Choline metabolism and membrane formation in rat hepatoma cells grown in suspension culture. III. Choline transport and uptake by simple diffusion and lack of direct exchange with phosphatidylcholine

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ABSTRACT The initial rate of incorporation of methyl-labeled choline into the acid-soluble pool (phosphorylcholine) of Novikoff hepatoma cells growing in suspension culture was investigated as a function of the choline concentration in the medium. Below, but not above, 20 μM, choline incorporation followed simple Michaelis-Menten kinetics at 24, 33, or 37°C with an apparent K_m of 4–7 μM, and the V_{max} values decreased with a Q_{10} of about 2.3 with a decrease in temperature. Between 20 and 500 μM, on the other hand, the rate of incorporation increased linearly with an increase in choline concentration in the medium, and the increase in incorporation rate with increase in choline concentration was about the same at all temperatures tested. The data suggest that at low concentrations choline is taken up mainly by a transport reaction, whereas at concentrations above 20 μM, simple diffusion becomes the principal mode of uptake. The energy of activation for choline transport was estimated from an Arrhenius plot of the V_{max} values as 67,000 J (16 kcal)/mole.

At concentrations below 20 μM, choline incorporation into membrane phosphatidylcholine also followed simple Michaelis-Menten kinetics, and the apparent K_m was about the same as that for choline transport. The data support the conclusion that the transport of choline into the cell is the rate-limiting step in the conversion of choline to phosphorylcholine and its incorporation into phosphatidylcholine. At concentrations above 100 μM, on the other hand, the ultimate rate of choline incorporation into phosphatidylcholine was independent of the choline concentration in the medium or the intracellular level of phosphorylcholine. Further, the rate of turnover of the choline moiety of phosphatidylcholine (half-life, 20–24 hr) either in whole cells or during incubation of isolated membrane fractions was unaffected by the presence of an excess of choline in the medium. The overall results indicate that a direct exchange between free choline and the choline moiety of phosphatidylcholine does not play a significant role in the incorporation of choline into phosphatidylcholine by Novikoff cells or in the turnover of the choline moiety of phosphatidylcholine, and that labeled choline therefore is a useful precursor in studying the synthesis and turnover of membrane phosphatidylcholine in these cells.

SUPPLEMENTARY KEY WORDS choline uptake · phosphatidylcholine synthesis and turnover · Novikoff rat hepatoma cells · Michaelis-Menten kinetics

CHOLINE is rapidly taken up by Novikoff rat hepatoma cells growing in suspension culture and accumulates in the cells as phosphorylcholine (1, 2). Results from pulse–chase experiments indicated that the accumulated phosphorylcholine then serves as pre-

Articles I and II in this series are Refs. 1 and 4, respectively.

Abbreviations: CDP, cytidine diphosphate; BM42, basal medium 42; PNF, postnuclear fraction; MMF, mitochondrial–microsomal fraction.

cursor in the synthesis of membrane phosphatidylcholine, probably via CDP choline ("Kennedy pathway" [3]), but CDP choline does not accumulate in the cells in detectable amounts (1). The results indicated that choline is a specific label for membrane phosphatidylcholine of Novikoff cells and a useful precursor for studies on the biosynthesis of cellular membranes. We have used radioactive choline to study the synthesis of phosphatidylcholine and its incorporation into various cellular membrane structures in uninfected (1, 4) and mengovirus-infected Novikoff rat hepatoma cells (5) and to measure the turnover of the choline moiety of phosphatidylcholine in these cells. Recent experiments with rat liver (6) and a soil amoeba (7), however, indicate that choline may enter cellular phosphatidylcholine by a direct base exchange. This conclusion was based on the finding that upon injection of rats with inorganic phosphate-^{32}P and choline-^{3}H, the $^{3}H/^{32}P$ ratio of phosphatidylcholine was significantly higher than that of phosphorylcholine (6). The authors estimated that choline entered phosphatidylcholine four times more rapidly by direct exchange than via the "Kennedy pathway." Choline is also incorporated into phosphatidylcholine by isolated liver mitochrondia and microsomes by a Ca^{2+}-activated, energy-independent reaction (8). Further, the studies with the amoeba showed that any labeled choline incorporated into phosphatidylcholine is rapidly displaced by incubating either whole cells or cell-free membrane fractions in medium containing unlabeled choline (7). The rate of displacement depends on the concentration of unlabeled choline in the medium.

It is clear that a direct choline exchange would complicate measurements of the biosynthesis and turnover of phosphatidylcholine using radioactive choline and the interpretation of results from the types of experiments we have conducted previously (1, 4, 5). We have, therefore, reinvestigated the mode of uptake of choline and its incorporation into membrane phosphatidylcholine by Novikoff cells and measured the turnover of phosphatidylcholine in the presence of various concentrations of choline in the medium both in vivo and in vitro. We found no evidence that extracellular choline enters the phosphatidylcholine of whole Novikoff cells at a significant rate by direct base exchange or that an exchange system is present in cell-free preparations. At low concentrations in the medium, choline is taken up by the cells by a transport reaction, but at higher concentrations, simple diffusion becomes the primary mode by which choline enters the cell. Choline is rapidly phosphorylated inside the cell and then used as precursor for the synthesis of phosphatidylcholine. The choline moiety of phosphatidylcholine turns over at a constant rate in whole cells or in cell-free membrane fractions regardless of the choline concentration in the medium.

MATERIALS AND METHODS

Materials

Methyl-^{3}H-labeled choline was purchased from Amersham/Searle Corp., Des Plaines, Ill. Buffer 2 (B2) was composed of 10 mM Tris–HCl (pH 7.4), 10 mM NaCl, and 1.5 mM $MgCl_2$; Buffer 14 (B14) was 0.25 M sucrose, 0.1 M phosphate buffer (pH 7.4), and 1 mM $CaCl_2$. Buffer 4 (B4) had the same composition as B2 except that it also contained 1 mM $CaCl_2$ and 1 mM triethanolamine.

Cell Culture

Novikoff rat hepatoma cells (subline NlSl-67) were propagated in suspension culture in Swim's medium 67, and the suspensions were monitored for cell concentration by tabulation in a Coulter Counter as described previously (9, 10). For studies of choline incorporation by whole cells, the cells were harvested from cultures in the late exponential phase, between 2×10^6 and 2.8×10^6 cells/ml, and they were suspended in choline-free BM42 (9) to the original cell density.

Incorporation of Choline

Methyl-^{3}H-labeled choline was added to suspensions of cells as indicated in the appropriate experiments. Replicate samples of suspension were analyzed for radioactivity in total cell material or in acid-insoluble material as described previously (1, 2). Briefly, samples of suspension were centrifuged and the cells were washed once in basal salt solution then analyzed for radioactivity (radioactivity in total cell material). Other samples were quickly frozen in a bath of solid CO_2 in ethanol, then later thawed and mixed with perchloric acid. The precipitates were washed repeatedly with perchloric and trichloroacetic acid and then analyzed for radioactivity (radioactivity in acid-insoluble material).

Extraction and Analysis of Acid-soluble Material and Lipids

Perchloric acid extracts were prepared from choline-labeled cells, and the lipids were extracted from the residue as described previously (1, 2). The acid extracts were analyzed by ascending paper chromatography (2), and the lipids were separated by thin-layer chromatography (1).

Cell Fractionations

Cells were collected by centrifugation from exponential phase cultures and suspended to $1.9–2.3 \times 10^7$ cells/

ml in B2 or B4. After 10 min at 0°C, the cells were disrupted by 10 strokes with a Dounce homogenizer. The lysate was centrifuged at 600 *g* for 5 min and the pellet was suspended in B2 or B4 to the original volume of suspension (nuclear fraction). Where indicated, a sample of the supernatant fluid (PNF) was centrifuged at 104,000 *g* for 1 hr and the pellet was suspended in B2 or B4 to the volume of PNF centrifuged (MMF). In some instances the PNF was first centrifuged at 10,000 *g* for 15 min and the supernatant fluid was centrifuged at 104,000 *g* for 1 hr. The pellets were suspended in B2 or B4 to the volume of PNF centrifuged (mitochondrial fraction and microsomal fraction, respectively). The final supernatant fluid was referred to as cell sap.

In an alternate procedure the cells were fractionated in a sucrose–phosphate–$CaCl_2$ solution (7, 8). In this procedure the cells were suspended in water and disrupted by five strokes with a Dounce homogenizer after 10 min at 0°C. Immediately thereafter, the suspension was supplemented with 2 M sucrose, 0.5 M phosphate buffer (pH 7.4), and 1 M $CaCl_2$ to final concentrations of 0.25 M, 0.1 M, and 1 mM, respectively. After another 10 min at 0°C, the suspension was again homogenized with 10 strokes. Further fractionation was conducted as described above, except that the sedimented membrane fractions were suspended in B14 rather than B2 or B4.

RESULTS

Transport and Simple Diffusion of Choline

The rate-limiting step in the formation of intracellular phosphorylcholine from extracellular choline by NISl-67 cells is the transport of choline into the cell (2). The cells, at least in the exponential phase of growth (4), possess an excess of choline kinase (2). Thus, any choline taken up by the cells is immediately trapped inside the cell by being phosphorylated. The rate of uptake of choline into the cell, therefore, can be estimated by determining the rate of incorporation of radioactive choline into the acid-soluble pool (2). Radioactivity in phosphorylcholine, although not removed by washing the cells with an isotonic salt solution, is solubilized by extraction of the cells with perchloric acid, whereas the phosphatidylcholine remains in the acid-insoluble fraction (1). Previous studies (1) showed that after labeling cells with 1–10 μM choline-^{3}H in the medium, significant amounts of radioactivity appear in phosphatidylcholine only after a delay of 30–60 min (see Fig. 4*B*). Thus, during the first 30–60 min of exposure to labeled choline, most of the incorporated label is present in phosphorylcholine (1). In praxis we have estimated the rate of choline uptake by the cells by measuring the rate of choline incorporation into total cell material (2), on the assumption that all choline incorporated into phosphatidylcholine must have passed through the acid-soluble pool. Studies on the effect of choline concentration on the initial rate of choline incorporation into total cell material (2) indicated that cultured NISl-67 cells, like tissue cells or slices taken directly from the animal (11–15), take up choline by a transport reaction with a K_m between 4 and 7 μM choline (see Figs. 1*B* and 2*B*). The transport system seemed to be saturated at 10–20 μM choline in the medium (1, 2). However, further studies have revealed that increasing the choline concentration in the medium above 50 μM resulted in further increases in the initial rate of choline incorporation into total cell material (Fig. 1*A*). The rate of choline incorporation increased linearly with the increase in choline concentration. The results, therefore, suggest that although choline is taken up mainly by a transport reaction at concentrations below 10–20 μM, at higher concentrations it enters the cell at a significant rate by simple diffusion. This conclusion is supported by the data in Fig. 2 which illustrate the effect of temperature on the initial rate of choline incorporation as a function of choline concentration. At concentrations below 20 μM, the rates of incorporation were markedly and progressively lowered with a decrease in temperature (Fig. 2*A*), though incorporation followed normal Michaelis-Menten kinetics at all temperatures down to at least 24°C (Fig. 2*B*). The finding that the apparent K_m values were approximately the same at 24, 33, and 37°C (4 μM), combined with the fact that the K_m of the choline kinase from NISl-67 cells is more than two orders of magnitude higher (700 μM, see Ref. 2) than that for choline incorporation by whole cells, suggests that over the temperature range tested, the rate of choline incorporation into the acid-soluble pool was a measure of the transport reaction. The apparent V_{max} for choline transport decreased from 0.52 nmole/10^6 cells/10 min at 37°C to 0.17 nmole/10^6 cells/10 min at 24°C with an average Q_{10} of 2.3. When the V_{max} values for choline transport were analyzed in an Arrhenius plot, a straight line was obtained (Fig. 3) from which an activation energy of 67,000 J (16 kcal)/mole was estimated.

In contrast to the marked effect of temperature on choline transport, the increase in the rate of choline incorporation with increase in choline concentration above 50 μM was little affected by changes in temperature (Fig. 2*A*). This finding supports the conclusion that this increase in the rate of choline uptake reflects simple diffusion. The initial rates of choline incorporation at 16°C, even below 20 μM, did not yield a straight line in a Lineweaver-Burk plot (not shown). Thus, the residual incorporation at 16°C was probably largely due to simple diffusion (see Fig. 2*A*). Simple diffusion was also

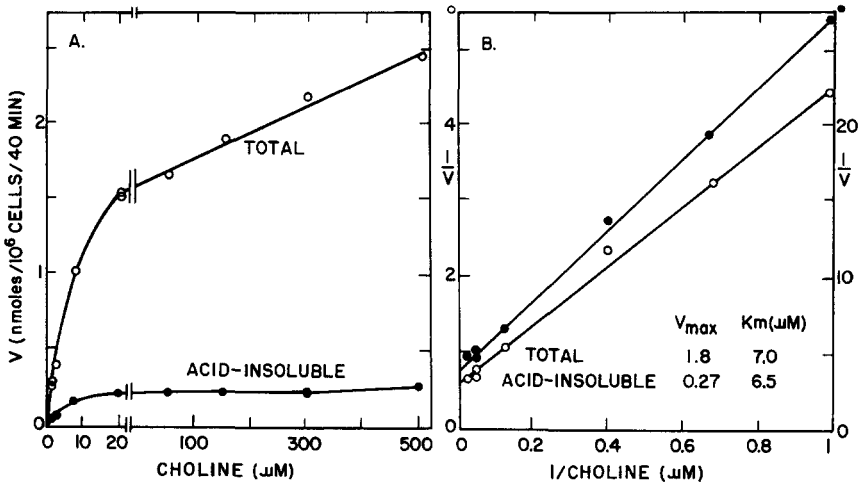

FIG. 1. Rate of incorporation of choline into total cell material and acid-insoluble material by N1S1-67 cells as a function of the choline concentration in the medium. (*A*) Samples of a suspension of 2 × 10^6 cells/ml of BM42 were supplemented with 1.0, 1.5, 2.5, 7.5, or 20 μM choline-^{3}H (60 cpm/pmole) or with 20, 50, 150, 300, or 500 μM choline-^{3}H (3.4 cpm/pmole) and incubated on a gyratory shaker at 37°C. At 5, 10, and 40 min, duplicate 1-ml samples of each suspension were analyzed for radioactivity in total cell material (○—○). Initial rates of choline incorporation into the acid-soluble pool were estimated from plots of amount of ^{3}H in total cell material as a function of time of incubation. Incorporation was linear with time for at least 40 min (see Fig. 4*A*). At 40 min, other duplicate 1-ml samples were analyzed for ^{3}H in acid-insoluble material (●—●). These values were used to estimate an "apparent rate" of choline incorporation into phosphatidylcholine (see text). All points represent averages of the duplicate samples. (*B*) Lineweaver-Burk plots of the values from *A* in the range of 1–50 μM choline.

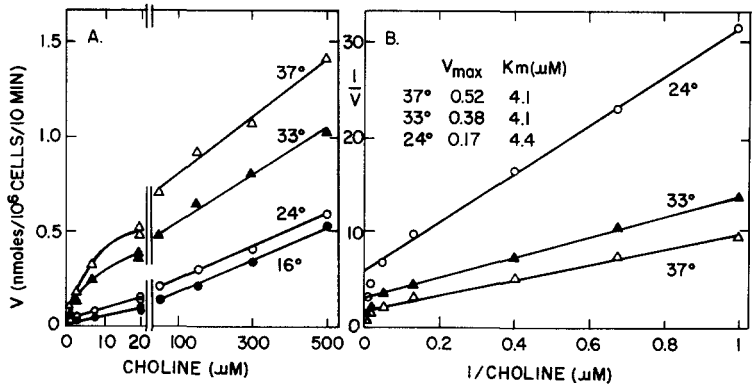

FIG. 2. Effect of temperature on the incorporation of choline into the acid-soluble pool. Samples of an exponential phase culture at 2.5 × 10^6 cells/ml were centrifuged, and the cells were suspended to 2 × 10^6 cells/ml in BM42 equilibrated to the indicated temperatures. After 2 min of incubation at the appropriate temperatures, 10-ml samples of each suspension were supplemented with 1.0, 1.5, 2.5, 7.5, or 20 μM choline-^{3}H (60 cpm/pmole) or 20, 50, 150, 300, or 500 μM choline-^{3}H (3.4 cpm/pmole) and incubated in reciprocal water bath shakers at the indicated temperatures. After 5 and 10 min, duplicate 1-ml samples of each suspension were analyzed for radioactivity in total cell material. The initial rates of incorporation (*V*) in frame *A* were estimated from these values and analyzed in Lineweaver-Burk plots in frame *B*. For technical reasons the experiment was conducted in four parts, one temperature at a time, but the entire experiment, except for the radioactivity analyses, was completed within 1.5 hr.

responsible for the divergence from linearity in the Lineweaver-Burk plots of the values obtained at choline concentrations above 20 μM (Fig. 2*B*).

Chromatographic analyses of acid-soluble extracts from cells after 40 min of labeling with choline showed that over 95% of the label was associated with phosphorylcholine regardless of the choline concentration in the medium (1–500 μM) or the temperature at which the cells were labeled (16, 24, 33, or 37°C). The remainder of the label was in residual choline not completely removed by the washing procedure (1). These results, coupled with the finding that the rate of choline incorporation increased linearly with an increase in choline concentration at all temperatures (Fig. 2*A*),

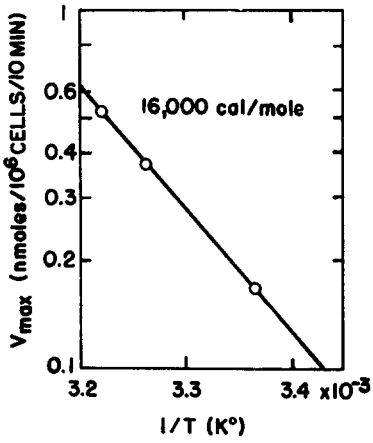

FIG. 3. Arrhenius plot of choline transport. The V_{max} values for the transport of choline used in the plot were estimated from the Lineweaver-Burk plots in Fig. 2*B*. The energy of activation for choline transport was 67,000 J (16 kcal)/mole.

indicate that the cells possessed sufficient choline kinase to rapidly phosphorylate all choline that entered the cells and thereby trap it, and that the choline kinase was active at the lower temperatures. It was shown previously (2) that the apparent V_{max} for the phosphorylation of choline by cell-free extracts (per 10^6 cells) is about 30 times larger than that for choline incorporation by whole cells.

Choline Incorporation into Phosphatidylcholine as a Function of Choline Concentration

The normal intracellular pool of phosphorylcholine fluctuates greatly during the growth cycle of NIS1-67 cells in medium 67, which contains approximately 0.06 mM choline (4). It increases rapidly to about 12–16 nmoles/10^6 cells early in the exponential phase, but then decreases progressively to 1–2 nmoles/10^6 cells in the stationary phase, because the rate of conversion of choline to phosphorylcholine decreases progressively during the latter part of the growth cycle. The reason for this decrease has not been established. The rate of phosphatidylcholine synthesis decreases much less during the growth cycle than the rate of choline incorporation into the acid-soluble pool (4).

When harvested during the late exponential phase of growth, cells contain 2–4 nmoles of phosphorylcholine/10^6 cells (4). Based on a total cell volume of 2 μl/10^6 cells (16), this amount is equivalent to an intracellular concentration of 1–2 mM phosphorylcholine. Thus, the 1.5–2 hr delay before choline incorporation into acid-insoluble material (phosphatidylcholine) attains a constant rate when labeling is conducted at low concentrations of choline in the medium (1) (see Fig. 4*B*) probably reflects the time period required for the equilibration of extracellular choline with the intracellular phosphorylcholine pool (see also below).

We estimated an "apparent initial rate" of choline incorporation into phosphatidylcholine from the amount of choline incorporated into acid-insoluble material after 40 min of incubation with labeled choline (Fig. 1*A*). The apparent rates increased with an increase in choline concentration from 1–20 μM, and when analyzed in a Lineweaver-Burk plot (Fig. 1*B*), a straight line was obtained from which a K_m of 6.5 μM was estimated. The finding that this K_m is similar to that for choline transport suggests that at low concentrations in the medium, the incorporation of extracellular choline into phosphatidylcholine is limited by the rate at which the choline is transported into the cell and thus enters the phosphorylcholine pool. This is also indicated by the fact that competitive inhibition of choline transport by phenethyl alcohol (2) results in a proportional decrease in the incorporation of choline into phosphatidylcholine (17).

A change in concentration from 50 to 300 μM, on the other hand, had little effect on the time course of choline incorporation into phosphatidylcholine (see Fig. 4*B*), and the apparent initial rate of incorporation was therefore constant (Fig. 1*B*), probably because the rate of choline uptake increased too little over this concentration range (Fig. 1*A*) to cause a significant decrease in the time required for choline equilibration with the phosphorylcholine pool. More rapid equilibration, however, occurred at concentrations above 300 μM. The data in Fig. 4*B* show that the lag period required at low choline concentrations to reach a constant rate of incorporation of label into phosphatidylcholine was markedly shortened when the medium was supplemented with 1 mM choline, and it was almost completely abolished when 5 mM choline was added. At the latter concentrations, labeled choline diffused so rapidly into the cells and was phosphorylated (4 nmoles/10^6 cells/10 min; Fig. 4*A*) that the equilibration with unlabeled intracellular phosphorylcholine (about 3 nmoles/10^6 cells) was very rapid. It can be estimated that after 30 min of labeling, at least 80% of the intracellular phosphorylcholine was derived from choline in the medium. It should be noted from the data in Fig. 4*B*, however, that the ultimate rate of incorporation of label into phosphatidylcholine was approximately the same (0.75–0.9 nmole/10^6 cells/hr) whether the medium was supplemented with 0.1, 1, or 5 mM choline, in spite of the fact that the phosphorylcholine pool of the cells increased markedly upon incubation with the higher concentrations of choline. After 6 hr of incubation with 0.1 mM choline, the cells contained 8.5 nmoles/10^6 cells of phosphorylcholine (4.25 mM) which had been derived from choline in the medium (difference between values in Fig. 4*A* and *B*). After 6 hr of incubation with 5 mM choline, the cells contained 20 nmoles/10^6 cells, or a concentration of

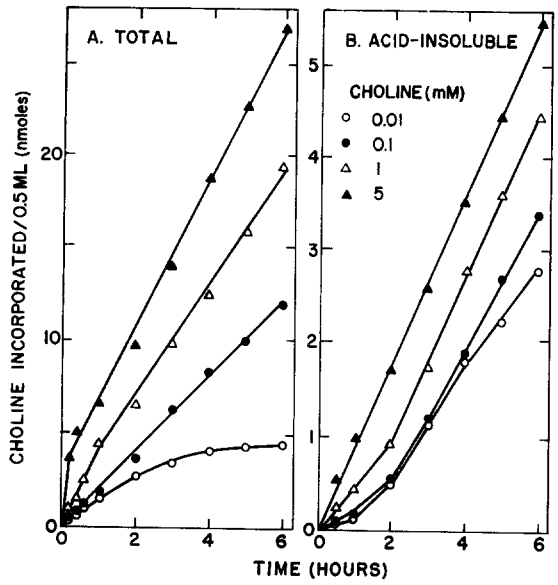

FIG. 4. Time courses of choline incorporation into total cell material (*A*) or acid-insoluble material (*B*) at various concentrations of choline in the medium. Samples of a suspension of 2×10^6 cells/ml of BM42 were supplemented with 1 μCi of choline-^{3}H/ml and unlabeled choline to the indicated concentrations. At various times of incubation at 37°C, duplicate 0.5-ml samples of suspension were analyzed for radioactivity in total cell or acid-insoluble material. Points represent averages of the duplicate samples.

10 mM of newly synthesized phosphorylcholine, which represents a 5–10-fold increase in the pool size from the original 1–2 mM. Even when labeling with 0.01 mM choline, the rate of incorporation into acid-insoluble material approached at 2–3 hr the maximum rate observed with 1 or 5 mM, but the rate then decreased (Fig. 4*B*) because the cells had exhausted the choline in the medium (Fig. 4*A*).

The overall results suggest that choline is incorporated into phosphatidylcholine by NIH-3T3 cells solely via phosphorylcholine, and that a direct base exchange does not play a significant role in this incorporation. This conclusion is supported by other results presented below. Further, the results indicate that the rate of choline incorporation into phosphatidylcholine is a true reflection of the rate of synthesis of phosphatidylcholine, and that the rate of synthesis is highly regulated and not influenced by the concentration of choline in the medium or by the intracellular level of phosphorylcholine. We had reached this conclusion in a previous study (4), since the density of the various cellular membrane fractions as analyzed by isopycnic centrifugation in sucrose density gradients was unaffected by the choline concentration in the medium between 1 and 10 μM, a concentration range which markedly influences the density of mitochondria of choline-requiring mutants of *Neurospora crassa* (18). In view of the finding that the phosphorylcholine pool of the cells can be rapidly expanded due to simple diffusion of choline into the cell at higher concentrations, we reinvestigated the effect of choline concentration on the density of cellular membranes. Cells were grown in the presence of either 0.06 or 5 mM ^{3}H-labeled choline. Then cytoplasmic fractions were prepared and analyzed by isopycnic centrifugation in sucrose density gradients as described previously (1, 4). The radioactivity profiles from both types of cells were very similar; about 50% of the radioactivity was associated with the mitochondrial band. Further, the densities of the mitochondria and the plasma membrane fragments as measured by the distribution of cytochrome oxidase and K^+- and Na^+-activated ATPase activities, respectively, were about the same, whether the medium contained 0.06 or 5 mM choline.

Turnover of Phosphatidylcholine in Vivo and in Vitro

The results in Fig. 5 indicate that the rate of turnover of the choline moiety of phosphatidylcholine in whole cells was unaffected by the concentration of choline in the medium up to 5 mM. Cells were uniformly labeled by growing them for 2 days in medium 67 containing choline-^{3}H. Then the cells were collected by centrifugation, washed with unlabeled medium, and further incubated in choline-free BM42 or BM42 supplemented with various concentrations of unlabeled choline. As

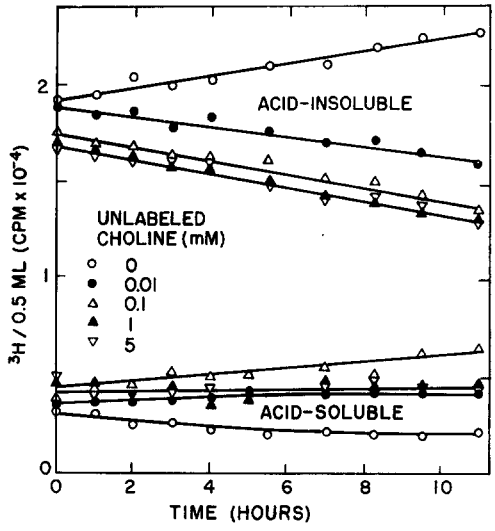

FIG. 5. Turnover of phosphatidylcholine in vivo. Cells were grown for 2 days in medium 67 supplemented with 0.2 μCi of choline-^{3}H (15.6 mCi/μmole)/ml and harvested at 2.1×10^6 cells/ml. The cells were washed once in BM42 and then suspended in choline-free BM42 to 2×10^6 cells/ml. Portions of this suspension were supplemented with the indicated concentrations of unlabeled choline and incubated on a gyratory shaker at 37°C. Duplicate 0.5-ml samples of each suspension were analyzed for radioactivity in total cell or acid-insoluble material. Radioactivity in acid-soluble material was calculated by subtracting the radioactivity in acid-insoluble material from the total radioactivity associated with the cells. All points represent averages of the duplicate samples.

mentioned already, after growing cells for 2 days in medium 67 containing labeled choline, the amount of label in the phosphorylcholine pool is relatively small compared with the amount of label in phosphatidylcholine (4) (see Fig. 5). Upon further incubation of the cells for 11 hr in choline-free BM42, about 50% of the labeled phosphorylcholine was transferred to phosphatidylcholine (Fig. 5). This transfer of radioactivity into phosphatidylcholine was prevented by the presence of unlabeled choline in the medium at a concentration as low as 0.01 mM due to the rapid dilution of labeled phosphorylcholine by newly synthesized unlabeled phosphorylcholine, thus allowing the measurement of the loss of label from phosphatidylcholine. The rate of loss of label from phosphatidylcholine was approximately constant with time and about the same whether the medium contained 0.01, 0.1, 1, or 5 mM unlabeled choline. These data are consistent with the view that a direct choline exchange does not play a significant role in the observed turnover of phosphatidylcholine in whole NlSl-67 cells. The results are quite different from those obtained in similar studies with amoebae (7), in which the concentration of unlabeled choline in the medium markedly influenced the rate of loss of label from phosphatidylcholine.

The pattern of loss of label from phosphatidylcholine of NlSl-67 cells also differed markedly from that observed by Pasternak and Bergeron (19) with neoplastic mast cells that had been uniformly labeled with either choline or inositol. Loss of the labels from these cells followed a biphasic pattern. It was estimated from the data presented by these investigators that the cells lost 30–50% of the labels during the first half of a population doubling period (about 8 hr). The rate of loss decreased progressively during the experimental period until about 60–80% of the label had been lost. The remaining labeled phospholipids seemed to be stable. In contrast, the loss of label from NlSl-67 cells seemed to be linear with time, at least over the first 11 hr of incubation, whether the cells were incubated in basal medium (Fig. 5), in which cell division ceases after 8–12 hr, or in growth medium (4). The loss of label from NlSl-67 cells was also much slower than that from the neoplastic mast cells. Only 20–25% of the label was lost during 11 hr of incubation, which represents close to one population doubling period (12 hr). The reasons for the different results with NlSl-67 and neoplastic mast cells in culture are not clear at present. Assuming that the loss of label from phosphatidylcholine of NlSl-67 cells continued at a constant rate, a half-life of 20–24 hr was estimated for the choline moiety of phosphatidylcholine by extrapolating the lines in Fig. 5. This half-life is somewhat greater than that estimated in a previous study (15 hr) (4).

Although the foregoing results on the effect of choline concentration on its incorporation into phosphatidylcholine and on the turnover of phosphatidylcholine indicate that whole NlSl-67 cells do not incorporate choline into phosphatidylcholine at a significant rate by a direct base exchange, they do not exclude the possibility that such an exchange system is present in the cells. For instance, a direct incorporation of choline into phosphatidylcholine may not be apparent with NlSl-67 cells because all choline entering the cell is rapidly phosphorylated and thus not available for a direct exchange. We have, therefore, determined the effect of unlabeled choline on the turnover of phosphatidylcholine during incubation of cellular membrane fractions in vitro. Membrane fractions from uniformly labeled cells were monitored for radioactivity in acid-insoluble material as a function of time of incubation at 37°C (Fig. 6*A*). The amount of label in the microsomal fraction remained relatively constant, but small amounts of label were lost from both the nuclear and mitochondrial fractions. The rate of loss, however, was the same whether or not the medium was supplemented with unlabeled choline or whether the suspension medium was B4 (Fig. 6*A*) or B14 (not shown), which supports in vitro choline exchange by mitochondrial or microsomal fractions from amoebae (7).

We also tested whether membrane fractions from NlSl-67 cells incorporated ^{3}H-labeled choline into

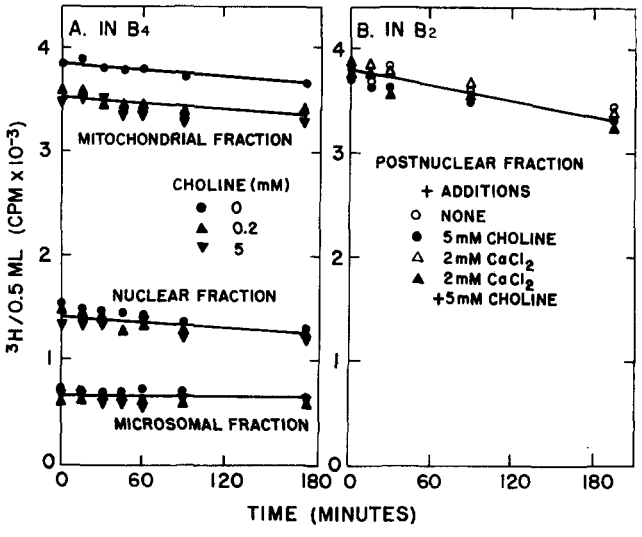

FIG. 6. Turnover of phosphatidylcholine in vitro. Cells were grown for 2 days in medium 67 containing 0.2 μCi of choline-^{3}H (550 μCi/μmole)/ml and harvested at 2.2 × 10^6 cells/ml. The cells were washed once in BM42 and fractionated in B4 (*A*) or B2 (*B*) as described in Materials and Methods. Portions of the cell fractions were supplemented with the indicated concentrations of unlabeled choline or $CaCl_2$, or both, and incubated at 37°C. At various times, duplicate 0.5-ml samples were analyzed for radioactivity in acid-insoluble material. Points represent averages of the duplicate samples.

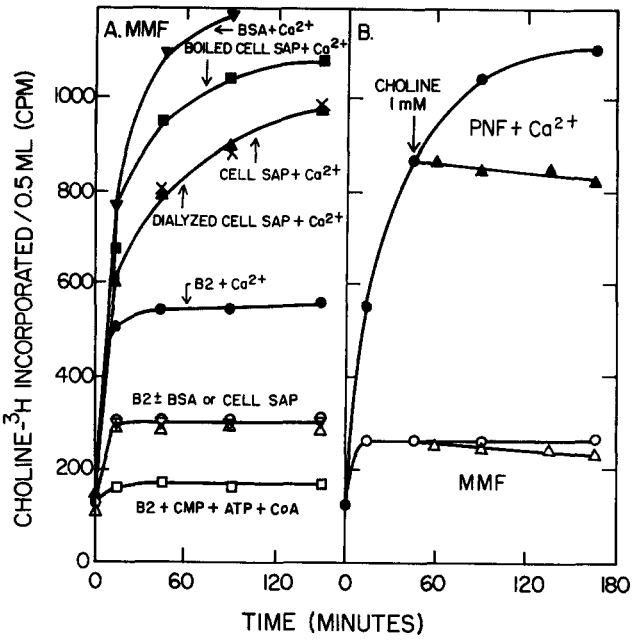

FIG. 7. Incorporation of choline into acid-insoluble material by cell-free fractions. (*A*) Portions of 20 ml of a PNF were centrifuged at 104,000 *g* for 1 hr and the pellets were suspended by homogenization in 20 ml of the following: B2 (○—○, ●—●); cell sap (△—△, ▲—▲); cell sap that had been heated in a boiling water bath for 10 min (■—■); cell sap that had been dialyzed twice against 2 liters of B2 for 1 hr at 4°C (×—×); B2 supplemented with 5 mg of bovine serum albumin (BSA)/ml (▽—▽, ▼—▼); and B2 supplemented with 0.5 mM CMP, 0.7 mM ATP, and 0.15 mM coenzyme A (□—□). One-half of each MMF was supplemented with 2.5 mM $CaCl_2$ (solid symbols and ×—×), and the other half was not (open symbols). Then both parts were supplemented with 4 μM choline-^{3}H (2×10^6 cpm/ml) and incubated at 37°C. At various times, duplicate 0.5-ml samples of each suspension were analyzed for radioactivity in acid-insoluble material. All points represent averages of the duplicate samples. At the end of the experiment, the remainder of each reaction mixture was mixed with an equal volume of 1 N perchloric acid at 0°C. After 10 min, the mixtures were centrifuged and the supernatant fluids (acid-extract) were analyzed chromatographically. The lipids were extracted from the acid-insoluble residue and analyzed by thin-layer chromatography as described in Materials and Methods. (*B*) A PNF in B2 supplemented with 2.5 ml of $CaCl_2$ (●—●) and a MMF in B2 without $CaCl_2$ (○—○) were incubated with choline-^{3}H and monitored for radioactivity in acid-insoluble material as in *A*. After 45 min of incubation, a portion of each suspension was supplemented with 1 mM unlabeled choline and further monitored for radioactivity in acid-insoluble material (▲—▲, △—△).

phosphatidylcholine (Fig. 7*A*). A mitochondrial–microsomal fraction (MMF) in B2 incorporated a small amount of choline into acid-insoluble material (○—○), but the reaction ceased within 10–15 min, when about 0.01% of the total choline in the reaction mixture had been incorporated. Inclusion of 2 mM $CaCl_2$ in the reaction resulted in a doubling of the amount of choline incorporated (●—●). When the mitochondrial–microsomal pellet was suspended in cell sap rather than B2 and supplemented with $CaCl_2$, the reaction proceeded for a longer period of time and the amount incorporated was increased 3–4-fold (▲—▲). In the absence of $CaCl_2$, however, cell sap had no effect on the MMF incorporation (△—△), and cell sap alone did not incorporate choline whether or not $CaCl_2$ was present (not shown). The Ca^{2+}-dependent stimulatory effect of cell sap probably resulted simply from a stabilization of the enzyme systems involved by proteins present in the cell sap, since boiled (■—■) or dialyzed (x—x) cell sap or bovine serum albumin (▼—▼) were at least as active in stimulating choline incorporation as untreated cell sap (Fig. 7*A*). Thin-layer chromatography of lipids extracted from the various reaction mixtures at 150 min showed that the choline was incorporated into phosphatidylcholine, and chromatographic analysis of acid extracts of the reaction mixtures showed that small amounts of choline (about 0.1% of the total) were converted to phosphorylcholine. Thus, the overall results suggest that the incorporation of choline into acid-insoluble material by the MMF represented de novo synthesis of phosphatidylcholine, rather than a direct base exchange. This is also indicated by the fact that the incorporated label was not chased out after addition of an excess of unlabeled choline to the reaction mixture, though this immediately stopped any further incorporation of choline (Fig. 7*B*). The presence of cell sap also had no effect on the rate of turnover of phosphatidylcholine that had been labeled in vivo (Fig. 6*B*). The loss of label from a PNF from choline-labeled cells was only slightly higher than that from the mitochondrial fraction (Fig. 6*A*) and was the same whether or not the PNF was supplemented with $CaCl_2$ or an excess of unlabeled choline, or both. The Ca^{2+}-dependent choline incorporation by the MMF was not inhibited by addition of 0.2 mM DL-serine (not shown). Further, the addition of 0.5 mM CMP, 0.7 mM ATP, and 0.15 mM coenzyme A did not stimulate choline incorporation by the MMF in B2 either with or without $CaCl_2$; on the contrary, it depressed the incorporation (Fig. 7*A*). Results similar to those presented in Fig. 7 were obtained with a MMF prepared in B14 rather than B2 (not shown).

DISCUSSION

Although at low concentrations in the medium choline is taken up by NlSl-67 cells mainly by a transport reaction (2), the present results indicate that at concentrations above 50 μM simple diffusion becomes the predominant mode of entry of choline into the cell. Similar observations have been made with various nucleosides (20) and glucose (21). The rate of simple diffusion is about the same for various substances. At a concentration of 0.5 mM in the medium, for instance, NlSl-67 cells take up adenosine or uridine (20), choline (Fig. 1), or glucose (21) by simple diffusion at a rate of approximately 1 nmole/10^6 cells/10 min. The transport reactions

are active at substrate concentrations the cells may encounter in nature, i.e., in serum or other body fluids. Simple diffusion, on the other hand, probably does not play a major role in the uptake of low molecular weight substances under natural conditions, since the rate of simple diffusion becomes significant only at relatively high concentrations. Simple diffusion, however, may complicate studies of the transport systems.

Our studies failed to detect any evidence that a direct exchange between free choline and the choline moiety of phosphatidylcholine, such as that reported for rat liver (6) and amoebae (7), plays a significant role in the incorporation of choline by NlSl-67 cells in culture. Similarly, Pasternak and Bergeron (19) failed to detect a direct choline exchange with phosphatidylcholine in cultured neoplastic mast cells by labeling simultaneously with choline and a second labeled precursor of phospholipids. Choline is a specific precursor for membrane phosphatidylcholine in NlSl-67 cells and seems to be incorporated solely via phosphorylcholine, i.e., via the "Kennedy pathway" (3). This conclusion was previously indicated by results from pulse–chase experiments (1) and has been confirmed by the studies on the effect of choline concentration on the rate of incorporation of choline into phosphatidylcholine (Figs. 1 and 4). At low concentrations in the medium, the rate of choline incorporation into phosphatidylcholine is limited by the rate of formation of phosphorylcholine, which in turn is limited by the rate with which choline is transported into the cell. At concentrations above 20 μM, the ultimate rate of choline incorporation into phosphatidylcholine and the density of the cellular membranes are independent of the choline concentration in the medium or the intracellular level of phosphorylcholine (Fig. 4). Thus, this incorporation rate of choline (0.8 nmole/10^6 cells/hr) probably reflects the absolute rate of synthesis of phosphatidylcholine minus the turnover rate, which proceeds at about 25% of the rate of biosynthesis (Fig. 5).

The failure of various concentrations of choline to have any effect on the rate of turnover of the choline moiety of phosphatidylcholine either in vivo or in vitro also argues against the occurrence of a direct choline exchange. The rate of turnover is slow and its physiological significance is not known. It has been suggested that the Ca^{2+}-stimulated choline exchange observed with isolated rat liver mitochondria or microsomes is catalyzed by a phospholipase C-like enzyme (8). The Ca^{2+}-stimulated incorporation of choline into phosphatidylcholine by MMFs from NlSl-67 cells, however, is probably not due to a direct base exchange but rather represents de novo synthesis, since the incorporated choline is not chased out by an excess of unlabeled choline, as is the choline incorporated by an amoebal cell-free fraction (7). Further, it is not inhibited by DL-serine, whereas the Ca^{2+}-dependent choline exchange by a similar fraction from rat liver is competitively inhibited by D- or L-serine (8). Choline incorporation by the MMF from NlSl-67 cells is probably catalyzed by a mitochondrial system and is dependent on internal cofactors, since the addition of CMP, ATP, and coenzyme A does not stimulate the reaction either with or without Ca^{2+}. Only small amounts of phosphatidylcholine, however, are synthesized and the reaction ceases rapidly unless the system is supplemented with large amounts or protein, which probably acts by protecting one or more components of the system from inactivation. It is not clear whether Ca^{2+} also acts as a stabilizing factor or is a required cofactor for the reaction. In contrast, the de novo synthesis of phosphatidylcholine by rat liver membrane fractions is stimulated by CMP and ATP plus coenzyme A, and is inhibited by Ca^{2+} (8).

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