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Salicylate-induced loose coupling: protonmotive force measurements

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The Surgeon General's report on salicylate and Reye Syndrome [1] emphasizes the concern about the role of aspirin in Reye Syndrome. A prospective study [2] found a 100% correlation between aspirin and Reye Syndrome in patients compared to 45% in carefully matched controls. A recent paper pointed out the histological similarity between salicylate hepatotoxicity and Reye Syndrome [3]. Salicylate has been known for many years to have effects on intact mitochondria. Early work [4] showed an uncoupling effect, and other early researchers [5] reported that salicylate stimulates mitochondrial adenosine triphosphatase (ATPase) activity. Jeffrey and Smith [6] found an inhibition of mitochondrial swelling with salicylates similar to that seen with 2,4-dinitrophenol. However, more recently You [7] found that salicylate produces swelling of mitochondria similar to that seen in patients with Reye Syndrome. Miyahara and Karler [8], using a Warburg technique, demonstrated an uncoupling effect of salicylate in mitochondrial fragments isolated from rat liver and brain. However, their paper emphasized the confusion in interpretation of salicylate effects. Different results were seen with alternate substrates, and the results of their study, as from many earlier studies, could have been explained by an ATPase-stimulating effect of salicylate which would produce the decreased ADP:O ratios seen. More recent studies in rat liver mitochondria demonstrated an effect on the adenine nucleotide exchange across the inner mitochondrial membrane as well as an uncoupling effect in the presence of salicylate [9]. Both the effects were half-maximal at about 3 mM extra matrix concentration. Oligomycin, an F_1 ATPase inhibitor, does not block the uncoupling effect of salicylate. It has been suggested that this uncoupling is closely related to that of dinitrophenol in spite of the other effects of salicylate [6].

One approach to resolving the mechanism of apparent uncoupling is to measure directly the protonmotive force across the inner mitochondrial membrane in the presence of salicylate. This paper describes the results of these experiments.

Rat liver mitochondria were prepared and polarographic assay was performed as previously described with the following modifications [10]. Polarographic assays were performed in a 384 μl thermostatically controlled glass microchamber of the authors' own design with a Teflon-coated Microflex stir bar. A Clark oxygen electrode (Radiometer E5046-0) was used with a Radiometer model PM72 pO_2 module as a polarographic system. Chamber volume was calculated by tritiated water dilution after scintillation counting of an aliquot. Control and salicylate-treated mitochondria were preincubated for 3 min before ADP was added. Final mitochondrial protein concentrations were in the range of 0.5 to 1.5 mg/ml. Liver mitochondrial protonmotive force and matrix volumes were measured with glutamate as substrate using a double-label equilibration technique with [^{14}C]triphenylmethyl phosphonium iodide, [^{14}C]acetate, [^{3}H]water and [^{3}H] mannitol. Our method is described in detail elsewhere [11]. The amounts of ADP added to the protonmotive force assay to achieve State 3 and State 4 rates were selected by polarographic assay of equivalent concentrations of mitochondria in protonmotive force assay mixture.

Salicylate (1 mM) produced an acceleration of state 4 rates of oxygen consumption with glutamate, succinate and α-ketoglutarate as substrates (Table 1). The modest state 3 rate inhibition reached significance only with α-ketoglutarate. Respiratory control ratios were reduced markedly for all substrates, reflecting both the acceleration of state 4 and the modest reduction of state 3. An increase in glutamate and α-ketoglutarate ADP/O ratios was seen although this is not thought to be of biological significance.

The protonmotive force in state 4 was reduced by 15% in salicylate (1 mM) treated mitochondria using glutamate as substrate (Table 2). There was also a small reduction in the transmembrane potential. The membrane proton conductance increased more than 4-fold from 0.595 to 2.669 nmoles of $H^+ \cdot min^{-1} \cdot$(mg mitochondrial protein)$^{-1} \cdot mV^{-1}$. No significant changes were seen in state 3.

Matrix volumes did not differ ($P > 0.05$, paired t-test) in control and salicylate-treated groups suggesting that salicylate effects are not the result of mitochondrial breakage. Matrix volumes (μl/mg protein, N = 6) were: control state 3, 1.40 ± 0.5 (means ± S.E.); salicylate-treated state 3, 1.47 ± 0.47; control state 4, 1.68 ± 0.46; and salicylate-treated state 4, 1.25 ± 0.39.

The acceleration of state 4 rates of oxygen consumption by salicylate which we observed in these polarographic studies was similar to results obtained with tissue slices and Warburg techniques by previous investigators [4, 6, 8]. This uncoupling effect was considered to be similar to that of 2,4-dinitrophenol [6]. Aprille [9] found in rat liver that the uncoupling effect is insensitive to oligomycin, suggesting that stimulation of the F_1 ATPase coupling enzyme is not the major mechanism of accelerated oxygen consumption.

Table 1. Effect of sodium salicylate on rat liver mitochondria: State 3 and State 4 rates of O_2 consumption,* respiratory control ratio (RCR) and the ADP:O ratio

Substrate (3.30 mM)	Control	Salicylate (1.0 mM)	Control	Salicylate (1.0 mM)
	State 3	*State 3*	*State 4*	*State 4*
Glutamate	86.2 ± 24.1 (5)	79.8 ± 23.6 (5)	16.4 ± 5.6 (5)	41.0 ± 14.0† (5)
Succinate	190.8 ± 39.9 (5)	170.4 ± 37.9 (5)	40.9 ± 13.3 (5)	88.3 ± 31.9‡ (5)
α-Ketoglutarate	69.3 ± 19.7 (5)	51.7 ± 21.3† (5)	14.9 ± 6.3 (5)	33.1 ± 9.2‡ (5)
	RCR	*RCR*	*ADP/O*	*ADP/O*
Glutamate	5.36 ± 0.75 (5)	2.16 ± 0.51‡ (5)	3.01 ± 0.51 (5)	3.57 ± 0.35† (5)
Succinate	4.87 ± 1.18 (5)	2.03 ± 0.47‡ (5)	2.09 ± 0.28 (5)	2.13 ± 0.41 (5)
α-Ketoglutarate	5.06 ± 1.36 (5)	1.51 ± 0.33‡ (5)	3.21 ± 0.46 (5)	5.67 ± 1.06† (4)

* Values are $nAO \cdot min^{-1} \cdot (mg\ protein)^{-1}$, summarized as means ± S.D.; N = number in parentheses.
† $P < 0.017$, Student's *t*-test (paired samples).
‡ $P < 0.008$.

Table 2. Protonmotive force data in isolated rat liver mitochondria after a 3-min incubation with 1 mM sodium salicylate*

	ΔP	ΔΨ	ΔpH	(C_{m,H^+})
Control State 3	191.5 ± 25.3 (6)	139.6 ± 13.7 (6)	−0.880 ± 0.265 (6)	3.52 ± 1.05 (6)
1 mM Salicylate State 3	170.2 ± 63.5 (6)	135.3 ± 41.1 (6)	−0.591 ± 0.416 (6)	3.72 ± 1.58 (6)
P (paired *t*-test)	>0.05	>0.05	>0.05	>0.05
Control State 4	201.8 ± 31.6 (6)	157.1 ± 17.2 (6)	−0.758 ± 0.309 (6)	0.595 ± 0.219 (6)
1 mM Salicylate State 4	171.3 ± 31.3 (6)	127.6 ± 17.8 (6)	−0.740 ± 0.305 (6)	2.669 ± 1.542 (6)
P (paired *t*-test)	0.018	0.013	>0.05	0.023

* Values are means ± S.D., N = number in parentheses. Abbreviations: protonmotive force (ΔP), transmembrane potential (ΔΨ), transmembrane pH difference (ΔpH) and membrane conductance (C_{m,H^+}). Units are ΔP and ΔΨ in mV. C_{m,H^+} is nmoles of $H^+ \cdot min^{-1} \cdot (mg\ mitochondrial\ protein)^{-1} \cdot mV^{-1}$. Glutamate was used as the substrate.

Salicylate does stimulate oligomycin sensitive ATPases in some preparations [5], but this is not the explanation for the state 4 acceleration with salicylate in rat liver. Our protonmotive force measurements showed a small reduction in protonmotive force and transmembrane electrical potential in state 3 which was not statistically significant. This protonmotive force was sufficient to sustain phosphorylation of ADP to ATP. However, in state 4 small changes in protonmotive force and transmembrane electrical potential were associated with a 2-fold increase in oxygen consumption rate and a 4-fold increase in proton conductance. This suggests that in state 4 an increased number of proton channels are available. It follows that increased oxygen and substrate consumption will be required to maintain the protonmotive force. These measurements provide direct evidence that the action of salicylate on mitochondria at this concentration is that of a proton ionophore.

The increased ADP/O ratios observed in this polarographic study with 1 mM salicylate may result from the 3-min preincubation in which matrix adenine nucleotides may be lost or translocase inhibition may become a significant factor.

The inhibition of state 3 rates of oxygen consumption (Table 1) which reached significance for α-ketoglutarate has been observed before [4, 8]. Possible explanations are an inhibition of α-ketoglutarate dehydrogenase which was first noted by Kaplan *et al.* [12] or, alternatively, an inhibitory effect on the ATP-ADP translocase [9].

Octanoate may also be of significance in the pathogenesis of Reye's syndrome and induces a similar "loose coupling" in rat liver mitochondria through an increase in state 4 proton conductance [13]. With both salicylate and octanoate increased, substrate consumption will result along with the accelerated oxygen consumption of a "loosely coupled" state 4. Both octanoate and salicylate have effects on mitochondrial oxidative phosphorylation which may induce or potentiate the mitochondrial damage occurring in Reye Syndrome.

In summary, recent evidence suggests that salicylate ingestion is associated with Reye Syndrome. We studied the effect of salicylate on isolated rat liver mitochondria, confirming previous work showing an uncoupling effect and investigating the mechanism of this action by use of a protonmotive force assay. Salicylate (1 mM) produced loose coupling of state 4 rates of oxidative phosphorylation allowing preservation of ATP production. The protonmotive force and the transmembrane electrical potential were reduced in state 4. Salicylate appeared to be acting as a proton ionophore as shown by a 4-fold increase in state 4 proton conductance.

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Tricyclic antidepressant inhibition of depolarization-induced uptake of calcium by synaptosomes from rat brain

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Release of neurotransmitters from nerve terminals is dependent upon an influx of extracellular calcium that is mediated by voltage-gated channels [1]. Depolarization-induced calcium uptake can be studied in synaptosomes isolated from mammalian brain using $^{45}Ca^{2+}$ as a radiotracer and elevated K^+ concentrations to decrease resting membrane potential [2]. This uptake is rapid (<1 min), saturable, and susceptible to inhibition by several divalent ions, including Co^{2+}, Mn^{2+} and Cd^{2+}, which have been shown to block Ca^{2+} currents in electrophysiological studies [3]. A number of psychoactive drugs interfere with synaptosomal calcium accumulation, including ethanol [4], barbiturates [5], neuroleptics [6], and benzodiazepines [7]. In the present paper, we report the inhibition by tricyclic antidepressant drugs of K^+-stimulated $^{45}Ca^{2+}$ uptake by rat brain synaptosomes.

Methods. Two buffered salt solutions were used: S buffer contained 130 mM NaCl, 5 mM KCl, 1.2 mM Na_2HPO_4, 0.1 mM $CaCl_2$, 10 mM glucose, and 20 mM Tris–Cl, pH 7.4. In K buffer, the NaCl was omitted and the KCl concentration was raised to 135 mM.

Synaptosomes were prepared by homogenizing the forebrains (telencephalon and diencephalon with the olfactory lobes removed) of 200–250 g male Wistar rats in 10 vol. of ice-cold 0.32 M sucrose. After centrifuging at 1000 *g* for 5 min, the supernatant fraction was centrifuged at 35,000 *g* for 25 min and the pellet was resuspended in 0.32 M sucrose. This crude synaptosomal-mitochondrial fraction was layered over a density gradient comprised of 15 ml of 0.8 M sucrose and 15 ml of 1.2 M sucrose and spun at 100,000 *g* for 1 hr in a swinging bucket rotor. The synaptosomes were recovered from the 0.8 to 1.2 M sucrose interface, slowly diluted with S buffer, spun at 30,000 *g* for 20 min and resuspended in S buffer at a concentration of 0.4 to 0.5 mg synaptosomal protein/ml. This final suspension was allowed to sit at room temperature for 60 min before uptake measurements were performed.

To measure calcium uptake, 500 μl of S buffer (for resting uptake) or K buffer (for stimulated uptake) containing 0.1 μCi $^{45}Ca^{2+}$ (New England Nuclear) was added to synaptosomes (0.2 to 0.3 mg protein) suspended in 500 μl S buffer. After an appropriate time (generally 20 sec), the samples were filtered through Whatman GF/B glass fiber filters. The filters were washed once with 2 ml of S buffer and their radioactivity content was determined by liquid scintillation counting. All uptake values were determined in triplicate. To assess the influence of various drugs on calcium uptake, the synaptosomes were preincubated with the drugs for 15 min in S buffer before the $^{45}Ca^{2+}$-containing influx medium was added.

Results and discussion. Dilution of synaptosomes with medium containing depolarizing concentrations of K^+ approximately doubled $^{45}Ca^{2+}$ uptake. This uptake reached 50% of the final level in about 15 sec and was 90% complete within 1 min. After 2 min, the calcium content of depolarized synaptosomes slowly decreased so that after 45 min the $^{45}Ca^{2+}$ content of synaptosomes diluted with S or K uptake medium was the same (data not shown). K^+-stimulated Ca^{2+} uptake was inhibited 50% by 3 mM Mn^{2+} and 0.28 mM Co^{2+} (not shown). The extent of $^{45}Ca^{2+}$ uptake at 2 min increased with K^+ concentration up to about 60 mM K^+. K^+-stimulated uptake was linearly related to tissue concentration up to at least 1 mg synaptosomal protein/ml.

The initial rate of K^+-stimulated Ca^{2+} uptake was estimated by measuring $^{45}Ca^{2+}$ uptake at 5 sec, at which time calcium uptake was 15–20% of the level reached by 1 min. A Lineweaver–Burk plot of this data was linear (Fig. 1) and indicated a maximum rate of uptake of 0.30 nmole $Ca^{2+} \cdot (\text{mg protein})^{-1} \cdot \text{sec}^{-1}$ and an apparent K_m of 0.37 mM Ca^{2+}.