (Table III), and, as presented in detail in the accompanying paper (19), by the antagonistic effects of exogenous free fatty acids and phenethylbiguanide on pyruvate oxidation. The functional consequences to the organism of such antagonism may be of considerable importance in the understanding of the hypoglycemic actions of these drugs.

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