Influence of Mitochondria on Phospholipid Synthesis in Preparations from Rat Liver

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1. The addition of mitochondria to an incubation system containing the soluble and microsomal fractions of rat liver enhances severalfold the incorporation of each of ethanolamine, phosphorylethanolamine and CDP-ethanolamine into phosphatidylethanolamine. 2. In the presence of microsomal, mitochondrial and soluble fractions, CDP-ethanolamine exhibits the greatest initial rate of incorporation (approx. 6 nmol/h per mg of protein), being slightly faster than that of phosphorylethanolamine (approx. 5 nmol/h per mg of protein). Incorporation of ethanolamine proceeds very slowly for the first 20 min and only after 30 min gives rates approaching those of the other two precursors. 3. By using a substrate 'dilution' technique it was shown that in the reconstituted system the affinity of each of the enzymes for their respective substrates is very high: 10 μ M for ethanolamine, 25 μ M for phosphorylethanolamine and 5 μ M for CDP-ethanolamine. 4. Isolation of the mitochondrial and microsomal fractions from the medium after incubation together with phosphorylethanolamine showed that about 70% of the total radioactivity was present in the microsomal fraction and about 30% in the mitochondria after only 20 min. Similar experiments with ethanolamine as precursor revealed that after 20 min only about 15% of the total radioactivity was present in the mitochondria but that after 40 min about 30% was present in this fraction. 5. Heating and phospholipase treatment of mitochondria, but not freeze-thawing, eliminated the stimulatory effect of mitochondria on phospholipid synthesis. 6. The reconstituted system exhibits an absolute requirement for Mg^{2+} (2 mM gave maximal rates) and is inhibited by very low concentrations of Ca^{2+} (100 μ M- Ca^{2+} produced half-maximal inhibition with 3 mM- Mg^{2+}). Further addition of Mg^{2+} overcame the Ca^{2+} inhibition, suggesting that the inhibitory effect is readily reversible. 7. The concept that modification of the Mg^{2+}/Ca^{2+} ratio is a means of controlling the rate of cellular phospholipid synthesis is introduced.

Substantial evidence supports the view that the enzymic machinery responsible for the synthesis of cellular phospholipids *de novo* is located in the microsomal and soluble (supernatant) fractions (Schneider, 1963; Wilgram & Kennedy, 1963; McMurray & Dawson, 1969; Williams & Bygrave, 1970). Despite the apparent absence of enzymes in mitochondria directly involved in phospholipid synthesis *de novo*, data in at least two reports have indicated that these organelles markedly stimulate phospholipid synthesis *in vitro* (McMurray & Dawson, 1969; Williams & Bygrave, 1970). These observations have been extended in the present work, in which the incorporation of ethanolamine, phosphorylethanolamine and of CDP-ethanolamine into phosphatidylethanolamine has been adopted as a measure of phospholipid synthesis *de novo*.

We show here that phospholipid synthesis by the microsomal fraction/supernatant system can be stimulated severalfold by the addition of mitochondria. Some properties of the reconstituted phos-

pholipid-synthesizing system are examined and a concept is introduced which considers a novel means of modifying phospholipid synthesis by using the bivalent-ion-accumulation properties of rat liver mitochondria.

Methods and Materials

Preparation of subcellular fractions from rat liver

Livers were obtained from adult Wistar-strain rats, each of which weighed about 200 g. The rats were provided with a normal diet, fed *ad libitum* and were not starved overnight before their use. After each rat was killed (usually by a blow on the head) the following precautions were taken in the removal of the liver; these precautions helped greatly in minimizing the extent of bacterial contamination in the subcellular fractions obtained. The outer skin of the abdomen was cut, layered back and the inner skin swabbed with ethanol. The inner skin was then cut longitudinally so as to expose the liver. This was removed and placed

in cold 0.25M-sucrose with sterilized scissors and forceps. These latter two steps were usually performed in a Clemco Contamination Control (model BCW-43C) work-station. The liver was washed and then minced into small pieces. Sufficient cold 0.25M-sucrose was added to give a 20% (w/v) mixture which was placed in a glass vessel and homogenized by using about seven up-and-down strokes of a Teflon pestle fitted to an electric motor, driven at 900 rev./min. An equal volume of 0.25M-sucrose was finally added to give a 10% (w/v) suspension. Fractionation of the suspension was carried out by the following procedure, which is essentially that previously described by Schneider (1948). The suspension was centrifuged at 800g for 10 min and the pellet discarded. The supernatant was centrifuged at 10000g for 8 min and the crude mitochondrial pellet obtained. This pellet was resuspended in about 80 ml of 0.25M-sucrose and centrifuged at 6000g for 6 min, followed immediately by 8000g for 2 min. This step was repeated to give the mitochondrial pellet. This procedure helps considerably to lessen microsomal contamination. The supernatant was then centrifuged in a Spinco model L preparative centrifuge with rotor no. 40 at 144000g for 30 min. The pellet obtained was designated the 'microsomal fraction' and the supernatant the 'soluble fraction'. Both the mitochondrial and microsomal fractions were resuspended in 0.25M-sucrose. The presence of bacteria in all subcellular fractions was checked as a routine; the number of bacteria present per reaction mixture usually did not exceed 1000.

Marker-enzyme assays

Glucose 6-phosphatase and cytochrome oxidase activities were used to determine the extent to which the microsomal and mitochondrial fractions were contaminated one with the other. Glucose 6-phosphatase was measured by the method of Swanson (1950), with a 20 min period of incubation. The cell fractions were usually about 2 h old when the assays were performed. The P_i liberated was determined by a modification of the method of Allen (1940). Cytochrome oxidase was measured by following the oxidation of reduced cytochrome *c* at 550 nm in a Unicam SP.800 recording spectrophotometer. The reaction volume contained 100 mM-potassium phosphate buffer (pH 7.4) and 0.066 mM-reduced cytochrome *c*. The final volume was 2.0 ml. KBH_4 was used to reduce the cytochrome *c*, care being taken to adjust the pH of the solution finally to 7.4. A molar extinction coefficient of $19.6 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ was used to calculate the activity of the enzyme.

Measurement of these enzyme activities in the two fractions in a number of separate experiments revealed that the mitochondrial fractions usually contained about 5% of microsomal fraction and that the

microsomal fractions were practically devoid of mitochondria.

Incubation conditions

Incorporation of radioactive ethanolamine, phosphorylethanolamine or CDP-ethanolamine into total phospholipid was used as a measure of phospholipid synthesis *in vitro*. The basic incubation medium contained 10 mM-Hepes [2-(*N*-2-hydroxyethyl)piperazin-*N'*-yl]ethanesulphonic acid] buffer (pH 7.4), 3 mM- MgCl_2 , 0.5 mM-CTP, 0.5 mM-ATP, $0.2 \mu\text{Ci}$ of $[2-^{14}\text{C}]$ ethanolamine (sp. radioactivity 30 mCi/ μmol), $0.2 \mu\text{Ci}$ of phosphoryl $[2-^{14}\text{C}]$ ethanolamine (sp. radioactivity 15 mCi/mmol), together with approx. 1–3 mg of cell-fraction protein. Protein was determined by a modified biuret method with bovine albumin (fraction V) as standard. The amount of non-radioactive precursor added and the relative proportions of microsomal fraction, mitochondria and supernatant present are indicated in the legends to Figures and in the Results section. The total osmolarity of the system was about 0.1, most of this being contributed to by the addition of sucrose. The final volume was 1.0 ml and incubations were usually for 30 min at 30°C. In most experiments the reaction was initiated by the addition of the combined cell fractions and terminated by the addition of 0.2 ml of 10% (w/v) trichloroacetic acid containing 2% (w/v) ethanolamine. These suspensions were then left overnight at 0–4°C. The sediment was thoroughly washed five times in the trichloroacetic acid–ethanolamine mixture with vigorous agitation to remove unincorporated radioactive precursor. Unless otherwise indicated, the washed sediment was collected on a glass-fibre disc which was placed in a glass scintillation vial and dried; 10 ml of scintillation fluid (Meli & Bygrave, 1972) was added and the contents were counted for radioactivity in a Beckman LS-100 liquid-scintillation counter.

Phospholipid analyses

The following procedure was adopted when the phospholipids were analysed. Chloroform–methanol (1:1, v/v) was added to the sediment obtained after washing out the non-incorporated radioactive ethanolamine. The mixture was vigorously agitated and then centrifuged at 12000g for 2 min to sediment the phospholipid-free protein pellet. The supernatant was collected. A further volume of chloroform–methanol was added to the protein pellet and the procedure repeated twice. The combined chloroform–methanol extracts were evaporated to dryness *in vacuo* at room temperature, taken up in a minimal volume of chloroform–methanol and chromatographed on instant-t.l.c. paper (Gelman ITLC; Gelman Instrument Co., Ann Arbor, Mich., U.S.A.) with a propan-2-ol–aq. NH_3 (sp.gr. 0.88) (100:9, v/v) solvent system. The phospholipids were detected by

I₂ vapour and further characterized by alkaline hydrolysis and paper chromatography as described by Dawson (1960).

All of the radioactivity located in the trichloroacetic acid-insoluble material was recovered in the phospholipid fraction, the bulk being in phosphatidylethanolamine.

Presentation of results

Each experiment was performed at least twice and usually three times. Variation between the results of triplicate experiments was usually 5–15% and often about 10%. Apart from experimental error these variations could also reflect changes in the endogenous concentration of the bivalent-metal ions and/or precursor pool size in the subcellular fractions.

Materials

All reagents used were of Analytic Reagent grade. Fine chemicals and enzymes were obtained from C. F. Boehringer und Soehne (G.m.b.H.), Mannheim, Germany. [2-¹⁴C]Ethanolamine hydrochloride was

obtained from The Radiochemical Centre, Amersham, Bucks., U.K. [1,2-¹⁴C]Phosphorylethanolamine was obtained from New England Nuclear Corp., Boston, Mass., U.S.A. CDP-[2-¹⁴C]ethanolamine was prepared from [2-¹⁴C]ethanolamine by the method of Kennedy (1956), by using the modifications outlined by Schneider *et al.* (1966).

Results

Reconstitution of phospholipid synthesis by rat liver-cell fractions

Data in Fig. 1 were collated from experiments in which the relative concentrations of supernatant, microsomal and mitochondrial fractions obtained from rat liver were varied with respect to one another and the ability to incorporate ethanolamine into total phospholipid was determined. Maximal incorporation *in vitro* takes place only when all three cell components are present together. When the concentrations of supernatant and of microsomal fraction are varied in the presence of fixed amounts of the mitochondria plus microsomal fraction and mitochondria

Fig. 1. Effect of varying the concentration of mitochondria, microsomal fraction and supernatant on phospholipid synthesis in rat liver extracts

Each incubation vessel contained 10mM-Hepes buffer (pH 7.4), 80mM-sucrose, 3mM-MgCl₂, 0.5mM-CTP, 0.5mM-ATP, in a final volume of 1.0ml. Ethanolamine (0.2 μ Ci) was the precursor. In Fig. 1(a), 0.47mg of mitochondria and 0.43mg of microsomal fraction were present, with various amounts of supernatant as indicated; in Fig. 1(b) 0.47mg of mitochondria and 0.89mg of supernatant were present, with various amounts of microsomal fraction as indicated; in Fig. 1(c) 0.43mg of microsomal fraction and 0.89mg of supernatant were present with various amounts of mitochondria as indicated. Incubations were for 30min at 30°C. Phospholipid analyses were carried out as described in the Methods and Materials section. □, Mitochondria; ○, microsomal fraction; △, supernatant; ▲, mitochondria plus microsomal fraction; ■, mitochondria plus supernatant; ●, microsomal fraction plus supernatant.

plus supernatant respectively, a sharp decline in the incorporation always occurs after maximal rates of incorporation have been attained. However, with mitochondria such a decline in activity beyond the optimum concentration is not observed. These data indicate that there is a critical ratio necessary between all three cell fractions to obtain optimum phospholipid synthesis *in vitro*. In the experiments reported below the concentrations of microsomal fraction, supernatant and mitochondria used in the reconstituted subcellular system were 0.43, 0.89 and 0.47 mg of protein respectively per 1.0 ml of final volume.

Time-course of incorporation of ethanolamine, phosphorylethanolamine and CDP-ethanolamine

The rates of incorporation of ethanolamine, phosphorylethanolamine and of CDP-ethanolamine by the reconstituted system from rat liver are shown in Fig. 2. The precursor incorporated most rapidly is CDP-ethanolamine, followed by phosphorylethanolamine; each is incorporated at a linear rate over the 55 min incubation period. However, ethanolamine incorporation always proceeds slowly at first and does not attain maximal rate until an incubation time of about 20–30 min has elapsed. Only then does the rate of incorporation approach that of the other two precursors. In other experiments, the results of which are not presented, it was established that the incorporation of all three precursors could be enhanced equally some 5–8-fold on the addition of the mitochondrial fraction. It can be calculated that in this reconstituted system the rate of phospholipid synthesis can approach values of 6–8 nmol/h per mg of cell protein.

Affinity of individual enzymes for their respective substrates in the reconstituted system

Information about the affinity of ethanolamine kinase for ethanolamine, ethanolamine cytidyltransferase for phosphorylethanolamine and ethanolamine phosphotransferase for CDP-ethanolamine in the reconstituted system was obtained as follows. In the first experiment, using radioactive ethanolamine, the concentrations of each of the three non-radioactive substrates were varied in the range 0–50 μ M. In the second and third experiments, the concentrations of non-radioactive ethanolamine, phosphorylethanolamine and CDP-ethanolamine were again varied in the range 0–50 μ M but with a fixed amount of radioactive phosphorylethanolamine and CDP-ethanolamine respectively.

From the 'dilution' data obtained in these experiments ' K_s ' values were determined (Table 1). The values obtained indicate that each of the three enzymes in the incorporating system have very high and similar affinities for their respective substrates. The

values shown were obtained in several identical experiments and lie within the concentration ranges known to exist in the liver cell (W. Bartley, personal communication). They are lower than those reported elsewhere from studies on the individual enzyme reactions in brain fractions (Porcellati *et al.*, 1970a,b) and developing liver (Weinhold & Rethy, 1972). The validity of this dilution method for determining the affinities is shown by the observation that ethanolamine had little effect on the incorporation of phosphorylethanolamine and of CDP-ethanolamine. Similarly, ethanolamine and phosphorylethanolamine had little effect on the incorporation of CDP-ethanolamine. These data also indicate that each of the three substrates influence only their respective enzymes.

Fig. 2. Time-course of incorporation of phospholipid precursors by reconstituted system from rat liver

The reaction mixture was as described in Fig. 1 except that the labelled precursor (including 50 μ M-non-radioactive precursor) was present as indicated. Mitochondria (0.47 mg), microsomal fraction (0.43 mg) and supernatant (0.89 mg) were present as indicated. Incubations were carried out at 30°C. ●, Ethanolamine; ○, phosphorylethanolamine; ▲, CDP-ethanolamine.

Table 1. Influence of added ethanolamine, phosphorylethanolamine and CDP-ethanolamine on incorporation of radioactive ethanolamine, phosphorylethanolamine and CDP-ethanolamine respectively, into phospholipid by reconstituted system from rat liver

Experiments were carried out in which radioactive ethanolamine, phosphorylethanolamine and CDP-ethanolamine were independently incubated with various amounts of non-radioactive substrate. Double-reciprocal plots of the data were constructed and the affinity of the substrates for each of the three enzyme steps of the incorporation pathway was determined. ' K_s ' refers to the concentration of non-radioactive substrate that produces 50% 'dilution' of the precursor incorporated.

Labelled precursor present	Non-radioactive substrate varied	K_s (μM)
Ethanolamine	Ethanolamine	10
	Phosphorylethanolamine	16
	CDP-ethanolamine	12
Phosphorylethanolamine	Ethanolamine	∞
	Phosphorylethanolamine	25
	CDP-ethanolamine	5
CDP-ethanolamine	Ethanolamine	∞
	Phosphorylethanolamine	∞
	CDP-ethanolamine	5

Involvement of the base-exchange reaction

Since ethanolamine can be incorporated into phosphatidylethanolamine by two mechanisms, i.e. Ca^{2+} -stimulated base exchange and synthesis *de novo* (see Dawson, 1966), control experiments were carried out in the absence of exogenous Ca^{2+} to find the relative contribution of each in the present study. It was found that non-radioactive CDP-ethanolamine completely diluted the radioactivity in phosphatidylethanolamine when phosphoryl[^{14}C]ethanolamine and CDP-[^{14}C]ethanolamine were used as precursors. However, dilution of [^{14}C]ethanolamine incorporation by non-radioactive CDP-ethanolamine displayed the characteristics of a two-component incorporating system, in which 90% of the total activity was sensitive to CDP-ethanolamine and the remaining 10% was insensitive to CDP-ethanolamine. The insensitive portion is most likely attributable to the action of low concentrations of endogenous Ca^{2+} that are presumably sufficient to activate the microsomal exchange mechanism, yet not large enough to inhibit significantly the Mg^{2+} -dependent synthesizing system *de novo* (see Fig. 4). By using this finding in conjunction with relevant data from Fig. 2 it can be calculated that only 0.25 nmol of ethanolamine is exchanged over a 30 min incubation period, this being the standard incubation time for most experiments including the above controls. The significance of this exchange component is further diminished on the establishment of steady-state equilibria. This is most readily seen when examined in the light of the time-course for CDP-[^{14}C]ethanolamine incorporation in which the 30 min incorporation value shows that

7.5 nmol has been converted into product, of which only 3% could be considered exchangeable were the precursor [^{14}C]ethanolamine and not CDP-[^{14}C]ethanolamine (Fig. 2).

Distribution of newly synthesized phospholipids in rat liver fractions

Having shown that maximum rates of phospholipid synthesis occur *in vitro* only when mitochondria are also present in the incubation mixture together with the microsomal and soluble fractions, we decided to determine the relative distribution of the newly synthesized phospholipid between the microsomal fraction and mitochondria. Data from experiments using ethanolamine and phosphorylethanolamine as precursors are presented in Fig. 3.

First, these data confirm points established earlier in this work, namely that incorporation of ethanolamine but not of phosphorylethanolamine exhibits a lag phase and that maximum incorporation of each precursor occurs only when mitochondria are also present. Secondly, resolution of the incubation system into the microsomal and mitochondrial fractions at the indicated times revealed that most of the radioactivity is associated with the microsomal fraction.

When ethanolamine is the precursor (Fig. 3a) only about 15% of the total radioactivity is associated with the mitochondria after 20 min of incubation. After 40 and 60 min of incubation about 25 and 30% respectively of the total radioactivity is associated with the mitochondria. Of interest is the finding that although the rate of incorporation of ethanolamine by the

microsomal fraction incubated only with the soluble fraction increases at a linear rate, that into the microsomal fraction incubated with both the soluble fraction and mitochondria increases exponentially (cf. Fig. 2).

When phosphorylethanolamine is used as precursor essentially similar data are obtained as with ethanolamine except that the rates of incorporation are linear with time (cf. Fig. 2). After 20, 40 and 60 min of incubation, approx. 30% of the total radioactivity is associated with the mitochondrial fraction. Again the amount of radioactivity associated with the microsomal fraction in the tripartite system is considerably greater than that associated with the microsomal fraction after incubation with only the soluble fraction.

Efforts to determine the means by which mitochondria induce a stimulation of phospholipid synthesis were made. The following inhibitors of protein synthesis were added and produced no effect: chloramphenicol, actinomycin D, puromycin and erythromycin. Dinitrophenol, an uncoupler of oxidative phosphorylation, likewise had no effect on the stimulation.

The following treatments of mitochondria caused the inhibitions indicated in parentheses: heating for 5 min at 100°C (100%), freezing and thawing three times (20%), treatment with each of phospholipases A, C and D (100%).

Effect of Mg^{2+} and Ca^{2+} on incorporation of phosphorylethanolamine by the reconstituted system

It is known that the individual enzymes involved in the biosynthesis of phosphatidylethanolamine exhibit little activity in the absence of Mg^{2+} (Kennedy & Weiss, 1956; Porcellati *et al.*, 1970a,b) and that Ca^{2+} inhibits some of these reactions. The influence of these ions on phospholipid synthesis in the reconstituted system was examined. Two points are brought out in data from three separate experiments. In the first experiment (Fig. 4a) the concentration of Mg^{2+} was varied in the range 0–8 mM. Little incorporation of phosphorylethanolamine occurs below 1 mM- Mg^{2+} . Above this concentration, however, there is a dramatic increase in incorporation, which is maximal around 2 mM- Mg^{2+} . Higher concentrations do not inhibit incorporation. In the second experiment the

Fig. 3. Distribution between mitochondria and microsomal fraction of newly synthesized phospholipid

The reaction mixture was exactly as described in Fig. 1 except that the volume was scaled up 5-fold. Ethanolamine and phosphorylethanolamine were the precursors in Figs. 3(a) and 3(b) respectively. At the times indicated a sample of the mixture that had been incubated at 30°C, was placed in cold 0.25 M-sucrose and centrifuged at 10000g for 10 min to sediment the mitochondria. The supernatant from this step was further centrifuged at 140000g for 30 min to sediment the microsomal fraction. Portions of each of these fractions were then analysed for radioactivity, protein content, glucose 6-phosphatase and cytochrome oxidase activity. The mitochondrial fraction obtained after re-isolation was found to be contaminated by microsomal fraction only to about 12% and the microsomal fraction by mitochondria to only about 5%. ●, Mitochondrial fraction obtained after incubation with mitochondria plus soluble fraction; ○, microsomal fraction obtained after incubation with soluble fraction only; □, mitochondria obtained after incubation with microsomal plus soluble fraction.

Fig. 4. Influence of Ca^{2+} and Mg^{2+} concentration on incorporation of phosphorylethanolamine by reconstituted system from rat liver

The reaction mixture was as described in Fig. 2 except that radioactive phosphorylethanolamine ($0.2 \mu\text{Ci}$) was present without added non-radioactive precursor. In Fig. 4(a) Mg^{2+} was varied as indicated without added Ca^{2+} ; Ca^{2+} was varied as indicated with 3 mM-Mg^{2+} present. In Fig. 4(b) Mg^{2+} was varied with and without $200 \mu\text{M-Ca}^{2+}$ present. Incubations were for 30 min at 30°C . (a): $\bullet$, Ca^{2+} ; $\circ$, Mg^{2+} ; (b): $\bullet$, $200 \mu\text{M-Ca}^{2+}$ present; $\circ$, Ca^{2+} absent.

concentration of Ca^{2+} was varied over the range $0\text{--}0.5 \text{ mM}$ in the presence of 3 mM-Mg^{2+} . The incorporation is clearly very sensitive to quite low amounts of added Ca^{2+} , with half-maximum and maximum inhibition at about $100 \mu\text{M-}$ and $200 \mu\text{M-Ca}^{2+}$ respectively.

In a third experiment (Fig. 4b) the ability of excess of Mg^{2+} to overcome the inhibition by Ca^{2+} was tested. The data show that the relative amounts of Mg^{2+} and Ca^{2+} present in the incubation medium (i.e. the $\text{Mg}^{2+}/\text{Ca}^{2+}$ ratio) can influence greatly the phospholipid-synthesizing capacity of the reconstituted system. Phospholipid synthesis is maximum when this ratio is large and minimum when it is small.

In summary, only very low concentrations of Ca^{2+} are needed to inhibit phospholipid synthesis in this system, and this inhibition can be overcome by increasing the Mg^{2+} concentration.

Discussion

The work presented in this paper has established that the addition of mitochondria to a system containing microsomal and supernatant fractions produces a considerable stimulation of cellular phospholipid synthesis *de novo* as measured by the incorporation of ethanolamine, phosphorylethanolamine and of CDP-ethanolamine into phosphatidylethanolamine. The incorporation of the latter two of the three precursors into membrane phospholipid will occur only via the '*de novo*' pathway described by Kennedy

(1961), whereas that of ethanolamine can occur as well via base-exchange reactions (see Dawson, 1966). That the incorporation of this precursor occurred mainly via the '*de novo*' pathway is indicated by the very considerable extent to which its incorporation was 'diluted' by added phosphorylethanolamine and CDP-ethanolamine (Table 1).

In other studies (J. B. Roberts & F. L. Bygrave, unpublished work) involving the incorporation of [^{14}C]ethanolamine into phosphatidylethanolamine by subcellular fractions of liver in the absence of added supernatant fraction, it was observed that the ethanolamine exchange was maximum at 8 mM-Ca^{2+} with a K_m of approx. 2 mM-Ca^{2+} . The 30 min incorporation value at 8 mM-Ca^{2+} was 0.5 nmol of ethanolamine exchanged for an equal amount of microsomal protein to that used in the present study. These data would further preclude any significant contribution to the net synthesis of phosphatidylethanolamine by the microsomal exchange mechanism in the reconstituted system. It is of note that the basic composition of the media employed in experiments on both exchange and synthesis *de novo* were essentially the same and that incubations were performed for 30 min at 30°C accordingly.

As pointed out in the introduction, McMurray & Dawson (1969) and ourselves (Williams & Bygrave, 1970) have observed a marked stimulation of $^{32}\text{P}_i$ incorporation into phospholipid on the addition of mitochondria to microsomal fraction and supernatant. Data from experiments designed to clarify

the means by which mitochondria stimulate cellular phospholipid synthesis in our study were inconclusive. The stimulation by mitochondria was eliminated by either heat or phospholipase treatment and was relatively resistant to freezing and thawing, suggesting the possible involvement of an enzyme(s) present in the mitochondria.

In those experiments in which stimulation of $^{32}\text{P}_i$ incorporation occurred in the presence of mitochondria (McMurray & Dawson, 1969; Williams & Bygrave, 1970), it can be argued that the role of mitochondria is simply to generate $[\text{P}]_{\text{ATP}}$ via oxidative phosphorylation (McMurray & Magee, 1972). However, in the present study the situation is somewhat different, as ATP was always added to the incubation system. Also the presence of dinitrophenol did not prevent the stimulation by mitochondria, further implying the non-involvement of any mechanism of oxidative phosphorylation. It is possible that stimulation by mitochondria of phospholipid synthesis occurs because these organelles allow for continuous enzymic formation of phosphatidate, which is known to occur in the outer membrane (Shephard & Hübscher, 1969). The product is an obligatory substrate in the final step of phosphatidylethanolamine biosynthesis from ethanolamine. Addition of 1,2-diglyceride to our incubation system, on the other hand, failed to stimulate incorporation of the three precursors. The possibility should be considered that mitochondria stimulate phospholipid synthesis *de novo* by providing acceptor sites for the newly formed phospholipid (see Fig. 3). The shape of the top curve in Fig. 1(c) would provide some support for this notion.

Kinetic data obtained in this study with the reconstituted system (Table 1) indicate that each of the enzymes involved in the incorporation of ethanolamine into phosphatidylethanolamine possess approximately equal apparent affinities for their respective substrates. These affinities were very high and lie well within the concentration range known to be present in the liver cell (W. Bartley, personal communication). They are considerably higher than those values obtained from studies on the individual enzymes (cf. Porcellati *et al.*, 1970a,b; Weinhold & Rethy, 1972). These differences may well be attributed to the presence of the mitochondria in the incubation system.

Despite the fact that in the reconstituted system each of the enzymes has an equal apparent affinity for its respective substrate (Table 1), we found in numerous experiments that the first step in the pathway, i.e. that involving ethanolamine kinase, was always rate-limiting in that steady-state kinetics were not attained until sufficient ethanolamine had been phosphorylated to enable net synthesis of phosphatidylethanolamine to proceed in a linear fashion (see, for example, Fig. 2). This finding is of interest in the

light of reports (see Porcellati *et al.*, 1970a,b) that the rate-limiting step in phospholipid synthesis is linked to the provision of diglyceride. Their studies, however, did not include an examination of the generation of phosphorylethanolamine from ethanolamine. In control experiments we had established that ATP was present in saturating quantities.

For some years we have advocated that Mg^{2+} and (in particular) Ca^{2+} play a central role in the regulation of cellular metabolism (Bygrave, 1964, 1966a,b, 1967; Meli & Bygrave, 1972; Spencer & Bygrave, 1972, 1973). The present work on phospholipid synthesis, which highlights both the absolute requirements of this system for Mg^{2+} and the high susceptibility to inhibition by Ca^{2+} (Fig. 4), reveals yet another vital cellular process whose activity is potentially conducive to ionic control. The following observations pertain to such a control of phospholipid synthesis in liver. First, the concentrations of Mg^{2+} and of Ca^{2+} which influence phospholipid synthesis are within the concentration range known to exist in the liver cell (see Thiers & Vallee, 1957). Second, liver mitochondria, as we have shown, are necessary for maximal phospholipid synthesis, rapidly accumulate Ca^{2+} but not Mg^{2+} (see Lehninger *et al.*, 1967) and so are able to modify the $\text{Mg}^{2+}/\text{Ca}^{2+}$ ratio in the cytoplasm where the enzymes involved in the synthesis are located (see McMurray & Magee, 1972). Third, the feasibility of mitochondria in modifying the activity of ion-sensitive cytoplasmic enzymes has already been demonstrated (Meli & Bygrave, 1972).

Thus we envisage that maintenance of a relatively high $\text{Mg}^{2+}/\text{Ca}^{2+}$ ratio in the cytoplasm by appropriate metabolic conditions will allow for high rates of cellular phospholipid synthesis. Conversely, metabolic circumstances which produce relatively low $\text{Mg}^{2+}/\text{Ca}^{2+}$ ratios in the cytoplasm will allow only low rates of phospholipid synthesis to occur. Such an interplay between the availability of Ca^{2+} and the extent to which Ca^{2+} -sensitive enzyme activities occur is obviously complex. However, we see this regulatory mechanism as being vital to the overall cellular metabolic network.

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