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Effects of Guanidine Derivatives and Oligomycin on Swelling of Rat Liver Mitochondria*

C. Bhuvaneswaran and K. Dakshinamurti†

ABSTRACT: The effects of guanidine derivatives and oligomycin on energy-dependent swelling of rat liver mitochondria have been evaluated. Guanidine derivatives are known to inhibit respiration. They also inhibit succinate-linked reduction of acetoacetate with both the aerobically generated high-energy intermediates or externally added ATP as the source of energy. Guanidines do not inhibit mitochondrial swelling mediated by energy derived from either substrate oxidation or by externally added ATP. Oligomycin, on the other hand, inhibits swelling supported by externally added ATP, while having no effect on swelling mediated by substrate oxidation.

Energy changes were restricted to any one of the three phosphorylation sites using a combination of inhibitors of electron transport (cyanide, antimycin A, and rotenone), and artificial electron donors (ascorbate plus *N,N,N',N'*-tetramethyl-*p*-phenylenediamine) and acceptors (phenazine methosulfate, and ferricyanide). Under these conditions swelling still occurred in the presence of guanidines. The results indicate that the mechanism of inhibition by the guanidines is similar to that of oligomycin. However the sites of inhibition are different.

Hollunger (1955) first observed that guanidine and its derivatives induced a unique and characteristic *in vitro* inhibition of the mitochondrial process associated with high-energy phosphate-bond formation. The slow release of the guanidine inhibition by 2,4-dinitrophenol (DNP)[1] as well as the profile of oxidized-reduced state of cytochrome *b* have led to the suggestion that the guanidines form a complex with the high-energy intermediates (Chance and Hollunger, 1963; Pressman, 1963a). However, little information is available regarding the nature of this complex.

We have examined the properties of four of the site-specific phosphorylation inhibitors. Three of these are guanidine derivatives, namely, 4-methyl-3-butenylguanidine (Galegine),[2] phenylethylbiguanide (DBI), and decamethylenediguanidine (Synthalin). In addition, oligomycin was chosen since this compound blocks ATP synthesis from all the three energy-conserving sites (Lardy *et al.*, 1958; Lardy and McMurray, 1959; Slater, 1963; Ernster and Lee, 1964). We present evidence here to suggest that the guanidines exert their influence between the high-energy intermediate and formation

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[1] The abbreviations used are: DNP, 2,4-dinitrophenol; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; BSA, bovine serum albumin; PMS, phenazine methosulfate.

[2] The commercial names used are: Galegine, 4-methyl-3-butenylguanidine; DBI, phenylethylbiguanide; Synthalin, decamethylenediguanidine.

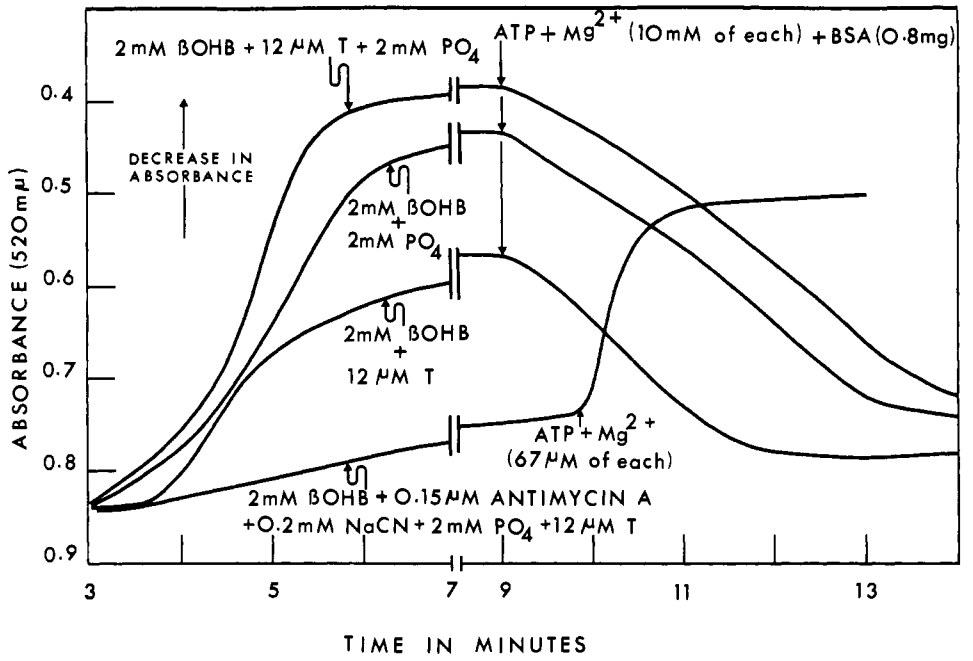

FIGURE 1: Effect of phosphate and thyroxine in inducing swelling of liver mitochondria. Mitochondria were suspended in 3 ml of 0.15 M KCl containing 20 mM Tris-HCl (pH 7.4) at 28° with 2 mM β-hydroxybutyrate as substrate. T, thyroxine; βOHB, β-hydroxybutyrate.

of ATP but at a site distinct from the site of action of oligomycin.

Materials and Methods

Preparation of Rat Liver Mitochondria. Liver mitochondria from stock Holtzman rats were prepared in 0.25 M sucrose essentially according to Schneider (1948). After washing the mitochondrial pellet with 0.25 M sucrose a second washing and final resuspension was made with a medium (pH 7.4) containing 0.175 M KCl and 0.025 M Tris-HCl. During washing the loose white fluffy layer sedimenting over the packed brown sediment was removed. The stock suspension of mitochondria contained approximately 25 mg of protein/ml. For the swelling experiments the stock suspension was adjusted so that 20 μl (~0.5 mg of protein) diluted to 3.0 ml in 0.15 M KCl containing 0.02 M Tris-HCl (pH 7.4, Tris-KCl medium) gave an initial absorbance at 520 mμ of 0.8–0.9.

Measurement of Swelling. Swelling of mitochondria was followed by continuous recording of the change in absorbance at 520 mμ in a Beckman DB-G spectrophotometer in silica cells of 1-cm light path at 28°. The reference cell contained all additions except the mitochondria. Recording of the absorbance was started immediately following the addition of mitochondria. The induction of swelling was brought about by 12 μM thyroxine and 2 mM phosphate (K-PO_4 buffer, pH 7.4). The stock solutions of thyroxine were made fresh every day by dissolving thyroxine in minimal amount of 0.05 N sodium hydroxide and then diluting to volume with water. The mitochondria were exposed to the inhibitors for 3 min before the addition of the swelling agents. β-Hydroxybutyrate or succinate at 5 mM final concentrations were used routinely as substrates. In some experiments ATP plus Mg^{2+} (60 μM of each) was used as energy source for swelling. The details of the experimental system are given in the legend for each figure.

Succinate-Linked Reduction of Acetoacetate in Rat Liver Mitochondria with Either Aerobically Generated High-Energy Intermediates or Externally Added ATP as the Source of Energy. For reduction of acetoacetate by energy derived from substrate oxidation, tightly coupled mitochondria from rat liver isolated in 0.25 M sucrose were incubated aerobically at 25° in the system described by Azzone *et al.* (1963). Acetoacetate was determined by the method of Walker (1954).

When externally added ATP served as the energy source, the mitochondria were isolated in 0.25 M sucrose containing 1 mM EDTA (pH 7.4). A second washing was carried out in a medium consisting of 0.175 M KCl–25 mM Tris-HCl (pH 7.4). Incubation was carried out at 25° in a medium of the following final concentrations: KCl, 0.15 M; Tris-HCl, 50 mM, pH 7.5; KCN, 2 mM; sodium succinate, 10 mM, pH 7.5; sodium acetoacetate, 4.5 mM; and ATP, 5 mM, pH 7.5. Sucrose and Mg^{2+} were found to be inhibitory and hence the above changes were made.

Protein was estimated using the biuret method of Layne (1957). Most of the experiments were finished within 2 hr after the preparation of the mitochondria.

Chemicals. Crystalline bovine serum albumin (BSA), ATP, rotenone, antimycin A, oligomycin, succinic acid, DL-β-hydroxybutyrate (sodium salt), 2,4-dinitrophenol (DNP), phenazine methosulfate (PMS), tris(hydroxymethyl)aminomethane (Tris), and L-thyroxine were obtained from Sigma Chemical Co; Galegine from Calbiochem; DBI from Arlington Laboratories, Montreal, Canada; and Synthalin from Upjohn Co., Kalamazoo, Mich. All other common chemicals were of analytical reagent grade obtained from British Drug House, Merck or Baker Chemicals. *N,N,N′,N′*-Tetramethyl-

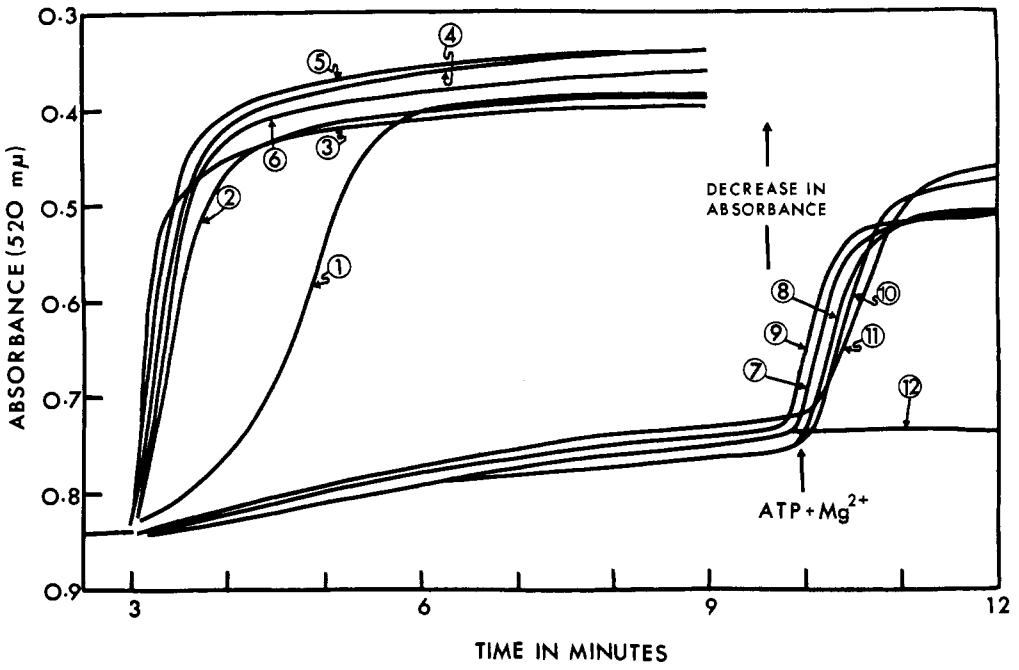

FIGURE 2: Effect of guanidine derivatives and oligomycin on phosphate plus thyroxine induced swelling of liver mitochondria. Mitochondria were suspended in 3 ml of 0.15 M KCl containing 20 mM Tris-HCl (pH 7.4) at 28° with 2 mM β-hydroxybutyrate as substrate. Swelling by phosphate (2 mM) plus thyroxine (12 μM) was started after a 3-min incubation with the following: (1) no further addition; (2) Galegine, 1 mM; (3) Synthalin, 0.2 mM; (4) DBI, 3 mM; (5) Galegine + Synthalin + DBI; and (6) oligomycin, 2 μg. In curves 7–12, the mitochondria were inhibited by 0.15 μM antimycin A and 0.2 mM NaCN for 10 min and the energy for swelling was supplied by 60 μM ATP containing 60 μM of Mg^{2+}; (7) no further addition; (8) Galegine, 1 mM; (9) Synthalin, 0.2 mM; (10) DBI, 3 mM; (11) Galegine + Synthalin + DBI; and (12) oligomycin, 2 μg.

p-phenylenediamine (TMPD) was obtained from Eastman Kodak. All solutions were made in double-distilled water. Rotenone, antimycin A, and oligomycin were used as ethanolic solutions.

Results

Inhibition of Substrate Oxidation by Guanidine Derivatives. We ascertained the effect of guanidine derivatives on substrate oxidation by mitochondria. In agreement with earlier reports (Chappell, 1963; Pressman, 1963b; Guillory and Slater, 1965), Galegine, DBI, and Synthalin inhibited ADP-stimulated oxidation of β-hydroxybutyrate, succinate, and ascorbate-TMPD, respectively. Concentrations of Galegine, DBI, and Synthalin needed for maximal inhibitions were 1.0, 3.0, and 0.2 mM, respectively. Addition of DNP to inhibited mitochondria brought about a release of the inhibition after a time lag. The rate of DNP-stimulated respiration in almost all cases was lower than that with the uninhibited mitochondria.

Induction of Swelling by Phosphate and Thyroxine in Tris-KCl Medium. The parameters of mitochondrial swelling used—extent, time of onset and rate of swelling—are as described by Connelly and Lardy (1964a). Thyroxine and phosphate have individually been used as mitochondrial swelling agents (Lehninger, 1962). The rate of swelling was improved (Figure 1) when we used a combination of thyroxine (12 μM) and phosphate (2 mM). The possibility that this improvement in the magnitude of swelling is simply a concentration-dependent effect has been excluded as increasing the concentrations of phosphate or thyroxine alone within reasonable limits beyond 2 mM and 12 μM, respectively, did not alter the swelling pattern. At relatively high concentrations of phosphate (20 mM) and thyroxine (25 μM) a decrease in extent of swelling was observed. These observations are in agreement with the results of Connelly and Lardy (1964b) and Connelly and Hallstrom (1966) for phosphate concentration and swelling. Almost complete contraction in terms of increase in optical density was achieved by addition of ATP and Mg^{2+} in the presence of bovine serum albumin (Figure 1). The results of Wlodawer *et al.* (1966), however, show that the contraction processes initiated by ATP is not a simple reversal of swelling. Hence, we have restricted our later experiments specifically to the energy dependent swelling. Swelling was completely blocked by 0.15 μM antimycin A plus 0.2 mM NaCN when added prior to the inducing agents. Addition of ATP (67 μM) plus Mg^{2+} (67 μM) to the antimycin A plus cyanide-inhibited system resulted in an active swelling of the mitochondria.

Effect of Galegine, DBI, Synthalin, and Oligomycin on Phosphate Plus Thyroxine Induced Swelling of Mitochondria. Galegine, DBI, Synthalin, and oligomycin at concentration which gave maximal inhibition of respiration were used in swelling studies, either singly or in combination. The results are presented in Figure 2. It is seen that the rate of swelling was affected. The time lag in the onset of swelling was also abolished. Galegine, DBI, and Synthalin did not have any effect on the ATP-induced swelling. Addition of oligomycin, as expected, completely prevented this type of swelling.

Effect of DNP on Induction of Swelling by Phosphate Plus Thyroxine. When DNP was added to a system where the

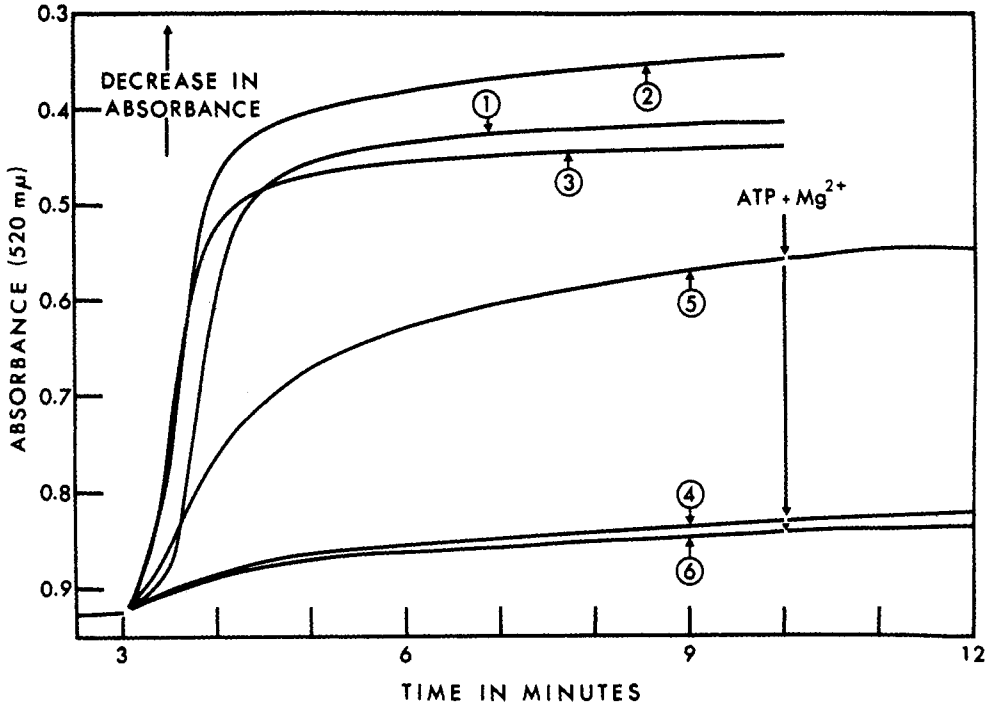

FIGURE 3: Effect of DNP on induction of swelling by phosphate plus thyroxine in liver mitochondria. Mitochondria were suspended in 3 ml of 0.15 M KCl containing 20 mM Tris-HCl (pH 7.4) at 28° with 2 mM β-hydroxybutyrate as substrate. Swelling by phosphate (2 mM) plus thyroxine (12 μM) was started after a 3-min incubation with the following: (1) no further addition; (2) Galegine, 1 mM + Synthealin, 0.2 mM + DBI, 3 mM; (3) oligomycin, 2 μg; (4) DNP, 60 μM; (5) DNP, 60 μM in presence of Galegine + Synthalin + DBI; (6) DNP, 60 μM in presence of oligomycin. ATP and Mg^{2+}, 60 μM of each was added to systems 4–6 at the end of 7 min after addition of phosphate plus thyroxine.

energy for swelling was derived from the oxidation of substrates swelling was completely abolished (Figure 3). When the guanidine derivatives were present in the incubation medium, swelling occurred. However, this was less than in the absence of DNP. Addition of oligomycin, on the other hand, did not alter the DNP effect. When ATP was the energy source, however, the swelling was completely abolished by DNP under all conditions.

Swelling Supported by Energy Conservation at Site I. Addition of antimycin A and cyanide effectively blocked electron transport between cytochrome *b* and c_1 as well as between cytochrome a_3 and oxygen. Under these conditions, addition of the artificial electron acceptor PMS supported oxidation of NAD-linked substrates as well as energy conservation at site I (Scott and Hunter, 1966). This method offered a convenient system to study swelling mediated by energy produced at site I specifically. The results presented in Figure 4 show that mitochondrial swelling was blocked by 0.15 μM antimycin A and 0.5 mM NaCN. If, however, 0.1 mM PMS were added to the above system swelling occurred. Addition of 10 μM rotenone did not eliminate the swelling supported by the bypass created with PMS. Addition of Galegine, an inhibitor specific for site I phosphorylation (Chappell, 1963) did not have any effect whatsoever on this swelling. In the absence of PMS swelling could be initiated by addition of 60 μM ATP and 60 μM Mg^{2+}. Addition of PMS to the control system with no inhibitors did not alter the swelling characteristics. DNP eliminated the swelling mediated by PMS both in the presence and absence of rotenone.

Evidence that we are dealing specifically with site I is based on the following observations. (1) Under the conditions used for energy conservations at site I, site II is not functional since no swelling takes place using the succinate–ferricyanide system in the presence of antimycin A and cyanide (see Figure 5). The antimycin A block of site II energy conservation has been reported by Lee *et al.* (1967). (2) Similarly under the same experimental condition, site III is also not functional, since ascorbate–TMPD system does not induce any swelling when cyanide is present in the assay system (see Figure 6). (3) The addition of DNP to system 5 or 7 in Figure 4 completely eliminated the swelling. (4) Polarographic estimation of ADP:O ratios for liver mitochondria using β-hydroxybutyrate and PMS in the presence and absence of antimycin A yielded values of 1.6 and 2.13, respectively, indicating that site I is functional. (5) Polarographic estimation of oxygen consumption by mitochondria under the condition employed for the swelling experiment using site I is difficult because of the rapid deviation from linearity with time during assay. However, the addition of DNP together with PMS at zero time stimulated the oxygen uptake (37.0 and 28.2 mμmoles of O_2 per min per mg of protein, respectively, in presence and absence of DNP). (6) Warshaw *et al.* (1966) have shown an energy-linked reversal of electron flow from dihydropyragallol–pyocyanine couple to NAD in the presence of antimycin A, cyanide, and ATP.

Swelling Supported by Energy Conservation at Site II. This was studied using the system described by Scott and Hunter (1966). In presence of 10 μM rotenone and 0.5 mM NaCN, oxidation of succinate by mitochondria can be sustained by $K_3Fe(CN)_6$. Addition of thyroxine and phosphate to such a system where sites I and III were blocked leaving only site II functional resulted in swelling (Figure 5). Addition of DBI,

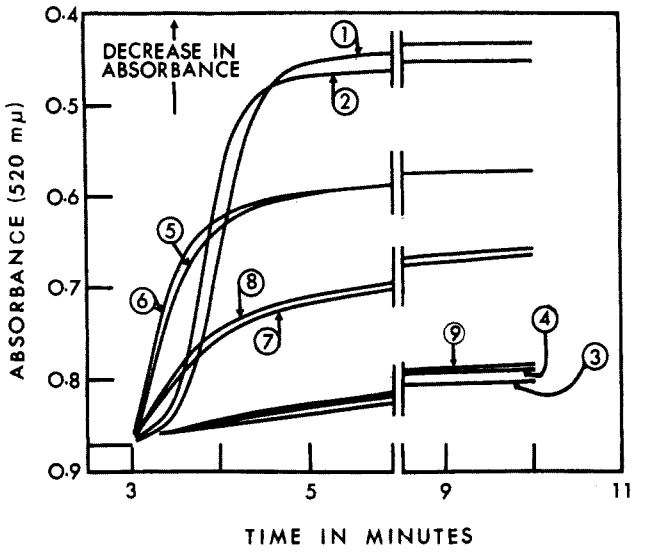

FIGURE 4: Induction of swelling by phosphate plus thryoxine in liver mitochondria supported by energy conservation at site I. Phosphate (2 mM) plus thyroxine (12 μM) added to mitochondria preincubated for 3 min with the following: (1) β-hydroxybutyrate, 5 mM; (2) Galegine (1 mM) added to system 1; (3) antimycin A (0.15 μM) plus NaCN, (0.5 mM) added to system 1; (4) Galegine (1 mM) added to system 3, (5) PMS (0.1 mM) added to system 3; (6) PMS (0.1 mM) added to system 4; (7) rotenone (10 μM) added to system 5; (8) rotenone (10 μM) added to system 6; and (9) DNP (60 μM) added to either system 5 or 7. Other details as represented in Figure 2.

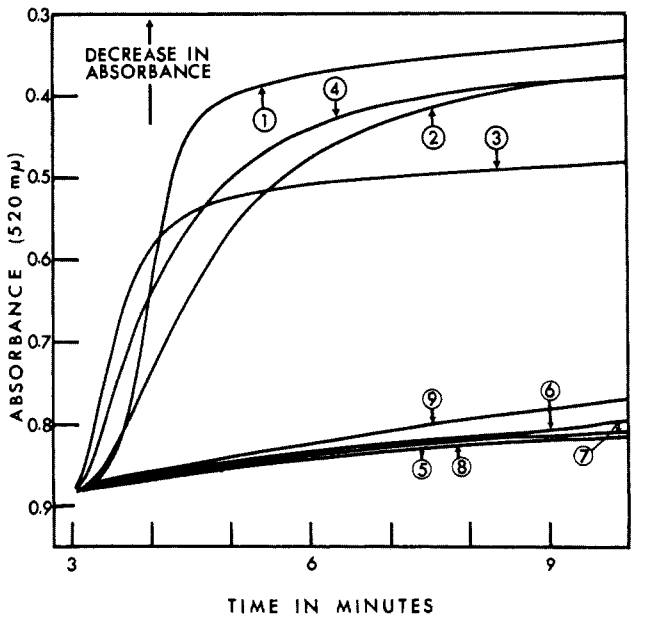

FIGURE 5: Induction of swelling by phosphate plus thyroxine in liver mitochondria supported by energy conservation at site II. Phosphate (2 mM) plus thyroxine (12 μM) added to mitochondria preincubated for 3 min with the following: (1) rotenone (10 μM) plus succinate (5 mM); (2) DBI (3 mM) added to system 1; (3) same as system 1 plus NaCN(0.5 mM) plus $K_3Fe(CN)_6$ (1 mM); (4) same as system 3 plus DBI (3 mM); (5) same as system 1 plus NaCN (0.5 mM); (6) same as system 5 plus DBI (3 mM); (7) same as system 5 plus antimycin A (0.15 μM); (8) same as system 7 plus DBI (3 mM); and (9) same as system 3 plus antimycin A (0.15 μM). Other conditions as described under Figure 2.

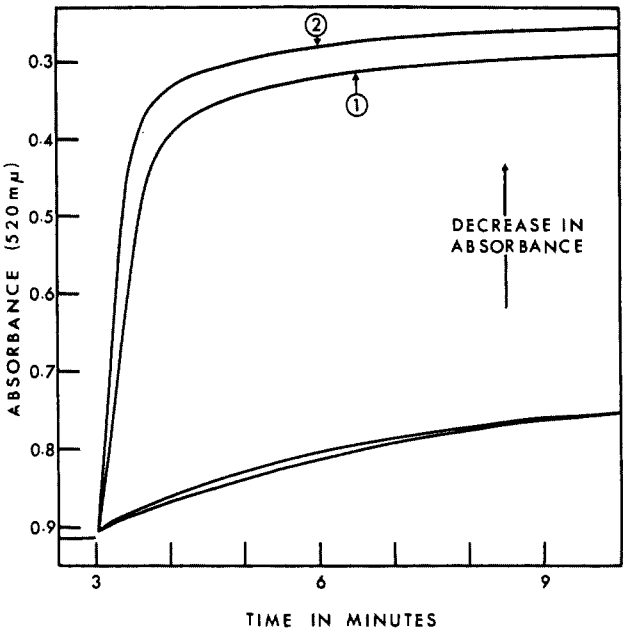

FIGURE 6: Induction of swelling by phosphate plus thyroxine in liver mitochondria supported by energy conservation at site III. Phosphate (2 mM) plus thyroxine (12 μM) added to mitochondria preincubated for 3 min with the following: (1) rotenone (10 μM), antimycin A (0.15 μM), sodium ascorbate (8 mM), and TMPD (100 μM); (2) same as system 1 plus Synthalin (0.2 mM); (3) same as system 1 plus NaCN (0.5 mM); (4) same as system 2 plus NaCN (0.5 mM). Other conditions as described under Figure 2.

an inhibitor for site II phosphorylation (Pressman, 1963a,b) had no effect on the swelling of mitochondria. Antimycin A eliminated swelling under these conditions. But swelling was again initiated by addition of 60 μM Mg^{2+} and 60 μM ATP. Addition of ferricyanide to the control system with no inhibitors did not alter the swelling characteristics.

Swelling Supported by Energy Conservation at Site III. Rotenone (10 μM) and antimycin A (0.15 μM) were used to block effectively the flux of electrons from both NAD-linked substrates as well as from succinate. Electrons were fed to the cytochrome *c*–cytochrome oxidase segment of the electron transport chain from ascorbate (8 mM) through TMPD (100 μM). Under these conditions only the segment from cytochrome *c* to oxygen was active. The results presented in Figure 6 show that ascorbate plus TMPD supported swelling. This was completely blocked by NaCN (0.5 mM).

Synthalin did not have any effect on the swelling mediated by site III phosphorylation. The addition of 60 μM each of ATP and Mg^{2+} to a cyanide-inhibited system promptly initiated swelling.

Effect of Guanidines on Energy-Linked NAD^+ Reduction. Azzone *et al.* (1963) have shown that β-hydroxybutyrate synthesis from acetoacetate can be a measure of the reduction of NAD^+ by succinate. In agreement with observations of Lee and Ernster (1966), Galegine and DBI inhibited the ATP-supported reduction of NAD^+ by succinate. We found this process was strongly inhibited by Synthalin as well. In addition we also observed that the three guanidine derivatives strongly inhibited the reduction of NAD^+ by succinate when aerobically generated high-energy intermediates served as the energy source. The results are presented in Figure 7.

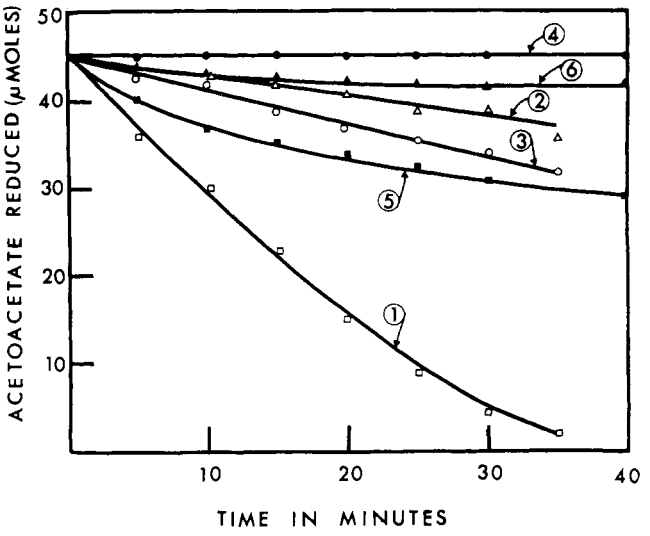

FIGURE 7: (a) Succinate-linked acetoacetate reduction in rat liver mitochondria with aerobically generated energy. Each vessel contained, in a final volume of 11.2 ml: mitochondria, 50 mg of protein; acetoacetate, 4.5 mM; $MgCl_2$, 8 mM; glycylglycine buffer (pH 7.5), 20 mM; KCl, 50 mM; sucrose, 75 mM; and succinate, 10 mM. Incubation was at 25°. At 5-min intervals 0.2-ml aliquots were taken and fixed with 0.1 ml of 1.5 M perchloric acid. (1) No further additions; (2) same as system 1 plus Synthalin (0.87 mM); (3) same as system 1 plus Galegine (0.87 mM) or DBI (2.61 mM). (b) ATP-supported succinate-linked acetoacetate reduction in rat liver mitochondria. Each vessel contained in a final volume of 11.4 ml: mitochondria, 85.5 mg of protein; acetoacetate (4.5 mM); succinate (10 mM); Synthalin when indicated (0.87 mM); KCN (2 mM); KCl (0.15 M); Tris-HCl buffer at pH 7.5 (50 mM); and ATP (5 mM). ATP was added to initiate the reaction. Incubation was at 25°; 0.2-ml aliquots were withdrawn at 5-min intervals and fixed with 0.1 ml of 1.5 M perchloric acid. Curve 4, all additions except Synthaline and ATP; curve 5, all additions except Synthalin; and curve 6, all additions including Synthalin.

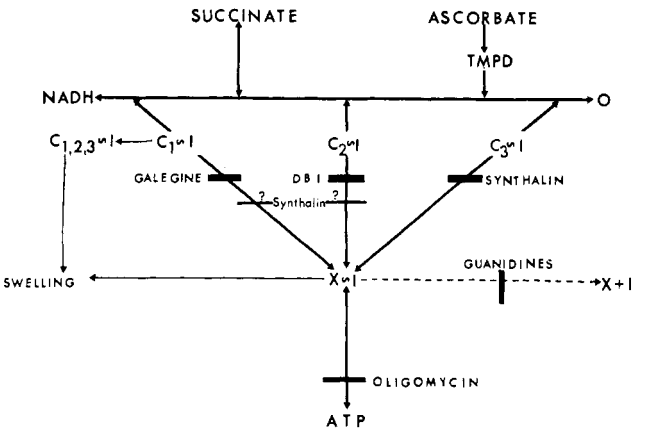

FIGURE 8: Hypothetic scheme of the energy-transfer system linked to the respiratory chain and the site of action of inhibitors. C_1, C_2, and C_3 indicate electron carriers at the energy coupling sites I, II, and III; I and X denote hypothetic energy-transfer carriers (see Lee and Ernster, 1966).

Discussion

The original indication that guanidines are inhibitors of phosphorylation was derived by their effect on substrate oxidation, which could be reversed by addition of DNP. However, the nature of the release of inhibition differed significantly from those of other phosphorylation inhibitors. It has been suggested by Chance and Hollunger (1963) and by Pressman (1963a) that guanidines react directly with the high-energy intermediate X~I to form X~I·guanidine. The slow release of inhibition by uncouplers was thought to be due to a slow displacement of the guanidine moiety by the uncoupler. Our observation on the effects of guanidines on the oxidation of substrates are in complete agreement with those of Pressman (1963a,b) and Chance and Hollunger (1963). In addition, the guanidines were found to be highly inhibitory for the succinate-linked reduction of NAD^+. It is assumed that the energy-linked reduction of NAD^+ by succinate is mediated through C_1~I, C_2~I, and X~I (Figure 8) (Lee and Ernster, 1966). The function of Synthalin, as well as Galegine and DBI when aerobically generated high-energy intermediate served as the energy source, may be to block the interconversions of the intermediates. When externally added ATP served as the energy source, Synthalin was still able to inhibit the succinate-linked reduction of acetoacetate (Figure 7). One possible explanation might be that Synthalin also exerts its inhibitory action at sites I and II. However, Haas (1964) using beef heart mitochondria has shown that Synthalin does not affect site I.

Our observations showed that swelling, which has been established as an energy-dependent process (Lehninger, 1962), was not affected by the guanidines. Possibility of any effect by guanidines *per se* was excluded as in the absence of a swelling agent guanidines caused no swelling. The guanidine compounds still exerted strong inhibition of respiration by mitochondria prepared and incubated in the medium used for swelling studies. Similarly, guanidines did not affect swelling induced by ATP under conditions where no electron transport takes place. Oligomycin, on the other hand, completely abolished the ATP-induced swelling. The function of oligomycin is understandable since its site of action is close to the ATPase. Guanidines, unlike oligomycin, have to act at a point distant from the site of phosphorylation. The noninterference by the guanidine derivatives in mitochondrial swelling mediated by aerobically produced high-energy intermediates or by externally added ATP would mean that the swelling process could derive the energy either from C~I or X~I, whereas the energy-linked reduction of acetoacetate depended on the production of X~I and its conversion into C_1~I. Thus, the site of action of guanidines should be between $C_{1,2,3}$~X and X~I. Also these compounds may exert an influence on the latent leak in mitochondria. Lee and Ernster (1968) found an improvement in P:O of mitochondria when low level of oligomycin was added to mitochondria. This was explained as due to the prevention of the leak and conservation of the high-energy intermediate X~I. With β-hydroxybutyrate (Figures 2 and 4) or ascorbate-TMPD (Figure 6) as substrate, we found that the delay in onset of swelling is virtually abolished in the presence of guanidines. This indicates conservation mechanism for C~I and other intermediates very similar to the action of oligomycin. When succinate (Figure 5, curves 1 and 2) was used as the substrate, addition of DBI (3 mM) reduced both the rate and extent of swelling in the absence of any other inhibitors. However, when the concentration of DBI was reduced to 0.3 mM, the results, although not shown here,

were very similar to those reported for β-hydroxybutyrate or ascorbate–TMPD system. The different sites of action of the guanidines are summarized in Figure 8.

In the absence of guanidines, addition of DNP virtually abolished all swelling. But when guanidines and DNP were present, there was some swelling although the extent and rate were diminished compared to a similar system where DNP was absent. When ATP served as the energy source for swelling, DNP completely abolished the swelling. This could be explained by a greater affinity of DNP for X∼I rather than for C∼I in the presence of guanidines. The nature of the release by uncouplers of the guanidine inhibition of respiration observed by Pressman (1963a,b) and by Chance and Hollunger (1963) is consistent with the above suggestion.

An interesting observation, unrelated to our main objective which rose from our study of swelling mediated by single sites, was the site of action of rotenone. It is seen from Figure 4 that rotenone does not abolish the swelling when PMS acts as the electron acceptor. The swelling mediated by PMS was less than that supported by intact respiratory system. This might be related to the lack of complete permeability of liver mitochondria to PMS. A similar situation has been reported when assaying for succinate–phenazine methosulfate reductase activities of intact mitochondria (Singer and Kearney, 1963; Bhuvaneswaran and King, 1967). The lack of inhibition by rotenone would place its site of action on the oxygen side of the high-energy production site.

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