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Activation and Inhibition of NADH Oxidation by Brain Mitochondrial Fragments

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The NADH oxidase activity of brain mitochondrial fragments is considerably greater when determined in salt solutions than in water or in 0.25 M sucrose solutions. Inhibition by dicumarol and amytal but not by chlorpromazine also depended on the addition of electrolytes in the incubation medium. Using phosphate as a representative ion it was found that retention of dicumarol and chlorpromazine by the mitochondrial fragments was correlated with the degree of inhibition. However, there was no retention of amytal. Dicumarol also inhibited succinate oxidation and to a greater degree in an incubation medium containing phosphate. It is proposed that an interaction of added electrolytes with lipids in the fragments is the underlying phenomenon responsible for the increased permeability to some solutes and an increase in mutual accessibility of components of the electron transport chain.

Previous work in this laboratory has demonstrated that the antimycin and amytal sensitive pathway of NADH oxidation in brain mitochondria undergoes a great increase of activity on fragmentation of the mitochondrial fraction and that a major part of this increased activity is only apparent when the assay is conducted in a medium containing salts[1]. Further investigation of this phenomenon has now revealed that a relationship may exist between the activation brought about by added electrolytes and the extent of inhibition observed with substances which inhibit electron transport from NADH *via* the antimycin sensitive respiratory chain. These effects have been studied using three classes of inhibitors, represented by dicumarol, amytal and chlorpromazine. A comparison has also been made of the effects of added electrolytes and inhibitors on succinate oxidation. The results presented in this paper suggest that added electrolytes including ATP and ADP alter the membrane permeability so that

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certain inhibitors and perhaps also substrates can more readily gain access to the organized respiratory chain embedded within the mitochondrial membrane.

MATERIALS AND METHODS

The chemical preparations used, the preparation of rat brain mitochondria and of mitochondrial fragments by sonic irradiation, the assays for NADH oxidase, NADH-cytochrome *c* reductase and succinate-cytochrome *c* reductase have been previously described[1,2]. In those experiments in which buffer was not added, all solutions were adjusted to pH 7.3–7.5. The pH (glass electrode) did not change beyond these limits during any experiment. Recording of the absorbancy spectrum of dicumarol, amytal and chlorpromazine, as described in the experimental results, was carried out with the Bausch & Lomb, model 505, spectrophotometer.

In the experiments of Figures 1–3, 1 ml of enzyme suspension catalyzed the oxidation of 940 mμmoles of NADH per minute in 0.1 M phosphate buffer, pH 7.3.

RESULTS

Stimulation of NADH oxidase by added electrolytes. The NADH oxidase activity of the mitochondrial fraction of rat brain homogenates prepared with 0.25 M sucrose is usually very low, ranging from 100 to 250 mμmoles per minute per gram (wet weight). The poor capacity of intact mitochondria to oxidize NADH has been reported also for other tissues[3–5]. Fragmentation of the brain mitochondrial fraction by sonic irradiation results in an increase of NADH oxidase activity up to 6 000 mμmoles per minute per gram. Much of this increased rate of oxidation depends on the addition of electrolytes in the incubation medium; in water or in 0.25 M sucrose solutions, NADH is oxidized by the mitochondrial fragments at only 6- to 8-fold as compared to intact mitochondria. Activating electrolytes previously reported include amino acids, neutral salts, ethylenediamine tetraacetate and various buffer solutions[1]. Extension of these studies to the adenosine polyphosphates as representative of cellular electrolytes demonstrated that ATP was also effective, ADP to a lesser degree and AMP had no effect. The data of Table 1, column 1, compare the NADH oxidase activity in water and in sucrose incubation media with the activity in some ionic media. The added electrolytes are at previously determined optimal concentrations. These experiments were carried out without added cytochrome *c*. Addition of cytochrome *c* stimulated NADH oxidation 2- to 3-fold in all experiments. Comparable data were obtained when NADH oxidase activity was measured by the change in absorbancy at 340 mμ, or cytochrome *c* reduction measured by the change in absorbancy at 550 mμ in the presence of KCN. The results to be described below, while referred to NADH oxidase without cytochrome *c* present, in general also apply to experiments with added cytochrome *c* whether measured at 340 or 550 mμ.

Effect of inhibitors. NADH oxidation in all incubation media was inhibited more than 95 per cent by antimycin A (1.72 μg/ml). During the course of our studies on the soluble dicumarol sensitive NADH oxido-reductases[6,7] *, it was observed that concentrations of dicumarol considerably in excess of that required to inhibit the soluble oxido-reductases were also inhibitory to the respiratory chain with either NADH or succinate as substrates. At a concentration of 1×10^{-4} M dicumarol, which is close to the solubility limit at the pH used,

* These enzymes have been referred to as diaphorases in previous publications. They are listed as menadione reductases (EC1.6.5.2) by the Enzyme Commission.

Table 1. Ion stimulation of NADH oxidation by fragmented mitochondria, and inhibitory effect by dicumarol, amytal and chlorpromazine. The incubation mixture contained 1×10^{-4} M NADH and activator or/and inhibitor at the final concentrations indicated in the table. Activity units are calculated as μmoles per min per g (wet weight) of brain.

Activator	Activity	Inhibition in % with		
		5×10^{-5} M dicumarol	2×10^{-4} M amytal	3.3×10^{-5} M chlorpromazine
none	800	0	0	52
0.1 M phosphate buffer	6 250	43	68	40
5×10^{-3} M ATP	2 200	15	10	45
5×10^{-3} M ADP	960	8	6	29
5×10^{-3} M AMP	755	2	0	34
5×10^{-2} M EDTA	5 750	50	60	55
0.25 M sucrose	800	0	0	32

inhibition of about 50 to 60 % was obtained. However, this inhibition was completely dependent on the addition of electrolytes to the incubation media, and there appeared to be a correlation between the degree of activation by the added ion and the degree of inhibition exerted by dicumarol in each ionic solution (Table 1, column 2). Similar experiments carried out with two other representative compounds, which are inhibitory to the respiratory chain, amytal[8–10] and chlorpromazine[11–13], demonstrated that amytal behaved like dicumarol, but that chlorpromazine was inhibitory in all incubation media, although the degree of inhibition varied (Table 1, columns 3, 4). In these experiments suboptimal concentrations of inhibitor were used, so as to permit the effect of the various added electrolytes to be seen more clearly. Sucrose media appeared to behave qualitatively like water; the addition of electrolytes to sucrose gave the same results as the addition of electrolytes to water.

Retention of inhibitors. The data in the literature indicating that phosphate promotes mitochondrial swelling by increasing the permeability of the membrane[14] and the evidence that sonic irradiation of mitochondria results in the formation of small, membrane-surrounded particles[15] suggested a study of the penetration of inhibitors into the mitochondrial fragments under the influence of phosphate and other added electrolytes. A feasible experimental approach was to compare the retention of the inhibitors by the mitochondrial fragments in water or sucrose with the retention in phosphate solutions. The characteristic optical absorption of each of these compounds in the ultraviolet region, permitted these experiments to be conducted readily. Suspensions of mitochondrial fragments were incubated in water, in 0.25 M sucrose or in 0.1 M phosphate solution, at room temperature for 10–15 minutes, with the inhibitor. The suspension was centrifuged at $105\,000 \times g$ for one hour and absorbancy of the supernatant determined in the region 230–350 mμ against a blank prepared in the same way without inhibitor present. Fig. 1 A compares the spectra of a dicumarol solution

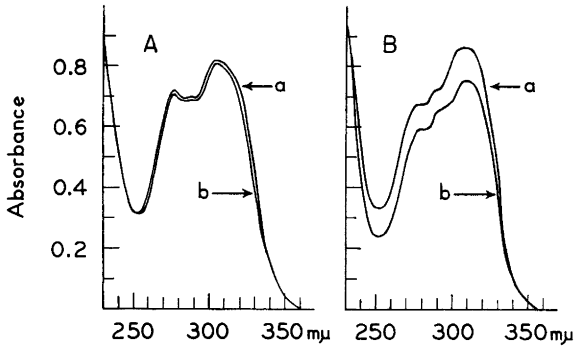

Fig. 1. Retention of dicumarol by sonicated mitochondria. Experimental details are given in the text. A: spectrum of dicumarol in water; *curve a* before incubation, *curve b* after incubation with mitochondrial fragments. B: same as A in phosphate buffer. Spectra recorded against blanks obtained after centrifuging off equal amounts of particles previously incubated with water and phosphate, respectively.

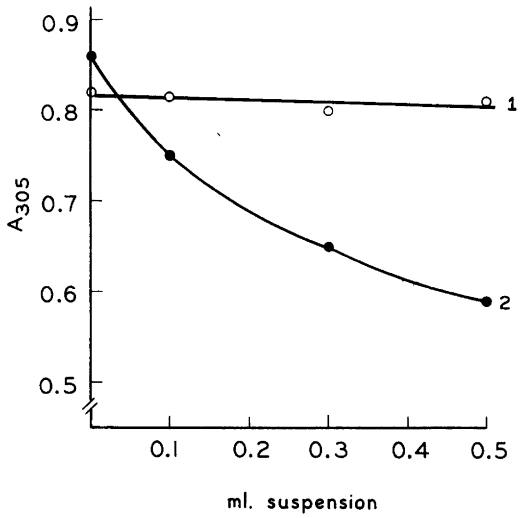

Fig. 2. Retention of dicumarol as a function of concentration of sonicated mitochondria. *Curve 1,* in water; *curve 2,* in phosphate buffer. Blanks as in Fig. 1.

(5×10^{-5} M) before and after incubation with fragmented mitochondria in water. No retention of dicumarol was observed under these conditions. By contrast, the incubation in 0.1 M phosphate resulted in the removal of about 13 % of the dicumarol by the particles (Fig. 1 B). Determination of the absorbancy changes at 305 mμ, where dicumarol absorbs maximally, was used to obtain the data presented in Fig. 2, which demonstrates that in water little or no dicumarol was removed with increasing concentrations of particles whereas in phosphate the removal of dicumarol depended on the concentration of fragments.

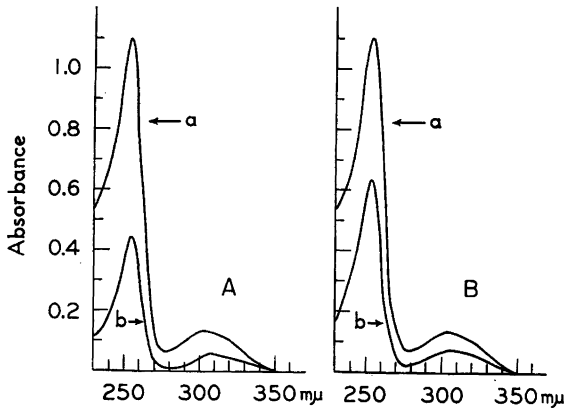

Fig. 3. Retention of chlorpromazine by sonicated mitochondria. A: spectrum of chlorpromazine in water; *curve a* before incubation, *curve b* after incubation with mitochondrial fragments. B: same as A in phosphate buffer. Blanks as in Fig. 1.

The same experiments carried out with chlorpromazine (3.3×10^{-5} M) revealed that under the same conditions this inhibitor was retained by the fragments when incubated in water, 0.25 M sucrose or phosphate (Fig. 3) and, indeed, that greatest retention was observed in the water incubation, and that a rough correlation existed between the retention of chlorpromazine and the degree of inhibition seen in Table 1.

In contrast to the results with chlorpromazine and dicumarol, which indicated a correlation between retention of the inhibitor by the fragments and degree of inhibition, amytal (2×10^{-4} M) was not retained when incubated with mitochondrial fragments in either water or sucrose on the one hand or in phosphate solution on the other.

Inhibition of succinate oxidation. Since succinate oxidation by heart and kidney preparations has been shown to be stimulated by phosphate and other added electrolytes[16–18], it was of interest to examine the effect of dicumarol and chlorpromazine on succinate oxidation with fragmented brain mitochondria. However, since saturating concentrations of succinate provided an ion concentration sufficiently high to activate the enzymatic system, it was necessary to carry out these experiments at substrate concentrations lower than optimal. At two different concentrations of succinate (2×10^{-3} M and 6×10^{-3} M) dicumarol inhibited the reduction of cytochrome *c* to the extent of 35 % in 0.1 M phosphate solutions and about 10 % in water. Chlorpromazine, as previously reported[12] and confirmed[13], did not inhibit succinate-cytochrome *c* reductase.

DISCUSSION

Dicumarol interferes with reduced nicotinamide adenine nucleotide oxidizing systems at different levels of concentration. The NADH and NADPH oxidoreductases are inhibited at a concentration of 10^{-8} M, and phosphorylation is uncoupled at a concentration of 10^{-5} M[6,7,19,20]. As shown in the experiments re-

ported here, 10^{-4} M dicumarol inhibits both NADH and succinate oxidation by the respiratory chain of fragmented brain mitochondria. Chlorpromazine has also been shown to have a similar spectrum of action. It inhibits brain NADH and NADPH oxido-reductases, uncouples oxidative phosphorylation and inhibits electron transport from NADH, succinate and NAD-linked dehydrogenases[7,11–14]. Amytal inhibits NADH oxidation but does not inhibit the oxidation of succinate. The effect of 0.1 M phosphate, as a representative ion, on each of these inhibitors is different. Thus phosphate, which is required for dicumarol inhibition, also promotes the retention of this compound by the mitochondrial fragments. With chlorpromazine, which inhibits in water or ionic media, retention is obtained in either media. Amytal, however, which also requires an ionic medium for inhibition, is not retained by the fragments. The assumption that phosphate increases the permeability to solutes of the membrane of the fragments, as has been shown for intact mitochondria, apparently is not true for chlorpromazine, although it could account for the effect on dicumarol and amytal. The latter two substances are acids, whereas chlorpromazine is a base. The undissociated form of amytal has been shown to be the active form in the inhibition of cell division of sea urchin eggs[21], and in the inhibition of NADH oxidation by brain mitochondrial fragments[22]. It is conceivable that the permeability to the unionized forms of acidic substances is promoted by the active electrolytes, whereas bases are easily permeable in either electrolyte or non-electrolyte media. In order to account for the retention of dicumarol and chlorpromazine compared to the non-retention of amytal, it would seem necessary to postulate that the former two substances after penetrating the membrane barrier are bound to some internal structure in sufficiently high concentration to be measured, whereas amytal is only bound to an active site of an enzyme involved in NADH oxidation, which enzyme is present in relatively low concentrations.

Chlorpromazine inhibition differs from that obtained with dicumarol in only one aspect, the lack of effect on the succinate-cytochrome *c* portion of the respiratory chain[12,13]. The data for the most part are in accord with the hypothesis that the inhibitory effects of dicumarol and chlorpromazine, in contrast to amytal, are the result of an interference with the physical structure containing the respiratory chain which causes a decrease in the mutual accessibility of some of the components of the chain.

The nature of this physical change is unknown. The inclusion of salts in the incubation medium results in a visible increase in turbidity of the suspension, perhaps indicating a contraction or agglutination of the particles. A similar increase in turbidity is observed when salts are added to a fine suspension of lecithin. The addition of dicumarol to the turbid suspension of mitochondrial fragments does not alter to any measurable extent the optical absorbancy of the suspension. It is conceivable that the increase in permeability, presumably brought about by added electrolytes, the hypothetical alteration of component accessibility and the visible increase in turbidity all reflect the same underlying phenomenon, an interaction of electrolytes with lipids in the particle structure.

The phenomenon described may be the same as or closely related to the activation of succinate and NADH oxidation, previously studied in heart and kidney preparations[16–18]. If further study indicates the two phenomena to be

indeed related, it would appear that the elucidation of the difference in inhibitory effect between dicumarol and chlorpromazine on the succinate-cytochrome *c* portion of the respiratory chain might aid in understanding the relationship of the common cytochrome *c* oxidase pathway to the convergent NADH-cytochrome *c* reductase and succinate-cytochrome *c* reductase pathways.

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