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Electron-transport and phosphorylation inhibitor in *Tetrahymena* and evidence for its formation by a phospholipase

Preparations of *T. pyriformis* were reported to form by enzymic action a heat-stable agent (or agents) which inhibits various respiratory enzymes of rat-liver mitochondria and *Tetrahymena* homogenate[1]. It has now been shown that this agent inhibits oxidative phosphorylation, activates latent ATPase, and promotes swelling of rat-liver mitochondria. In addition, evidence implicating a phospholipase in its production has been obtained.

Particle preparations (made from homogenates of the GL strain), aged 24 h at 0° and then boiled to destroy the enzyme which produces the inhibitor, uncouple phosphorylation and depress O_2 uptake in the presence of succinate (Table I). Usually, preparations containing 30–50 μg N inhibit phosphorylation 100% and respiration 55–80%. Particle preparations, boiled when fresh to reduce inhibitor formation, also suppress phosphorylation and decrease O_2 uptake but much larger amounts are required (Table I). Crystalline bovine serum albumin prevents the effect of the GL

TABLE I

INHIBITION OF OXIDATIVE PHOSPHORYLATION AND OXYGEN UPTAKE OF LIVER MITOCHONDRIA BY A *T. pyriformis* PREPARATION AND PROTECTION BY ALBUMIN

The reaction mixture contained: 40 μmoles potassium phosphate buffer, pH 7.4; 20 μmoles $MgCl_2$; 5 μmoles ATP; 60 μmoles glucose; 140 K.M. units hexokinase; 100 μmoles sodium succinate; 0.042 μmole cytochrome *c*; and 0.28 mg rat-liver mitochondrial N in 63 μmoles sucrose; final volume, 2.8 ml; 0.2 ml 20% KOH in centre well; temp. 30°; duration of experiment 15 min plus 7 min equilibration; phosphate uptake assayed by change in inorganic P by method of LOWRY AND LOPEZ[2]. 7 day-old culture of *T. pyriformis* GL homogenized as described[1]; a portion was immediately boiled 20 min; both preparations were stored 24 h at 0° and the unboiled portion was then boiled 20 min. Both preparations were centrifuged 30 min at 105,000 × *g* to obtain the particles (1.1 mg N/ml).

Additions	*ΔO μatoms*	*ΔP μmoles*	*P/O*
None	6.0	10.8	1.8
0.025 ml GL particles, aged (0°) and boiled	4.9	6.5	1.3
0.05 ml GL particles, aged (0°) and boiled	3.1	0.5	0.2
0.1 ml GL particles, aged (0°) and boiled	2.1	0.5	0.2
0.2 ml GL particles, aged (0°) and boiled	0.6	0	0
0.05 ml GL particles, boiled and aged (0°)	6.8	11.0	1.6
0.1 ml GL particles, boiled and aged (0°)	6.1	8.6	1.4
0.2 ml GL particles, boiled and aged (0°)	4.1	3.3	0.8
0.3 ml GL particles, boiled and aged (0°)	3.9	0	0
0.05 ml GL particles, aged (0°) and boiled + 5 mg crystalline bovine albumin	6.8	10.9	1.6

Abbreviations: ATP, adenosine triphosphate; Tris, tris(hydroxymethyl)aminomethane.

particles on phosphorylation and oxidation. Albumin likewise prevents inhibition of *Tetrahymena* succinic oxidase by the particles. Recently, EDWIN AND GREEN[3] also reported that albumin—and α-tocopherol or ubiquinone—reverses the inhibition of liver-homogenate succinic oxidase induced by a *Tetrahymena*-inhibitor preparation. In other experiments, it was found that the inhibitor completely uncouples phosphorylation linked to α-ketoglutarate or β-hydroxybutyrate oxidation; however, O_2 uptake by the latter system is not decreased even with relatively high levels of the inhibitor preparation.

Aged and boiled GL particles activate the Mg-stimulated latent ATPase activity of liver mitochondria made in 0.25 *M* sucrose and assayed at 30° for 10 min (0.025 *M* Tris, pH 7.5). With higher levels of particles, activity is decreased, and with still higher levels, it is again increased. Relatively large amounts of particle preparations, boiled when fresh, also activate ATPase. ATPase activity of mitochondria treated to annul the latency, *i.e.*, suspended in water and pre-incubated for 15 min at 30°, is not enhanced by the GL preparations. ATPase activity of particles obtained from *T. pyriformis* S or GL in 0.25 *M* sucrose is not latent, does not respond to dinitrophenol, and is not activated by the GL preparations.

In experiments to be described elsewhere, aged and boiled GL particles have been found to promote the swelling of liver mitochondria suspended in 0.125 *M* KCl or 0.25 *M* sucrose and 0.02 *M* Tris, pH 7.4, while preparations which are boiled immediately are not as effective.

The data in Table II show that incubation of "inactive" GL homogenate with crotoxin, a crystalline lecithinase A from *Crotalus t. terrificus*, followed by prolonged boiling to destroy the lecithinase, results in a mixture which inhibits the succinic oxidase of *Tetrahymena* (and liver) almost as markedly as does the active GL homogenate. Purified ribonuclease, deoxyribonuclease, hyaluronidase, trypsin, chymotrypsin, papain, ficin, or wheat-germ lipase do not produce inhibitory substance(s) on incubation with "inactive" GL homogenate. In addition, with crude soy-bean

TABLE II

EFFECT OF LECITHINASE A (CROTOXIN) ON FORMATION OF INHIBITORY SUBSTANCE IN BOILED *T. pyriformis* PREPARATION

1 ml GL homogenate (1.27 mg N), either boiled 30 min when fresh and then aged 7 days at 0° ("inactive") or aged 7 days at 0° (active), was mixed with 0.5 ml water in (1) and (2), respectively, with 0.5 ml lecithinase (20 μg) in (3), and with 0.5 ml lecithinase previously boiled 1 h in (4); in (5), 1 ml water was mixed with 0.5 ml lecithinase. All mixtures were incubated in glass homogenizers 2h at 37°, boiled 1 h, and thoroughly homogenized. 0.5 ml of each mixture was added in duplicate to Warburg vessels containing 150 μmoles sodium succinate, 100 μmoles potassium phosphate buffer, pH 7.1, and *Tetrahymena* S homogenate (about 1 mg N); final volume, 2.8 ml; 0.2 ml 20% KOH in centre well; temp. 30°. Results are given for the first 10 min and for the first h after closing taps and are expressed as % inhibition as compared to control vessels without additions.

No.	*Additions to succinic oxidase system of* T. pyriformis *homogenate*	*Inhibition (%)*	
		10 min	*60 min*
1	Active Gl homogenate	93	95
2	"Inactive" GL homogenate	15	18
3	"Inactive" GL homogenate + lecithinase	63	75
4	"Inactive" GL homogenate + boiled lecithinase	22	35
5	Lecithinase	0	0

lecithin as substrate, the presence of a lecithinase (presumably a phospholipase A) has been demonstrated in homogenates of the GL, S, and W strains of *Tetrahymena*.

These results suggest that the *Tetrahymena* agent(s) which inhibits electron transport, uncouples phosphorylation, and stimulates latent ATPase of rat-liver mitochondria is a fatty acid(s) (and possibly a lysophosphatide) originating from the breakdown of particulate phospholipid(s) by the protozoan phospholipase. A comparison of the patterns of inhibition of various liver and *Tetrahymena* respiratory enzymes produced by oleic acid, lysolecithin, and the protozoan preparation, as well as other studies—all of which will be detailed elsewhere—supports this view. Thus it appears that the inhibitor(s) from *Tetrahymena* is similar to the uncoupling agents extracted by organic solvents from the mitochondria of blow-fly thoraces[4], wax-moth larvae[5], and rat liver[6], and from mitochrome[7].

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The enzymic degradation of heparitin sulfate

Extracts of flavobacteria had been found to degrade various mucopolysaccharides[1]. When the organisms were adapted to either heparin or heparitin sulfate, the "induced enzyme" degraded both substrates[2]. Heparitin sulfate resembles heparin in optical rotation and composition but has only one sulfate group per disaccharide unit. Approximately one-half of the hexosamine units are N-sulfated, the other half are N-acetylated[2].

It has been observed in all cases where "induced enzyme" was used that a loss of carbazole reaction for uronic acid accompanied the hydrolysis. In the case of hyaluronic acid and the chondroitin sulfates, this was shown to be due to the formation of an α-keto acid[3].

When heparitin sulfate was degraded by "induced enzyme", paper chromatography showed the presence of four major products (Fig. 1). The fastest moving component appeared to be N-acetylglucosamine. The three other components, Compounds I, II and III (Fig. 1) were isolated from a cellulose column[4] using butanol–acetic acid–water (50:12:25) as eluant. From 650 mg of a digest, 100 mg N-acetylglucosamine, 200 mg Compound I, 50 mg Compound II and 120 mg Compound III were