STIMULATION OF CHOLINE INCORPORATION INTO A MEMBRANE FRACTION OF RAT LIVER BY BARBITALS IN VITRO

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SUMMARY A cytoplasmic post-mitochondrial extract (Sephadex-IP) from rat liver was active in incorporating [^{14}C]methyl labeled choline into acid insoluble membrane material. The result of aralysis of labeled phospholipid showed that most of the radioactivity was in lecithin. When Sephadex-PM was pre-incubated with hexobarbital, the choline incorporating activity was stimulated. Phenobarbital, but not 3-methylcholanthrene, also stimulated the incorporation. And the degree of the stimulation was dependent on the concentration of the drugs.

INTRODUCTION

It is well known that drugs such as phenobarbital and 3-methylcholanthrene, when injected into a rat, induce drug metabolizing enzymes of the endoplasmic reticulum (ER) of the liver and proliferation of membranes of ER (1). At the present time, our knowledge is still incomplete as to the mechanisms of the induction of enzymes and synthesis of membranes. The earliest event detectable thus far is a binding of drug to ER (2), possibly to cytochrome P450 in membranes (3,4), observed a few minutes after administration of the drug. The next event that can be detected is increased synthesis of phospholipids of membranes, as demonstrated by the rapid incorporation of [^{32}P]-orthophosphate (4). This step does not seem to require the actual synthesis of new enzymes or messenger RNA (5).

Accepting the fluid mosaic model of the membrane proposed by Singer and Nicolson (6), increased phospholipid synthesis could be envisioned as a spatial redistribution of membrane components caused by attachment of a drug, resulting in an

increased activity of phospholipid-synthesizing enzymes associated with membranes of ER (7).

If this were the case, increased incorporation of a precursor to phospholipid might be expected to be observed *in vitro* by addition of the drug to the membrane fraction. In the present communication, we report stimulation of incorporation of [^{14}C]methyl labeled choline into membranes by incubating the cytoplasmic extract with hexobarbital and phenobarbital *in vitro*.

MATERIALS AND METHODS

The cytoplasmic extract (referred to as Sephadex-PM) was prepared from a rat liver by filtering the post-mitochondrial fraction through Sephadex G-25 as described previously (8). The incubation system was a modification of that reported by McMurray and Dawson (9). It consisted of 50mM HEPES*-KOH, pH 7.6, 70mM KCl, 5mM MgCl2 and 4mM DTT** ("buffer and salt") and [^{14}C]methyl choline (New England Nuclear, 0.1mCi/0.47mg), 20mM Na-pyruvate, 1.3mM Na-malate, 1.7mM ATP (Sigma), 0.25mM CTP (Sigma), 0.1mM CoA (Sigma) and 1.7mM CMP (Sigma) ("the choline incorporating system") and about 1.5mg protein of Sephadex-PM with or without hexobarbital. In some experiments, "the choline incorporating system" was added after pre-incubation of Sephadex-PM in "buffer and salt" with or without hexobarbital for 1-2hr. Stimulatory effect of other drugs, phenobarbital and 3-methylcholanthrene, was also examined in some experiments. The final volume was 0.25ml and the incubation was carried out at 37°C. After the incubation for various time periods, an aliquot was removed and trichloroacetic acid insoluble material was collected on a glass fibre disc (GF/C, Whatman) and counted by a scintillation counter. In some cases, lipid was extracted by the method of Bligh and Dyer (10) and analysis of phospholipids was carried out by thin layer chromatography, followed by radioautography of the chromatogram. The solvent system consisted of chloroform, methanol and acetic acid (130:50:20).

RESULTS AND DISCUSSION

In Fig. 1, a time course of incorporation of [^{14}C]methyl choline into trichloroacetic acid insoluble material is presented. The rate of incorporation accelerated for 30min, and then attained a linearity. Using the same kind of the reaction system, Soto et al (11) proved that choline was incorporated

*HEPES; N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid,
**DTT; dithiothreitol

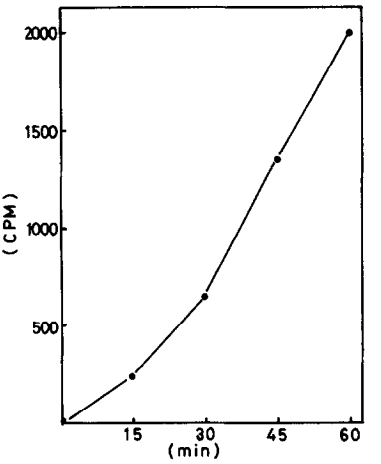

Fig. 1. A time course of incorporation of [^{14}C]choline into acid insoluble fraction by the cytoplasmic extract.

1.5mg protein of Sephadex-FM was incubated in the complete reaction mixture of 0.25ml at 37°C. At each time after the incubation, 50µl of the reaction mixture was removed and trichloroacetic acid insoluble radioactivity was determined.

not by an exchange of base but by de novo synthesis of phosphatidyl choline, by showing that the amount of radioactive choline once incorporated was not reduced during further incubation with cold choline. De novo synthesis of phosphatidyl choline was confirmed in the present experiment by showing that the incorporation was inhibited in the presence of $CaCl_2$ (Table I). It is known that the enzyme which catalyzes formation of lecithin from CDP-choline and diglyceride is extremely sensitive to calcium ions (12). Table I also shows that "the choline incorporating system" (pyruvate, malate, ATP, CTP, CoA and CMP) was indispensable for the incorporation. Thin layer chromatographic analysis of lipid after the incubation proved that practically choline was incorporated only into lecithin.

In the next experiment, the effect of hexobarbital was examined. As it was expected that the postulated hexobarbital modification of the structure of membranes might require a finite period of time, "the choline incorporating system" was

Table I Basic characterization of [^{14}C]choline incorporating system in vitro.

1. Complete	100% (577cpm)
2. Complete - "the choline incorporating system"	8%
3. Complete + $CaCl_2$ (3mM)	7%

1.5mg protein of Sephadex-PM was incubated with or without "the choline incorporating system" or $CaCl_2$ for 30min. 50µl of each reaction mixture was removed and acid insoluble radioactivity was determined.

Table II Effect of hexobarbital on the incorporation of [^{14}C]choline into acid insoluble fraction.

Pre-incubation	0hr	1hr	2hr
1. -hexobarbital	519cpm	1108cpm	1171cpm
2. +hexobarbital	407cpm	1481cpm	2368cpm

1.5mg protein of Sephadex-PM was pre-incubated for 0, 1 or 2hr without "the choline incorporating system" in the presence or absence of hexobarbital (2mM), and then "the choline incorporating system" was added and incubated for 1hr. 25µl of the reaction mixture was removed and acid insoluble radioactivity was determined. Each figure represents an average of two determinations.

added after incubation of Sephadex-PM with hexobarbital for 0, 1 or 2hr. As shown in Table II, hexobarbital stimulated the incorporation of choline by the cytoplasmic extract, only when both were pre-incubated for 1 or 2hr. The stimulation of choline incorporation during pre-incubation without hexobarbital (the first line of Table II) is a reflection of the accelerating rate of incorporation already noted in Fig. 1.

In Table III, it is shown that phenobarbital had the stimulatory effect on choline incorporation comparable with hexobarbital. It may be expected, however, that under some different conditions, different stimulatory effect could be detected with hexobarbital and phenobarbital, since it is known that hexobarbital is shorter active and more rapidly metabolized

Table III Effect of drugs of different concentrations on the incorporation of [^{14}C]choline into acid insoluble fraction.

	Pre-incubation	% of control
1.		100%
2.	+hexobarbital (3.3mM)	145
3.	+hexobarbital (6.7mM)	161
4.	+hexobarbital (10mM)	184
5.	+phenobarbital (3.3mM)	119
6.	+phenobarbital (6.7mM)	155
7.	+phenobarbital (10mM)	182
8.	+olive oil (2µl)	105
9.	+olive oil (6µl)	98
10.	+olive oil (10µl)	105
11.	+3-MC (2µl)	97
12.	+3-MC (6µl)	99
13.	+3-MC (10µl)	100

Pre-incubation was for 1.5hr and incubation was for 1.5hr. 3-Methylcholanthrene (3-MC) was dissolved in olive oil to the concentration of 25mg/ml. The other experimental conditions were the same as shown in the legend of Table II.

than phenobarbital *in vivo*. When the concentration of hexobarbital and phenobarbital was increased, greater stimulation was observed as shown in Table III. This might be related to the observation that the phenobarbital-induced increase of the microsomal contents of phospholipids was, within certain limits, proportional to the amount of phenobarbital injected (13). Table III also shows that neither olive oil nor 3-methylcholanthrene dissolved in olive oil had any effect on incorporating activity. It is not clear, however, whether the failure of 3-methylcholanthrene to stimulate choline incorporation in this system was due to low solubility of this drug in aqueous solution and slow interaction with membranes.

The degree of stimulation of choline incorporation by drugs was not constant with different preparations of Sephadex-PM. And it was observed that previous treatment of rats with drugs *in vivo* enhanced the stimulation of the incorporation by drugs

in vitro (unpublished data). The analysis of the distribution of [^{14}C]choline incorporated into membranes between rough and smooth ER in the reaction mixture pre-incubated with or without hexobarbital showed that hexobarbital stimulated the incorporating activity of both rough and smooth ER to the same extent (unpublished data).

Although the mechanisms of the stimulation of [^{14}C]choline incorporation by pre-incubation with hexobarbital and phenobarbital should be clarified by further investigation, the data presented in this paper may give us clues to the mechanisms of drug-induced membrane proliferation and drug metabolizing enzyme systems.

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