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Effects of Guanidine Derivatives on Mitochondrial Function.

IV. Changes in Citric Acid Cycle Intermediates and NADH

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Abstract

Steady-state concentrations of citric acid cycle compounds accumulating during state 3 oxidation of pyruvate by guinea pig heart mitochondria have been measured using isotopic and fluorometric techniques; incubations partially inhibited with several guanidine derivatives and other inhibitors were compared with controls. The changes in levels of intermediates which occurred with guanidine derivatives were quantitatively and qualitatively identical to those with amytal; this pattern of intermediates was therefore not limited to those compounds which possess hypoglycemic properties *in vitro*. With antimycin, rotenone and nigericin the pattern of intermediates was specific for each agent, and differed from that with guanidine derivatives and amytal.

Reduced pyridine nucleotides were also estimated at progressively increasing degrees of respiratory inhibition by these same agents. Lower concentrations of phenethylbiguanide and amytal produced identical increases in state 3 level of reduced pyridine nucleotide, while higher phenethylbiguanide concentrations were associated with a phosphate-dependent decrease in reduced pyridine nucleotide level in both state 3 and state 4 which was not observed with amytal, and not accompanied by a stimulation of respiratory rate. These changes resemble the metabolic condition termed state 6, and are consistent with a calcium-like activity of guanidine derivatives. Changes in level of reduced pyridine nucleotide were observed which were specific to rotenone and nigericin; these changes, combined with the patterns of citric acid cycle intermediates observed with these inhibitors, are useful in interpreting the effects of these agents on the functional state of intact mitochondria.

Introduction

Respiration of isolated mitochondria is inhibited by guanidine and its derivatives. Although the characteristics of this inhibition have been examined in some detail [1-7], no studies of the citric acid cycle intermediates which accumulate during biguanide-inhibited respiration have been reported. In the present studies, which are part of a series of investigations on guanidine derivatives [5, 6, 8], the pattern of metabolic intermediates accumulating during state 3* incubations of guinea pig heart mitochondria was analyzed and compared with incubations inhibited to varying degrees with a series of mono- and biguanide derivatives. A pattern of intermediates emerged which was common to all of these agents, but which was not specific for inhibitors with hypoglycemic activity *in vivo,* since an identical pattern was seen with two inhibitors which are devoid of hypoglycemic action. The levels of TCA (tricarboxylic acid) cycle intermediates observed with several other unrelated inhibitors, antimycin, rotenone and nigericin, were completely different from those found with guanidine derivatives, which indicates that the sensitivity and specificity of the incubation and assay procedures was adequate to detect important differences in functional states of the mitochondria.

To help elucidate the mechanisms responsible for the observed changes in TCA cycle acids, the endogenous NADH content of the mitochondrial suspensions was measured. Although the changes in NADH level during inhibition with lower concentrations of phenethylbiguanide and amytal were indistinguishable from one another, striking differences emerged at higher inhibitor concentrations. Furthermore, although the TCA-cycle patterns with rotenone and nigericin inhibition were very similar to each other, endogenous NADH levels changed in a manner which was highly specific for each inhibitor.

Materials and Methods

Guinea pig heart mitochondria were prepared as described previously [5]; passage of mannitol, sucrose and glucose solutions over a mixed bed ion exchange resin, AG 501-x8 (Bio-Rad Laboratories, Richmond, Cal.), prior to their use in isolation, suspension and incubation media significantly improved respiratory control of the mitochondria. Incubations with radioactive substrate were carried out in small Warburg flasks at 37° C, and oxygen uptake measured in a Gilson differential respirometer. The basic incubation medium contained 110 mM sucrose, 5 mM $MgCl_2$, 4 mM ATP, 60 mM glucose, 10 mM sodium phosphate

*Metabolic states are as defined by Chance and Williams [9].

buffer, pH 7.2, 1.5 mg/ml fatty acid-free bovine serum albumin, 2.4 mM pyruvate and 0.5 mM malate. Malate has been found necessary in other mitochondrial systems to sustain linear rates of pyruvate oxidation beyond the first few minutes of incubation [10, 11]. Since preincubation for 15 min is required to induce maximal biguanide inhibition with the current guinea pig heart mitochondrial preparation [5], the requirement for malate was examined under these preincubation conditions. In the absence of malate, state 3 respiratory rates were actually lower than the state 4 rate of the same preparation, and continued, rapid pyruvate oxidation in state 3 was found to be completely dependent on malate; this requirement was the same when twice-distilled pyruvic acid was used as the substrate. Malate was also necessary for the induction of phenethylbiguanide inhibition, apparently because an energized state of the mitochondrion is required to maintain intramitochondrial accumulation of phenethylbiguanide against a concentration gradient [2, 3, 8]. The apparent K_m for malate was about 0.05 mM; accordingly 0.5 mM malate was added in most subsequent studies to ensure rapid oxidation rates without excessive dilution or trapping of radioactivity or interference with measurement of changes in the steady-state level of total malate. In some experiments, 80 mM Na N-tris (hydroxymethyl) methyl-2-aminoethane sulfonic acid (TES), pH 7.2 and 55 mM KCl were included in the incubation medium. All inhibitors were added to the main compartment of the flask, and the incubation started by adding guinea pig heart mitochondria.

All flasks were then preincubated with shaking for 15 min, to allow for full induction of phenethylbiguanide inhibition [5]. At this time, 0.024 mg hexokinase (Type VI, Sigma Chemical Co., St. Louis, Mo.), in 0.1 ml of 350 mM sodium phosphate buffer, pH 7.2, was added from the sidearm to make up the final incubation volume of 1.25 ml; incubation was continued for 5 min, then measurement of oxygen uptake was begun. To minimize variation in the pool size of labelled substrate present at the beginning of state 3 oxidation, the labelled substrate was added to all flasks from the sidearm after the preincubation period, along with the phosphate buffer and hexokinase; 5-7 x 10^6 cpm of either pyruvate-2 or 3-^{14}C with a specific activity of 3-4 mc/mmole was used per flask. Control incubations were continued in state 3 for at least 15 min, and measurement of oxygen uptake was continued until approximately 90% of the total pyruvate added was decarboxylated, a calculation based on the previous observation that decarboxylation in this system proceeds about twice as rapidly as citric acid cycle turnover in state 3 [5]. Incubations without and with inhibitor were carried out until the total oxygen uptake was the same before terminating the reaction, thus ensuring that the total flux of substrate through the citric acid cycle was approximately equal in control and inhibited incubations at the time of comparison. The reaction was terminated by rapid transfer

of the incubation mixture to chilled tubes containing 0.05 ml of 70% perchloric acid, denatured protein was centrifuged down, and the supernatant used for assays.

Separation of organic acids was achieved using ion-exchange column chromatography on Dow-1 (Cl^-) according to a modification of the technique of von Korff [12]; elution was initiated with 0.03 N HCl input into the reservoir until malate was eluted from the column, then the input changed to 0.15 N HCl until fumarate emerged. Calculated recovery of radioactivity from the column ranged from 93-107%. On most runs, an internal marker standard of ^{3}H-lactate was included to facilitate identification of the peaks. Assays of radioactive intermediates from a series of state 4 and state 3 incubations with pyruvate-^{14}C and unlabelled malate gave rise to seven major peaks. From their relative positions on elution [10, 12], rechromatography of each major radioactive peak on cellulose thin-layer plates using known unlabelled carrier standards, and column chromatography of single standard ^{14}C- or ^{3}H-labelled acids, the identity of the peaks was established as follows: I: alanine plus acyl carnitine; II: β-hydroxybutyrate (plus acetate); III: succinate; IV: malate; V: citrate plus pyruvate; VI: α-ketoglutarate; VII: fumarate. In this system, citrate eluted slightly prior to pyruvate but was generally so small relative to residual pyruvate and overlapped pyruvate to such an extent that it could not be quantitated; fumarate similarly was poorly resolved from α-ketoglutarate. The lactate marker eluted just after β-hydroxybutyrate.

For analysis of citric acid cycle acids by thin-layer chromatography, plates coated with cellulose were prepared as follows: cellulose powder MN 300 (Brinkmann Instruments, Westbury, N.Y.) was slurried in water at top speed in a Waring blender for 2 min. The slurry was then adjusted to pH 9.0 with NaOH [13] and spread on glass plates at a thickness of 0.375 mm. When dry, the plates were activated at 105° for 10 min before use. An aliquot of sample containing approximately 2×10^6 cpm/ml of radioactive products plus residual substrate was neutralized to approximately pH 6.5 with K_2CO_3, and the potassium perchlorate removed by centrifugation. To the supernatant were added unlabelled citrate, malate, α-ketoglutarate, fumarate and succinate as carrier, and 20 λ of sample, containing a known amount of radioactivity, was streaked along the origin of a single plate. The plate was then placed in a chromatography jar containing *n*-propanol : eucalyptal : formic acid : water, 50 : 50 : 20 : 4 [14] plus 4 mg% of 2′,7′-dichlorofluorescein [13] and equilibrated for 48 h before developing; this prolonged equilibration step was found to be essential to achieve good definition and clear separation of the individual acids. After developing, the plates were dried in a warm air stream, and the streaks containing each acid were identified under ultraviolet light. Each streak was scraped into a counting vial, 0.1 cc of water and 10 cc of scintillator solution [5] were

added, and the samples counted in a scintillation spectrometer with correction for quenching. Recovery of radioactivity from control chromatograms using known quantities of ^{14}C-labelled standards was quantitative for these five major citric acid cycle intermediates. When ^{14}C-labelled pyruvate was chromatographed, however, no radioactivity could be recovered, indicating that this acid was degraded by the solvents into volatile products which evaporated from the plate. This phenomenon permitted chromatographic analyses without interference from residual labelled substrate. By this technique, the approximate R_f's of the major metabolic intermediates were as follows: amino acids, 0.00; citrate, 0.30; aconitate, 0.37; malate, 0.40; isocitrate, 0.48; α-keto-glutarate, 0.53; β-hydroxybutyrate, 0.71; succinate, 0.74; fumarate, 0.89 (Fig. 1).

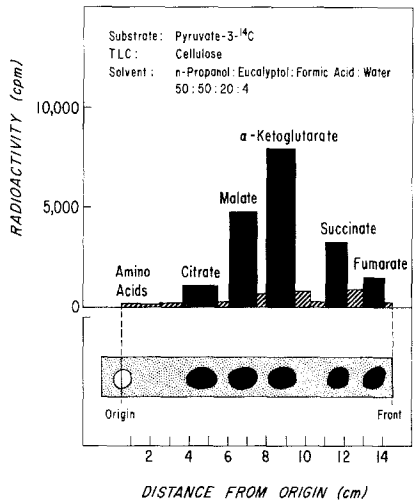

Figure 1. Separation and quantitation of citric acid cycle intermediates by cellulose thin-layer chromatography. Labelled acids were recovered from a control incubation of guinea pig heart mitochondria with pyruvate-3-^{14}C in state 3 and chromatographed as described in Materials and Methods.

Citric acid cycle intermediates were assayed fluorometrically according to the techniques described by Williamson and Corkey [15], using an Eppendorf fluorometer with a baseline-compensating voltage in the output to the recorder. Changes in endogenous NADH (reduced nicotinamide adenine dinucleotide) levels were measured directly on mitochondrial suspensions by the technique of Estabrook *et al.* [16].

In each experiment, duplicate or triplicate flasks were run for each incubation condition, and the results averaged; experiments with each inhibitor were repeated several times, as indicated in the text.

Nigericin was the generous gift of Dr. M. Gorman, Eli Lilly Co., Indianapolis, Indiana; enzymes for fluorometric assays were obtained from Boehringer-Mannheim Corporation, New York, N.Y.

Results

(1) Analysis of Citric Acid Cycle Intermediates by Chromatographic Methods

During prolonged state 4 oxidation in the presence of pyruvate-3-^{14}C and unlabelled malate, β-hydroxybutyrate-^{14}C accumulated to a greater extent than any other product (Table I). The very small quantity of radioactivity recovered in malate apparently reflects the very small flux through the citric acid cycle; obviously the specific activity of malate did not begin to approach that of the added pyruvate under state 4 conditions.

In contrast, in state 3 incubations, radioactive succinate, malate and αketoglutarate accumulated in major quantities. Phenethylbiguanide led to striking changes in the relative quantities of these three acids: a drop in α-ketoglutarate and in succinate, and a rise in malate. Antimycin produced a very different pattern, with a small drop in α-ketoglutarate, major increases in succinate and β-hydroxybutyrate and no change in malate.

Analysis by column chromatography did not permit separation of citrate from pyruvate nor fumarate from α-ketoglutarate, but these separations were possible using thin-layer methods. Thin-layer analysis of the paired control flask from the experiment described in Table I revealed the following levels of metabolic products (in nanomoles/ml): total amino acids plus acyl carnitine, 15.4; citrate, 55.7; malate, 132; α-ketoglutarate, 248; succinate plus β-hydroxybutyrate, 94; and fumarate, 46.8; these values were very similar to those found in several other control experiments. Quantitative agreement between the column and TLC assays is thus excellent and the complementary use of these two procedures permits a relatively complete analysis of radioactivity appearing in the major metabolic products.

Using the TLC technique, phenethylbiguanide was noted to cause an increase in fumarate, in addition to the changes in malate and α-ketoglutarate seen on column analysis, while citrate was the same as in the control (Fig. 2); according to the thin-layer assay phenethylbiguanide did not suppress the level of succinate apparently because of the inclusion of β-hydroxybutyrate, which increased with phenethylbiguanide (Table I), in the succinate area of the thin-layer plate. Antimycin produced an increase in citrate, in addition to the increase in succinate and the fall in α-ketoglutarate seen on column analysis; fumarate and malate remained close to the control levels. Changes in

TABLE I. Accumulation of mitochondrial metabolites during oxidation of pyruvate-3-^{14}C as determined by ion-exchange chromatography

Each flask in experiment 1 contained 1.1 mg mitochondrial protein; measurement of oxygen uptake was started immediately after temperature equilibrium and allowed to proceed to 35 μl. In experiments 2-4, each flask contained 0.4 mg protein; following a 15-min pre-incubation, state 3 respiration was initiated with hexokinase, oxygen uptake measurement was begun after 5 min for temperature equilibration and continued until 100 μl were consumed. Calculations were based on the assumption that all products had reached the same specific activity as the added pyruvate-^{14}C; alanine was not included in the table since it constituted a small percentage of the radioactive products.

Experiment	Additions	Metabolic state	Respiratory rate	Metabolic products							
				Total radioactive products	β-hydroxy butyrate + acetate		Succinate		Malate		α-keto glutarate + fumarate	
			μl/min/mg protein	% of added radioactivity	μM	% total	μM	% total	μM	% total	μM	% total
1	None	4	0.39	3.5	55	62.5	8.7	9.9	4.8	5.4	4.6	5.2
2	None	3	7.71	17.3	16.6	3.0	70.1	12.7	139	25.2	326	59.0
3	Phenethyl-biguanide 1×10^{-4}M	3	3.12	17.3	48.0	8.7	47.2	8.5	301	54.5	146	26.5
4	Antimycin 16 ng/ml	3	4.07	23.3	97.6	13.1	222	29.8	140	18.8	281	37.8

intermediates with antimycin inhibition qualitatively similar to those shown in Fig. 2 were seen in other experiments with as little as 10% decrease in respiratory rate, increasing progressively as respiratory rate fell further, indicating that the effects of the inhibitor did not change qualitatively or become less specific as the degree of respiratory inhibition was varied.

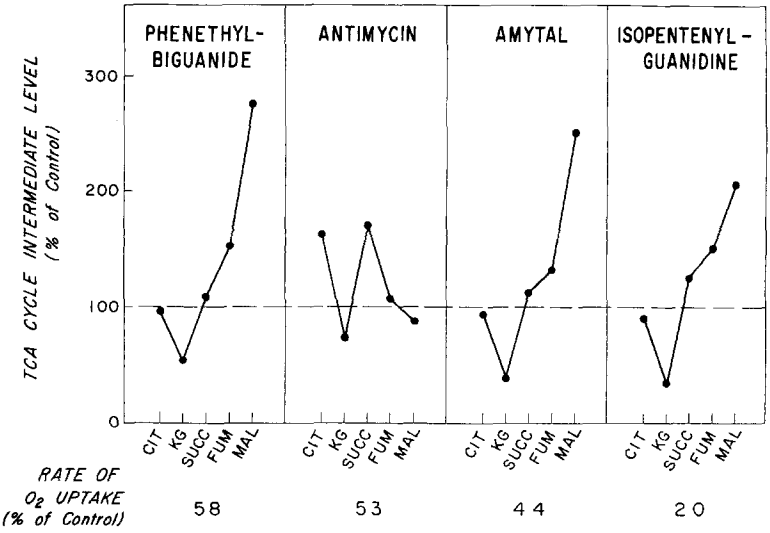

Figure 2. Changes in levels of citric acid cycle intermediates with various inhibitors as analyzed by thin-layer chromatography. To normalize the data for comparison, the radioactivity in each of the five major products was calculated as the percent of the total products, and is expressed relative to the percent which that intermediate constituted in the control flasks. Recovery of radioactivity in the major products ranged from 31-37% of the total loaded onto the thin-layer plates, the remainder being in pyruvate and therefore lost during chromatography (see Materials and Methods). Concentrations of inhibitors were: phenethylbiguanide, 1×10^{-4} M; antimycin, 16 n/ml; amytal, 6×10^{-4} M; isopentenyl guanidine, 0.9×10^{-4} M.

The patterns of citric acid cycle intermediates accumulating during inhibition with amytal and isopentenyl guanidine were also examined in similar experiments by the thin-layer technique. Qualitatively and quantitatively, these patterns were essentially identical to that seen with phenethylbiguanide (Fig. 2).

Following the state 3 incubation in one experiment, one-half of the suspension was centrifuged to remove the mitochondria, the radioactive products in the supernatant analyzed and compared with those in the whole suspension; recovery of radioactive products was quantitative in the supernatant, and the patterns on TLC analysis from the supernatant and the whole suspension were indistinguishable.

(2) *Fluorometric Assay of Citric Acid Cycle Intermediates*

Use of the fluorometric method permitted direct and selective assay of the total level of each intermediate without the need for qualifying assumptions about specific activities, as with the isotopic methods. In addition, this technique made possible assays of isocitrate and oxaloacetate.

The quantities of citric acid cycle intermediates accumulating in control incubations as determined by the fluorometric technique were as follows (mean, nmoles/ml ±SEM [17]; figure in parenthesis represents number of experiments): citrate, 103 ±16 (11); isocitrate, 4.4 ± 0.8 (12); α-ketoglutarate, 169 ± 19 (14); fumarate, 93 ± 23 (12); malate, 178 ±17 (16); oxaloacetate, 3.1 ± 0.3 (13).

Agreement was good between the absolute and relative quantities of malate, fumarate and citrate determined in control incubations by isotopic and fluorometric methods. The level of α-ketoglutarate was generally somewhat lower by the fluorometric assay, but since the data were obtained by the two methods in separate experiments it was not possible to draw firm conclusions concerning this discrepancy. Since, however, in each experiment, the inhibited incubation mixture was always compared to its own control, the changes resulting from respiratory inhibition represent internally consistent data.

The changes in levels of intermediates induced by phenethylbiguanide and amytal inhibition closely resembled those found using isotopes and chromatographic separations, except that in the fluorometric assay the increases in fumarate level were more concordant with those of malate (Fig. 3). By fluorometric assay, significant changes in levels of α-ketoglutarate, fumarate and malate were already apparent at very small degrees (~10%) of overall respiratory inhibition by phenethylbiguanide or amytal (Fig. 4), and became progressively greater with increasing inhibition; similar data were obtained with isopentenyl guanidine. In contrast, with increasing phenethylbiguanide or amytal inhibition, citrate, isocitrate and oxaloacetate levels did not differ significantly from the control (Fig. 4). When succinate replaced malate as the four-carbon acid added to the incubation mixture, the resulting patterns of intermediates, malate and oxaloacetate in particular, were unchanged. The patterns of citric acid cycle intermediates accumulating during inhibition with methyl-, butyl- and dodecyl-biguanide were also analyzed fluorometrically and found to be identical to the pattern with phenethylbiguanide.

The time-course of changes in malate and α-ketoglutarate levels was also studied. The data presented in Fig. 5 establish several important points: first, changes in the size of the malate and α-ketoglutarate pools during the state 4 pre-incubation were small and could not account for the differences between control and inhibited suspensions during the

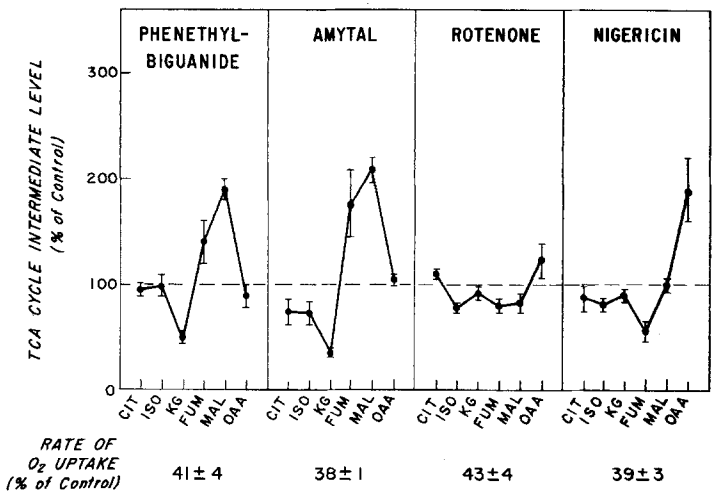

Figure 3. Fluorometric analysis of changes in citric acid cycle intermediates accumulating with various inhibitors. Concentration of rotenone was 0.4×10^{-6} M and nigericin, 1.3×10^{-9} M; other inhibitor concentrations were as described for Fig. 2. In incubations with nigericin, all reagents were present as sodium salts, while some incubations with other inhibitors included potassium. Total number of separate experiments ranged from three to eight for each inhibitor; identical bars represent SEM.

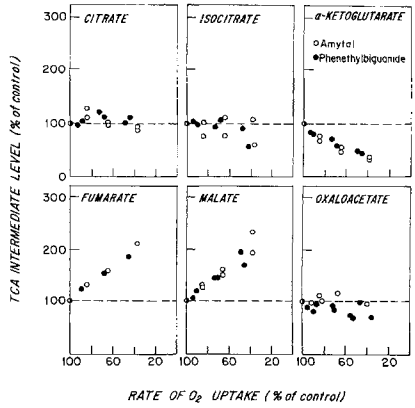

Figure 4. Fluorometric assay of intermediates with increasing degree of phenethylbiguanide and amytal inhibition. Each point represents average from paired flasks in one experiment, expressed relative to control level for that experiment.

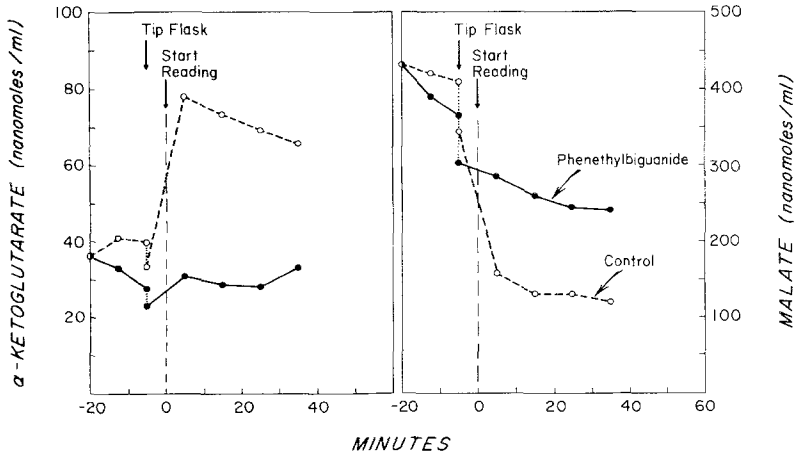

Figure 5. Time course of malate and α-ketoglutarate levels during state 4 and state 3 incubations with phenethylbiguanide. Malate and α-ketoglutarate were analyzed fluorometrically. Drop in concentrations indicated at −5 min was due to dilution with hexokinase-phosphate solution introduced from the sidearm.

subsequent state 3 incubation; second, differences between control and inhibited incubation mixtures were established rapidly, i.e. within the first 10 min of state 3 metabolism; and finally, measurements of these intermediates in state 3 at time points beyond 15 min approached steady state levels in both control and inhibited incubations.

Rotenone [18] and nigericin [19] both inhibit mitochondrial respiration between NADH and cytochrome *b*, which is close to the locus affected by amytal and several of the guanidine derivatives [2-4]. The effects of rotenone and nigericin inhibition on citric acid cycle intermediates were therefore of interest: rotenone had very little effect on metabolite levels; with nigericin the major change was an increase in oxaloacetate, although fumarate appeared to decrease somewhat (Fig. 3).

(3) *Endogenous NADH Levels During Inhibition by Phenethylbiguanide and Other Agents*

Although the pattern of citric acid cycle metabolites which accumulated during phenethylbiguanide inhibition was essentially identical to that with amytal, the very different chemical and pharmacological characteristics of these two compounds suggested that more subtle differences might be revealed on examination of other parameters such as levels of reduced pyridine nucleotide which are related to control of substrate flux.

The behavior of NADH levels with phenethylbiguanide and amytal was identical up to the point of about 75% inhibition of state 3 respiration (Figs. 6 and 7); NADH levels increased progressively in state 3

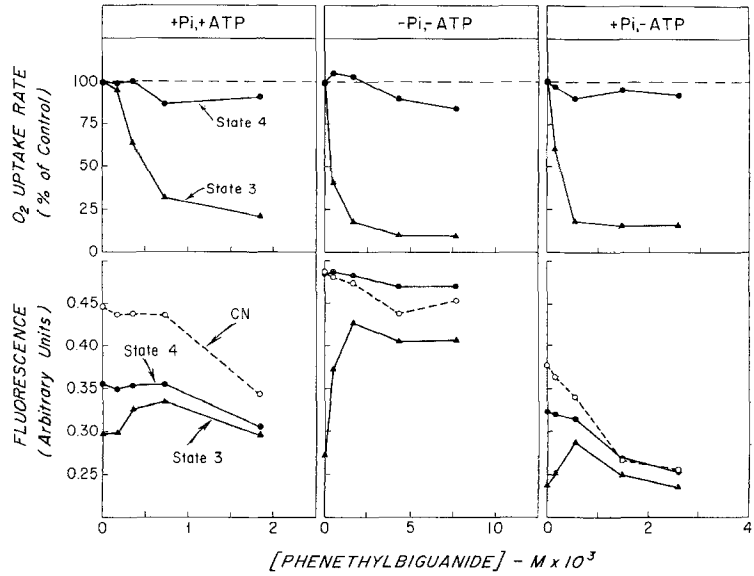

Figure 6. NADH levels of mitochondrial suspensions with increasing degrees of respiratory inhibition. Incubations, in a total volume of 2.75 ml of potassium-free basic medium, were carried out at 20° instead of 37° C to prevent exhaustion of O_2 during fluorometric measurements. Each sample contained a total of 2.5 mg guinea pig heart mitochondrial protein. Only mitochondria with a respiratory control ratio (state 3/state 4) of greater than 4 were employed for these studies. Preincubations with all inhibitors were for 20 min. A portion of the sample was then introduced into the fluorometer cuvette; after a steady level of fluorescence was established (state 4), 1.25 mM ADP plus 17.5 mM phosphate (final concentrations) were added and the state 3 level of fluorescence determined; finally, 1 mM CN^- was introduced into the cuvette. The remaining portion of the suspension was simultaneously placed in a polarigraphic cell, and respiratory rates determined with a Clarke electrode before (state 4), during (state 3) and after (state 4) introduction of 0.3 mM ADP and 5 mM phosphate.

when compared with the controls, while no significant change in NADH occurred in state 4 or after blockade of electron flow with CN^-. However, upon increasing the concentrations further, a major difference between the two inhibitors became apparent: a concentration of phenethylbiguanide four times that required to produce 50% inhibition caused a consistent and reproducible decrease in NADH under all three conditions, the level falling toward that found in the controls in state 3 (Fig. 6), while the opposite, i.e. a rise toward the CN-blocked level of the control, was observed with high levels of amytal (Fig. 7).

Further investigation of the decrease in NADH levels produced by higher concentrations of phenethylbiguanide revealed that this effect was strongly dependent on phosphate (Fig. 6): in the absence of phosphate

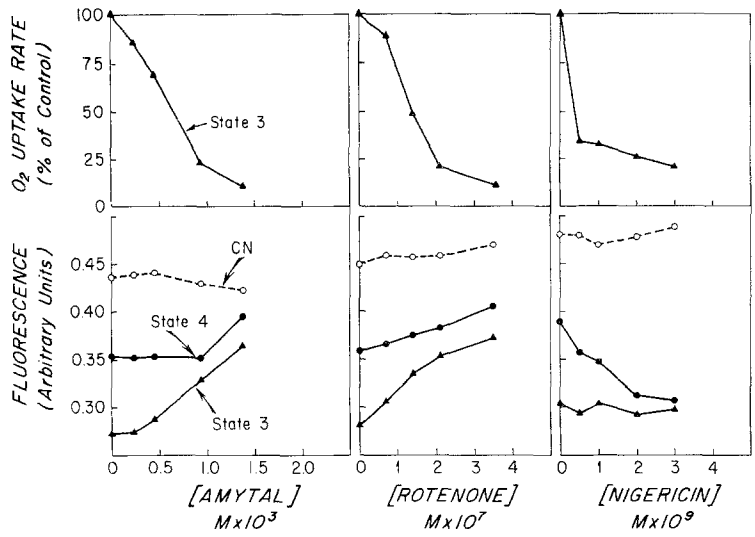

Figure 7. NADH levels with amytal, rotenone and nigericin. Incubation and assay conditions were identical to those described in Fig. 6 and identical P_i and ATP.

and ATP in the pre-incubation mixture, phenethylbiguanide, at concentrations 4-16 times that required for 50% respiratory inhibition, produced little fall in state 4 NADH level, while the state 3 level of NADH rose even further than at lower biguanide concentrations. The NADH level after CN^- blockade diminished slightly at these high biguanide concentrations, but it should be noted that the mitochondria were exposed to phosphate for the duration of the state 3 incubation (about 2 min) which preceded the addition of CN^-. With phosphate but no ATP in the pre-incubation mixture, the concentration of phenethylbiguanide required to produce 50% respiratory inhibition was reduced to about 1/3-1/2 of that needed in the absence of phosphate, and the progressive oxidation of NADH by increasing phenethylbiguanide concentrations was very pronounced (Fig. 6). NADH was extracted from mitochondria in state 4, state 3 and with CN^- immediately following the steady-state fluorometric readings and assayed enzymatically [15]; the changes in NADH content by this technique exactly paralleled those observed in the intact suspension, thus ruling out artifactual fluorescence changes as the cause of the biguanide effect. It is also of importance to note that the rate of oxygen uptake in state 4, measured prior to the induction of state 3 respiration, was not increased by phenethylbiguanide, whether or not phosphate or phosphate and ATP were present in the pre-incubation mixture, and in fact the state 4 rate was generally slightly depressed (Fig. 6).

Increasing amounts of rotenone progressively raised the level of NADH in both states 4 and 3, and even the CN^--blockaded level increased slightly. Nigericin, on the other hand, caused a very obvious and progressive decrease in state 4 NADH until it approached the state 3 level of the control; NADH continued to be maximally reduced in the presence of CN^-, even at high levels of nigericin (Fig. 7), although the T 1/2 needed to reach maximal reduction was markedly prolonged (at least four-fold).

Discussion

As noted by LaNoue *et al.* [10], interpretation of patterns of citric acid cycle intermediates accumulating during *in vitro* incubations of mitochondria is complex, because of changing concentrations of substrates and products and limitations on free exchange of various anionic metabolites across the mitochondrial membrane. Malate plays an important role in facilitating these exchanges [20], particularly with α-ketoglutarate and succinate. However, other studies in this laboratory (F. Davidoff, unpublished) support the view that in the present mitochondrial system the major importance of malate in maintaining rapid pyruvate oxidation is in controlling the rate of citrate synthetase reaction: pyruvate alone, added to mitochondria previously depleted of substrate, then blocked with antimycin, produced only slight reduction of pyridine nucleotide; subsequent addition of malate permitted full reduction of the pyridine nucleotides. Although this effect of malate could have been due to further introduction of reducing equivalents from malate oxidation or to stimulation of pyruvate entry through a malate-pyruvate exchange, these explanations seem unlikely since addition of carnitine instead of malate caused an identical effect. It thus appears that in the absence of malate, the endogenous level of oxaloacetate was too low to permit generation of citrate, and acetyl CoA therefore accumulated to a level which inhibited pyruvate dehydrogenase [21]. Analysis of the radioactive products accumulating in state 4 in the presence of carnitine, using the column technique, revealed a large conversion of pyruvate-2-^{14}C to acyl carnitine (F. Davidoff, unpublished), indicating that acetyl units were continuously transferred to carnitine and lost across the membrane into the medium, thus lowering the steady-state level of acetyl CoA and relieving pyruvate dehydrogenase inhibition.

An analysis of the inhibitor-induced control mechanisms at individual steps of the citric acid cycle may be attempted from the present data, since at the time of measurement the levels of those intermediates which underwent significant changes approximated the steady state (Fig. 5). In general terms it is clear that under these conditions, the overall flux

through the citric acid cycle must be controlled by the rate of input into the cycle [22]. In the presence of malate, the oxaloacetate level in the medium was above the K_m for citrate synthetase [23] and was not significantly affected by biguanide or amytal inhibition. Control of the input step to the cycle, the citrate synthetase reaction, was apparently not due to changes in intramitochondrial oxaloacetate level, but may have been exerted by a decrease in acetyl CoA concentration; the rate of acetyl CoA generation by pyruvate dehydrogenase was probably limited by the rise in NADH/NAD ratio which accompanied both phenethylbiguanide and amytal inhibition (Fig. 6), since NADH is also an allosteric inhibitor of pyruvate dehydrogenase [21]. This increase in NADH/NAD ratio was probably also important in preventing a decline in isocitrate (and citrate) and in increasing malate (and fumarate) levels despite a diminished rate of flux through the citric acid cycle. The decreased level of succinate with phenethylbiguanide, and of α-ketoglutarate with biguanide, amytal and antimycin inhibition, suggested that the dehydrogenases which metabolize these intermediates may have been operating according to Michaelis-Menten kinetics, unencumbered by the effects of allosteric modifiers [10, 22].

While with increasing degrees of biguanide and amytal inhibition the correlation was excellent between rising NADH levels and the changes in citric acid cycle substrate redox couples, with rotenone a marked discrepancy was obvious between the increasingly reduced state of NADH and the essentially complete lack of change in the citric acid cycle intermediates. These data are consistent with known differences in the binding characteristics of the two types of agents. Thus, phenethylbiguanide appears to be accumulated reversibly by mitochondria in a process which is energy dependent and which is also sensitive to the transmembrane pH gradient [8]; at any given biguanide concentration, therefore, all the mitochondria in the suspension would be partially inhibited, and their citric acid cycle function would be altered to reflect the increased level of NADH. In contrast, rotenone binds essentially irreversibly to mitochondria [18]; the present data further indicate that this binding is cooperative, such that once inhibition of a mitochondrial respiratory unit is initiated the remainder of units in that mitochondrion are then inhibited preferentially over those mitochondria in which inhibition has not yet occurred. The result appears to be the complete functional removal of inhibited mitochondria from the metabolism of the suspension, although the reduced state of pyridine nucleotides in these completely inhibited mitochondria would of course contribute to the measured increase in NADH level of the whole suspension.

The decrease in endogenous NADH which accompanied nigericin inhibition (Fig. 7) has been observed by others [24] and has been ascribed to a diminished rate of entry of substrate anions across the inner mitochondrial membranes, secondary to loss of intramitochondrial K^+

[19, 24]. As expected with such a mechanism, pyridine nucleotides in state 4 became more and more oxidized, as long as electron flow was permitted to continue between NADH and oxygen; however, when the flow was completely blocked by CN^-, the level of NADH rose, albeit more slowly, to its maximum, indicating that while the rate of substrate entry was diminished by nigericin it was not reduced to zero. The increase in oxaloacetate level noted during nigericin-inhibited state 3 respiration may reflect the limited supply of acetyl CoA resulting from diminished substrate influx since the redox state of pyridine nucleotides in inhibited state 3 did not differ significantly from the control.

Finally, it is of interest that no differences whatsoever in the patterns of citric acid cycle intermediates were found between agents with hypoglycemic properties *in vivo*, e.g. phenethylbiguanide, isopentenyl guanidine and methylbiguanide, and several with no hypoglycemic activity, e.g. amytal and dodecylbiguanide. This observation lends further support to the hypothesis that the hypoglycemic effect of guanidine derivatives is due to some action other than respiratory inhibition *per se* [5, 6]. The oxidation of pyridine nucleotides with high levels of phenethylbiguanide does indicate a fundamental difference in some of the biological properties of biguanides and amytal, however, an observation which may therefore be more relevant to hypoglycemic activity than is effectiveness as a respiratory inhibitor. This pyridine nucleotide oxidation is not due to simple uncoupling, as has been described with high concentrations of guanidine [1], decamethylene-diguanide [1] and long-chain alkylbiguanides in a succinate system [7, 25], since phenethylbiguanide did not increase state 4 respiration (Fig. 6), although a combination of uncoupling and inhibitory activities in the same molecule could account for this effect [4, 26]. However, the dependence of biguanide-induced pyridine nucleotide oxidation on phosphate is strongly reminiscent of the influence of phosphate on the mitochondrial effects of calcium which under certain conditions is a potent respiratory inhibitor [27], suggesting a different mechanism for guanidine derivative effects on mitochondria, i.e. a calcium-like activity. Pyridine nucleotides may become oxidized during respiratory inhibition by calcium [27] or manganese [28], a distinctive metabolic condition which has been termed state 6 [28]. This effect is observed only in the absence of permeant anions such as phosphate or acetate, probably because these anions permit Ca^{++} to move out of its inhibitory membrane binding sites and on into the mitochondrial matrix [27]. During state 6 resulting from respiratory inhibition with Ca^{++}, however, pyridine nucleotides may remain reduced [28], a state which is probably analogous to the respiratory inhibition observed with phenethyl-biguanide in the absence of phosphate (Fig. 6). Addition of phosphate does not appear to alter the binding of phenethylbiguanide, at lower concentrations, to sites which are primarily inhibitory to electron flow

(Fig. 6), but phosphate does appear to permit biguanides at higher concentrations to gain access to elements of the energy conservation mechanism which leads to pyridine nucleotide oxidation. It has been shown that phosphate movement into mitochondria accompanies the uptake of guanidine [2] as it does calcium uptake.

Among the other interactions of calcium with mitochondria which resemble those of guanidine derivatives are the following: (1) calcium binds to mitochondria in the absence of energy, but binding is markedly increased by energy [29], as observed for phenethylbiguanidine [8]; (2) the relative affinity of calcium for binding sites in mitochondria from different species [29] parallels the relative effectiveness of respiratory inhibition by phenethylbiguanide in these same species [5]; (3) calcium interacts with the energy conservation mechanism to prevent ADP from its normal access to this mechanism [28], as does guanidine and its derivatives [2-4]; (4) calcium affects all three energy conservation sites [27], and although individual guanidine derivatives show some selectivity for specific sites, this apparent selectivity is in part concentration dependent, and various guanidine derivatives have been shown to inhibit all three sites [30]; (5) uptake of calcium into mitochondria is accompanied by proton ejection [29], and changes in intramitochondrial pH as observed by the bromthymol blue [31] and dimethyloxazolidine dione [32] techniques, changes which are very similar to those observed with biguanides [8, 33]. The recent demonstration that phenethylbiguanide and related guanidine derivatives inhibit purified pyruvate kinase in a manner which kinetically is identical to calcium inhibition [34] greatly strengthens the suggested parallel between the biological effects of calcium and guanidine derivatives.

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