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SOME NEW ASPECTS ON THE INTERACTION OF HYPOGLYCEMIA-PRODUCING BIGUANIDES WITH BIOLOGICAL MEMBRANES

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Abstract— Effects of biguanides on gluconeogenesis, on mitochondrial ion uptake and oxidative phosphorylation, and on properties of artificial membranes were investigated and correlated. The following observations have been made: (1) Biguanides inhibit glucose formation in cellular systems, like kidney tubule cells, lowering the respiratory rate and the ATP/ADP ratio. In subcellular particulate systems, like mitochondria, the formation of phosphoenolpyruvate from precursors is inhibited, even when energy supply is not rate-limiting. (2) In isolated mitochondria biguanides inhibit oxidative phosphorylation; the inhibition is released by low concentrations of long-chain fatty acids. The respiration-dependent proton pump of mitochondria is also inhibited; electroneutral uptake of H^+ in presence of uncouplers is not affected. Biguanides inhibit the energy-linked uptake of K^+ or Ca^{2+} into liver mitochondria. (3) Binding of biguanides causes a positive shift of a membrane's surface charge. This shift can be reversed by binding of fatty acids. Hydroxylated metabolites of biguanides exhibit much lower binding affinity. They are much weaker inhibitors as well. The biguanide induced shift of surface charge causes a fluidity change of membrane lipids in membrane models and in liver mitochondria. By decreasing the net surface charge, the membrane lipid assembly is rendered more rigid. It is postulated that biguanides act only on membranes and by alteration of membrane properties in general. These alterations are based on the demonstrated generation of a positive surface potential. This, for the first time, provides a unique molecular basis for an understanding of the large variety of biguanide effects. The hypoglycemic effect may be considered as an accidental result rather than a principle of drug action.

The extraordinary number of studies on the blood sugar lowering effect and on metabolic interactions of biguanides is extensively discussed in different recent reviews by Söling and Ditschuneit [1] and by Beckmann [2]. Although these reviews summarize the relevant observations in the field, a unique concept for the mechanism of action could not be developed.

On the metabolic level, three different phenomena, which have been studied extensively *in vitro*, can be distinguished. They serve as an explanation of most toxic as well as useful drug effects. These are: (1) the inhibition of mitochondrial oxidative phosphorylation and respiration [3–8]; (2) the inhibition of glucose production in gluconeogenetic tissues [9–14] and (3) the inhibition of intestinal glucose uptake [15–18].

Most of the reported *in vitro* studies on biguanides have been performed with phenformin or with buformin. For systematic studies on a molecular basis it is reasonable, however, to compare a larger variety of compounds and to modify the interaction of a drug with any presumed receptor site. In the simplest way this may be achieved by varying the length of a hydrocarbon side-chain, a technique which has been applied in the present study. Derivatives ranging from 1,1-dimethylbiguanide to *n*-octylbiguanide and phenethylbiguanide have been used in the following experiments, which will be presented in three groups: the first group dealing with gluconeogenesis as an integrated process, the second group adding some new information on interaction of biguanides with respiration, phosphorylation and ion-transport in isolated mitochondria. The third group comprehends binding studies and physico-chemical aspects of biological membranes and synthetic membrane models.

Abbreviations: ANS = 1-anilino-naphtalene-8-sulfonic-acid; DBI = phenethylbiguanide; FCCP = *p*-trifluormethoxy-carbonylcyanide-phenylhydrazone.

MATERIALS AND METHODS

Most methods employed in the present investigation are standard procedures and have been described in detail elsewhere [19]. Any modifications are mentioned in the legends to tables and figures.

Isolation of cells and mitochondria. Kidney tubules were isolated from the pooled kidneys from 4–5 male Wistar rats after 18 hr of starvation, essentially following the technique of Burg *et al.* [20] with the modification reported by Guder *et al.* [21]. During collagenase digestion of kidney cortex particles the state of the preparation was continuously followed by phase contrast microscopy. Collagenase was purchased from Worthington. The buffer used was an oxygen saturated Krebs–Henseleit medium in which bicarbonate buffer was replaced by triethanolamine.

Kidney cortex mitochondria were prepared from rat kidneys after short perfusion of the organ with isolation medium to remove blood cells. The medulla was removed and the cortex of half kidneys was cut into small pieces. All further procedures were identical to those applied for liver mitochondria.

Liver mitochondria from male rats, guinea pigs, and pigeons were prepared according to standard procedures by differential centrifugation. The isolation medium consisted of 0.18 M mannitol and 0.07 M sucrose in presence of 0.1 mM EDTA and 1 mM triethanolamine. The mitochondrial pellet was washed twice and finally suspended in EDTA-free isolation medium.

All protein determinations were performed with a biuret method [22] using "Lab-Trol" as standard for calibration.

Incubation procedures. Rat kidney tubules were incubated in a Krebs Henseleit medium under oxygen saturation as described above. Osmolality was 300 mOsm/l. The incubations were carried out in total volumes of 2 ml containing 1.5–3.0 mg tissue protein at 37° in a shaking water bath. The incubations were terminated by addition of perchloric acid followed by centrifugation and neutralization with potassium bicarbonate.

Mitochondrial incubations were carried out in a medium also used for respiratory control measurements containing 0.25 M sucrose, 10 mM KCl, 5 mM $MgCl_2$, 5 mM K_2HPO_4, 0.2 mM EDTA, 10 mM TRA. The concentration of substrates and other additions are given with the individual experiments. The method for measurement of phosphoenolpyruvate synthesis essentially follows the procedure of Nordlie and Lardy [23, 24].

Determinations. Glucose, ADP, ATP phosphoenolpyruvate, malate, oxaloacetate, pyruvate, and lactate were determined enzymatically in the deproteinized supernatants of incubations by standard methods as published by Bergmeyer [25, 33].

Oxygen uptake of kidney tubule suspensions and mitochondria was continuously monitored polarographically using a commercial equipment of Eschweiler & Co. The experiments were started by addition of tubule suspensions (1–2 mg) or mitochondria (1–3 mg) in a total volume of 2.6 ml at room temperature.

Measurement of ion fluxes. Mitochondrial uptake or release of H^+ ions was measured with a specially designed glass electrode (Ingold) of low resistance, connected to a Keithley-610 electrometer. The electrometer output was monitored with a rapid recorder or a storage oscilloscope. The half-time of the set up was about 100 msec. In this case the incubation medium contained 0.25 M sucrose, 0.5 mM EDTA, 10 mM Na succinate as a substrate and as a minimum buffer. The miniature electrode fitted a thermostated glass chamber of 0.9 ml volume equipped with a high-speed magnetic stirring device. Additions were made with Hamilton syringes through a capillary opening.

For measurement of K^+ uptake the H^+ electrode was replaced by a K^+ electrode (Beckmann 39047).

For recording H^+ uptake in oxidative phosphorylation the buffer was supplemented with 0.5 mM triethanolamine, pH 7.3, and 1 mM inorganic phosphate and was oxygen saturated. The reaction was initiated by addition of aliquots of ADP. Inhibitors were added 2 min ahead of ADP.

Membrane models. Phospholipid vesicles were prepared by sonification of purified egg lecithin or of dipalmitoylphosphatidic acid in 50 mM Tris buffer pH 7.2 or in water at a final concentration of 1.3 mM. For sonification, a Branson sonifier was used at maximum power output; the treatment was carried out under nitrogen.

Fluorescence measurements. Fluorescence of 8-anilino-naphtalene-1-sulfonic acid (ANS) was measured using a modified Eppendorf fluorimeter (high intensity lamp) and appropriate filter combinations. The method of study for ANS binding was as described by Azzi [34].

Biguanides. Phenethylbiguanide (phenformin, DBI), *n*-butylbiguanide (buformin), and 4-hydroxyphenethylbiguanide were a generous gift of Dr. R. Beckmann (Chemie Grünenthal, Aachen); 1-anthrylbiguanide and *n*-octylbiguanide (C_8) were synthesized as described [35]. ANS was obtained from Sigma Corp. All chemicals were obtained from commercial sources and of analytical grade.

RESULTS

A. Biguanides and gluconeogenesis. The overall reaction of glucose formation has been studied in suspensions of isolated rat kidney tubules, providing a sensitive model for control of gluconeogenesis [22, 36]. Figure 1 shows the influence of phenformin on glucose production from various precursors. The rate of glucose production decreases markedly in all cases and it should be added that the same could be observed with kidney tubules from guinea pig. Succinate and lactate proved to be the best precursors in this model. The dependence of the inhibition on phenformin concentration is shown in Fig. 2, which also indicates that the cellular ATP level decreases concomitantly with glucose production.

An analogue experiment using buformin is summarized in Table 1. In this case the ATP/ADP ratio has been determined and appears well-correlated to the degree of inhibition. The right part of the table outlines data on phenformin and reveals an almost 3-fold increase over the endogenous rate of glucose generation and of the ATP/ADP ratio on addition of substrate. Phenformin yields a depression of the respiratory rate by a factor of 0.5 together with decrease in ATP/ADP ratio to 1.7. Gluconeogenesis,

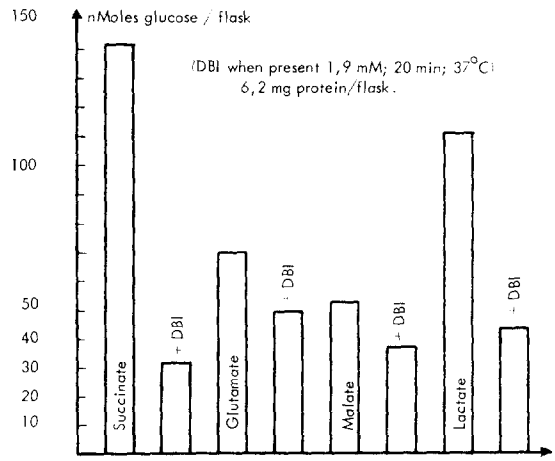

Fig. 1. Glucose formation from various precursors in isolated rat kidney tubules. Concentration of substrates 5 mM; inhibitor concentration 1.9 mM; 37°; 6.2 mg protein flask. (DBI = phenethylbiguanide). (Expt. 220770).

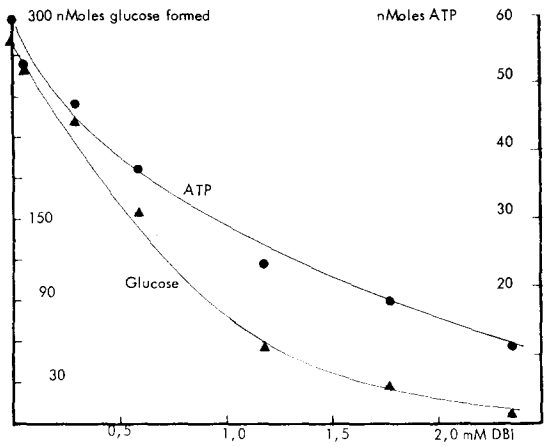

Fig. 2. Dependence of glucose formation and cellular ATP level in isolated rat kidney tubules on concentration of phenethylbiguanide (DBI). Conditions given in Methods; 1.6 mg protein/flask, total vol. 2.65 ml. (Expt. 20/210770).

however, is suppressed to 1/10. Pertaining to the high concentrations of inhibitor used in the above experiments it should be emphasized that on preincubation of the cells with biguanides the same extent of the inhibition can be achieved with 10-fold lower concentrations [14, 37].

As an additional model, isolated mitochondria from kidney cortex, guinea pig liver, and pigeon liver have been used. The latter tissue exhibits an extraordinary rate of gluconeogenesis as shown by perfusion studies [38].

Figure 3 shows the time dependence of formation of phosphoenolpyruvate from rat kidney cortex mitochondria. The data are presented as a balance of C_3-compounds formed (pyruvate, lactate, phosphoenolpyruvate) versus C_4-compounds used (malate, oxaloacetate). The formation of phosphoenolpyruvate is demonstrated separately. Only in the absence of phenformin does a continuous increase of phosphoenolpyruvate result. This may be mainly due to the lack of oxaloacetate in presence of the inhibitor which causes a concentration dependent increase of the malate/oxaloacetate ratio as shown in Fig. 4. These experiments have been carried out in uncoupled mitochondria with ITP added as an energy source. ATPase has been blocked by oligomycin. Obviously biguanides inhibit phosphoenolpyruvate production also in a system which is independent of ATP regeneration by the respiratory chain.

This result could be confirmed using guinea pig liver mitochondria either in presence or in absence of an uncoupler. Figure 5 shows that in coupled mitochondria which may reflect the mitochondrion *in vivo*, the inhibitory effect is much more pronounced. The inhibition can still be observed, however, in the completely uncoupled system. From this it is also concluded that, in addition to ATP production, another effect contributes to the inhibition. We ascribe this to an inhibition of anion exchange (see below).

A final series of metabolic experiments was carried out with pigeon liver mitochondria. Figure 6 gives a single experiment on the concentration dependence of the inhibition of phosphoenolpyruvate formation by *n*-heptylbiguanide. Part (b) of the figure shows for a fixed inhibitor concentration the relative effectiveness of different biguanides. It is important to note that exactly the same relation has been observed with the other models reported above. A simultaneous measurement of mitochondrial respiration and of phosphoenolpyruvate formation allows calculation of the efficiency of the system expressed as the amount of oxygen atoms used per mole of phosphoenolpyruvate formed (Fig. 7). The system becomes less effective if the inhibitor concentration increases, and more respiratory energy is necessary to produce one molecule of glucose.

In summary, the above experiments clearly demonstrate that the effect of biguanides on gluconeogenesis can be localized in the primary energy consuming reactions and in the negative shift of the NADH/NAD redox potential.

B. Biguanides and energy-linked functions of mitochondria. The general phenomenology of biguanide interaction with oxidative phosphorylation and related processes is demonstrated by the following experiments. Figure 8 shows the inhibition of mitochondrial ADP-stimulated respiration. This inhibition can be released by uncouplers and as also shown by long chain fatty acids [39, 40]. In the present case, 1-anthrylbiguanide has been applied and is seen to resemble the effects of other biguanides. The release of the inhibition by fatty acids has been interpreted by others as an uncoupling effect [40, 41] or as a displacement

Table 1. Influence of *n*-butylbiguanide (C_4) and phenethylbiguanide (C_6) on glucose formation, respiration, and ATP/ADP ratio in isolated rat kidney tubules

(a) *n*-Butylbiguanide			(b) Phenethylbiguanide			
Conditions	Glucose formation (nmoles)	ATP/ADP	Conditions	Respiration nA [O]/min per mg	Glucose formation (nmoles)	ATP/ADP
Control	451	3.0	No substrate	14.2	151 ± 1.8	1.4
+ 1.19 mM C_4	370	1.26	+ 20 mM lactate	23.4	472 ± 7.3	3.4
+ 1.77 mM C_4	242	1.03	+ 20 mM lactate + 1.2 mM C_6	12.5	47 ± 2.7 (n = 5)	0.5
+ 2.30 mM C_4	142	0.72				

(a) 20 mM lactate, 37 °, 20 min. incubation, inhibitor concentration as indicated, 3.9 mg protein/ml; (b) 37 °, 25 min. incubation, 3.6 mg protein/ml. Other conditions as described in methods. (Expts. 270770/290970).

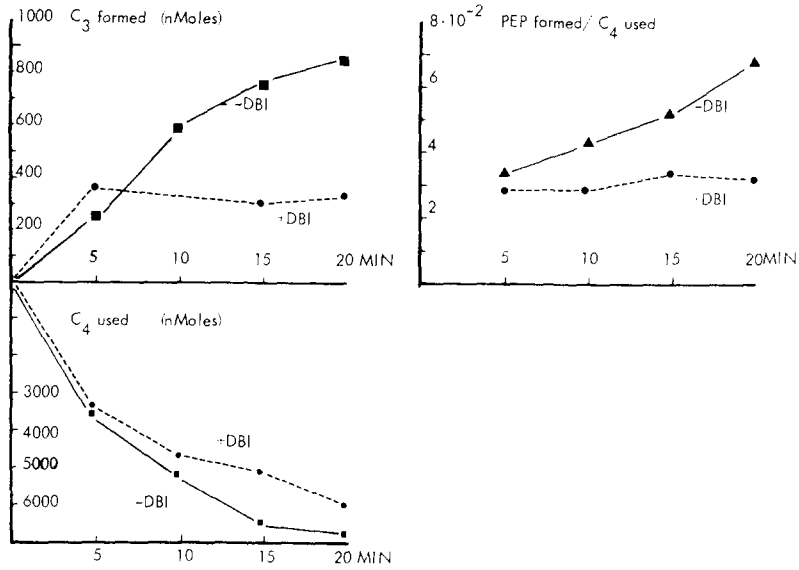

Fig. 3. Balance of formation of C_3 units formed and C_4 units used in isolated rat kidney mitochondria under the influence of phenethylbiguanide (DBI). Total vol. 2.78 ml, containing 7.4 mg mitochondrial protein, 40 μg oligomycin, 14 mM malate as substrate, 54 μM 2,4-dinitrophenol, 30 μM ITP, and 1.9 mM phenethylbiguanide when present. (Expt. 120870).

of the inhibitor [40]. We disagree with this interpretation as will be established below.

In a recent report we have shown that biguanides interfere with membrane ion transport rather than with the chemical reaction of oxidative phosphorylation [19, 42]. The latter involves the mitochondrial uptake of hydrogen ions. By increasing concentrations of phenethylbiguanide [43] this proton uptake during ADP phosphorylation is successively inhibited. The concomitant inhibition of ADP-stimulated respiration has been interpreted by us as a result thereof and not as the source.

Further experiments made obvious, that there exists a direct interaction between biguanides and migration of cationic charges across the mitochondrial membrane. The influence on the mitochondrial proton pump is described in Fig. 9. Anaerobic mitochondria respond to pulses of oxygen by a fast liberation of protons via an electrogenic proton pump [44, 39]. These protons are reabsorbed in a much slower reaction when the oxygen pulse is exhausted. Biguanides diminish the rate as well as the extent of this electrogenic proton translocation. The rate constant for the proton ejection is diminished.

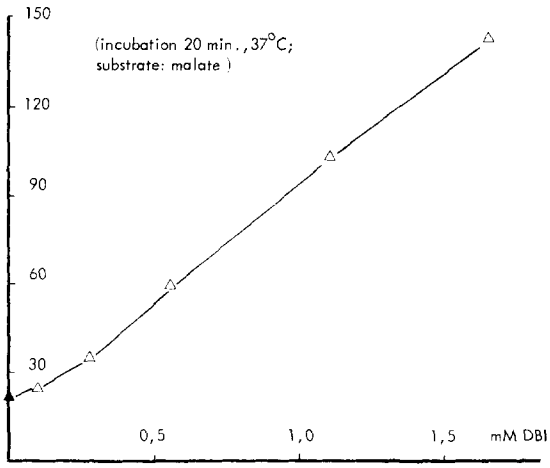

Fig. 4. Effect of phenethylbiguanide concentration on malate/oxaloacetate ratio in rat kidney mitochondria. Conditions as given in Fig. 3, 8.5 mg mitochondrial protein, total vol. 2.68 ml; incubation for 20 min. (Expt. 060870).

An analogue result could be obtained regarding the valinomycin-mediated uptake of potassium into respiring liver mitochondria. From Table 2 it is seen that *n*-octylbiguanide or phenethylbiguanide cause a concentration-dependent decrease of the initial velocity of K^+-uptake.

Fast kinetic studies of Ca^{2+}-uptake into rat liver mitochondria in presence of *n*-octylbiguanide made it evident that the rate constant is lowered and can be titrated by *n*-octylbiguanide (Fig. 10). Moreover, the inhibitory effect shows saturation characteristics which follow the binding properties of biguanides to mitochondrial membranes. The reciprocal plot of A/k vs biguanide concentration yields a straight line and allows calculation of an indirectly derived affinity constant for *n*-octylbiguanide which perfectly agrees with data obtained by other methods [45]. The observed inhibition of cation transport has been shown to result from the membrane surface-charge generated by adsorbtion of positively charged guanidinium or (biguanide) ions to the mitochondrial membrane [19, 42].

C. Biguanide binding and the state of membranes. In previous studies a rather unspecific affinity of biguanides to phospholipid containing membranes has been shown [42–47], whereas protein binding is negligible [48].

Regarding mitochondria, our concept that the membrane itself is the site of action for biguanides is supported by the close correlation of biguanide

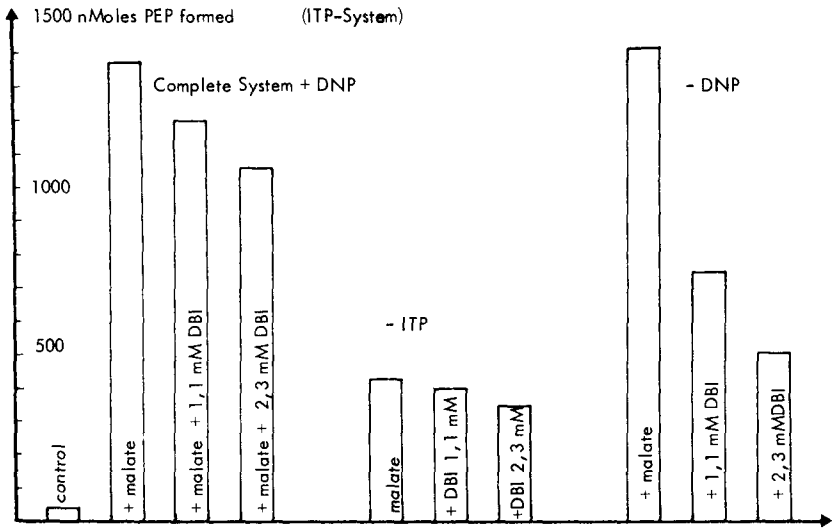

Fig. 5. Influence of phenethylbiguanide on phosphoenolpyruvate formation from malate in guinea pig liver mitochondria. 8.3 mg mitochondrial protein/2.69 ml total volume. Other conditions as in Fig. 3, except that ITP has been omitted in one set of experiments and 2,4-DNP has been omitted in another set of experiments. Control was without malate as substrate. Incubation for 20 min at 37 . Each column represents the average of four determinations. (Expt. 210870).

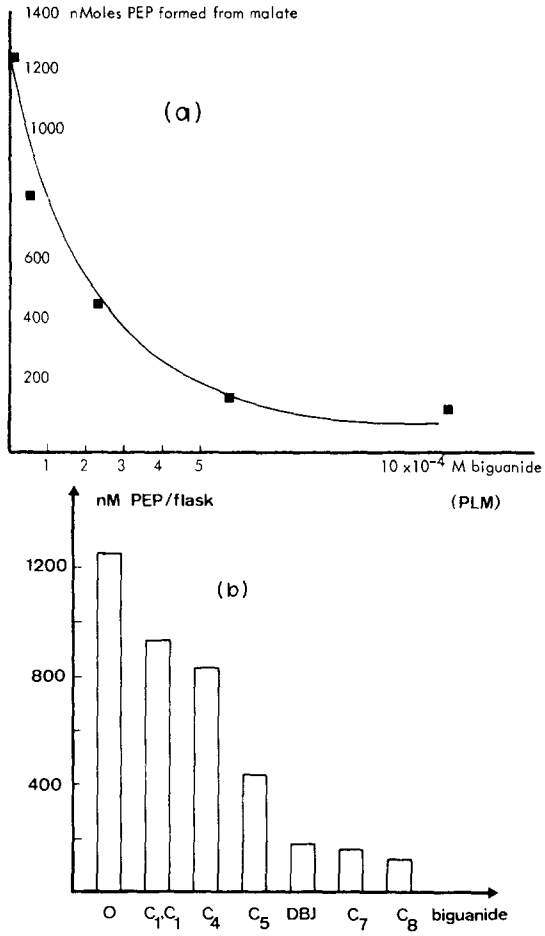

Fig. 6. Inhibition of phosphoenolpyruvate formation by biguanides in pigeon liver mitochondria. Part (a): concentration dependence with *n*-heptylbiguanide. Part (b): comparison of different biguanides at a concentration of 0.59 mM. Conditions as given in Fig. 3; incubation for 20 min at 37°. Expt. (251170).

binding and cytochrome content. Table 3 gives the binding data for three different biguanides and four mitochondrial species which differ markedly in cytochrome content. Since cytochromes are integral constituents of the membrane their amount represents a relative measure of mitochondrial inner membrane area. This is in line with the morphological structure of mitochondria from different sources [49]. From the above we conclude, therefore, that the capacity for biguanide binding is a function of membrane area and of membrane phospholipid content.

From the effect of biguanide binding on the electrical conductance of phospholipid bilayer membranes the generation of fixed positive surface-charges by these drugs could be shown [19]. Since reversible binding of ANS also reflects changes of a membrane's surface-charge [39, 50–52], the latter can be directly monitored, illustrating the antagonistic effect of biguanides and of fatty acids.

Figure 11 shows the fixation of positive charges by addition of biguanide to electroneutral lipid vesicles. It is followed by a large increase of ANS fluorescence. When stearic acid (or other long chain fatty acids) are added, the surface potential is driven more negative by adsorption of the fatty acid anions followed by a release of ANS and a concomitant decrease of fluorescent intensity. The effect is reversed again by further addition of biguanide. Part (b) of the figure shows that liver mitochondria respond in exactly the same way. This observation becomes relevant for the interpretation of the release of biguanide inhibition by long chain fatty acids, which may simply be explained by counteracting the biguanide induced positive shift of membrane surface charge.

Titration of ANS fluorescence has been used to determine binding constants of biguanides [46]. Because the hydroxylated metabolites of biguanides have no or only weak inhibitory and blood sugar lowering activities, it was of interest to compare their

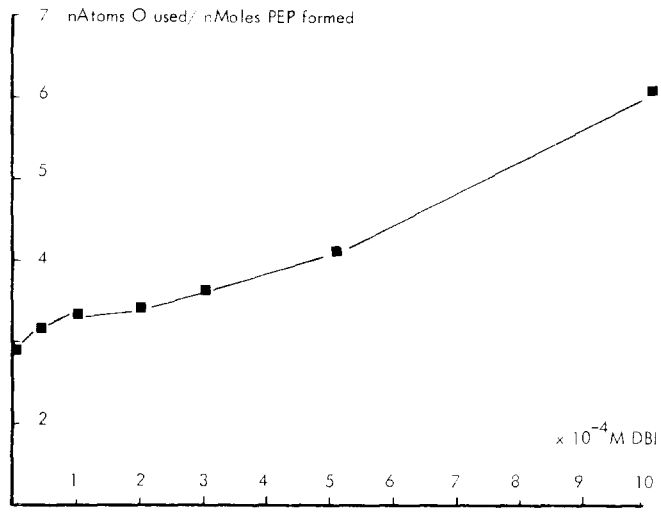

Fig. 7. Oxygen consumption per mole of phosphoenolpyruvate, formed from malate in pigeon liver mitochondria. Dependence on concentration of phenethylbiguanide (DBI). Conditions as in Fig. 3; 3.4 mg mitochondrial protein/flask. The reaction was started by addition of malate. Oxygen uptake was measured polarographically; the mixture was deproteinized in the same moment when oxygen was exhausted. Biguanide was added two min before addition of substrate. (Expt. 210171).

binding properties. As an example 4-hydroxyphenformin was available. An affinity constant to mitochondria and to phospholipid micells of 0.185 mM^{-1} was found compared with 0.63 mM^{-1} for phenformin. The relative inhibitory activity on mitochondrial oxidative phosphorylation agrees well with the affinity ratio of both compounds. This result strongly supports the molecular theory of biguanide action communicated in a commentary [53].

It has been established [54, 55, 56] that the charge density of a lipid membrane is of critical importance for the membrane structure. In this case independent of the sign of the charge, the absolute amount of charge is the relevant parameter. Due to the electrostatic interaction between the polar head groups of lipids an increase of the charge density normally is reflected by a higher fluidity of a membrane; or in other words, the phase transition temperature T_t, at which a membrane undergoes transition from the ordered state to the fluid state of lipids, is lowered [55]. Since most biological membranes bear a negative surface-charge the adsorption of biguanides

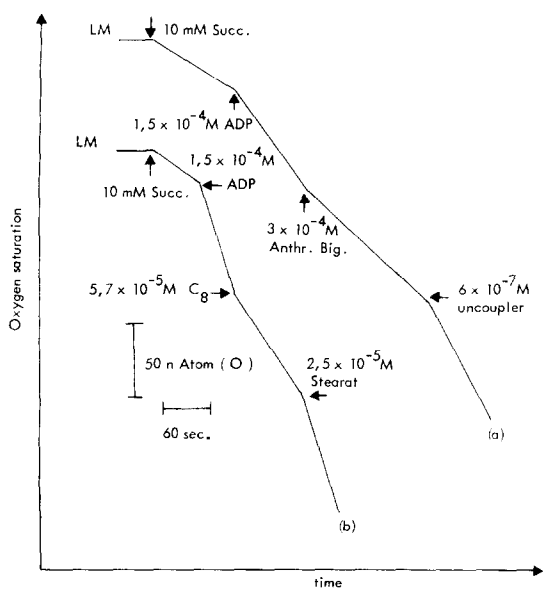

Fig. 8. Release of biguanide inhibition of oxidative phosphorylation by uncouplers and by fatty acids. (a) 0.95 mg protein/ml, inhibition by 1-anthrylbiguanide (this compound strongly fluoresces when bound to mitochondrial membranes), release by FCCP. (b) 1.6 mg protein/ml, inhibition by *n*-octylbiguanide (C_8), release by stearate. 25°. (Expt. 210375).

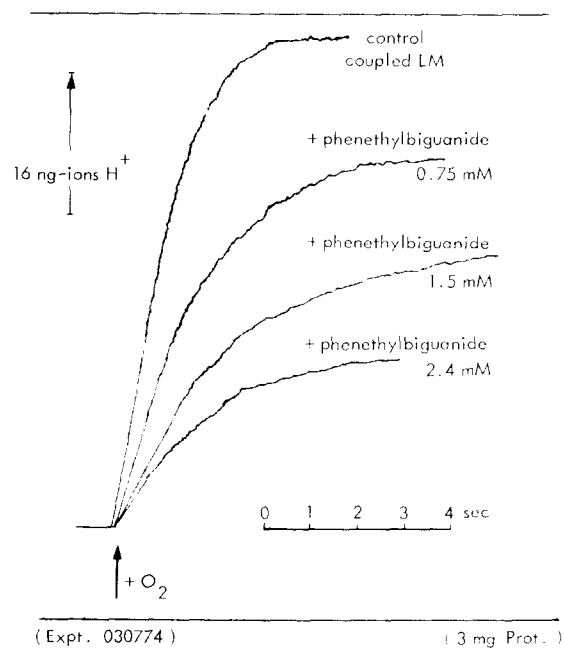

Fig. 9. Effect of phenethylbiguanide on respiration induced H^+ extrusion in coupled rat liver mitochondria. Conditions and registration as described in Methods. 3 mg protein in a total vol of 0.9 ml at 25°. (Expt. 030774).

Table 2. Effect of surface-potential-modifying biguanides on initial velocity of K^+-uptake by aerobic rat liver mitochondria.

μM C_8	v_0* (%)	k (sec^{-1})	μM C_6	v_0† (%)	k (sec^{-1})
0	100	0.022	0	100	0.021
30	47	0.023	120	80	0.017
58	34	0.023	290	43	0.018
117	21	0.022	600	26	0.023
176	11	0.021	880	20	0.023

* v_0 control 44.5 nmoles K^+/sec.
† v_0 control 39.4 nmoles K^+/sec. (Expt. 240974)
C_8 = *n*-octylbiguanide; C_6 = phenethylbiguanide.
t = 20°, 6.7 mg protein/ml, substrate 5 mM succinate, other conditions as given in methods.

is expected to reduce the charge density and to increase the phase transition temperature of membrane lipids.

This is exactly what has been found with synthetic phospholipid membranes and with mitochondria. Figure 12 illustrates that at the high concentration of phenethylbiguanide applied in this experiment, the phase transition is completely absent. It has been shown that it can be gradually shifted from lower to higher temperatures by titration with biguanide [39, 57]. The phase transition was monitored via the temperature dependence of the respiration-induced H^+ liberation from mitochondria (see Fig. 11).

Two important conclusions were drawn from these findings: first, that the principles derived from studies on model systems apply equally well to phospholipid containing biological membranes, and second, that binding of biguanides diminishes the fluidity of membrane lipids turning the membrane structure more rigid.

DISCUSSION

In line with our previous suggestions [19, 42] the experiments presented on biguanide binding and physical structure of membranes provide additional proof for the hypothesis that biguanides induce a positive shift of surface potentials. With respect to the physiological meaning, two criteria have to be distinguished: (1) The absolute charge density on a membrane, which significantly influences the fluidity of lipid phases as demonstrated above; (2) The sign of the electrostatic surface-charge, which determines the activity of ionic solutes at the membrane surface, becoming the relevant parameter for all systems where cation transfer is considered, as for example Ca^{2+} or K^+ uptake into mitochondria.

In any particular case the actual concentration of biguanides and the chemical composition of the membrane will determine whether the effect on ion activities in the membrane–aqueous interface or the effect on membrane fluidity is dominating. Moreover, both effects work synergistically. Regarding the inhibition of oxidative phosphorylation we recall that in tightly coupled mitochondria the respiratory rate is controlled by phosphorylation of ADP, an H^+-consuming process. A positive shift of the membrane surface-charge causes a gradual decrease of H^+ ion activity at the membrane surface until the availability of H^+ becomes rate-limiting.

The release of this inhibition by free fatty acids (in concentrations which do not uncouple oxidative phosphorylation) is explained by reverse of the biguanide-induced positive shift of surface potential (see Fig. 11). The release by uncouplers is explained by the function of uncouplers as electroneutral or negatively charged carriers of protons [58], providing a proton by-path which is independent of the surface potential.

It may be added that at very high biguanide concentrations (> 5 mM), or with derivatives of high

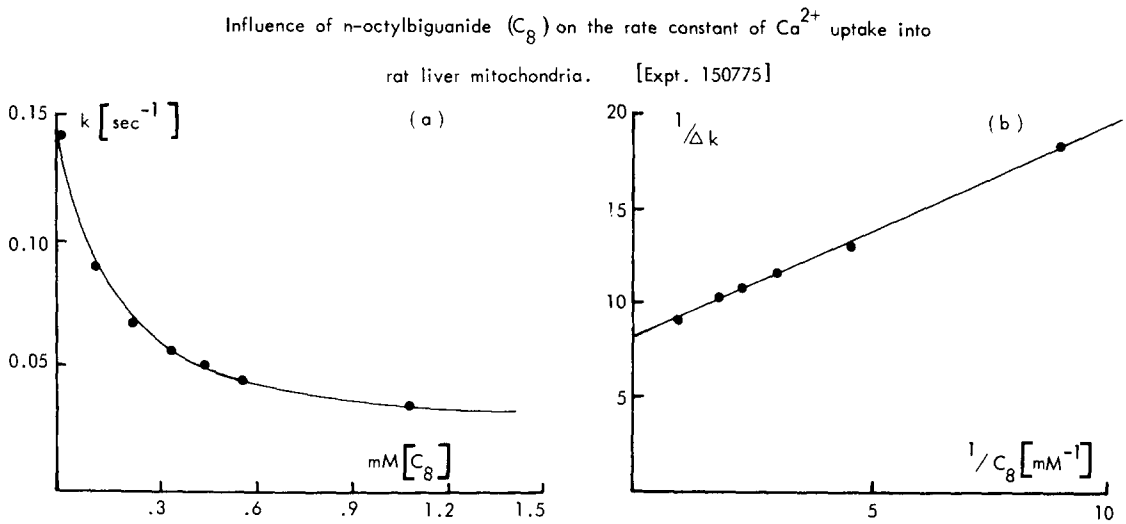

Fig. 10. Influence of *n*-octylbiguanide on the kinetics of Ca^{2+} uptake in rat liver mitochondria. Mitochondrial protein/ml in 0.25 M mannit/sucrose, 20 mM Tris–Cl-buffer, 10 mM succinate, 13 μM murexide; the kinetics of Ca^{2+} uptake were monitored by absorbance change of murexide (540–510 nm) in a dual-wavelength spectrophotometer at 25°; start of reaction by addition of 300 μM $CaCl_2$. (a) first-order rate constant of Ca^{2+} uptake; (b) reciprocal plot of change in rate constant versus inhibitor concentration. (Expt. 110675).

Table 3. Comparison of biguanide uptake and cytochrome content of mitochondria from various tissues.

Source of mitochondria	Guinea pig liver	Rat liver	Pigeon liver	Rat kidney
Cytochrome *c* (nmoles/mg protein)	0.216	0.270	0.392	0.450
Ratio $(c + c_1)/aa_3$	1.1	1.1	1.02	1.7
Uptake of *n*-butylbiguanide	12	13	20	
Uptake of phenethylbiguanide	17	23	31	42
Uptake of *n*-heptylbiguanide	24	36	58	

Biguanide uptake was measured by isotope distribution as described (G. Schäfer *et al.* [64]). The cytochrome content was measured with a a dual-wavelength spectrophotometer according to Schollmeyer and Klingenberg [50].

membrane affinity, a partial inhibition of electron transport persists not releasable by uncouplers. This is presumably due to structural alterations of the membrane as already discussed with respect to membrane fluidity.

More generally, an agent which interferes with the distribution of protons on ordered structures—like membranes—is therefore expected to exert a large variety of effects which in many cases may hardly be resolved. In mitochondria and other subcellular or cellular membranes, the translocation of protons is a primary process which drives the exchange-transport of cations as well as the uptake of anions by means of a pH gradient across the membrane [59–62]. Application of a positive surface potential to a membrane is equivalent to an increase of the local pH and a lowering of the pK_a of intrinsic dissociable groups in a membrane; the latter has been well established by studies with membrane-bound indicator dyes [63]. From this it follows that biguanides not only inhibit the uptake of protons in oxidative phosphorylation but also lower the electrochemical gradient of protons over the membrane, thus diminishing the rate of substrate ion exchange as well. The inhibition of phosphoenolpyruvate formation (Fig. 5) in a system which is independent of ATP synthesis by oxidative phosphorylation may be interpreted under this particular aspect.

Turning to metabolic studies of this and related papers (cited in ref. [1] and [2]), it is too easy to make the inhibition of ATP formation alone responsible for the inhibition of gluconeogenesis. Certainly the decrease of ATP/ADP ratio may contribute to the inhibition of energy-requiring metabolic processes. As already mentioned above, however, the exchange of substrates and the turnover rate of the respiratory chain which regulates the redox state at least of the intramitochondrial pyridine nucleotide-pool—will also essentially contribute to the overall effect, as seen from the experiments with isolated mitochondria.

It has to be specially emphasized that none of the

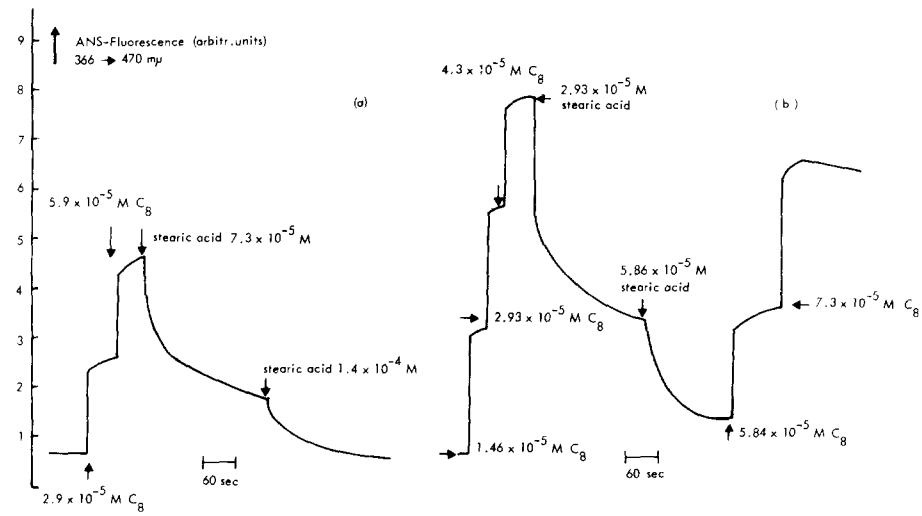

Fig. 11. Indication of changes in membrane surface potential by ANS-fluorescence. Effects of *n*-octylbiguanide and of stearic acid on surface charge. (a) synthetic phospholipid micells; (b) rat liver mitochondria. Micells were suspended in 20 mM Tris-buffer, mitochondria in 0.25 M sucrose/20 mM Tris-buffer, pH 7.2. (Expt. 260675).

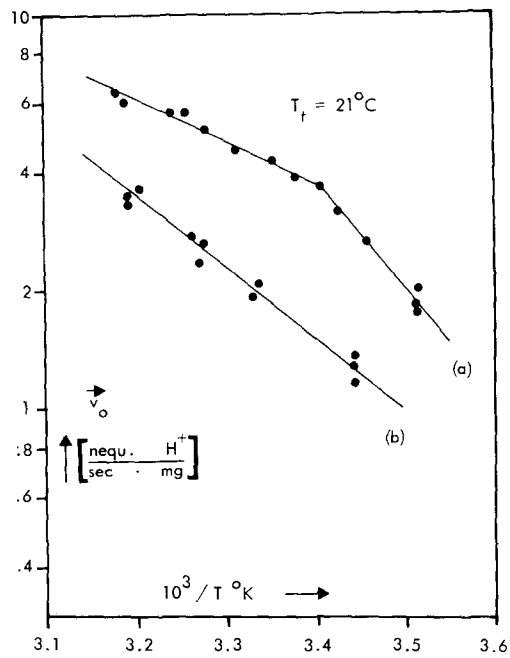

Fig. 12. Arrhenius plots of respiration-induced H^+ ejection from rat liver mitochondria. v_0 denotes the initial velocity. (a) control; (b) 3.0 mM phenethylbiguanide present. Protein concentration varied from 1.4 to 1.7 mg/ml. Other conditions as given in Methods. (Expt. 131174/131274).

reactions involved in our model experiments could be inhibited by biguanides when the relevant enzymatic systems were investigated in solubilized form. Not only some of the involved enzymes, but also the regulation of gluconeogenesis as an integrated process, depend on the presence of divalent cations [24, 36, 64, 65]. It follows from our experiments that biguanides certainly will affect the intracellular distribution of cations and it has also been shown that divalent cations were released from the endoplasmic reticulum (microsomal fraction) on addition of biguanides [66, 67]; again an effect strictly depending on membrane structures.

Comparative studies on blood sugar lowering activity with laboratory animals were reported [2] which indicate a maximum of pharmacological activity for buformin and phenformin. One should keep in mind, however, that the deviation from a straight correlation to membrane binding affinity does not contradict our concept, because in whole laboratory animals many other parameters will be involved in a structure–function correlation. Species differences have to be considered and have been described for the blood sugar lowering activity long ago [2, 63]. In addition, the tissue distribution and the pharmacokinetics of single compounds may certainly be different.

Under these aspects an important observation made with isolated tissue preparations, as well as with intact organisms, is the inhibition of intestinal uptake of glucose [15–18] or of aminoacids [68]. Like other metabolic effects this has been ascribed to the biguanide-induced decrease of cellular ATP/ADP ratio and the respiratory inhibition. This may be an over simplification, although such an effect may be involved. Sugar and aminoacid transport have been shown to be linked to cation transport [69, 71], suggesting a dependence on the membrane surface-potential, like the transport of other cationic or anionic species. This effect has to be considered independent of energy supply. In fact, a very recent report makes it likely that in presence of biguanides the activity of Na^+ in the microenvironment of the intestinal glucose carrier is reduced [18]. This lends substantial support to our interpretation of biguanides as modifiers of surface potentials.

Taking into account that after oral application the highest local concentrations of biguanides are found in the intestinal tract, in liver and in kidney [2], inhibition of gluconeogenesis may be essential for production of hypoglycemia, but it may enhance the development of lactate acidosis as well. Accumulation of blood lactate observed after high doses of biguanides [2, 72], may not only result from stimulation of glycolysis following respiratory inhibition but also from a decreased rate of removal by the gluconeogenic tissues of liver and kidney. From this it becomes clear that the operational bandwidth is very small between intoxication and useful intervention of carbohydrate metabolism.

It is not the intention of this paper to present a complete resolution of the blood sugar lowering mechanism. Too many divergent observations have been reported which can never be discussed in particular. It was intended, however, to direct attention to the fact that the targets for biguanide action are biological membranes in a rather unspecific manner. Together with former data the new information on biguanide effects presented here provides a solid basis for a more general theory of biguanide action on a molecular level. In this regard the observations on membrane surface-potentials and their modification by biguanides are of significance. This molecular theory will be communicated in a separate commentary [53] which will also discuss the problem of specificity of these drugs in more detail.

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