

# Effects of Guanidine Derivatives on Mitochondrial Function

## II. REVERSAL OF GUANIDINE-DERIVATIVE INHIBITION BY FREE FATTY ACIDS

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**ABSTRACT** Long chain free fatty acids interfere with the inhibitory action of phenethylbiguanide and related compounds on mitochondrial respiration in vitro. This interference depends on binding of fatty acids to mitochondria and diminishes with decreasing chain length. Reversal of guanidine-derivative inhibition by fatty acids differs from that caused by dinitrophenol in that the effect of fatty acid is achieved without alteration in coupling or respiratory control. The binding of phenethylbiguanide to mitochondria is inhibited by both fatty acid and dinitrophenol. Serum albumin potentiates the inhibitory potency of guanidine derivatives, probably by removing endogenous mitochondrial free fatty acids.

### INTRODUCTION

From extensive studies of the inhibition of mitochondrial respiration and phosphorylation, it has been inferred that guanidine derivatives bind to energy conservation sites in mitochondria, the major functional locus of binding depending upon the structure of the guanidine compound (1). The modifications of structure which enhance the inhibitory potency of the guanidine derivatives and alter site-binding specificity are alterations in the nonpolar hydrocarbon side chain, a fact which has suggested that binding depends in large part on nonpolar interactions with mitochondrial con-

stituents. The inhibitory effects of these agents can be reversed by various uncoupling agents which are also lipophilic in nature (2), a finding which lends further support to the inference that both groups of compounds operate at loci in a relatively nonpolar environment.

It was of particular interest, therefore, to find that the inhibitory potency of phenethylbiguanide on mitochondrial preparations in vitro could be strikingly enhanced by the presence of low concentrations of fatty acid-free serum albumin (3), bringing the effective inhibitory concentrations of phenethylbiguanide in vitro into the range which is effective in producing hypoglycemia in vivo. As suggested in the previous paper, this effect of albumin may reasonably be ascribed, at least in part, to the binding and removal from the mitochondrion of endogenously generated mitochondrial free fatty acids (4, 5). The present paper presents further evidence to support the antagonism between both endogenous and exogenous long chain free fatty acids and guanidine derivatives. The interaction of free fatty acids with mitochondria under the special conditions of these experiments has been characterized in some detail and appears to involve binding to relatively specific mitochondrial sites. Binding of phenethylbiguanide to mitochondria has also been examined directly and found to be affected by the presence of free fatty acid. The mechanism whereby this bound fatty acid interferes with the inhibitory action of guanidine derivatives differs significantly

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from dinitrophenol-induced reversal of guanidine-derivative inhibition.

## METHODS

Individual fatty acids were obtained from Applied Science Laboratories, Inc., State College, Pa., Fisher Scientific Company, Pittsburgh, Pa., and Calbiochem, Los Angeles, Calif. They were analyzed as the methyl esters by gas-liquid chromatography and contained less than 5% of other fatty acid impurities. Decamethylene diguanidine (DDG) carbonate was obtained from K & K Laboratories, Inc., Plainview, N. Y., galegine sulfate and guanidine HCl from Calbiochem, phenethylbiguanide and dimethylbiguanide (DMB) from Aldrich Chemical Co., Inc., Milwaukee, Wis. Radiochemicals were obtained from New England Nuclear Corp., Boston, Mass. and were stored as previously described (3). Fatty acid-free bovine serum albumin (BSA) and fatty acid albumin complexes were prepared as described in the accompanying paper (3). Phenethylbiguanide- $^3\text{H}$  was prepared by catalytic exchange tritiation, and the product was purified by preparative paper chromatography. The resultant product was radiochromatographically homogenous in two different solvent systems on paper (6).

**Incubation procedures.** Preparation of mitochondria, incubations, measurement of respiration, and collection and counting of  $^{14}\text{CO}_2$  were carried out as described in the accompanying paper (3). P:O ratios were determined by stopping the reaction with 0.1 ml of 25% trichloroacetic acid, centrifuging off the precipitated protein, and assaying an aliquot of the supernatant for inorganic phosphate (7). Control incubations were carried out to determine the amount of inorganic phosphate present at zero time. P:O ratios were calculated by dividing the phosphate esterified, in micromoles, determined as the difference between zero time and final inorganic phosphate, by the amount of oxygen consumed, in microatoms.

Fatty acids were added to the reaction mixture as the fatty acid:albumin complex usually 7:1, prepared as described in the preceding paper (3).

Binding of fatty acids to mitochondria was measured directly by incubating mitochondria with  $^{14}\text{C}$ -labeled fatty acids of known specific activity complexed to albumin. At appropriate time intervals, aliquots of the incubation mixture were placed in chilled centrifuge tubes and spun at 12,000 *g* for 3 min. Since bound fatty acids are not removed by washing mitochondria in the absence of serum albumin (4), the supernatant was removed, and the mitochondrial pellet washed twice by resuspension in sucrose-ethylenediaminetetraacetate (EDTA)-KCl buffer, followed by centrifugation. Control experiments indicated that less than 10% of the radioactivity remaining after two washings was removed by further washings. The mitochondrial pellet was then dissolved in a small quantity of Hyamine hydroxide base (*p*-[diisobutyl-cresoxy-ethoxyethyl]-dimethylbenzyl ammonium hydroxide), the mixture taken up in scintillator solution (3), and radioactivity determined in a scintillation spectrometer with corrections for quenching.

The direct determination of phenethylbiguanide- $^3\text{H}$  binding to mitochondria was complicated by the much greater water solubility of phenethylbiguanide, making it likely that unless the binding were relatively tight, an unknown quantity of bound inhibitor would be removed during the process of washing out the medium trapped in the mitochondrial pellet. In order to determine bound phenethylbiguanide- $^3\text{H}$  without washing the pellet, a tracer quantity of polyethylene glycol- $^{14}\text{C}$ , molecular weight 4000, which is probably excluded from the intramitochondrial water (8), was added to the incubation mixture. After centrifugation and removal of the supernatant, the amount of  $^{14}\text{C}$  and  $^3\text{H}$  in the unwashed pellet was determined; from these figures, and from the known amounts of  $^3\text{H}$  and  $^{14}\text{C}$  in the supernatant, correction could be made for extramitochondrial medium trapped in the pellet; the total amount of  $^3\text{H}$  present in the pellet and finally the  $^3\text{H}$  bound to the mitochondria could then be calculated. Corrections were made for quenching throughout, using channels ratios, external or internal standards where appropriate.

## RESULTS

**Potential of phenethylbiguanide inhibition by fatty acid-free serum albumin.** If albumin potentiates the inhibitory potency of phenethylbiguanide by binding and removing some mitochondrial component such as long chain free fatty acid, the degree of potentiation by albumin should depend on the ratio of albumin to mitochondria, rather than on the absolute concentration of albumin in the medium.

As seen in Fig. 1, as little as 40  $\mu\text{g}$  of albumin per ml of medium (0.65  $\mu\text{moles/ml}$ ) significantly potentiated phenethylbiguanide inhibition of pyruvate oxidation by guinea pig heart mitochondria equivalent to 0.22 mg of protein per ml. The potentiating effect of albumin rose asymptotically to a maximum at about 0.5 mg/ml. 50% of this maximum potentiation was achieved at an albumin concentration of about 0.7  $\mu\text{moles/ml}$  for this quantity of mitochondria. When one-half the quantity of mitochondria was employed, the same degree of potentiation was achieved with one-half as much albumin. This relationship was consistent with the hypothesis that a constant amount of mitochondrial free fatty acid was present per milligram of mitochondrial protein, and was probably bound to a limited class of fatty acid binding sites on albumin.

The amount of fatty acid per milligram of mitochondrial protein removed by albumin to produce maximal potentiation can be estimated from these

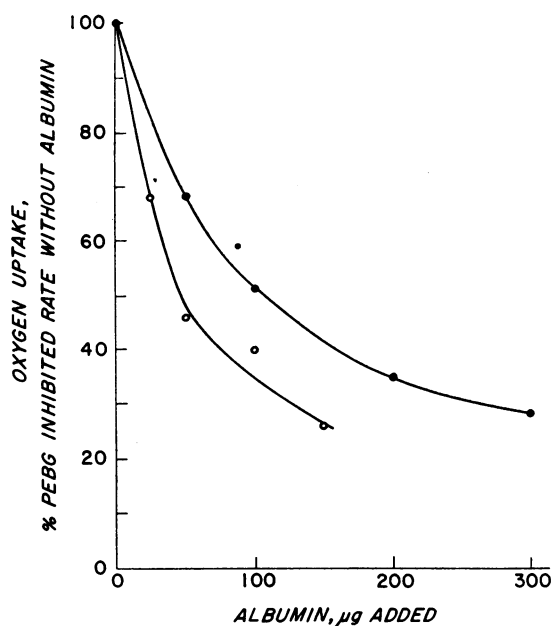


FIGURE 1 Potentiation of phenethylbiguanide (PEBG) inhibition by increasing quantities of fatty acid-free serum albumin. Each Warburg incubation flask contained  $4 \times 10^{-3}$  M adenosine triphosphate (ATP),  $5 \times 10^{-3}$  M  $\text{MgCl}_2$ , 0.1 M glucose,  $3.6 \times 10^{-3}$  M pyruvate,  $8 \times 10^{-4}$  M fumarate, 0.1 M sucrose, 0.15 M KCl,  $0.5 \times 10^{-3}$  M  $\text{Na}_2$  ethylenediaminetetraacetate (EDTA), fatty acid-free bovine serum albumin in the amount indicated, and  $1 \times 10^{-4}$  M phenethylbiguanide. Closed circles indicate flasks with 0.27 mg of guinea pig heart mitochondrial protein per flasks, open circles, 0.14 mg of mitochondrial protein/flask. After 15 min of preincubation, 6  $\mu\text{g}$  of hexokinase in 0.1 ml of 0.35 M potassium phosphate buffer, pH 7.2, was added from the side arm to make a total volume of 1.25 ml. 8 min later the first reading was taken. Incubations were for 50 min, with 0.27 mg of mitochondrial protein, 70 min with 0.14 mg of protein.

Control flasks (not shown) for each set were incubated without phenethylbiguanide or albumin simultaneously with experimental flasks. Oxygen uptake in these non-inhibited controls containing 0.27 mg of mitochondrial protein was 152% of the corresponding flasks with phenethylbiguanide and no albumin, and 140% in the analogous controls with 0.14 mg of mitochondrial protein.

data. Assuming that only the two albumin binding sites per albumin molecule with the greatest affinity for fatty acids (9) are capable of competing successfully with mitochondrial binding sites for fatty acids, it is probable that at full potentiation the number of these tightest albumin binding sites available roughly equals the amount of fatty acid removed, while at 50% potentiation, about one-half this number of tightest albumin sites is avail-

able. 3.3  $\mu\text{moles}$  of albumin are needed to achieve half potentiation for mitochondria equivalent to 1 mg of protein; hence full potentiation is probably achieved by the removal of about 13.2  $\mu\text{moles}$  of fatty acid per mg of mitochondrial protein. If the second class of albumin binding sites is also assumed to be capable of stripping fatty acid from the mitochondria, the figure then becomes 59.4  $\mu\text{moles}$  of fatty acid removed per mg of mitochondrial protein at full potentiation.

BSA which has not been extracted with heptane-acetic acid and which contains about 1 mole of fatty acid per mole (10) of albumin was significantly less effective in potentiating the inhibitory effects of phenethylbiguanide. Mitochondria prepared without the use of bacterial proteinase exhibited the same degree of sensitivity to phenethylbiguanide and the same albumin potentiation of phenethylbiguanide effect as did mitochondria prepared with proteinase.

**Characteristics of fatty acid reversal of phenethylbiguanide inhibition.** Phenethylbiguanide inhibition of pyruvate oxidation could be almost completely prevented by inclusion of stearate:albumin in the preincubation medium before the induction of state 3<sup>1</sup> (Table I and Fig. 2). When the stearate:albumin complex was added at zero time (i.e. along with the hexokinase), the inhibi-

<sup>1</sup> Metabolic states and respiratory control ratios as defined by Chance and Williams (14) are outlined in the accompanying paper (3).

TABLE I  
Prevention of Phenethylbiguanide Inhibition by Increasing Amounts of Stearate

Phenethylbiguanide present	Stearate	BSA	Stearate: albumin ratio	Oxygen uptake	
	concentration $\times 10^3$ (M)			$\mu\text{l/hr}$	% of control
0	0	1.2	0	109	100
+	0	1.2	0	51	47
+	0.4	1.3	0.3:1	58	53
+	0.8	1.3	0.6:1	65	60
+	1.3	1.4	0.9:1	76	70
+	2.0	1.5	1.3:1	84	77
+	4.0	1.8	2.2:1	92	85
+	6.0	2.1	2.9:1	92	85

BSA; bovine serum albumin.

Conditions of incubation were as described in the legend to Fig. 1 except that fatty acid and albumin were varied as described in the Table and were present in the main compartment during preincubation. Concentration of phenethylbiguanide was  $6 \times 10^{-4}$  mole/liter. Each flask contained 0.27 mg of guinea pig heart mitochondrial protein. Incubations were for 60 min after zero time reading.

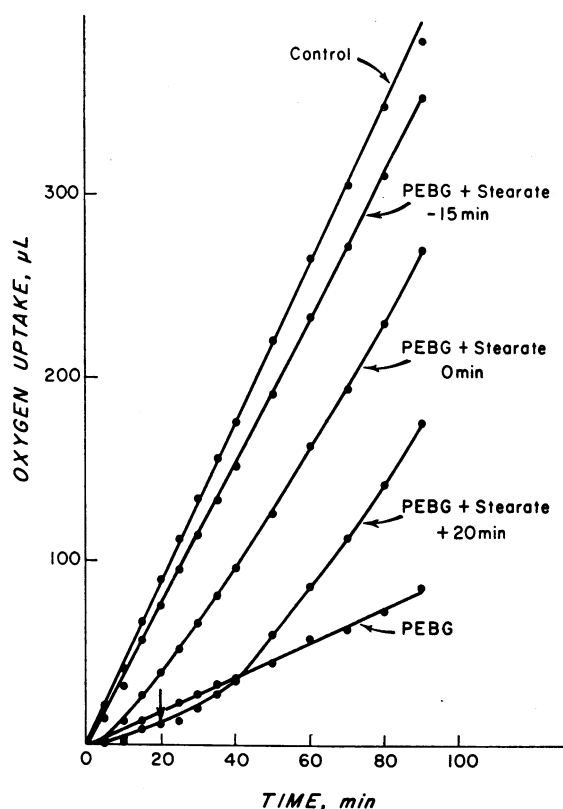


FIGURE 2 Stearate reversal of phenethylbiguanide-inhibited pyruvate oxidation in guinea pig heart mitochondria. Conditions of incubation were as described in Legend to Fig. 1 except that 15-ml double side arm Warburg flasks were used, and final volume of incubation mixture after all additions was 2.5 ml. All flasks contained, in addition,  $0.56 \times 10^{-4}$  M fatty acid-free bovine serum albumin and, where indicated, phenethylbiguanide was present in the main compartment at a concentration of  $8 \times 10^{-6}$  mole/liter. Each flask contained 12  $\mu$ g of hexokinase in 0.2 ml of 0.35 M potassium phosphate buffer. In the flasks marked *Stearate*, -15 min, 1  $\mu$ mole of sodium stearate plus an additional 0.14  $\mu$ mole of fatty acid-free bovine serum albumin was present in the main compartment during preincubation. In the flasks marked *Stearate*, 0 min, the same amount of stearate:albumin was tipped in from the second side arm at the time of induction of state 3 (zero time) by addition of hexokinase-phosphate from the first side arm. In the flasks marked *Stearate*, +20 min, the stearate:albumin was tipped in from the second side arm 20 min after the induction of state 3, as indicated by the arrow.

tion was progressively reversed, the final respiratory rate not quite reaching the control rate. When the fatty acid:albumin complex was added during the course of the incubation, at times up to 20–30 min after initiation of state 3 respiration, reversal

of phenethylbiguanide inhibition occurred relatively abruptly after a lag period of about 15–20 min after addition of the fatty acid. The final rate of respiration achieved after such late reversal approximated the final rate achieved when fatty acid was added at zero time (Fig. 2).

Restoration of phenethylbiguanide-inhibited respiration depended more on the ratio of fatty acid to albumin than on the absolute concentration of free fatty acid. Thus, when the concentration of albumin was low ( $1.2 \times 10^{-5}$  mole/liter) the addition of as little as  $4 \times 10^{-6}$  M stearate produced significant reversal of phenethylbiguanide inhibition; 50% reversal was achieved by  $1.3 \times 10^{-5}$  M stearate (Table I). Since in this experiment stearate was added with albumin in a ratio of 7:1, the final stearate:albumin ratio at 50% reversal was 0.9:1, while near maximal reversal the ratio was 2.2:1. When the initial concentration of albumin was higher ( $9 \times 10^{-5}$  mole/liter), proportionately larger amounts of stearate were necessary to reverse phenethylbiguanide inhibition to a comparable extent, and the fatty acid:albumin ratio again approached 2:1 at the point of near maximal reversal (Table II). Identical restorative effects on respiratory rate were achieved with palmityl-CoA and oleyl-CoA as substrates when long chain free fatty acids were present along with phenethylbiguanide in the incubation medium.

The prevention and reversal of phenethylbiguanide-induced respiratory inhibition by fatty acid:albumin complexes at ratios below 2:1 suggests that long chain free fatty acids may saturate a small number of high affinity mitochondrial bonding sites, since the affinity constant of the tightest binding sites on albumin for stearate is about  $10^8$  (9). Alternatively, the number of binding sites for fatty acid on the mitochondrion may be very large but with an affinity for fatty acid which is lower than that of the tightest albumin sites; in this instance, fatty acid would be bound to only a very small percentage of the total mitochondrial sites, but this small quantity of bound fatty acid would be adequate to produce an alteration in sensitivity to phenethylbiguanide inhibition.

Restoration of respiration to the control rate was easily achieved with stearate when the degree of unmodified phenethylbiguanide inhibition was of the order of 30–40% (Fig. 3, left). When the initial inhibition was greater, the control rate of

TABLE II  
*Effect of Increasing Concentrations of Saturated and Unsaturated Long Chain Fatty Acids in Preventing Phenethylbiguanide Inhibition of Pyruvate Oxidation by Guinea Pig Heart Mitochondria*

Phenethyl- bigua- nide present			Fatty acid: albumin ratio	Fatty acid added											
				18:0*	18:1	18:1	16:0	14:0	12:0	18:0	18:1	18:1	16:0	14:0	12:0
					<i>cis</i>	<i>trans</i>					<i>cis</i>	<i>trans</i>			
				Oxygen uptake											
concentration $\times 10^5$ M			$\mu\text{l/hr}$						% of control						
0	0	0	152	120	120	146	146	146	100	100	100	100	100	100	
+	0	0	51	19	19	56	56	56	34	16	16	38	38	38	
+	10	1.0:1	—	46	56	76	72	52	—	38	47	52	49	36	
+	20	1.7:1	136	70	72	99	80	53	89	58	60	68	55	36	
+	40	2.8:1	159	94	98	111	78	57	104	78	80	76	53	39	

Conditions of incubation were as described in legend to Table I. Concentration of phenethylbiguanide was  $8 \times 10^{-5}$  mole/liter in experiments with oleate (18:1 *cis*) and elaidate (18:1 *trans*), and was  $6 \times 10^{-5}$  mole/liter in all other experiments. Control flasks contained  $9 \times 10^{-5}$  M albumin. Incubations were for 45 min. Note that concentration of fatty acids and albumin was an order of magnitude greater than in experiments described in Table I. In these experiments, a single flask was used to determine each experimental value.

\* Fatty acids are designated by chain length: number of double bonds; thus abbreviation for stearate is 18:0.

respiration was reached asymptotically as the concentration of fatty acid increased (Fig. 3, left).

Restoration of phenethylbiguanide-inhibited respiration by long chain free fatty acids appeared to be due to a quantitative alteration in sensitivity to

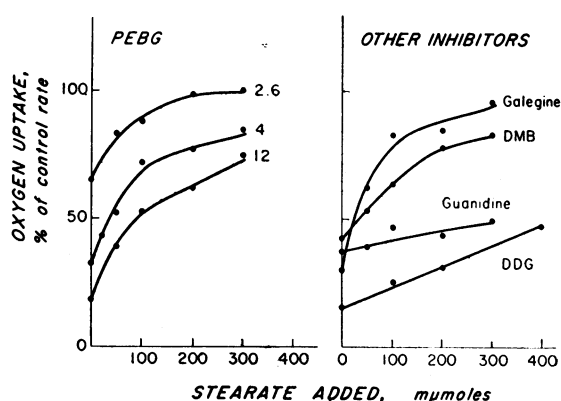


FIGURE 3 Quantitative aspects of stearate restoration of guanidine derivative-inhibited respiration. Conditions of incubation were as described in legend to Fig. 1. Control flasks contained no inhibitor and  $1.7 \times 10^{-5}$  M serum albumin. Flasks with inhibitor contained stearate: albumin added as the complex in a ratio of 7:1 in the amount noted, in addition to the same amount of albumin as that present in control flasks. Left: phenethylbiguanide (PEBG) was present in each experiment as noted, expressed as concentration  $\times 10^5$  moles/liter. Right: Guanidine-related inhibitors were present in individual experiments in the following concentrations: guanidine,  $12 \times 10^{-3}$  mole/liter; dimethylbiguanide (DMB),  $3 \times 10^{-3}$  mole/liter; galegine,  $2 \times 10^{-3}$  mole/liter; decamethylene diguanide (DDG),  $3 \times 10^{-3}$  mole/liter.

the action of the inhibitor, rather than to total loss of susceptibility to inhibition. Thus, by increasing the phenethylbiguanide concentration high enough, it was possible to produce the same degree of inhibition of respiration in the presence of long chain fatty acid as in its absence; i.e., in the presence of free fatty acid, 50% inhibition was achieved at about  $2 \times 10^{-4}$  instead of  $3-5 \times 10^{-5}$  M phenethylbiguanide (Fig. 4). It is of interest that this difference in sensitivity to phenethylbiguanide inhibition with and without added fatty acid is of the same order of magnitude as that between mitochondria incubated in the absence *versus* the presence of fatty acid-free serum albumin alone (Fig. 4). (See also reference 3, Figs. 2 and 3.)

*Influence of fatty acid structure on phenethylbiguanide antagonism.* Fatty acid structure was found to influence strongly the effectiveness of fatty acid antagonism to phenethylbiguanide inhibition. As the chain length of the fatty acid diminished, reversal of phenethylbiguanide inhibition became less apparent (Tables II and III). Concentrations of shorter chain fatty acids equimolar to submaximal concentrations of stearate had less preventive effect on phenethylbiguanide inhibition than longer chain acids (Table III). In addition, the maximal phenethylbiguanide reversal achieved with shorter chain acids appeared to be much less than that achieved with stearate; laurate and decanoate demonstrated very little effect (Tables II and III). Unsaturation generally reduced the

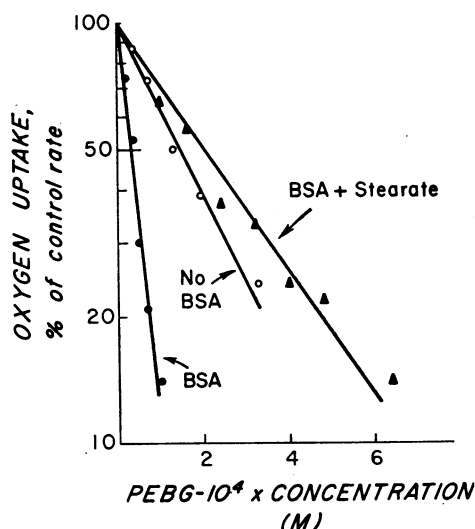


FIGURE 4 Phenethylbiguanide (PEBG) inhibition of pyruvate oxidation in the presence and absence of serum albumin and of stearate. Conditions of incubation were identical with those described in Fig. 1 except that flasks contained either no serum albumin,  $1 \times 10^{-5}$  M fatty acid-free bovine serum albumin (BSA), or  $6 \times 10^{-5}$  M potassium stearate and  $2 \times 10^{-5}$  M albumin. Phenethylbiguanide was present in the main compartment during preincubation. Each flask contained 0.27 mg of guinea pig heart mitochondrial protein; incubations were for 50 min.

efficacy of phenethylbiguanide reversal, although palmitoleate and palmitate were roughly equal in this regard. The *cis* and *trans* isomers of octadecenoic acid (oleate and elaidate) appeared to have almost identical effects (Table II).

**Metabolic fate of free fatty acids added for reversal of phenethylbiguanide inhibition.** Carnitine was found not to be necessary for the fatty acid-induced reversal of phenethylbiguanide inhibition (Fig. 2) and, in fact, had essentially no effect on this phenomenon. Since, in the presence of adenosine triphosphate (ATP), carnitine is obligatory for fatty acid oxidation by guinea pig heart mitochondria (3), as it is for mitochondria from other sources, (11, 12), the lack of a carnitine requirement for fatty acid antagonism to phenethylbiguanide inhibition strongly suggested that the fatty acid effect did not entail activation or oxidation of the fatty acid. For the same reason, the apparent restoration of respiration by fatty acid in mitochondrial preparations exposed to phenethylbiguanide was not likely to be due to the substitution of fatty acid oxidation for that portion of pyruvate oxidation which has been inhibited by

phenethylbiguanide. These hypotheses were confirmed directly by measuring the oxidation of pyruvate-1- $^{14}\text{C}$  and -2- $^{14}\text{C}$  to  $^{14}\text{CO}_2$ . From the data in Table IV, it may be seen that with both labeled substrates  $^{14}\text{CO}_2$  evolution was restored in parallel with oxygen uptake when phenethylbiguanide inhibition was prevented by fatty acid. When the experiment was repeated under identical conditions but with palmitate-1- $^{14}\text{C}$  replacing the fatty acid, and with unlabeled pyruvate as substrate, the amount of palmitate converted to  $^{14}\text{CO}_2$  and to soluble radioactive products in the presence of phenethylbiguanide accounted for less than 1% of the total oxygen uptake, despite the restoration of oxygen uptake to 80% of control values.

**Reversal of phenethylbiguanide inhibition by long chain fatty acids in mitochondria from other tissues of guinea pig and rat.** Phenethylbiguanide-induced inhibition of pyruvate oxidation by mitochondria from guinea pig skeletal muscle and liver was also prevented by stearate. When  $^{14}\text{C}$ -labeled pyruvate was used, the increase in oxygen uptake by mitochondria from these tissues occurring when phenethylbiguanide inhibition was relieved by stearate was accounted for entirely by the increase in pyruvate oxidation. Long chain free fatty acids also produced a reversal of phenethyl-

TABLE III  
Effectiveness of Various Fatty Acids in Preventing Phenethylbiguanide Inhibition of Pyruvate Oxidation by Guinea Pig Heart Mitochondria

Phenethylbiguanide present	Fatty acid	Fatty acid: albumin ratio		Oxygen uptake	
		concentration $\times 10^5$ (M)		$\mu\text{l/hr}$	% of control
0	0	0	0	144	100
+	0	0	0	26	18
+	Stearate	4	1.7:1	102	71
+	Stearate	6	2.3:1	120	83
+	Oleate	4	1.7:1	80	56
+	Linoleate	4	1.7:1	68	47
+	Palmitate	4	1.7:1	94	65
+	Palmitoleate	4	1.7:1	61	42
+	Myristate	4	1.7:1	42	29
+	Decanoate	4	1.7:1	36	25

Incubation conditions were identical with those in legend to Table I, except that fatty acid and albumin additions were made as indicated in the Table. Control flasks contained albumin at a concentration of  $1.7 \times 10^{-5}$  mole/liter. Phenethylbiguanide concentration was  $8 \times 10^{-5}$  mole/liter. Each flask contained 0.27 mg of guinea pig mitochondrial protein. Incubations were for 40 min after zero time reading.

TABLE IV  
*Oxidation of Pyruvate-1-<sup>14</sup>C and -2-<sup>14</sup>C to <sup>14</sup>CO<sub>2</sub> by Guinea Pig Heart Mitochondria: Inhibition by Phenethylbiguanide and Reversal of Inhibition by Long Chain Fatty Acids*

Label added	Metabolic state*	Phenethylbiguanide present	Stearate	Pyruvate- <sup>14</sup> C oxidized to <sup>14</sup> CO <sub>2</sub>		Oxygen uptake	
			concentration × 10 <sup>4</sup> (M)	μmoles	% of control	μatoms	% of control
Pyruvate-1- <sup>14</sup> C	State 4	0	0	0.55	22	0.4	5
	State 3	0	0	2.46	100	7.4	100
	State 3	+	0	1.03	42	2.1	29
	State 3	+	1.5	1.55	63	5.1	69
Pyruvate-2- <sup>14</sup> C	State 4	0	0	0.06	3	1.1	11
	State 3	0	0	1.87	100	10.4	100
	State 3	+	0	0.40	21	3.6	35
	State 3	0	4	2.03	108	10.9	104
	State 3	+	4	1.62	87	10.1	97

Conditions of incubation were identical with those described in legend to Table I except that flasks without fatty acid contained  $0.5 \times 10^{-4}$  M fatty acid-free bovine serum albumin, while flasks with fatty acid contained  $0.9 \times 10^{-4}$  M albumin in experiment with pyruvate-1-<sup>14</sup>C and  $1.2 \times 10^{-4}$  M albumin in experiment with pyruvate-2-<sup>14</sup>C (fatty acid:albumin ratios of 1.7:1 and 3.3:1, respectively). Each flask contained either  $3.8 \times 10^4$  cpm of pyruvate-1-<sup>14</sup>C or  $6.8 \times 10^4$  cpm of pyruvate-2-<sup>14</sup>C. Concentration of phenethylbiguanide was  $6 \times 10^{-5}$  mole/liter with pyruvate-1-<sup>14</sup>C and  $8 \times 10^{-5}$  mole/liter with pyruvate-2-<sup>14</sup>C. <sup>14</sup>CO<sub>2</sub> was collected and counted as described previously (3). Corrections for <sup>14</sup>CO<sub>2</sub> evolved during the preincubation were made by subtracting the number of counts released as <sup>14</sup>CO<sub>2</sub> during the state 4 preincubation from the final result for each other flask in the experiment.

\* Metabolic states as described by Chance and Williams (14) are outlined in the accompanying paper (3).

biguanide-induced inhibition in rat heart mitochondria with pyruvate as substrate; a maximum reversal of about 60% of the inhibition induced by  $3.2 \times 10^{-4}$  M phenethylbiguanide was achieved with  $6 \times 10^{-4}$  M stearate (fatty acid:albumin ratio of 4:1). Myristate was a less effective antagonist, achieving a maximal reversal of only about 35% of the inhibition.

*Comparison of long chain fatty acids with dinitrophenol in the protection against phenethylbiguanide inhibition.* Since uncoupling agents such as halophenols are known to prevent and to reverse the inhibitory effects of guanidine derivatives on mitochondria (1, 2), it seemed particularly important to determine whether the uncoupling properties of long chain fatty acids (13) were obligatory for their antagonism toward guanidine-derivative-induced inhibition.

The respiratory control ratio with pyruvate as substrate is a sensitive indicator of mitochondrial integrity and of the tightness of coupling between oxidation and phosphorylation (14). The control ratio was markedly lowered in the presence of

2,4-dinitrophenol (DNP), due largely to the increase in state 4 rate (Table V). With larger amounts of DNP, the respiratory rate in the presence of hexokinase (state 3) fell, as observed by others (13), and actually became lower than the rate in the absence of hexokinase (state 4). In contrast, the presence of stearate at a concentration of  $4 \times 10^{-4}$  mole/liter and a fatty acid:albumin ratio of 3:1 produced insignificant alterations in state 4 and state 3 respiratory rates for pyruvate with consequent preservation of good control ratios (Table V, experiment 1). Even at a fatty acid concentration of  $10 \times 10^{-4}$  mole/liter with a fatty acid:albumin ratio of 4.7:1, the control ratio fell only moderately, mainly as the result of a small absolute increase in state 4 rate (Table V, experiment 2). It should be recalled that 95% of the inhibition induced by  $6 \times 10^{-5}$  M phenethylbiguanide could be reversed by a stearate concentration of  $6 \times 10^{-5}$  mole/liter, at a stearate:albumin ratio of 2.6:1, well below the concentration and fatty acid:albumin ratio which affected respiratory control ratio.

TABLE V  
Respiratory Control Ratios and P:O Ratios for Guinea Pig Heart Mitochondrial Oxidation of Pyruvate:  
Effects of Fatty Acid:Albumin, Phenethylbiguanide, and Dinitrophenol

Expt. No.	Stearate	Fatty acid: albumin ratio	Phen- ethyl- bigua- nide	DNP	Oxygen uptake		Control ratio	Pi ester- ified, state 3	P:O ratio
					State 4	State 3			
					<i>μatoms</i>				
	<i>con- cen- tration × 10<sup>4</sup> (M)</i>		<i>con- cen- tration × 10<sup>5</sup> (M)</i>	<i>con- cen- tration × 10<sup>4</sup> (M)</i>					
1	0	0	0	0	1.1	11.3	10.3	30.6	2.9
	0	0	0	1	6.0	9.9	1.7	22.1	2.3
	0	0	0	1.5	8.2	5.9	0.7	15.3	2.6
	4	3:1	0	0	1.1	11.3	10.3	33.8	3.3
2	0	0	0	0	0.8	8.4	10.4	—	—
	10	4.7:1	0	0	1.4	8.2	6.0	—	—
3	0	0	0	0	0.6	8.6	13.9	22.2	2.6
	0	0	6	0	0.3	2.5	9.0	4.9	2.0
	0	0	6	1	2.8	6.2	2.2	13.8	2.2
	4	2.7:1	6	0	0.8	7.3	8.9	20.3	2.8

DNP, dinitrophenol; Pi, inorganic phosphate.

Conditions of incubation were identical in all experiments and were the same as in legend for Fig. 1, except for additions as noted. Flasks without fatty acid all contained  $0.7 \times 10^{-4}$  M fatty acid-free bovine serum albumin. Pi esterified and P:O ratios were determined as described in Methods. All measurements of respiratory rates and phosphate determinations were carried out in duplicate or triplicate flasks and results averaged for each experimental value.

Phenethylbiguanide alone produced only a minimal effect on the control ratio, since both state 4 and state 3 respiratory rates were reduced to the same extent (Table V, experiment 3). When phenethylbiguanide-inhibited state 3 respiration was restored with DNP, control ratios were lowered to the range induced by DNP alone. In contrast, stearate-induced restoration of phenethylbiguanide-inhibited respiration was consistently accompanied by preservation of control ratios close to the values for uninhibited respiration.

At the concentrations of DNP used in these studies, significant lowering of the P:O ratio could not be consistently demonstrated (Table V, experiment 1). P:O ratios were, however, slightly but reproducibly lowered when substantial respiratory inhibition was induced with phenethylbiguanide alone; when phenethylbiguanide-inhibited respiration was restored with DNP, the P:O ratio was not increased to normal (Table V, experiment 3). Stearate alone did not alter the P:O ratio appreciably; reversal of phenethylbiguanide-inhibited respiration by stearate restored the P:O ratio toward the control value, in contrast to the lack of effect of DNP (Table V, experiments 1 and 3).

*Inhibition of respiration by other guanidine derivatives: effects of serum albumin and long chain free fatty acids.* The inhibitory properties of several other guanidine derivatives of guinea pig heart mitochondrial pyruvate oxidation were also studied. These compounds exhibit some functional specificity in locus of inhibition (1): the monoguanidine derivatives appear to inhibit largely at the first energy conservation site; hence they affect the metabolism of substrates oxidized via pyridine nucleotide-linked reactions. Increasing the chain length of the alkyl substituent of guanidine up to 12 carbons markedly enhances the inhibitory potency but does not appear to affect the locus of action (18). Phenethylbiguanide, in contrast, appears to affect mainly the second energy conservation site. When the two guanidine residues are separated by increasing numbers of methylene groups, however, the second energy conservation site is much less strongly affected, and inhibition at the third site becomes predominant.

The relative potentiating effect of serum albumin for each inhibitor appeared to vary in exact parallel with the preventive effect of long chain free fatty acid against inhibition by that same

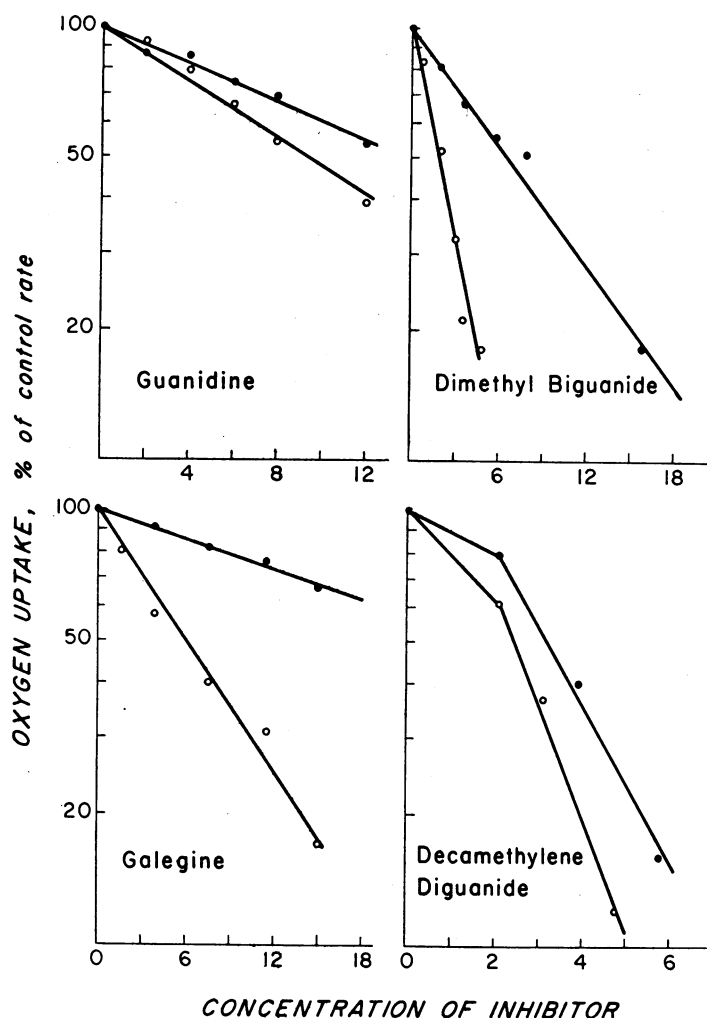


FIGURE 5 Inhibition of guinea pig heart mitochondrial respiration by guanidine derivatives in the presence and absence of albumin. Conditions of incubation were as described in legend to Fig. 1 except that inhibitors were present in the main compartment in each experiment as indicated. Concentrations are expressed as follows: guanidine,  $\times 10^8$  moles/liter; dimethylbiguanide,  $\times 10^8$  moles/liter; galegine,  $\times 10^8$  moles/liter; decamethylene diguanide,  $\times 10^8$  moles/liter. Closed circles, no added albumin; open circles,  $1.7 \times 10^{-5}$  M fatty acid-free serum albumin.

compound. Thus, by the criterion of the change in concentration needed to achieve 50% inhibition, decamethylene diguanide inhibition was potentiated by serum albumin by only about 30%, guanidine by 40%, dimethylbiguanide by 300%, galegine by about 350% (Fig. 5), and phenethylbiguanide by 5–600% (Fig. 4). The relative ability of stearate to prevent the inhibition induced by these compounds increased in the same order (Fig. 3, left and right). It is of interest that, with the exception of decamethylene diguanide, the over-all inhibitory potency of this series of compounds also increased in the same order.

These data indicate that the interaction of fatty acids and guanidine derivatives is not limited to a single energy conservation site. This interaction appears to be governed rather by the structure of the inhibitor, a nonpolar side chain generally in-

creasing the susceptibility of the inhibition to reversal by free fatty acid in addition to enhancing the inhibitory potency (18).

**Binding of long chain free fatty acids to guinea pig heart mitochondria.** When stearate  $1\text{-}^{14}\text{C}$  was added to the incubation mixture as the fatty acid:albumin complex, a relatively small but highly reproducible quantity of fatty acid radioactivity was recovered in the mitochondrial pellet which could not be removed by repeated washings in albumin-free medium. When carried out at  $37^\circ\text{C}$  under state 4 conditions, the binding increased progressively with time, reaching a maximum within 2–3 min and then remaining constant. Maximal binding at  $0^\circ\text{C}$  was only about 15–20% as great as that at  $37^\circ\text{C}$  and increased only very slightly with time. Binding of fatty acid did not appear to depend on the presence of substrate;

however, significantly less fatty acid was bound after induction of state 3 than during the pre-incubation in state 4. When fatty acid-free serum albumin was included in the washing medium, between 40 and 85% of the bound radioactivity was removed in two washes, suggesting that the bulk of the bound fatty acid remained in the unesterified form.

When the bound radioactive lipid was extracted by the method of Bligh and Dyer (15), chromatographed with carrier lipids on thin-layer plates of silica gel G in a solvent system of petroleum ether-diethyl ether-acetic acid (90:10:1), and individual spots eluted with diethyl ether, no radioactivity was detected at the origin where the phospholipids remain. All the radioactivity was found in the spot corresponding to the free fatty acids; total recovery of radioactivity was 60% in this procedure.

Binding of fatty acid was extremely sensitive to the fatty acid:albumin ratio. At low fatty acid:albumin ratios, (5:1 or less), the amount of fatty acid bound reached an apparent maximum at relatively low concentrations of fatty acid, a fact which suggests saturation of high affinity mitochondrial binding sites (Fig. 6a). At higher fatty acid:albumin ratios, i.e. 9:1, binding continued

to increase with increasing concentrations (Fig. 6b). About 10–12  $\mu$ moles of stearate were bound per mg of mitochondrial protein under conditions which produced 50% reversal of the inhibition by  $4 \times 10^{-5}$  M phenethylbiguanide (cf. Fig. 3).

Fatty acid chain length also exerted a strong influence on the magnitude of binding to mitochondria. The quantity of fatty acid bound became progressively less as the chain length decreased, the binding of laurate being only about 5% as great as that of stearate when mitochondria were exposed to equimolar amounts of fatty acid at the same fatty acid:albumin ratio (Fig. 7). This decrease in mitochondrial binding could not be due to greater binding to serum albumin, since the affinity of serum albumin for fatty acids decreases as the chain length diminishes (9). These incubations were carried out in state 4 and in the absence of carnitine, making it most unlikely that the differences in recovery of bound radioactivity were due to differential oxidation of the shorter chain acids. The relative magnitude of binding of the different chain length fatty acids closely paralleled the effectiveness of these fatty acids as antagonists of the inhibitory action of phenethylbiguanide (Tables II and III). Finally, addition of phenethylbiguanide in concentrations up to  $10^{-3}$  mole/

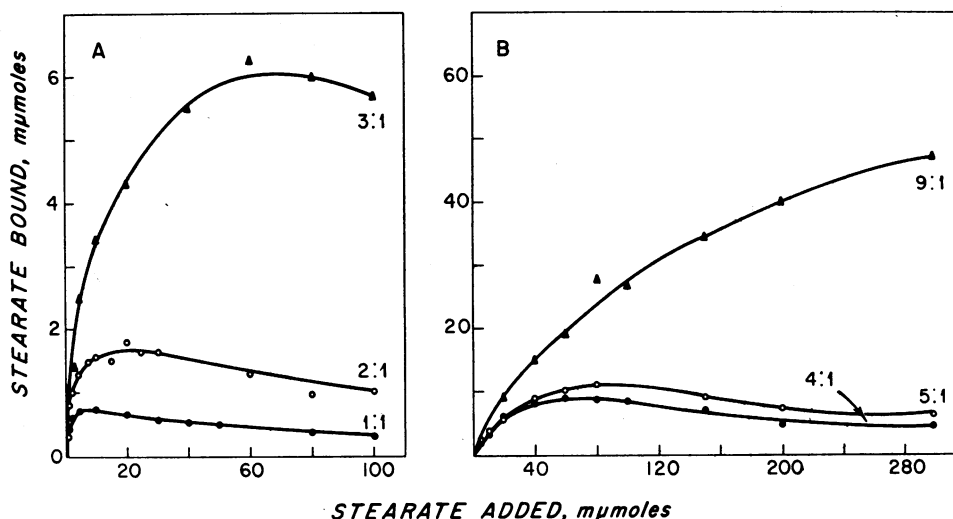


FIGURE 6 Binding of stearate-1-<sup>14</sup>C to guinea pig heart mitochondria at different fatty acid:albumin ratios. Conditions of incubation were the same as described in the legend to Fig. 1, except that stearate-1-<sup>14</sup>C and fatty acid-free bovine serum albumin were present in the concentrations and ratios indicated. Each flask contained 0.55 mg of mitochondrial protein. Incubations were carried out for 15 min in the absence of hexokinase, the mitochondria centrifuged and washed, and radioactivity determined as described in Methods.

liter was found to cause no detectable alteration in the binding to guinea pig heart mitochondria of stearate-1- $^{14}\text{C}$  added as the 1:1, 2:1, or 4:1 fatty acid:albumin complex.

**Binding of phenethylbiguanide- $^3\text{H}$  to guinea pig heart mitochondria.** Phenethylbiguanide binding to mitochondria did not appear to reach saturation in the same manner as did the binding of fatty acids, but continued to increase in quantity over the range of concentrations tested. Binding was dependent to some degree on the presence of substrate. The total quantity of phenethylbiguanide bound was less than that of stearate, generally in the range of 2–3  $\mu\text{moles}/\text{mg}$  of mitochondrial protein, at concentrations of phenethylbiguanide in the range which produced about 50% inhibition of respiration (Fig. 8).

Most striking, however, was the decrease in binding of phenethylbiguanide to mitochondria in the absence of BSA, an over-all reduction of about 60% (Fig. 8, left). The magnitude of this change

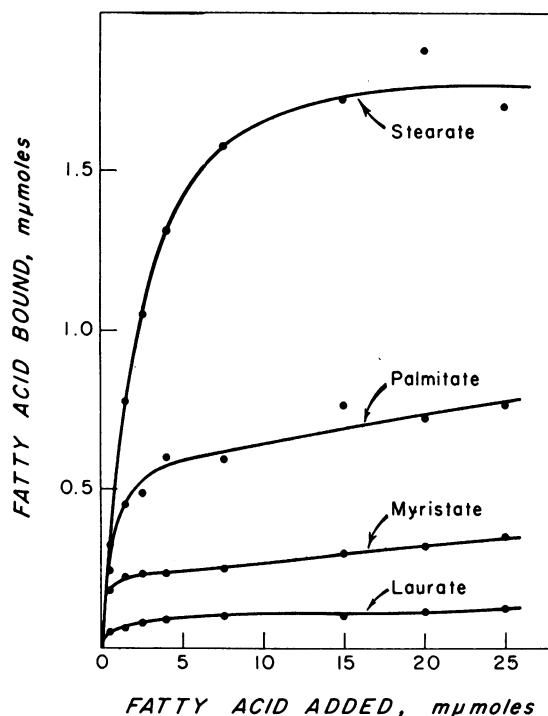


FIGURE 7 Binding of saturated fatty acids of different chain lengths to guinea pig heart mitochondria. Incubations were carried out under state 4 conditions and radioactivity determined as described in legends to Figs. 1 and 6. Fatty acids, all labeled with  $^{14}\text{C}$  in the carboxyl carbon, were added as the fatty acid:albumin complex at a ratio of 2:1.

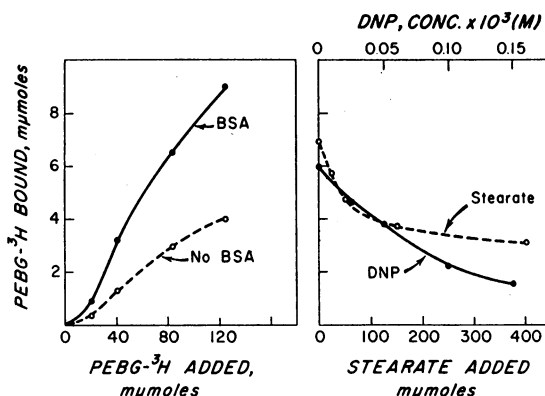


FIGURE 8 Binding of phenethylbiguanide (PEBG)- $^3\text{H}$  to guinea pig heart mitochondria. Mitochondria were incubated with phenethylbiguanide- $^3\text{H}$  with a specific activity of 0.34  $\text{mc}/\text{mmole}$  under state 4 conditions as described in the legend to Fig. 1. The amount of bound biguanide was determined as indicated in Methods. Each experimental point was determined in duplicate or triplicate. Each flask contained 1.1  $\text{mg}$  of mitochondrial protein. **Left:** Binding of phenethylbiguanide- $^3\text{H}$  at increasing phenethylbiguanide concentration. Flasks for curve marked BSA contained  $1.6 \times 10^{-5} \text{ M}$  fatty acid-free bovine serum albumin. **Right:** Binding of phenethylbiguanide- $^3\text{H}$  in the presence of increasing concentrations of stearate:albumin or of dinitrophenol (DNP). Stearate was added to flasks in the total amount indicated at the 7:1 fatty acid:albumin complex, so that the final fatty acid ratio increased progressively, the ratio being 5:1 with 400  $\mu\text{moles}$  of stearate added. All flasks contained 124  $\mu\text{moles}$  of phenethylbiguanide- $^3\text{H}$ .

corresponded roughly with the decrease in the inhibitory effect of phenethylbiguanide on respiration brought about by omitting albumin from the incubation. Similarly, the decrease in phenethylbiguanide binding brought about by increasing amounts of stearate paralleled the restoration of respiratory rate by the fatty acid (Fig. 3, left and 8, right). The binding of phenethylbiguanide was diminished by DNP in a manner which exactly paralleled its effects on uncoupling of respiration (unpublished data) as well as on reversal of phenethylbiguanide inhibition (Fig. 8, right).

## DISCUSSION

**Binding of phenethylbiguanide and the effects of free fatty acids.** Diminution of the inhibitory potency of biguanide inhibitors by long chain fatty acids in these studies was obtained under conditions in which the fatty acids exerted no discernable effects on the respiratory rate, P:O ratio, or

respiratory control. In fact, were it not for the alteration in sensitivity to guanidine-derivative inhibition, there would have been no reason to suspect any functional interaction of long chain free fatty acids with mitochondria under these conditions. In this respect, the current experiments resemble others in which low concentrations of exogenously added free fatty acids ( $3 \times 10^{-5}$  mole/liter) inhibited ( $^{32}\text{P}_i$ )-ATP and  $\text{P}_i$ - $\text{H}_2^{18}\text{O}$  exchange reactions while not affecting basal ATPase activity or causing mitochondrial swelling (16). Similar low concentrations of exogenous fatty acids prevented the activation of ATPase by DNP (5). This evidence plus studies of fatty acid- $\text{H}_2^{18}\text{O}$  exchange (17) has been interpreted to mean that the carboxyl group of long chain free fatty acids may interact with the high energy intermediates of mitochondria, possibly undergoing transient covalent bonding with these intermediates. Direct analysis has also established that small amounts of endogenous long chain free fatty acids accumulate in mitochondria, probably as the result of endogenous mitochondrial phospholipase activity; these fatty acids are responsible for the progressive inactivation of DNP-stimulated ATPase which takes place in mouse liver mitochondria during incubation at  $37^\circ\text{C}$  (4).

In contrast, relatively high concentrations of exogenously added fatty acids ( $5 \times 10^{-4}$  mole/liter) uncouple respiration when added in vitro and in even higher concentrations ( $2 \times 10^{-3}$  mole/liter) inhibit respiration, probably as the result of the detergent properties of aqueous solutions of fatty acid soaps (13). The presence of serum albumin in the experiments of the current studies limited the concentration of unbound free fatty acid in solution to the range of  $10^{-7}$  mole/liter (9); it was therefore not surprising that uncoupling or inhibitory effects of fatty acids were not observed.

Fatty acids may bring about the apparent decrease in inhibitory potency of the guanidine-derived inhibitors by a number of mechanisms.

(a) *Charge neutralization in solution.* This possibility is rendered highly unlikely by the kinetics of restoration of respiration when fatty acid is added to an inhibited mitochondrial system (Fig. 2), since charge neutralization might be expected to occur rapidly and without a lag period. If simple charge neutralization were a

significant mechanism, equimolar concentrations of shorter chain fatty acids should have been as effective in preventing guanidine-induced inhibition as longer chain acids, and the fatty acid effect should have depended on the absolute fatty acid concentration rather than on the fatty acid:albumin ratio; neither of these effects was observed.

(b) *Competition of fatty acid and guanidine derivative for a single mitochondrial binding site.* Since the quantity of bound fatty acid did not change significantly even after addition of very large quantities of phenethylbiguanide, competition for a single binding site does not appear to constitute the mechanism of antagonism between phenethylbiguanide and long chain fatty acids. These experiments were run under conditions in which the quantities of bound phenethylbiguanide and fatty acid were very similar (cf. Figs. 6 and 8), under which circumstances displacement of the fatty acid should have been easily detected.

(c) *Simultaneous binding of fatty acid and guanidine derivative to adjacent mitochondrial sites with consequent charge neutralization.* The kinetics of restoration of phenethylbiguanide-inhibited respiration by fatty acid militates against this hypothesis (Fig. 2), since charge neutralization would be expected to occur much more rapidly. The lag in restoration of respiration could result from a delay in binding of fatty acid induced by the presence of phenethylbiguanide. However, further experiments (unpublished data) have shown that phenethylbiguanide does not interfere with the time course of binding of stearate-1- $^{14}\text{C}$ , thus ruling out this possibility.

The major evidence against this hypothesis stems from the observation of decreased binding of phenethylbiguanide in the presence of bound fatty acid (Fig. 8). Not only is the magnitude of this decrease sufficient to explain most of the difference in inhibition, but also bound fatty acid might be expected actually to increase the amount of bound phenethylbiguanide if electrostatic interactions on the surface of the mitochondrion were involved in the fatty acid-induced change in inhibitory potency of the biguanide.

(d) *Alteration by fatty acids of the number of binding sites for guanidine derivatives.* The reversal of phenethylbiguanide inhibition by DNP has been taken as evidence that guanidine-derived inhibitors bind to "high energy intermediates" (1,

2, 18), since it is also generally assumed that DNP allows dissipation of these high energy intermediates. In this scheme, the interference by DNP with biguanide-induced inhibition would be explained by a diminution in the number of biguanide binding sites. Since under the conditions of the current experiments long chain free fatty acids induced no discernible changes in the creation or dissipation of such high energy intermediates, an analogous hypothesis does not seem likely to explain the fatty acid effect.

It is worth pointing out that some kinetic evidence does not support the concept that DNP allows "dissipation" of high energy intermediates (19) but rather may prevent their formation. In addition, Dicoumarol, despite its considerable activity as an uncoupler, does not reverse the inhibition induced by octylguanidine, whereas DNP does, an observation which has been interpreted to indicate energy conservation site specificity of the uncouplers (2). However, these data can also be taken simply as a further demonstration of a more general dissociation of uncoupling activity from the ability to reverse guanidine-derivative inhibition; octyldinitrophenol, which is six times more potent as an uncoupler than is DNP itself, resembles Dicoumarol in its inability to reverse octylguanidine inhibition (2). Finally, the lag period observed during the reversal of biguanide-induced inhibition by DNP has previously been attributed to a "position effect" of the sites of action of these two compounds in relation to the electron transport chain (18). The resemblance of the DNP lag period to that seen with fatty acid-induced reversal of inhibition, however, suggests that the two compounds may both affect biguanide inhibition by similar mechanisms, and that this ability to reverse guanidine-derivative inhibition may not be directly related to uncoupling activity.

(e) *Alteration of the accessibility of inhibitor to the mitochondrial binding sites, or alteration of binding site affinity, possibly by alteration in membrane structure.* On the basis of the binding studies reported here with fatty acids and phenethylbiguanide, this hypothesis seems the most plausible to explain the interference of fatty acids with biguanide and other guanidine-derivative inhibition. The structural requirements for effective binding of fatty acid and the parallel effects of

fatty acid structure on guanidine-derivative inhibition indicate that considerable nonpolar interaction is involved in the binding of fatty acid to mitochondria. However, once the fatty acid is bound, the presence of the carboxyl group appears to be the determining factor in the interference with biguanide binding and inhibition, since the shorter chain fatty acids are just as effective as the longer chain acids *per mole of fatty acid bound*. The observed lag period before respiratory rate is restored by free fatty acid is consistent with the time needed for change in conformation of a protein or membrane structure after the binding of the fatty acid. The nature of the binding of biguanide or guanidine-derivative inhibitors is not known, but again probably involves considerable nonpolar interaction, since increases in the length of the nonpolar side chain increase the potency for respiratory inhibition; further study is necessary to determine whether the biguanide inhibitors are actively transported across mitochondrial membranes, admitted by carrier-mediated diffusion, or simply bound to surface structures.

The interference with phenethylbiguanide inhibition and phenethylbiguanide binding by endogenous fatty acids appears to be fully as great as that produced by exogenous acids, a finding which suggests that the binding sites for fatty acids are freely accessible to exchange with fatty acids from both sources. The effectiveness of serum albumin in the medium in enhancing the inhibitory potency of the guanidine inhibitors is consistent with this formulation. These effects of endogenous fatty acids must be taken into account in future studies of uptake and binding of guanidine derivatives to mitochondria and possibly to other cell components.

*General effects of free fatty acid binding.* The binding of free fatty acids to mitochondria at sites which exhibit high affinity and selectivity for fatty acid structure but without evident effect on those functions of mitochondria immediately related to energy conservation raises the question of the possible physiologic function of such fatty acid binding *in vivo*. It has become increasingly clear that mitochondrial membranes are selectively impermeable to such small molecular substances as alkali metal cations (20), long chain fatty acyl-CoA derivatives (21), and citric acid cycle intermediates (22-24). This rate-limiting barrier may

constitute a major site of control for the rate of lipogenesis (25, 26), gluconeogenesis (22), and glycolysis (27) through control of the flux of citrate,  $\alpha$ -ketoglutarate, and amino acids into the cytoplasmic compartment. Several experimental observations suggest that exogenous fatty acids, introduced into the medium bound to albumin, or endogenous fatty acids, may increase the permeability of mitochondrial membranes to long chain fatty acyl-CoA substrates (28) and to  $Mg^{++}$  (29). The stimulation of gluconeogenesis (30) and inhibition of glycolysis (27) produced by long chain fatty acids in perfusion studies may in part be explained by such effects on mitochondrial permeability. The current binding experiments with phenethylbiguanide- $^3H$  support the role of long chain free fatty acids in altering the accessibility of intramitochondrial spaces to certain small molecules.

Conversely, as suggested in the preceding paper (3), the mechanism whereby the guanidine derivatives lower the blood sugar may well relate to their ability to alter the transmembrane flux of these same metabolic intermediates, but in a manner opposite to the changes induced by free fatty acids. In this respect, the guanidine derivatives may resemble hypoglycin (L- $\alpha$ -amino- $\beta$ -methylenecyclopropane propionic acid), the hypoglycemic activity of which has been postulated to result from inhibition of the palmitoyl-CoA:carnitine acyltransferase reaction (31) in hepatic mitochondria, with consequent restriction of gluconeogenesis. Butylbiguanide, in concentrations too low to interfere with respiration, has been shown to inhibit the efflux of amino acids,  $K^+$ ,  $P_i$ , and urea from isolated, perfused rat liver (32), and octylguanidine interferes with the efflux of  $K^+$  from rat liver mitochondria induced in vitro by histones (33). In a functional sense, then, guanidine derivatives may be free fatty acid antagonists at a mitochondrial level. It is possible that the differences in sensitivity of diabetic *versus* nondiabetic humans to the hypoglycemic activity of these agents may spring at least in part from differences in the tissue levels of free fatty acids or in the sensitivity of mitochondria to the effects of these fatty acids.

It should be emphasized that the possible existence of guanidine derivative-sensitive trans-mitochondrial membrane metabolite transfer does not

rule out an independent, direct inhibitory effect of guanidine derivatives on electron transport or energy conservation, as suggested by previous work (2, 18). Rupture of the intact mitochondrion might be expected to eliminate the "permeability" component of guanidine derivatives inhibition while not affecting the "energy conservation" component of inhibition. Such "tightly coupled" sub-mitochondrial particles have been found to be susceptible to respiratory inhibition by phenethylbiguanide and octylguanidine; DNP was no longer capable of reversing inhibition (34) in these particles, however, consistent with the hypothesis that the major effect of DNP in intact mitochondria may be on the "permeability" inhibition by biguanides.

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