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Chelating Agents, Barbiturates, and Stimulation of Rat Brain Adenosinetriphosphatase. (21251)

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Recent studies have indicated that barbiturates may, *in vitro*, interfere with oxidative phosphorylation(1). The significance of this interference is difficult to assess, since many compounds of apparently unrelated biological activity and structure exhibit the same effect. However, further understanding of the manner in which oxidative phosphorylation is interrupted may reveal points of departure among the various compounds involved. In a previous discussion(2), it was pointed out that certain broad-spectrum antibiotics, as well as 2,4-dinitrophenol(3), increased the adenosinetriphosphatase (ATPase)* activity of rat liver homogenates and mitochondria. It was also observed(2) that the ATPase activity of rat brain homogenates was not stimulated by either the antibiotics or dinitrophenol. The results obtained in the present study indicate a possible role of chelation effects in ATPase stimulation.

Materials and methods. The purities of Sigma sodium adenosinetriphosphate (ATP), Sigma sodium adenosinediphosphate (ADP), and Pabst sodium uridinetriphosphate (UTP) were confirmed by analyses of labile and total phosphorus. The barbiturates† and other chemicals used were the purest obtainable from various commercial sources. Ten per cent homogenates of rat liver or brain (frontal lobes) were made in 0.25 M sucrose at 0°C by use of a glass homogenizer(5). Tubes were set up containing the following: 0.04 M KCl, 0.0001 M $MgSO_4$, 0.05 M tris(hydroxymethyl)amino-methane buffer (pH 7.4), 0.003 M ATP (or other substrate), 0.1 ml homogenate, barbiturate solution or other additions if desired, total volume 1.0 ml. All solutions were made in glass-redistilled water and adjusted to pH 7.4. Substrate was added after the other components of the reaction mixture had been equilibrated in the water bath for 5 minutes. Following 10 minute incubation at 30°C, proteins were precipitated by addition of 0.1 ml 50% trichloracetic acid. Orthophosphate was determined(6) in the supernatant after centrifugation. Corrections were made for phosphorus liberation in the absence of homogenate and in the absence of substrate. The level of magnesium ion used in these experiments was purposely set far below the optimum in order to allow for maximum expression of stimulatory and chelation effects.

Results. In these experiments, the reproducibility of results when duplicate determinations were made with the same homogenate was quite satisfactory, a variation of more than ±5% being unusual. However, the activities and stimulatory responses of tissues from different animals were often subject to considerable quantitative variation. For this reason, comparisons of stimulatory activity

* This term is used to identify enzyme(s) catalyzing release of inorganic phosphate from ATP. The exact mechanism involved may be more complex than that of a direct hydrolysis of substrate(4).

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TABLE I. Barbiturate Stimulation of Rat Liver ATPase.

Barbiturate (1×10^{-3} M)	% increase in activity, avg (range)
Barbital	15 (8- 22)
Phenobarbital	31 (23- 33)
Pentobarbital	44 (33- 59)
Thiamylal	90 (77-100)
Thiopental	155 (116-195)

among various compounds are drawn from experiments in which all of the compounds were tested with the same homogenate, and the results are most conveniently expressed as the per cent increase in phosphorus liberated over the control homogenate. The average values and ranges given represent experiments with at least 3 animals for each compound listed.

Barbiturate stimulation of liver ATPase. As shown in Table I, there were definite variations in potency among the barbiturates tested. While this observation was of interest in itself, it was felt that it would be more appropriate to examine effects of barbiturates on brain tissue reactions. However, it was not possible to influence the ATPase activity of brain homogenates by addition of high levels of barbiturates, chloramphenicol, chlortetracycline, or 2,4-dinitrophenol. The same negative results were obtained with homogenates prepared from intestinal muscle, spleen, and bone marrow.

Stimulation of brain ATPase. The report of Chappell and Perry(7) that pigeon muscle mitochondria isolated in the presence of ethylenediamine-tetraacetic acid (EDTA) exhibited a lowered ATPase activity which could be stimulated by dinitrophenol led to the trial of this technic with brain homogenates. The results, summarized in Table II, clearly indicated that homogenization of the brain in the presence of 1 x 10^{-3} M EDTA resulted in a lower ATPase level which could then be increased by stimulating agents which had been found effective with liver ATPase. The one exception among the compounds tested was oxytetracycline, which was apparently still inert in the brain system. It was also determined that it was not necessary to add the EDTA previous to homogenization. Similar results were obtained when 1 x 10^{-4} M EDTA was added after homogenization in sucrose. This latter procedure was followed as a matter of convenience in many subsequent experiments.

Effect of other chelating agents. The following compounds had little or no effect on the initial brain ATPase level, nor did their presence bring about significant stimulation by 1 x 10^{-3} M pentobarbital, 1 x 10^{-4} M dinitrophenol, or 1 x 10^{-4} M chlortetracycline: 1 x 10^{-3} M citric acid, carboxymethylmercaptosuccinic acid, acetylacetone, ammoniadiacetic acid, N,N-bis(2-hydroxyethyl)glycine, N,N-bis(carboxymethylethanolamine, and 8-hydroxyquinoline. Ammoniatriacetic acid, 1 x 10^{-3} M, and 1,2-diaminocyclohexane-N,N'-tetraacetic acid, 1 x 10^{-4} M, gave results which were comparable to those obtained with EDTA.

Other substrates. Under the conditions used in these experiments with brain homogenates, there was no measurable production of orthophosphate from pyrophosphate or adenylic acid. ADP was degraded about half as rapidly as ATP, and stimulatory effects of dinitrophenol, chlortetracycline, and pentobarbital were of the same order of magnitude (Table III). UTP degradation by liver homogenates was exceedingly sensitive to all these stimulators, while only chlortetracycline produced a marked increase in the activity of the brain-EDTA homogenates.

Discussion. The 3 chelating agents which were effective in reducing ATPase activity and allowing subsequent stimulation are not-

TABLE II. Stimulation of Rat Brain ATPase.*

Agent	Concentration (M)	% increase in activity, avg (range)
Barbital	1×10^{-3}	15 (7- 20)
Phenobarbital	"	26 (25- 27)
Thiamylal	"	50 (40- 60)
Amytal	"	76 (62- 93)
Pentobarbital	"	91 (86-100)
1,3-Dimethyl-butyl-ethyl barbiturate	"	79 (73- 86)
2,4-Dinitrophenol	1×10^{-4}	93 (67-133)
Chlortetracycline	"	104 (75-133)
Chloramphenicol	1.55×10^{-3}	59 (58- 60)

* Homogenates prepared in presence of 1×10^{-3} M EDTA. Control homogenates produced avg of 15 γ P (13-19) as compared to 33 γ P (28-43) for homogenates prepared in absence of EDTA.

TABLE III. Stimulation of Adenosinediphosphate and Uridinetriphosphate Degradation in Rat Brain and Liver Homogenates.

Substrate	Tissue*	Stimulator (M)		γ P liberated: Stimulator Absent	γ P liberated: Stimulator Present
Adenosinediphosphate	Brain	2,4-Dinitrophenol,	1×10^{-4}	8	15
		Chlortetracycline,	"	8	18
		Pentobarbital,	1×10^{-3}	8	14
Uridinetriphosphate	"	2,4-Dinitrophenol,	1×10^{-4}	9	10
		Chlortetracycline,	"	9	18
		Pentobarbital,	1×10^{-3}	9	11
	Liver	2,4-Dinitrophenol,	1×10^{-4}	6	41
		Chlortetracycline,	"	6	25
		Pentobarbital,	5×10^{-4}	6	15

* Brain homogenates made in presence of 10^{-3} M EDTA; liver homogenates in 0.25 M sucrose only.

able for their enormous affinity for magnesium ion: log K^a values(8) are 7.0 for ammoniatriacetic acid, 8.69 for EDTA, and 10.3 for 1,2-diaminocyclohexane-N,N'-tetraacetic acid. The possibility was considered that the stimulating agents might act by a modification of chelate stability. No indication for such an effect of chloramphenicol or 2,4-dinitrophenol on the EDTA-Mg^{++} chelate could be seen in titration studies patterned after those of Schwarzenbach and Ackermann(9), nor could any evidence be obtained in this way for the formation of Mg^{++} complexes by the various stimulating agents employed. Obviously such negative results are inconclusive and a different approach to the problem may be more rewarding.

Speculation as to the possible significance of ATPase stimulation by barbiturates is restrained by the finding that the convulsive compound(10), 1,3-dimethyl-butyl-ethyl-barbiturate, was also active in this respect, as it is in the uncoupling of oxidation and phosphorylation(1). The greatest value to be derived from these observations at the present time is an awareness of the fact that various compounds may stimulate the breakdown of ATP (and ADP and UTP) under certain conditions and by so doing may influence the course of other reactions dependent on a supply or balance of high-energy phosphate compounds.

Summary. 1. The ATPase activity of rat brain homogenates prepared in 0.25 M sucrose was not affected by 1 x 10^{-4} M 2,4-dinitrophenol, 1 x 10^{-4} M chlortetracycline, 1.55 x 10^{-3} M chloramphenicol, or 1 x 10^{-3} M levels of various barbiturates, in contrast to the effects of these compounds on liver homogenates prepared in the same way. 2. If EDTA was added either before or after homogenization, so that the final concentration in the reaction mixture was 1 x 10^{-4} M, the ATPase activity of the brain homogenates was considerably reduced and susceptible to stimulation by the above compounds.

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