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EFFECTS OF SYNTHALIN ON MITOCHONDRIAL FUNCTIONS

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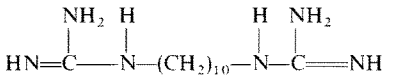
Abstract—Synthalin, the decamethylene derivative of diguanide,



has been used in the treatment of diabetes for several years. However, its use has been limited due to its liver toxicity. The present study was devoted to biochemical studies in an attempt to reveal some fundamental effects on isolated mammalian heart mitochondria. Isotopic studies demonstrated that synthalin uptake results in inhibition of respiration and that the process is energy-dependent. The uptake is not carrier-mediated, since no evidence of saturation was observed, as would have been expected for a carrier-mediated process. It has also been established that, when present in reaction medium, synthalin prevented energy-dependent Ca^{2+} uptake and addition of Ca^{2+} failed to stimulate proton ejection into the suspending medium. In the presence of synthalin at a concentration of 2 μg/mg of mitochondrial protein, lysocephalin and lysolecithin spots were not detected on the chromatogram.

Although guanidine derivatives inhibit the respiration of isolated mitochondria, respiratory inhibition is not the critical parameter for the hypoglycemic effect. It was reasoned that, even though respiratory inhibition might prove to be an artifact of the highly artificial conditions of mitochondrial studies *in vitro*, close examination of the mitochondrion–guanidine interaction might provide important insight into those biological properties of biguanides relevant to their hypoglycemic action. The studies reported in this paper were therefore directed to the fundamental questions of the mechanism of intramitochondrial accumulation of the hypoglycemic agent, synthalin, and its effect on energy-dependent uptake of calcium ions.

The current studies demonstrate that synthalin accumulates within vervet monkey heart mitochondria against a concentration gradient. The uptake at low concentration of biguanidine is slow, and even high guanidine concentrations do not saturate the uptake rate. It has also been demonstrated that synthalin depletes lysocephalin, lysolecithin and mitochondrial peptidolipid from the mitochondrial membrane.

MATERIAL AND METHODS

Young male vervet monkeys (*Cercopitecus aetiops*) which weighed about 4 kg were sacrificed within 4 hr after their capture. Heart mitochondria were isolated by a modification of the method developed by Schneider *et al.* [1]. The medium used for isolation consisted of 0·21 M mannitol–0·07 M sucrose–0·01 M disodium EDTA–0·01 M Tris–KCl, pH 7·2; isolated mitochondria were suspended in 0·25 M sucrose–0·01 M Tris–KCl–0·0001 M disodium EDTA, pH 7·2, at a final concn of 8·4 mg mitochondrial protein/ml. The mitochondrial suspension was kept at 0° and used within 4 hr. Polarographic measurements of oxygen uptake by mitochondria under different conditions were made with a Teflon-coated Clark oxygen electrode connected to a Sargent SRG recorder via a voltage divider. The acceptor control ratio of all preparations was determined with a Clark oxygen electrode in a medium of sodium succinate (5 mM), sodium chloride (80 mM), sodium phosphate (4·5 mM), magnesium chloride (10 mM), and Tris–KCl, pH 7·4 (10 mM), in a total volume of 2·0 ml at 25°. The mitochondria (4·6 mg protein) were added to the medium and respiration was stimulated by addition of 250 nmoles ADP (adenosine diphosphate). After return of the system to state 4, a second addition of 250 nmoles ADP was made. The average acceptor control ratio from the two respiratory jumps was taken. Preparations having a ratio of less than 4·0 were discarded, since they yielded unsatisfactory results. The concentration of mitochondrial protein was determined by the procedure of Gornall *et al.* [2]. To determine the extent of calcium translocation into the intramitochondrial space, radioactive calcium chloride ($^{45}CaCl_2$) was used and isotopic studies were conducted as follows: calcium chloride (400 nmoles) was ejected into a reaction medium made up as described for the determination of acceptor control ratio. The reaction was allowed to proceed for a period of 2 min. From the reaction medium, 0·4 ml was carefully withdrawn by use of a

microsyringe. The mitochondria were then quickly sedimented by use of Beckman/Spinco Microfuge (No. 152). The supernatant contained labeled calcium which had not been translocated into the mitochondrial matrix, while the accumulated divalent ion was in the mitochondrial pellet. Exactly 100 μl of the supernatant was inoculated into 10 ml of scintillation mixture in a glass counting vial and a Packard Tri-Carb liquid scintillating spectrometer model 3320 was used for counting.

To investigate the extent of synthalin accumulation by isolated mammalian mitochondria, labeled synthalin (^{14}C-) was employed for isotopic studies. Isolated mitochondria were incubated in a reaction medium consisting of 0·25 M sucrose–0·01 M Tris–KCl–0·0001 M disodium EDTA at pH 7·2, under given conditions. A known quantity of radioactive synthalin was then inoculated into the vessel and incubation allowed to proceed for a specified period of time (see legends for Fig. 1 and Table 1). After incubation for the specified period, 0·4 ml of the incubation mixture was carefully withdrawn by use of a microsyringe and mitochondria were sedimented by use of a Beckman/Spinco Microfuge (No. 152). The supernatant was used for counting as already described in respect to radioactive calcium.

The pH changes in the mitochondrial suspensions were followed with a combination of a glass electrode (A. H. Thomas, Inc., No. 4858-L15) linked to a Beckman Expandomatic pH meter and a Sargent SR recorder. The quantity of (H^+) protons ejected into or absorbed from the mitochondrial suspension was determined by adding standard HCl externally. Chromatographic separation of mitochondrial phospholipids was conducted according to the technique of Curri *et al.* [3].

To separate the mitochondrial phospholipids, a chromatographic mixture of 30 ml ethanol, 65 ml chloroform, and 5 ml water was used. Mitochondria which had been (a) freshly prepared, (b) incubated for 30 min at 20°, (c) incubated with phospholipase A, or (d) freshly prepared and treated with synthalin were spotted on cellulose F plates by use of lambda pipettes. After running for about 2 hr, the plates were dried in air and then exposed to iodine gas to locate the spots.

The scintillation mixture was prepared as follows: 1,4-bis-2-(4-methyl-5-phenyloxazolyl)benzene, 0·6 g; 2,5-diphenyloxazole, 9 g; ethanol, 300 ml; and redistilled toluene to make the final volume to 1000 ml.

The chromatographic plates and sucrose used in these experiments were products of E. Merck AG-Darmstadt (Reagenzien DV-Ferting Plattern Cellulose F Cat. 5728/0050, serial No. 8520345 and 8609538). ADP was a pure compound from BDH Chemical Ltd, Poole, England. Radioactive calcium chloride and synthalin were purchased from Sigma Chemicals Co., Missouri, U.S.A.

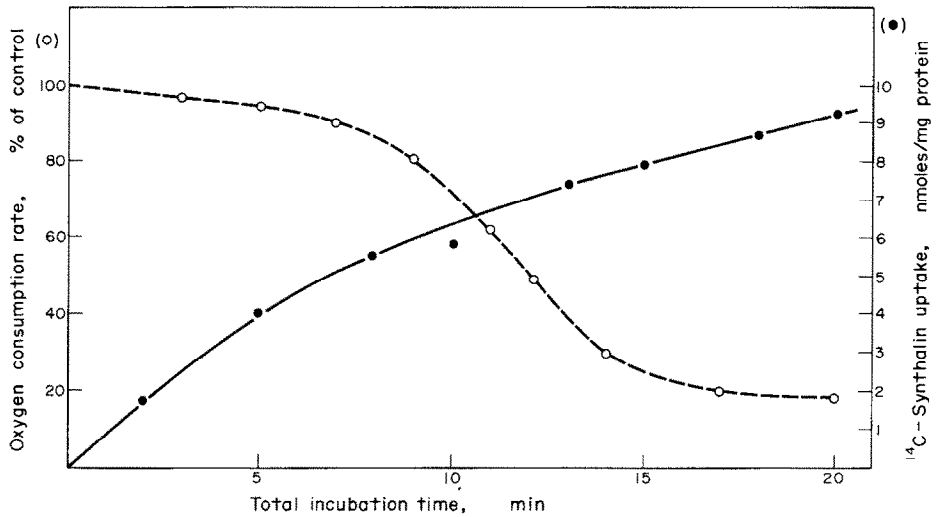


Fig. 1. Parallel measurements of onset of synthalin-induced respiratory inhibition and uptake of labeled synthalin. Incubation conditions were as described in Material and Methods, except that reagents were added as potassium salts. For determination of respiratory rate, mitochondria equivalent to 1·7 mg of protein were added to previously warmed medium containing synthalin, $5{\cdot}6 \times 10^{-5}$ M, and the mixture was incubated in a metabolic shaker at 37° for increasing time intervals before transferral to the oxygraph cell. After 2 min for equilibrium, 2·5 μmoles ADP was added and the respiratory rate determined, using the interval from 20 to 50 sec after addition of ADP. Total preliminary incubation time includes the time between addition of mitochondria to the medium and addition to ADP. For measurement of ^{14}C-synthalin uptake, $1{\cdot}20 \times 10^5$ count/min of ^{14}C-synthalin was included in the mixture in a parallel set of flasks, and samples were sedimented at the times indicated in Materials and Methods.

RESULTS

Characteristics of time course of synthalin uptake and its relationship to onset of respiratory inhibition. A major distinguishing characteristic of mitochondrial respiratory inhibition by synthalin *in vitro* is its slow progressive action, which very much resembles that of phenethylbiguanide [4–6]. Intramitochondrial levels of synthalin have been examined during the dynamic state of the induction of respiratory inhibition.

Figure 1 indicates that synthalin uptake by mitochondria continued throughout the period of induction of inhibition. The biguanide accumulation slightly preceded the inhibition. A similar observation has been reported by Davidoff [7] in regard to the respiratory inhibition of phenethylbiguanide. The degree of respiratory inhibition correlated well with the level of intramitochondrial biguanide. Furthermore, a permeability barrier to biguanide uptake apparently existed, which at this concentration range was only slowly permeated by biguanide; extramitochondrial biguanide exerted no influence on respiration.

From Fig. 1 it can be seen that an uptake of about 6 nmoles biguanide/mg of mitochondrial protein caused 60 per cent inhibition of respiration. Table 1 contains data which illustrate that withdrawal of the respiratory substrate from the reaction medium resulted in failure of uptake of synthalin. In addition, the presence of respiratory chain inhibitors or oligomycin plus rotenone and antimycin A resulted in inhibition of biguanide uptake. Clearly, the uptake of synthalin by mitochondria is energy-dependent.

Previous studies with alkylmonoguanidines demonstrated that as the concentration of inhibitor in the medium was increased, the lag period of onset of inhibition decreased [8]. This observation indicated that the uptake rate of guanidine derivatives, including biguanides, was probably limited by a membrane permeability barrier rather than a metabolic process; however, the available data did not establish whether this transmembrane movement was carrier-mediated or diffusion-limited, or whether uptake was energy-dependent or passive.

As an initial approach to this problem, in regard to synthalin, measurements were made of the rate of ^{14}C-synthalin uptake over a wide range of biguanide concentrations. These studies indicated that the rate of synthalin uptake, measured at 1-min intervals, increased linearly with increasing biguanide concentrations over nearly a 10^4-fold range of concentrations. Carrier-mediated uptake of biguanide thus seemed extremely unlikely, since no evidence of saturation of uptake was observed, as would have been expected for a carrier-mediated process.

Effects of changes in energized state on ^{14}C-synthalin accumulation. Data presented in Table 1 demonstrated that where substrate oxidation was inhibited with antimycin A several times greater than that needed for inhibition of state 3 respiration, with or without depletion of endogenous substrate, the decrease in biguanide uptake was about 50 per cent. Omission of ATP alone had a relatively modest effect on synthalin uptake. When all energy supplies were withdrawn, by omitting both substrate and ATP or by omitting ATP and adding rotenone, biguanide uptake was significantly further reduced. Addition of ADP, even after a steady state level of biguanide uptake was achieved, decreased synthalin uptake substantially, while inclusion of 2,4-dinitrophenol during the preliminary incubation prevented uptake almost completely. It is clear from these results that synthalin uptake is in some sense dependent on energization of the mitochondria.

Figure 2 shows a typical chromatogram of intact freshly isolated mitochondria as compared with synthalin-treated, aged, and phospholipase A-treated organelles. In this experiment mitochondrial phospholipids were separated by a procedure developed by Curri *et al.* [3]. According to this method, direct thin-layer chromatography of mitochondria without any previous extraction step resolved the phospholipid fractions into their components just as does the thin-layer chromatography of chloroform–methanol extracts of mitochondria.

Table 1. Accumulation of ^{14}C-synthalin by vervet monkey heart mitochondria—Factors affecting uptake*

Experimental conditions	Total synthalin uptake (nmoles/mg protein)	(% of control)
Complete	7·43	100
−Pyruvate, malate	3·22	43
−ATP	5·25	71
−Pyruvate, malate, ATP	0·74	10
+Antimycin A	1·07	14·4
+Oligomycin	6·74	91
+DNP	0·94	13
+Rotenone, antimycin A, and oligomycin	0·32	4·3
Pyruvate, malate, −ATP		
Control	5·28	100
+Antimycin A	0·97	18·6
+Rotenone	0·74	14
Complete	5·20	100
+Antimycin A	0·69	14·4
+Dinitrophenol	0·80	18
+Antimycin A and dinitrophenol	0·34	8
+Antimycin A, rotenone, and oligomycin	0·28	5·3

* Incubation conditions were as outlined under Material and Methods. Each flask contained 1·70 mg of vervet monkey heart mitochondrial protein; ^{14}C-synthalin, $6{\cdot}4 \times 10^4$ count/min, was present in all flasks at final concentrations of $2{\cdot}4 \times 10^{-5}$ M in all experiments. Substrate was pyruvate–malate. Antimycin (0·1 μg), oligomycin (2·5 μg), 200 nmoles DNP and 2 μg rotenone were added where indicated. All samples were incubated for 15 min with shaking at 37°, sedimented, and the uptake of labeled synthalin determined as described under Material and Methods.

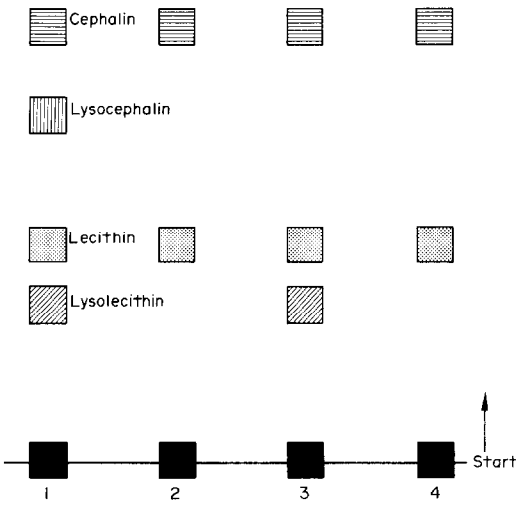


Fig. 2. Chromatogram of mitochondrial phospholipids. Fresh mitochondria = 1; synthalin-treated mitochondria = 2; aged mitochondria = 3; phospholipase A-treated mitochondria = 4.

Inhibition by synthalin of energy-dependent calcium accumulation by isolated mitochondria. Data presented in Table 2 show that calcium uptake by isolated mitochondria was severely inhibited by synthalin. In fact, when the concentration of synthalin present in the reaction medium was 2 μg/mg of mitochondrial protein, accumulation of calcium dropped from 64 to 4 nmoles/mg of protein. It is significant that when isolated mitochondria were incubated with synthalin at a concentration of 2 μg/mg of mitochondrial protein, lysocephalin and lysolecithin were depleted. This latter effect was similar to the action of phospholipid A (Fig. 2). However, in the presence of 2,4-dinitrophenol or respiratory chain inhibitors, lysocephalin and lysolecithin spots appeared on the chromatogram when mitochondria incubated with synthalin were used.

Table 2. Synthalin-induced inhibition of Ca^{2+} uptake by vervet monkey heart mitochondria*

Experimental conditions	Accumulated calcium (nmoles/mg protein)
Pyruvate, malate	64 ± 4·2
Pyruvate, malate, dinitrophenol	18 ± 3
Succinate	43 ± 2
Succinate + dinitrophenol	14 ± 4
Pyruvate, malate + synthalin (1 μg/mg protein)	8 ± 3
Pyruvate, malate + synthalin (2 μg/mg protein)	4 ± 1·7
Pyruvate, malate + synthalin (4 μg/mg protein)	4 ± 2
Succinate + synthalin (1 μg/mg protein)	10 ± 1·9
Succinate + synthalin (2 μg/mg protein)	3 ± 0·6
Succinate + synthalin (4 μg/mg protein)	3 ± 0·8

* The reaction medium consisted of the following: 31·25 mM NaCl–10 mM Pi 7·5 mM $MgCl_2$–75 mM KCl; 1·2 × 10^5 count/min of labeled calcium at a concentration of 150 nmoles in each flask. Where indicated, 5 mM pyruvate, 7·5 mM malate, 10 mM succinate and 200 nmoles DNP were added to the reaction medium. All samples were incubated for 10 min with shaking at 37°. Other experimental details were as described in Material and Methods.

Effect of synthalin on proton ejection into the suspending medium. In the present study, an attempt has been made to correlate the inhibition of electron transport, together with the concomitant Ca^{2+} uptake, with proton ejection during the electron flux. Recent studies have clearly demonstrated that ejection of H^+ ions into the suspending medium accompanies energy-linked accumulation of divalent cations such as Ca^{2+} by isolated mitochondria [9, 10]. Figure 3 illustrates that with synthalin in the reaction medium, addition of Ca^{2+} failed to stimulate ejection of protons into the reaction medium. In addition, the degree of inhibition was related to the concentration of biguanide present in the reaction medium. When 5 nmoles biguanide/mg of mitochondrial protein was present, it caused 60 per cent inhibition of proton ejection, while 10 nmoles caused complete inhibition.

Thus, the inhibition of proton ejection by synthalin is related to inhibition of respiration and energy-linked

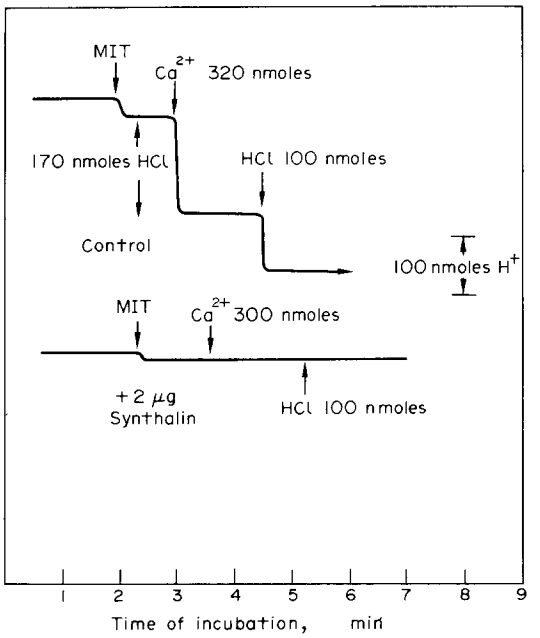


Fig. 3. Inhibition of proton ejection by synthalin. The basic reaction medium consisted of the following: 31·25 mM NaCl, 10 mM Pi, 7·5 mM $MgCl_2$, 75 mM KCl, 10 mM Na pyruvate and 1·7 mg mitochondrial protein. The determination of pH changes was as described in Material and Methods.

uptake of calcium ions by isolated mitochondria. Electron flux through the respiratory chain (respiration) is the source of energy for movement of ions during active transport and is accompanied by ejection of protons [9, 10].

DISCUSSION

The present experiments demonstrate that, after incubating mitochondria together with synthalin for a period of approximately 15 min, a strong inhibition of substrate oxidation was exerted (Fig. 1). Further incubation revealed that a substantial accumulation of synthalin by mitochondria continued and its intramitochondrial concentration increased from 8 to 9 nmoles/mg protein. Uptake of synthalin by mitochondria in the absence of respiration strongly suggested that ATP formed prior to inhibition of substrate oxidation was responsible for the accumulation. This was supported by two subsequent observations: (1) that when oligomycin was present in the reaction medium, synthalin uptake by mitochondria was severely inhibited (Table 1), and (2) that in the absence of respiratory substrate or ATP, synthalin uptake was also inhibited. In addition, 2,4-dinitrophenol, rotenone or antimycin A had a substantial inhibitory effect on the entry of synthalin into the mitochondrial matrix. It is also important that, under those conditions where synthalin uptake was inhibited either by uncoupling agents, respiratory inhibitors or by withdrawal of respiratory substrate in the reaction medium, synthalin had no effect on mitochondrial phospholipids. Thus, lysocephalin and lysolecithin spots from mitochondria treated with synthalin in the presence of either 2,4-dinitrophenol, rotenone or antimycin A, appeared on the chromatographic plate. Similarly, mitochondria incubated with oligomycin in the presence of ATP yielded lysocephalin and lysolecithin on the chromatographic plate.

The evidence available would therefore allow us to conclude that synthalin requires energy for translocation into the intramitochondrial space. The process occurs in two phases. The first phase is characterized by utilization of energy derived from reactions of the respiratory chain and is therefore inhibited by 2,4-dinitrophenol or the respiratory chain inhibitors. The second phase utilizes ATP as the energy source and is consequently inhibited by oligomycin. Since in the absence of sythalin accumulation lysolecithin and lysocephalin are not depleted from the mitochondrial membrane, we can safely conclude that synthalin destroys the membrane in the course of its translocation into the mitochondrial matrix.

Although the process of initial cation–proton exchange was energy-independent [11], the maintenance of cation uptake in the steady state was apparently dependent on small endogenous energy supplies, since the exclusion of energy (electron transport inhibitors, dinitrophenol) led to prompt release of cation and uptake of protons.

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