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COMMENTARY

ON THE MECHANISM OF ACTION OF HYPOGLYCEMIA-PRODUCING BIGUANIDES. A REEVALUATION AND A MOLECULAR THEORY

G. SCHÄFER

Department of Biochemistry, Medizinische Hochschule Hannover, Germany

For more than fifty years it has been known that guanidine itself [1] as well as substituted guanidines, diguanidines [2, 3], and biguanides [4, 5] exhibit blood sugar lowering activity with various laboratory animals and men. A great line of compounds has been developed on the basis of the guanidine structure, most of which exert intolerable side effects. Only a few compounds appear in the narrow range between toxicity and useful drug action and are still widely used in treatment of diabetes mellitus. An extraordinary number of studies has been carried out on their mechanism of action. Reviews have appeared [6, 7] which summarize the observed effects and the multiple interpretations on the blood sugar lowering mechanism. The reader is left suspicious, however, as long as a large number of explanations exists instead of a unique concept.

In the ideal case a drug is required to interact specifically with a metabolic reaction or a regulatory process as for example antibiotics or—regarding the treatment of diabetes—the sulfonylureas. This requirement is not fulfilled by biguanide. None of the observations on metabolic systems *in vitro* may explain the integrated action on whole organisms. In contrast, most of the observed effects are inhibitory ones which much better explain the toxic side-effects observed with higher doses of biguanides.

Thus the provocative question may be raised of whether the hypoglycemic effect is only an accidental one, resulting from superposition of a number of minor toxic interactions which an organism answers by mobilizing various regulatory mechanisms. In other words: a specific drug action is put in doubt.

On the metabolic level biguanides were found *in vitro* to inhibit: (1) mitochondrial oxidative phosphorylation and respiration, (2) glucose production in gluconeogenetic tissues and intestinal glucose uptake (for review see [6, 7, 8]). Nevertheless, the latter findings so far provide the biochemical basis for an explanation of many of the clinical observations on drug action, as well as for their toxicity.

As has been suggested by our studies [8–12] a more common basis may be the interaction of these drugs with biological membranes. Such an assumption is justified because most of the processes sensitive to guanidines or biguanides are either directly localized in membranes or involve membrane-linked reactions as rate-limiting steps. Inhibitory actions of guanidine derivatives have been observed on the following processes, for example: respiration and oxidative phosphorylation; specificity to site I or II in oxidative phosphorylation; turn over of TCA-cycle, ^{14}C-incorporation into liver lipids; gluconeogenesis in liver and kidney; intestinal glucose uptake; ^{32}P/ATP exchange in isolated mitochondria; energy transfer in chloroplasts; phosphorylation in chromatophores; cation exchange at the mitochondrial membrane (the relevant literature is summarized in [13]); cation uptake of yeast cells [14].

Moreover, no biguanide effects became known on soluble enzymatic systems. Only higher organized structures were sensitive to biguanides, a fact which is in support of our assumption that membrane interactions play a significant role.

The present review emphasizes that in fact all guanidine and biguanide derivatives share a common and unique property, altering the functional and the physical structure of membranes. This is the change of the electrostatic surface potential (ψ_0) of a membrane. The latter is defined as the electrical potential between the immediate membrane surface and the aqueous bulk phase in which a membrane structure is immersed. It will be shown that practically all observations on biguanide action *in vitro* may be reduced to this physical phenomenon.

A. BINDING OF BIGUANIDES TO MEMBRANES

Although it is apparent from the entire literature on biguanides that they are effective only in membraneous or particulate systems, it was the aim of many investigations to find specific interactions of these drugs with catalytic proteins of different metabolic importance. Many years' investigation were spent without detailed knowledge of binding and distribution of the inhibitors on a subcellular level, until specific studies were carried out in our laboratory [9, 15]. We were the first to put forward the concept that rather than proteins itself it is the membrane structure, building the natural environment for a large number of enzymes, which is affected by these inhibitors [10, 11, 13].

It was shown with ^{14}C-labeled compounds that biguanides bind firmly to mitochondrial membranes and that binding is slightly modified by the energy state of mitochondria [15, 16]. Binding constants could be obtained which agree well with those obtained with another method using the fluorescence of ANS as an indicator of biguanide binding. An example is given in Fig. 1. Mitochondria were incubated with a constant amount of ANS in presence

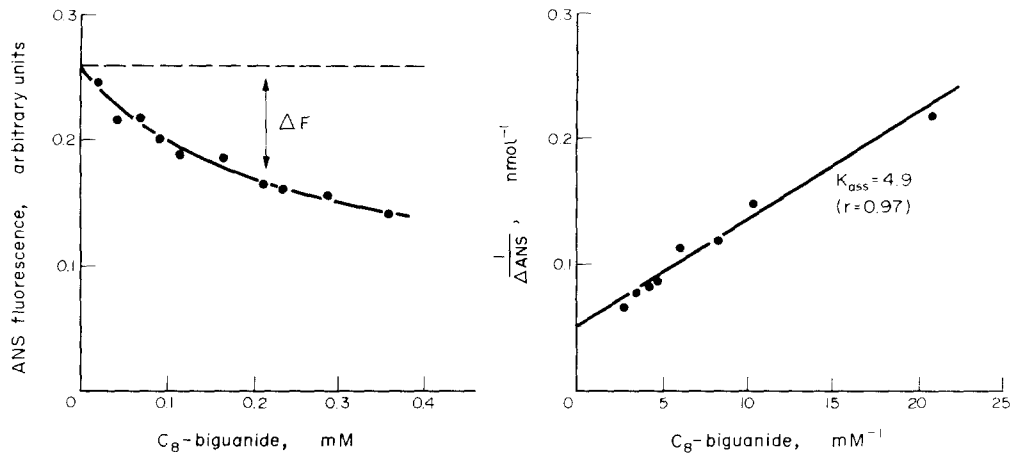

Fig. 1. Influence of biguanide on binding of ANS to rat liver mitochondria. (Data from G. Schäfer [9].) Left hand: decrease of ANS content in mitochondrial supernatant. Right hand: Lineweaver–Burk plot of ANS binding data.

of various concentrations of biguanide. After equilibration the mitochondria were separated from the incubation medium and ANS was determined in the particle-free supernatant by its fluorescence as described [9]. Binding can be saturated and the reciprocal plot of bound ANS vs biguanide concentration yields the affinity constant.

Bound ANS shows a much higher fluorescence yield than free ANS. Biguanide binding could therefore be directly measured by monitoring ANS fluorescence in membrane suspensions which are titrated with biguanides. Table 1 refers to these experiments and shows that the affinity constants obtained with different methods are in good agreement and that all biguanides have a high membrane affinity which can be directly correlated with their partition coefficient. The correlation indicates that there is almost no electrostatic contribution to binding; in other words, binding is exclusively hydrophobic and represents the fixation of the guanidinium group to a membrane by its hydrophobic side chain. It became clear very soon that biguanides bind equally well to membranes of completely different origin, as summarized in Table 2. The close relationship of binding to natural membranes or synthetic phospholipid structures leads us to assume that membrane phospholipids are the natural binding sites [9]. Evidence for this could be accumulated by successive lipid extraction from mitochondrial membranes resulting in a gradual decrease of biguanide binding. It was demonstrated that the affinity constants did not change by this treatment. The number of binding sites, however, was strictly correlated with the phospholipid content as summarized in Table 3.

From comparison of results it is evident that the inhibitory effectiveness of biguanides closely resembles their affinity for membrane phospholipids. It emerges also from this investigation that membrane binding is completely unspecific. Protein binding of biguanides is negligible [17], and regarding the effective concentrations with mitochondria it can be estimated that these are on a stoichiometric basis close to the membrane lipid content and far above the content of respiratory enzymes. On a protein basis, mitochondrial phospholipid content differs by three orders of magnitude from cytochrome content. Actually, the maximum capacity for biguanide binding fits very well the range of phospholipid content. For example liver mitochondria containing approximately 170 nmole phospholipid/mg protein were found to bind 50–100 nmole biguanides/mg protein, whereas the cytochrome content is only 0.2–0.25 nmole/mg.

Table 1. Binding affinity of biguanides to mitochondrial membranes.

Biguanide	$C_{1,1}$	C_4	C_5	C_6*	C_7	C_8
K_{ass} ANS (mM^{-1})	0.012	0.312	0.505	0.681	1.49	3.18
K_{ass} ^{14}C (mM^{-1})	—	0.312	—	0.63	1.46	4.38
P_0 (*n*-octanol/phosphate buffer)	0.037	0.063	0.096	0.148	0.247	0.605

* C_6 signifies phenethylbiguanide (DBI).

Data were obtained either by ANS fluorescence titration, or by ^{14}C distribution with labeled biguanides. P_0 signifies the distribution coefficient between *n*-octanol and phosphate buffer at pH 7.2. (From G. Schäfer *et al.* [15].)

Table 2. Affinity constants of biguanides towards different types of membranes. (From G. Schäfer *et al.* [15].)

Compound	C_6*	C_7	C_8	Type of membrane
K_{ass}† ANS	0.603	1.49	3.38	Mitochondrial (liver)
K_{ass} ^{14}C	0.63–0.68	1.46	4.38	Mitochondrial (liver)
K_{ass} ANS	0.521			Sub. mitochondrial particles
K_{ass} ANS	0.51–0.63		4.96	Liposomes
K_{ass} ANS	0.4	1.64	4.39	Microsomes

* The index gives the number of carbon atoms in the side chain; C_6 = phenethylbiguanide (DBI).

† The affinity constants are given as mM^{-1}.

Table 3. Correlation of phospholipid content and capacity for biguanide binding of liver mitochondria. (From G. Schäfer [9, 13]).

Phospholipid content		Number of binding sites			
Protein (μg/mg)	Per cent of control	Phenethylbiguanide (n_{relat}).*	nmole/mg†	*n*-octylbiguanide (n_{relat})	nmole/mg
12.6	100	1.0	82	1.0	130
4.2	33.4	0.40	33	0.47	62
3.1	24.6	0.35	29	0.38	50
1.8	14.3	0.25	20	0.32	41

* Data taken from ANS fluorescence titrations.
† Data based on [^{14}C]biguanide binding to normal rat liver mitochondria (= 100%).

Data presented in this issue [8] also support the contention that the capacity for biguanide binding to mitochondria is related to the available mitochondrial inner membrane area (Table 3 of ref. [8]). A model experiment on bulk-phase distribution of ^{14}C-labeled biguanide lends further support to this assumption. The experiment, in addition, contradicts the hypothesis that biguanides are distributed across a membrane by nonionic diffusion following a pH gradient across the membrane [18]. It is shown in Fig. 2 that in a three-compartment system where two aqueous phases A and B are separated by a lipid-containing nonaqueous phase C the distribution of phenethylbiguanide is determined only by the phospholipid content of the nonaqueous phase and not by the pH gradient across this phase. The experiment was started with 100 per cent radioactivity present in compartment A. Almost all of the biguanide extracted from compartment A is found in the phospholipid-containing hydrophobic phase and less than 10 per cent is extracted from there into the acidic compartment B, despite a 1000-fold higher activity of hydrogen ions. A very similar distribution was when the pH was 8 in both aqueous compartments. In this model the hydrophobic lecithin-containing phase resembles the membrane. The result is in accordance with the assumption that practically all of the biguanide taken up by a membrane structure from a solution remains membrane-bound. Independent support is derived from the observation that even large amounts of biguanides taken up into mitochondria are osmotically inactive [9].

The question of where the adsorbed biguanide molecules reside in the membrane may be answered from the response of ANS. It is known that ANS binds to the aqueous/lipid interface in the region of the phosphoester bonds [19] within the headgroups of polar phospholipids. Because biguanide binding is strictly hydrophobic, it is justified to assume that the hydrocarbon part of the biguanide molecule is inserted between the hydrocarbon chains of the phospholipid molecules, with the polar guanidinium-group localized near the phosphoester bonds similar to ANS. Whereas the hydrocarbon chain of alkyl or aralkyl biguanides is responsible for membrane binding, the toxophoric function has to be ascribed to the biguanide structure. These molecules are strong bases [20–23] with pK_a values ranging from 11.3 to

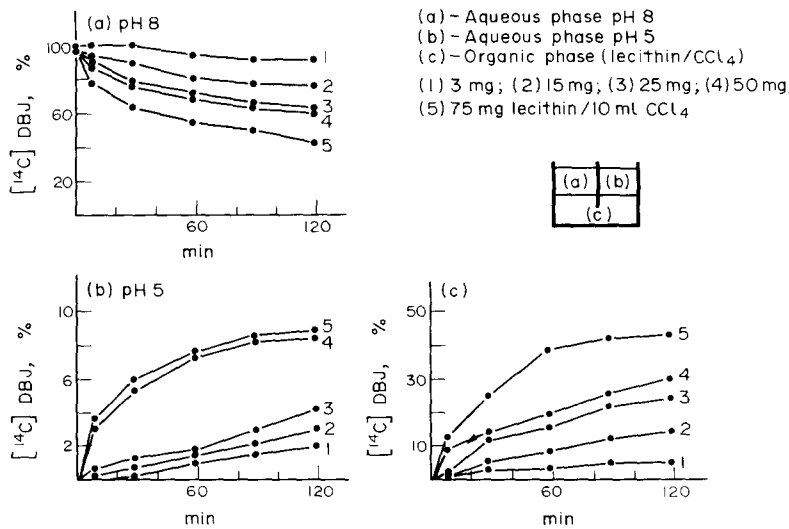

Fig. 2. Bulk phase distribution of [^{14}C]phenethylbiguanide. Influence of lecithin concentration on distribution kinetics. (Data from G. Schäfer [9]).

12.8 for the dissociation of the protonated form according to equation (1).

$$H_2O + BH^+ \rightleftarrows B + H_3^+O. \quad (1)$$

At acidic pH a second proton can be taken up with a pK_a around 2.8. At physiological pH these compounds exist to more than 99.9999 per cent in the protonated form and the free energy of dissociation is about $\Delta G = +16.0$ kcal/mole at room temperature. Therefore it is reasonable to assume that biguanides exist exclusively in the cationic form in aqueous solutions and that they maintain the positive charge also in the membrane adsorbed state.

From this it becomes clear that adsorption of biguanides to any high molecular structures and membranes is equivalent to hydrophobic fixation of positive charges.

At this stage the question has to be asked of the consequences of biguanide binding on the structural and functional properties of membranes.

B. BIGUANIDES AND PHYSICAL PROPERTIES OF MEMBRANES

From the above it was concluded that membrane lipids are the domain of biguanide interaction. That means the molecular arrangement of lipid structures forming the matrix of most biological membranes may be disturbed by these drugs. The perturbation of membrane structure and physical membrane properties may then be transmitted to integral membrane proteins and their catalytic function. This working hypothesis could have been verified during the past two years and convincing evidence accumulated that the primary event in biguanide action is a positive shift of electrostatic surface potential of membranes [10, 11, 13, 24].

In order to study some physico-chemical principles of biguanide interaction synthetic phospholipid membranes have been used as a valuable model. It is very probable, moreover, that the results derived from these studies also apply to biological models.

Assuming that adsorbed biguanidinium ions represent fixed positive charges on a membrane, the electrical properties should reflect such a change in surface potential $\pm\psi_0$. This has been demonstrated by measurements of the electrical conductance of black phospholipid membranes as reported elsewhere [10]. As theoretically derived from the diffuse double-layer theory [25, 26], electrical conductance and surface potential ψ_0 are related by equation (2). The negative sign denotes the conductance mediated by a membrane-permeable anionic species of effective charge z_i.

$$G^{(-)}_{charged} = G^{(-)}_0 \exp(z_i F \psi_0 / RT). \quad (2)$$

where $G^{(-)}_{charged}$ signifies the conductance of the charged membrane and $G^{(-)}_0$ the conductance of membrane with electroneutral surface. An example is referred to in Fig. 3. Twenty μM *n*-octyl biguanide was found to induce a surface potential of $\psi_0 = +42$ mV. It was also found that the magnitude of the surface potential could be titrated by binding of biguanides and that the relative effectiveness of different biguanides exactly follows their binding affinity to phospholipid membranes [9, 10, 13]. Surface potentials of up to 80 mV could be measured.

The potential profile across a membrane and the influence of surface potentials are schematically represented in Fig. 4. It shows a membrane M, separating the aqueous solutions I and II. I′ and II′ denote the unstirred aqueous layers present at each membrane surface. Between these and the bulk solution any solute distributes according to a partition coefficient α. Across the membrane there exists an electrochemical potential V, and the surface potentials $+\psi_1$ and $+\psi_2$ are assumed on the respective sides. Since the electrical gradient Φ is the actual driving force for transport of charged species through the membrane, it can be seen that variation of surface potentials modifies this gradient and the transport kinetics.

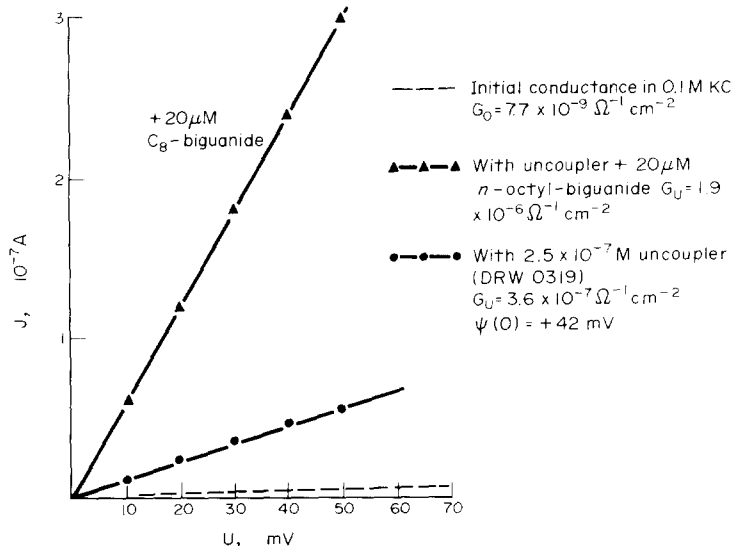

Fig. 3. Current voltage diagram of a black phospholipid membrane. Influence of *n*-octylbiguanide on the membrane surface-potential. The uncoupler DRW 0319 served as an anionic carrier of electrical charge through the membrane. (From G. Schäfer *et al.* [10, 13]).

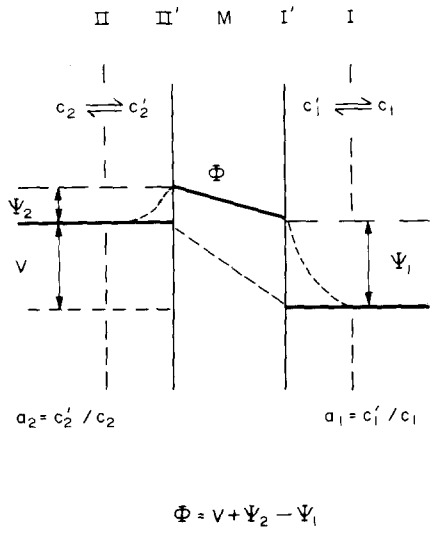

Fig. 4. Schematic diagram of an electric potential profile across a membrane, including the surface potentials on both sides. For convenience all terms were taken positive.

respectively. The activity $[c]$ of ionic species in the unstirred layer depends on the magnitude of the surface potential and is described for a positive species on side I' by equation (3):

$$[c] = \alpha [c_1] \exp(-\psi_1/kT), \quad (3)$$

where $[c_1]$ is the ion activity in the bulk solution on side I for example. It becomes clear that by means of surface potentials the availability of ionic species for interaction with specific membrane binding sites is controlled. It is now well documented that reversible binding of ANS to lipid membranes also reflects changes of a membrane's surface potential [27, 11, 28–30]. Therefore, the fluorescence of ANS can be taken as a relative measure of surface potential changes. In fact, it was shown by us [8] that the surface potential of model membranes and of mitochondria can be alternatively shifted positive or negative by biguanides or by long chain fatty acids, respectively (Fig. 11, ref. [8]). Thus, our findings on electrical conductance of lipid membranes and on biguanide-induced changes of ANS fluorescence are in excellent mutual agreement.

C. BIGUANIDES AND ENERGY-LINKED FUNCTIONS OF MITOCHONDRIA

It has long been assumed that biguanides and guanidines in general directly interact with the primary reactions of energy conservation in mitochondrial oxidative phosphorylation (reviewed in [13, 7]). An oligomycin-like effect [31] and even some site-specificity [32, 33] have been described, localizing the inhibition in the terminal reactions of phosphate transfer and apart from the electron transfer itself. A series of objections against this concept may be raised, however. On one hand, a high-energy intermediate according to the chemical hypothesis of oxidative phosphorylation [34, 35], which might interact with biguanides, could never be identified. In addition, the inhibitory concentrations are much higher than expected on the basis of a stoichiometric interaction with any functional intermediate. On the other hand, the effect of biguanides and guanidines was difficult to explain in terms of the chemiosmotic theory of energy conservation [36]. Our present view overcomes these objections.

The phenomenology of biguanide interaction with oxidative phosphorylation and the release of the inhibition by fatty acids and uncouplers is demonstrated in the following paper [8].

According to equation (4), mitochondrial phosphorylation of ADP at pH 7.2 involves the uptake of hydrogen ions and can easily be followed by means of a sensitive pH electrode in a weakly-buffered mitochondrial suspension.

$$ADP^{3-} + HPO_4^{2-} + H^+ \rightleftarrows ATP^{4-} + H_2O. \quad (4)$$

It was found that the mitochondrial proton uptake during ADP phosphorylation is successively inhibited by increasing concentrations of phenethyl biguanide [10].

Concomitantly with the inhibition of phosphorylation-linked proton uptake, mitochondrial state-3-respiration (ADP present [35]) is titrated back to the state 4 level resting respiration in absence of ADP [35], as shown in the lower part of Fig. 5 for two different biguanide derivatives. The upper part gives the fluorescence increase of ANS (8-anilino

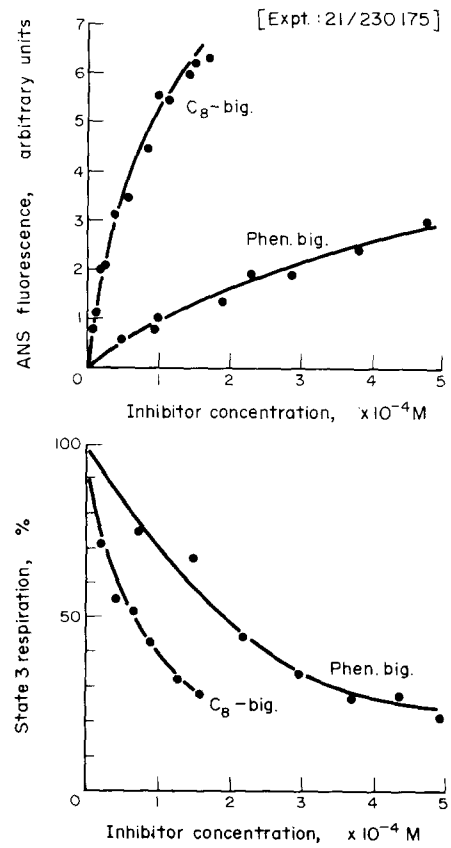

Fig. 5. Influence of biguanides on state-3 respiration of rat liver mitochondria and on ANS fluorescence, indicating the positive shift of surface potential. (From G. Schäfer *et al.* [12]).

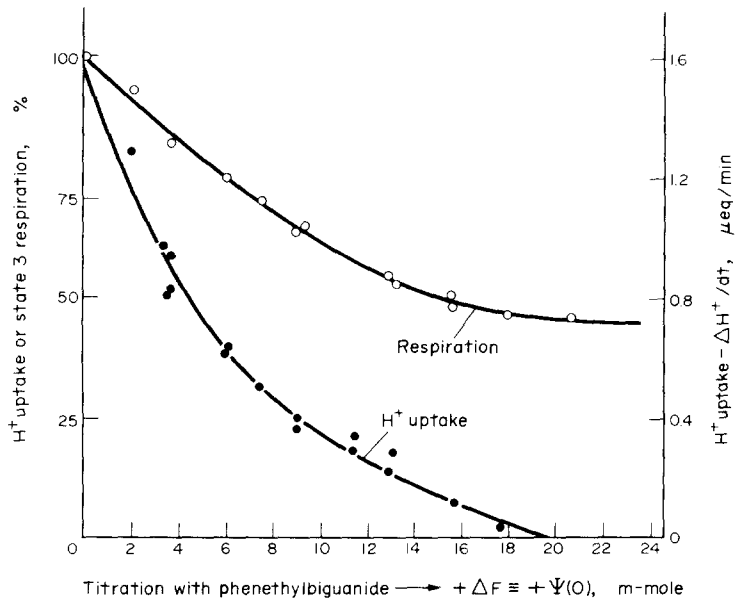

Fig. 6. Rate of H^+ uptake and of state-3 respiration of rat liver mitochondria during ADP phosphorylation as a function of surface-potential. The increase of surface-potential was monitored by ANS fluorescence (measured in arbitrary units) and achieved by titration with phenethylbiguanide. (From G. Schäfer *et al.* [12]).

naphtalene-1-sulfonic acid) as measured under corresponding conditions with the same preparation. As outlined above, this increase of ANS-fluorescence is not only a measure of biguanide binding but also of the positive change in membrane surface-potential. Obviously respiratory rate and ANS fluorescence are dependent in an inverse manner on biguanide concentration. Data as given in Fig. 5, together with the rate of mitochondrial proton uptake during phosphorylation, can be plotted vs the fluorescence of ANS as a relative measure of $+\Delta\psi_0$. This leads to a plot shown in Fig. 6, making it clear that at a certain positive shift of surface-potential the uptake of H^+ ions for ADP phosphorylation is totally suppressed. At the same stage the respiratory rate levels off, the residual respiration representing the resting state of electrontransport which proceeds independently of ADP phosphorylation in coupled mitochondria (according to Chance [35], state-4 respiration). Our interpretation is that a positive shift of ψ_0 causes a gradual decrease of H^+ ion activity at the membrane surface until their availability becomes rate-limiting. A comprehensive view is given in Fig. 7 for a series of different biguanide derivatives. The inhibition of proton uptake is correlated with the respiratory rate, yielding a straight line intercepting the respiratory axis at the average state-4 rate. The positive surface potential increases from the upper right to the lower left along the regression line and has been modified by the indicated biguanides in varying concentrations.

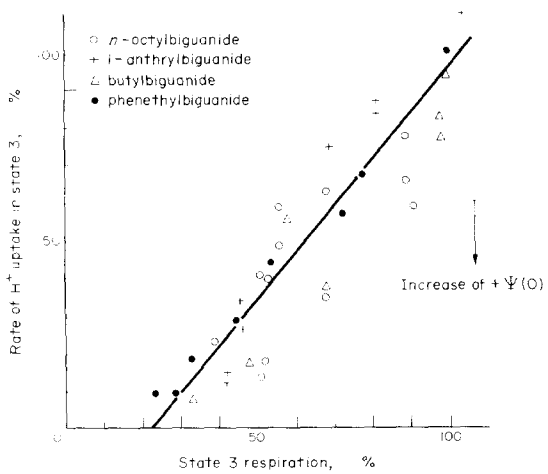

Fig. 7. Correlation of state-3 respiration and H^+ uptake during ADP phosphorylation under the influence of increasing positive surface-potential. The symbols indicate different derivatives of biguanide; data from G. Schäfer *et al.* [12]. Rates of H^+ uptake and of respiration are normalized with 100% being the rate in absence of biguanide.

The important conclusion has to be drawn that all biguanides qualitatively exert the same effect; quantitatively their activity is strictly correlated to their binding affinity. The release of biguanide inhibition by free fatty acids may thus be nothing other than a reverse of the biguanide-induced positive shift of surface-potential.

Further experiments have shown [11] that the kinetics of mitochondrial K^+ or of Ca^{2+} uptake are also inhibited by biguanides. On the above basis evoking equation (3), we simply understand these effects as a result of the more positive surface-potential in presence of these drugs.

As to the electrogenic mitochondrial proton pump it was shown that the translocation of protons is also inhibited by biguanides [8, 24]. It is again the more positive surface-potential which can be considered responsible for this effect.

A reduced flux of protons, however, may be of significance for the flux of substrate anions across the mitochondrial membrane. Anion uptake is driven by the electrochemical gradient of protons across the membrane [37-39] via coupling to the energy-linked uptake of inorganic phosphate. Any lowering of the gradient of H^+ may thus induce a subsequent inhibition of metabolic activities, such as the primary reactions in gluconeogenesis (see below).

Regarding enzymatic catalysis more generally, a positive shift of ψ_0 causes a displacement of H^+ ions which means a positive shift of the very local pH or – which is equivalent – a decrease of the pK_a of intrinsic dissociable groups at the membrane.

D. BIGUANIDES AND MEMBRANE FLUIDITY

The surface potential ψ_0 of a membrane has been discussed for systems where the sign of the charge is the critical parameter. In such systems we have to include all experiments where ion transfer has been measured. Binding of biguanides reduces, by screening of negative sites on a membrane, the number of binding sites for cations or even results in a displacement of divalent cations from subcellular membranes [40, 41]. It has also been established [42–44] that the charge density σ of a lipid membrane is of critical importance for the membrane structure. The absolute amount of charge is the relevant parameter, in this case independent of the sign of the charge. It is related to the molecular area per lipid molecule f by $\sigma = \sigma'/f$ where σ' denotes the charge per molecule. The concept implies that electrostatic interaction between the polar headgroups of lipids largely determines the intermolecular distance. In these terms f reflects the molecular mobility and therefore is an important parameter for membrane fluidity, keeping in mind that at physiological temperatures the lipids of membranes are considered as bilayer-like liquid-crystaline structures. Normally an increase of σ results in a higher fluidity of a phospholipid structure, which is reflected by a decrease of the temperature T_t at which the ordered → fluid transition occurs [43]. Figure 8 shows that in model membranes such a shift of the transition temperature T_t could be verified [24] in the presence of biguanides. What has been observed, however, was an increase of T_t, which we have also reported for liver mitochondria [8] using the temperature dependence of the proton pump as an indicator of the membrane fluidity state.

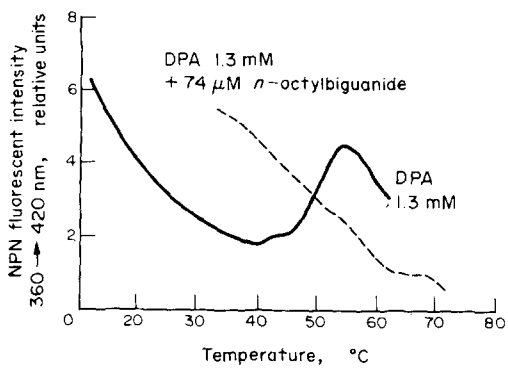

Fig. 8. Temperature-induced phase transition of dipalmitoylphosphatidic acid micells, monitored by fluorescence of NPN. Influence of 74 μM *n*-octylbiguanide (dashed line). (From G. Schäfer *et al.* [24]).

In fact, this is in line with the theory outlined so far. Owing to the high content of negatively charged lipids in these membranes [45], biguanide binding causes a decrease of the absolute density of net charges of these membranes and thus has to increase the transition temperature. From this it follows that at negatively charged lipid membranes the adsorption of biguanides makes the membrane structure more rigid. This is considered of significant importance for the dynamic function of membranes.

E. BIGUANIDES AND GLUCONEOGENESIS

Using different models (reviewed in [7, 8]), it has been shown that gluconeogenesis or its partial reactions can be strongly inhibited by biguanides. The specificity of this effect, however, is questionable, since only lack of energy supply may be the cause. Gluconeogenesis as an energy-demanding process responds sensitively to withdrawal of ATP. The relevant energy-requiring reactions are the carboxylation of pyruvate (equation (5)) and the formation of phosphoenolpyruvate from oxaloacetate (equation (6)).

$$\text{Pyruvate} + CO_2 + \text{ATP} \rightarrow \text{Oxaloacetate} + \text{ADP} + \text{Pi} \quad (5)$$

$$\text{Oxaloacetate} + \begin{Bmatrix}\text{ITP}\\ \text{GTP}\\ \text{ATP}\end{Bmatrix} \rightarrow \text{Phosphoenolpyruvate} + CO_2 + \{\text{IDP, GDP, ADP}\} \quad (6)$$

The turnover of these key reactions also controls the redox state of the pyridine nucleotide pool, which determines the steady-state concentration of pyruvate and oxaloacetate, for instance, (equation (7)).

$$\begin{Bmatrix}\text{Malate}\\ \text{Lactate}\end{Bmatrix} + NAD^+ \rightleftharpoons \begin{Bmatrix}\text{Oxaloacetate}\\ \text{Pyruvate}\end{Bmatrix} + NADH + H^+ \quad (7)$$

Because the ATP/ADP ratio and the NADH/NAD ratio are mutually related via the respiratory chain, the rate of gluconeogenesis critically depends on mitochondrial metabolism. Owing to species differences reaction (6) may be localized intra- or extramitochondrially.

There is no doubt of course that lowering of ATP-supply may be one reason for the rate of glucose formation decreasing in gluconeogenetic tissues. Another reason is the (already discussed) interference of biguanides with substrate anion transport across intracellular membranes (see section C) may contribute to a considerable degree. Like the uptake of phosphate, the intra/extra mitochondrial distribution of aspartate is also energy-linked [46], and thus depends on the electrochemical gradient of H^+ ions as pointed out above. The key role of the aspartate/malate shuttle in gluconeogenesis should be mentioned in this context as a regulatory functional unit [47].

Although the specific contribution of single processes to dependence of gluconeogenesis on membrane functions can hardly be resolved, little doubt remains that biguanides are active by way of their

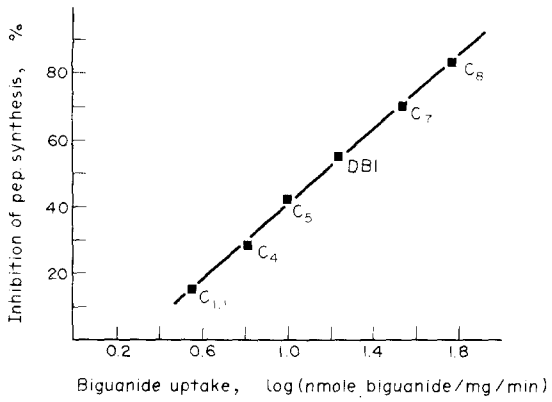

Fig. 9. Correlation of biguanide inhibition of phosphoenolpyruvate synthesis vs mitochondrial capacity for biguanide binding in pigeon liver. [15].

membrane-modifying properties. This is documented in Fig. 9, compiling data available from our laboratory. It clearly follows that the relative effectiveness of six different biguanides in inhibiting phosphoenolpyruvate synthesis (cf. equation (5, 6)) is strictly related to their membrane-binding affinity.

GENERAL CONCLUSIONS

The above discussion is based on the modification of the physical membrane structure by biguanides. It has been established that biguanide binding to membranes causes a positive shift, or the generation of a positive surface potential, providing a unique molecular basis for discussion of the large variety of metabolic activities of guanidines. Thereby the modifying influence of the biguanide-dependent surface-potential on charge transfer and on membrane fluidity may act synergistically, depending on the particular composition of a membrane.

There is no reason to assume any chemical reaction as the basis of biguanide action because biguanides are very stable and chemically inert compounds. They are eliminated from the organism either unaltered, or hydroxylated in the hydrocarbon side-chain [48–50]. Other biological reaction products are not known. The latter products are biologically inactive, suggesting that any increase of polarity in the hydrocarbon part abolishes the observed interaction with membranes, and as shown recently [8].

Large-scale alterations of mitochondrial membranes detectable by electron microscopy have only been observed at very high biguanide concentrations *in vitro*.* They may easily be interpreted in terms of surface-potential changes as well.

It is certainly impossible to discuss in every detail all models on which the effect of biguanide has been tested. We suggest instead that when reviewing the literature one should be aware that all systems sensitive to biguanides involve the function of membranes. Nevertheless, a few important observations should be mentioned illustrating the broad and unspecific activity of biguanide action on biological structures.

* G. Schäfer, unpublished observations (1972).

One should remember, for example, that biguanides as well as guanidines were found to exhibit antibacterial activity [57–59]. Bacteria are in many respects similar to mitochondria and the energy-conserving system is located in the membrane. It is not surprising that similar inhibitory actions could be shown. Furthermore, a fungicide activity of biguanides was reported by several authors [60, 61]. In this case the observation on yeast cells that the proton cation exchange across the cell membrane is inhibited by these drugs is of interest [14]. An affinity of guanidine and biguanide for sodium channels in nerve has also been shown [62]; biguanide is almost impermeable, but the alkylated derivatives may bind to the membrane and screen the cation-specific pores as assumed for yeast cells. All these observations can be explained on the basis outlined in this paper for mitochondria. This extends further to chloroplasts and bacterial chromatophores, which differ only with respect to the energy source but fulfill all other requirements such as the development of a proton gradient across the membrane and the transport of cations and anions by means of this gradient. For a series of biguanides even an antiviral activity [63] or an inhibition of tumor cell growth [64] has been reported.

It should be stressed that these latter activities do not necessarily imply a blood sugar lowering activity by the same compounds [7]; some compounds, however, develop both drug actions.

No doubt even the active transport of electroneutral solutes is subject to changes in surface-potentials. The biguanide inhibition of intestinal aminoacid and glucose uptake is the best known example (for review see [7, 8]). Both processes are intimately linked to cation transport [65, 66] and thus depend on the surface-charge of the membrane. A recent report on a decreased activity of Na^+ in the microenvironment of the intestinal glucose carrier system in presence of biguanides [67] is a straightforward support of the molecular theory presented here. The fact that biguanide concentrations reach their highest values in the intestinal tract, besides liver and kidney [51–53], makes it most likely that inhibition of glucose absorption and of gluconeogenesis mainly contribute to drug action *in vivo* according to the mechanism outlined. But certainly there also exist interferences with the endocrinic system including the action of insulin (for review see [7]). Since receptor binding is the first step of insulin action, it is again a membrane function which may be influenced by biguanides.

As an additional new aspect, the potential function of the guanidine structure as a "chaotropic" agent should be discussed. These are large univalent ions, like ClO_4^-, I^-, guanidinium$^+$ and SCN^- for example, which are known to disturb the ordered structure of water [54]. Bilayer-like phospholipid surfaces contain a definite number of adsorbed water molecules which are in a "non-normal" physical state [55] compared with ordinary water as regards melting point and crystal structure. Properties and arrangement of these water molecules could be perturbated by bound biguanide. It must be clarified by further experimentation whether biguanides represent hydrophobically fixed "chaotropic" ions.

The question may be asked why only biguanides and guanidines have blood sugar lowering effects but not other organic bases mounted as headgroups on a hydrocarbon chain. One answer is that guanidine and biguanide are much stronger bases than most other organic bases and that the property discussed in this paper is fundamentally related to the high pK_a of the protoated form. An additional effect is the size of the toxophoric group in the molecule. Other examples for drug action on the basis of double-layer potentials have been presented, however: these are the salicylates and the local anaesthetics of the procaine type [56].

This leads to the problem of structure–function correlations. We have only considered derivatives of different lipophilic properties. As has been mentioned, introduction of polar groups like hydroxyl into the side chain abolishes the drug action. At least regarding the blood sugar lowering activity, even minor modifications by introduction of additional substituents into the biguanide structure or into the side chain cause a loss of activity (for review see [7]).

From the known variations of the molecule it follows that introduction of any electron-attracting centers close to the biguanide group diminishes drug potency. This may be due to a lowering of the pK_a on the one hand; on the other hand, it will cause an alteration of the resonance structure and thereby of the charge distribution within the molecule. It seems that a firmly localized positive charge on a small planar or linear molecule is necessary, forming, together with a lipophilic side-chain, a configuration which can be easily inserted into bilayer-like areas of biological membrane structures.

In summary, it appears evident that the targets for biguanide action are biological membranes in a rather unspecific manner. From their molecular mechanism of interaction with membranes a large multiplicity of possible consequences is self-evident, which does not necessarily imply that biguanides or related compounds have to possess blood sugar lowering activity. In contrast, the hypoglycemic effect appears as an accident rather than as a principle of drug action. This agrees with the fact that only a few compounds among biguanides are useful blood sugar lowring agents: pronounced species differences exist, and many biguanides are without effects on blood sugar. The side-effects of higher concentrations of biguanide and toxicity, however, may equally well be explained on the basis of their membrane-modifying capability.

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REFERENCES

1. C. K. Watanabe, *J. biol. Chem.* **33,** 253 (1918).
2. E. Frank, M. Nothmann and A. Wagner, *Klin. Wschr.* **5,** 2100 (1926).
3. F. Bischoff, M. Sahyun and M. L. Long, *J. biol. Chem.* **81,** 325 (1928).
4. E. Hesse and G. Taubmann, *Naunyn-Schmiedeberg's Arch. exp. Path. Pharmak.* **142,** 290 (1929).
5. K. H. Slotta and R. Tschesche, *Ber. dtsch. chem. Ges.* **62,** 1398 (1929).
6. H. D. Söling and H. Ditschuneit, *Handbuch des Diabetes mellitus* (Ed. E. F. Pfeiffer) Vol. **1,** pp. 685–714. Lehmann-Verlag, München (1969).
7. R. Beckmann, *Handbook of expl. Pharmac.* (New Series), Vol. **29,** (Ed. H. Maske) pp. 439–596. Springer-Verlag, Berlin-Heidelberg, New York (1971).
8. G. Schäfer, *Biochem. Pharmac.* **25,** 2105 (1976).
9. G. Schäfer, *Eur. J. Biochem.* **45,** 57 (1974).
10. G. Schäfer, E. Rieger and M. Speichert, *Eur. J. Biochem.* **46,** 613 (1974).
11. G. Schäfer, *Hoppe-Seyler's Z. Physiol. Chem.* **335,** 1247 (1974).
12. G. Schäfer and G. Rowohl-Quisthoudt, *FEBS Lett.* **59,** 48 (1975).
13. G. Schäfer, in *Biomembranes* (Ed. L. Packer) pp. 231–259. Academic Press, N.Y. (1974).
14. A. Peña, *FEBS Lett.* **34,** 117 (1973).
15. G. Schäfer and D. Bojanowski, *Eur. J. Biochem.* **27,** 364 (1972).
16. G. Schäfer, D. Bojanowski and E. Schlimme, in *Biochemistry and Biophysics of Mitochondrial Membranes* (Eds. G. F. Azzone *et al.*) pp. 201–214. Academic Press, N.Y. (1972).
17. H. G. Shepherd and H. J. McDonald, *Clin. Chem.* **4,** 496 (1958).
18. F. Davidoff, *J. biol. Chem.* **246,** 4017 (1971).
19. W. Lesslauer, J. Cain and J. K. Blasil, *Biochim. biophys. Acta* **241,** 547 (1971).
20. J. C. Gage, *J. chem. Soc.* 221 (1949).
21. P. Ray, *Chem. Rev.* **61,** 313 (1961).
22. R. L. Dutta and N. R. Sengupta, *J. Indian Soc.* **38,** 741 (1961).
23. D. A. Doornbos, *Pharm. Weekbl.* **102,** 269 (1967).
24. G. Schäfer and G. Rowohl-Quisthoudt, *J. Bioenergetics* (1976).
25. B. Neumcke, *Biophysik* **6,** 231 (1970).
26. S. G. A. McLaughlin, G. Szabo, G. Eisenman and S. M. Ciani, *Proc. natn. Acad. Sci. U.S.A.* **67,** 1268 (1970).
27. G. K. Radda, *Curr. Topics in Bioenergetics* **4,** 81 (1971).
28. D. H. Haynes, *J. Membrane Biol.* **17,** 341 (1974).
29. M. B. Feinstein and H. Felsenfeld, *Biochemistry* **14,** 3041 (1975).
30. A. Azzi, in *Methods of Enzymology* (Eds. S. Fleischer, L. Packer) Vol. 32, pp. 234–246. Academic Press, N.Y. (1974).
31. G. Schäfer, *Biochem. Z.* **339,** 46 (1963).
32. G. Schäfer, *Biochim biophys. Acta* **93,** 279 (1964).
33. B. C. Pressman, in *Energy linked functions of Mitochondria* (Ed. B. Chance) pp. 181–203. Academic Press, N.Y. (1963).
34. E. C. Slater, *Nature*, Lond. **172,** 975 (1953).
35. B. Chance and G. R. Williams, *Adv. Enzymol.* **17,** 65 (1956).
36. P. Mitchell, *Nature, Lond.* **191,** 144 (1961).
37. P. Mitchell, *Biol. Rev.* **41,** 445 (1966).
38. F. Palmieri, E. Quagliariello and M. Klingenberg, *Eur. J. Biochem.* **17,** 230 (1970).
39. J. D. McGivan and M. Klingenberg, *Eur. J. Biochem.* **20,** 392 (1971).
40. R. M. Bayer, R. Haeckel and E. Schnabel, *Fedn Proc.* **28,** 835 (1969).
41. H. Hauser and R. M. C. Dawson, *Biochem. J.* **109,** 909 (1968).
42. D. E. Graham and E. J. Lea, *Biochim. biophys. Acta* **274,** 286 (1972).
43. H. Träuble and H. Eibl, *Proc. natn. Acad. Sci. U.S.A.* **71,** 214 (1974).
44. H. J. Galla and E. Sackmann, *J. Am. chem. Soc.*, in press (1975).
45. M. Levy, R. Toury, M. T. Sauner and J. André, in *Mitochondria. Structure and Function* (Eds. L. Ernster, Z. Drahota) pp. 33–42. Academic Press, N.Y. (1969).
46. K. F. LaNoue and J. F. Williamson, *Metabolism* **20,** 119 (1971).

47. J. H. Anderson, W. J. Nicklas, B. Blank, C. Refino and J. R. Williamson, *Regulation of Gluconeogenesis* (Eds. H. D. Söling and B. Willms) pp. 293–314. Georg Thieme, Verlag, Stuttgart (1971).
48. R. Beckmann, *Archs int. Pharmacodyn.* **160,** 161 (1966).
49. R. Beckmann, *Diabetologia* **3,** 368 (1967).
50. P. J. Murphy, A. N. Wide, *J. pharm. Sci.* **57,** 1125 (1968).
51. C. M. Bertrand, *Press méd.* **78,** 13 (1970).
52. Y. J. Yoh, *Jap. J. Pharmac.* **17,** 439 (1967).
53. A. N. Wick, P. W. Mobley, C. J. Stewart, *Diabetes* pp. 154. Excerpta Medica, Amsterdam (1970).
54. Y. Hatefi and W. G. Hanstein, *Proc. natn. Acad. Sci.* **62,** 1131 (1969).
55. B. D. Ladbrooke and D. Chapman, *Chem. Phys. Lipids* **3,** 304 (1969).
56. S. McLaughlin, *Nature, Lond.* **243,** 234 (1973).
57. J. P. Brown, *Chem. Abstr.* **66,** 1371 (1967).
58. A. Davies and B. S. Field, *Biochem. J.* **106,** 46 (1968).
59. E. D. Weinberg, *Ann. N.Y. Acad. Sci.* **148,** 587 (1968).
60. G. R. Gale, A. M. Welch and J. B. Hynes, *J. Pharmac. exp. Ther.* **138,** 277 (1962).
61. L. A. Kuselev, V. E. Ruchkin, N. M. Osipova, N. N. Melinkow and K. D. Shvetsova-Shilovskaya, *Z. Org. Khim.* **2,** 2186 (1966).
62. B. Hille, *J. gen. Physiol.* **58,** 599 (1971).
63. Y. Shimizu, A. Tsunoda and N. Ishida, *Proc. Soc. exp. Biol. Med.* **123,** 488 (1966).
64. M. Rikimaru, T. Nishikawa, Y. Shimizu and N. Ishida, *J. Antibiot.*, Tokyo **18,** 196 (1965).
65. P. F. Curran and S. G. Schultz, *Physiol. Rev.* **50,** 637 (1970).
66. G. Wiseman, in *Handbook of Physiol.* (Ed. C. F. Code) Vol. **3,** Section 6 pp. 1277–1307 (1968).
67. M. Kessler, W. Meier, C. Storelli and G. Semenza, *Biochim. biophys. Acta* **413,** 444 (1975).