A STIMULATORY FACTOR FOR MITOCHONDRIAL NADH OXIDASE*

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This laboratory has reported previously that fragmentation of brain mitochondrial fractions resulted in a 7-25 fold increase of the antimycin A-sensitive NADH oxidase, provided that ionic compounds such as inorganic phosphate were present in the incubation media used for enzymatic determination (Giuditta and Strecker, 1959). The oxidation of NADH and of succinate by the Keilin-Hartree heart muscle preparation has been shown also to be stimulated by ionic compounds and by globin (Keilin and Hartree, 1949; Slater, 1949; Bonner, 1954). Similar observations have been made now with liver and heart mitochondria. Evidence is presented to indicate that the stimulatory effects obtained with added ionic compounds depends at least partially on interaction with a heat stable non-dialyzable factor, which appears to be present exclusively in the mitochondrial fractions of heart, liver or brain homogenates.

Methods - Rat livers, hearts and brains were minced with a fine scissor and ground by hand with 0.25 M sucrose solution, pH 7.5, using a glass homogenizer. The heart suspension was reground with a motor-driven teflon homogenizer. Mitochondrial fractions were obtained essentially according to Schneider and Hogeboom (1950). Sonically disrupted mitochondria were prepared as described previously (Giuditta and

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Strecker, 1959). When required, this disrupted mitochondrial fraction was centrifuged at 105,000 x g for 1 hour to yield a firmly packed pellet (P) and a clear supernate (S). With the aid of a glass homogenizer the pellet was resuspended in 0.25 M sucrose solution of the same volume as that of the original mitochondrial suspension.

NADH oxidase was measured as described previously (Giuditta and Strecker 1959). All solutions used in the incubation mixture were adjusted to pH 7.3 - 7.5. The pH (glass electrode) did not change beyond these limits in any experiment including those not containing buffer. The incubations were carried out at 28° - 30° in cells of 1-cm light path, in a total volume of 3 ml. Absorbancy changes were recorded automatically with the aid of a Gilford Optical Density Converter. The enzymatic activities summarized in Table I and II are expressed in millimicromoles NADH oxidized per min. per ml of enzyme suspension. The protein concentration of these suspensions varied from 3 to 5 mg/ml for brain, 5 - 10 mg/ml for liver and 0.5 to 0.9 mg/ml for heart.

Results — Disruption of the mitochondrial fractions of brain, heart and liver yielded fragments which, when incubated with NADH in water or sucrose solution, oxidized the substrate at the most 4 times as fast as did intact mitochondria. Addition of ionic compounds to the incubation medium caused a 7-25-fold increase in the rate of oxidation of NADH. Table I presents some results obtained with optimum levels of potassium phosphate solution. Inorganic phosphate was not unique; other active electrolytes tested included ethylenediaminetetraacetate, tris (hydroxymethyl) amino-methane, ATP, ADP, potassium, lithium, sodium, and magnesium halides and sulfates, borate, proline, leucine, glycine, lysine, arginine, glutamate, aspartate, histidine, cysteine, pyrophosphate and glycylglycine. The stimulation obtained varied with the ionic species used. Phosphate was most effective; the alkali halides least.

In all instances the NADH oxidase activity was inhibited 90-95 per cent by antimycin A (1.7 μg/ml).

Table I

Effect of sonic disintegration and of inorganic phosphate on brain, liver and heart

Tissue	Untreated Mitochondrial Fraction*	Disintegrated Mitochondrial Fraction	
		No phosphate	With phosphate (0.1 M)
Brain	7.2	28	182
Liver	48.2	44.4	458
Heart	43.4**	24.1	301

*Incubations were conducted in 0.25 M sucrose solution.
**It is probable that some damage was present because of the extra homogenization required.

Further studies of the stimulatory effect of these ionic substances using phosphate as a representative ion, and brain, as the tissue of choice since it showed the greatest increase of activity after fragmentation, revealed that additional components in the mitochondrial fraction were also involved. Centrifugation of the disrupted mitochondrial fraction at 105,000 x g for 1 hour followed by resuspension of the sedimented pellet (P) in sucrose solution resulted in a loss of NADH oxidase activity ranging from 20 to 60 per cent. Activity was almost fully restored by addition of the fragmented mitochondrial supernate (S) to the incubation cuvette together with the resuspended sediment (P). The supernatant showed little or no activity alone. Both loss of activity and restoration by the supernate were observed only when the determination was carried out with added electrolytes (Table II).

The stimulatory factor present in the supernate (S) obtained from the disrupted mitochondrial fraction appeared to be limited to this fraction. Other subcellular fractions were without effect. Heating the supernate (S) at 100° for 10 minutes did not affect the stimulatory activity, although there was destruction of the small residual NADH oxidase activity which sometimes was present. The stimulatory effect was retained also after dialysis of the supernate (S) against 0.25 M sucrose solution.

Table II

Stimulation of NADH oxidase of fragmented brain mitochondria

Preparation	No phosphate	With phosphate (0.1 M)
Disintegrated mitochondrial fraction	62.8	337
Pellet (P), 105,000 x g, 1 hr	62.8	145
Supernatant (S), 105,000 x g, 1 hr	0	23.2
(P) + (S)	62.8	337

During the course of these studies it was noted that heart preparations of NADH oxidase were far less stable than preparations obtained from brain.

Figure 1 demonstrates the loss of NADH oxidase activity of heart preparations with duration of storage at -15°. The rate of NADH oxidation (Curve A) catalyzed by the disrupted mitochondrial fraction decreased in two days to the same level of activity as was obtained with the same fraction in the absence of added ionic substances. This low level of activity (of either the total fragmented mitochondrial fraction or the resuspended pellet (P)) measured in media lacking added electrolytes did not change over a period of 50 hours (Curve D). The sediment obtained by high speed centrifugation of this fraction lost activity even more rapidly (Curve B) so that the minimal level was reached in less than one day of storage. When the disrupted mitochondrial supernate fraction (S) from brain was combined with the resuspended heart pellet (P) the activity of the heart preparation was better preserved, so that after 2 days of storage about 50 per cent of the activity remained (Curve C).

Bovine serum albumin, hemoglobin, native globin, denatured globin, and protamine were tested as possible substitutes for the factor present in the mitochondrial fractions. Although some stimulation of activity was observed with each of these proteins,

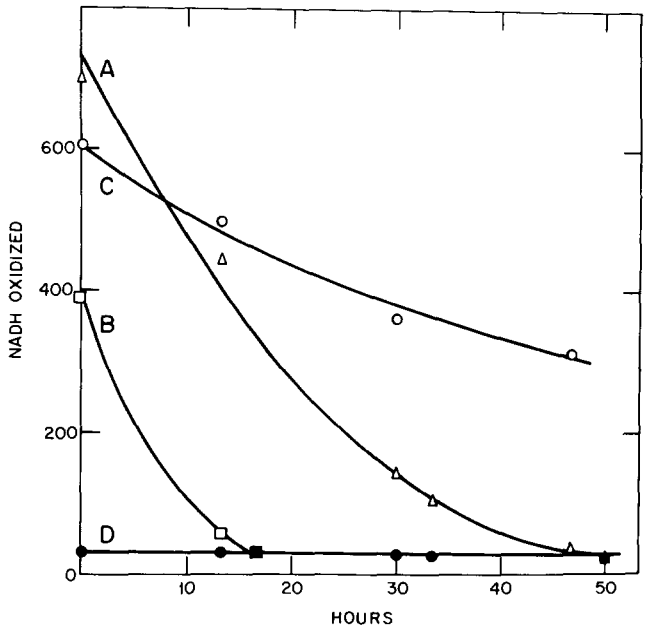

Fig. 1 Effect of storage on NADH oxidizing activity of heart preparations. Curve A (Δ — Δ), disrupted mitochondrial fraction assayed with 0.1 M potassium phosphate, pH 7.5. Curve B (□ - □), resuspended pellet (P), assayed with phosphate. Curve C (o — o), (P) combined with supernate (S) from disrupted mitochondrial fraction of brain, assayed with phosphate. Curve D (• — •), the preparations of Curve A and Curve B assayed without phosphate. All preparations were suspended in 0.25 M sucrose solution and maintained at -15° between assays.

this stimulation did not require added ions. Cytochrome c at the concentrations present in brain mitochondrial extracts had no effect.

The results obtained indicate that at least part of the stimulation of mitochondrial NADH oxidase brought about by added ions depends on interaction with a heat-stable, non-dialyzable component present exclusively in the mitochondrial fraction. In addition, this component or other components in the same fraction stabilize the mitochondrial NADH oxidase which is measurable only when ionic compounds are added.

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