# Intracellular sites of lipid synthesis and the biogenesis of mitochondria

**Edward A. Dennis* and Eugene P. Kennedy**

Department of Biological Chemistry, Harvard Medical School, Boston, Massachusetts 02115

**Abstract** Experimental data are presented on the intracellular localization in rat liver of three enzymes which are involved in the biosynthesis of phosphatidylethanolamine and diphosphatidylglycerol. These enzymes are phosphatidylserine decarboxylase, CDP-diglyceride-L-α-glycerophosphate phosphatidyl transferase, and phosphatidylethanolamine-L-serine phosphatidyl transferase. It was found that the first two enzymes are primarily mitochondrial while the latter enzyme is primarily microsomal. The intracellular sites for the biosynthesis of phosphatidylcholine, phosphatidylethanolamine, and diphosphatidylglycerol are discussed, and the implications of their sites of biosynthesis on the assembly processes involved in the biogenesis of mitochondria are considered.

**Supplementary key words** assembly · CDP-diglyceride-L-α-glycerophosphate phosphatidyl transferase · diphosphatidylglycerol · exchange enzyme · phosphatidylcholine · phosphatidylethanolamine · phosphatidylethanolamine-L-serine phosphatidyl transferase · phosphatidylserine decarboxylase

THE MOST ABUNDANT PHOSPHOLIPIDS found in rat liver mitochondria are phosphatidylcholine, phosphatidylethanolamine, and diphosphatidylglycerol (cardiolipin) (1). Since these lipids are major components of mitochondrial membranes, the intracellular localization of their sites of biosynthesis is a central problem in efforts to elucidate the assembly processes involved in the biogenesis of mitochondria. In this paper, we present some new experimental data on the intracellular localization in rat liver of three enzymes which catalyze essential steps in the formation of phosphatidylethanolamine and of diphosphatidylglycerol. These enzymes are phosphatidylserine decarboxylase, CDP-diglyceride-L-α-glycerophosphate phosphatidyl transferase, and phosphatidylethanolamine-L-serine phosphatidyl transferase ("exchange enzyme"). The exchange enzyme is found to be almost entirely microsomal, while the other two enzymes are primarily localized in the mitochondrial fraction. The implications of these findings, and of other studies on the intracellular localization of phospholipid biosynthesis, for models of mitochondrial biogenesis are discussed.

* Present address: Department of Chemistry, University of California at San Diego, La Jolla, Calif. 92037.

## MATERIALS AND METHODS

### Subcellular fractionation of rat liver

White male rats were obtained from the Charles River Breeding Laboratories, Inc., North Wilmington, Mass., and were maintained on the high-carbohydrate, fat-free diet of Wooley and Sebrell (2). Rats weighing about 200 g were decapitated by guillotine, and the livers were removed and fractionated according to the procedure of Wilgram and Kennedy (3). The liver was homogenized in 0.25 M sucrose with a Potter-Elvehjem homogenizer fitted with a Teflon pestle; the procedure resulted in the following fractions: nuclei and debris (repeated centrifugations at 600 *g* for 12 min at 0°C), mitochondria (well-washed pellet from centrifugation at 8500 *g* for 12 min at 0°C), intermediate fraction containing mitochondria and microsomes (unwashed pellet from centrifugation at 8500 *g* for 12 min at 0°C), microsomes (pellet from centrifugation at 100,000 *g* for 60 min at 0°C in a Beckman model L ultracentrifuge), and soluble supernatant (supernatant from centrifugation at 100,000 *g* for 60 min at 0°C). The final fractions were taken up in 0.25 M sucrose and frozen rather than dialyzed against buffer as in the procedure of Wilgram and Kennedy (3). Protein was determined by the method of Lowry et al. (4). Two similar fractionations were carried out; 2.7 g of fresh rat liver was used for fractionation 1 and 3.2 g for fractionation 2.



Downloaded from www.jlr.org by guest, on June 19, 2012

### Assay of phosphatidylserine decarboxylase

The phosphatidylserine decarboxylase was assayed by a procedure based on the direct measurement of $^{14}CO_2$ production (5) from dipalmitoyl-L-α-glycerophosphoryl-L-[1-$^{14}$C]serine prepared according to the methods of Baer and Maurukas (6); this procedure is described in detail elsewhere (7). Assays were conducted for 30 min at 37°C in a system of 0.4 ml containing 0.05 M imidazole, pH 7.1, 1 mM EDTA, 2 mg/ml Triton X-100, 0.125 M sucrose, 0.2 mM dipalmitoyl-L-α-glycerophosphoryl-L-[1-$^{14}$C]serine, and protein. Control values observed without added enzyme were subtracted from the values reported herein.

### Assay of phosphatidylethanolamine-L-serine phosphatidyl transferase

The assay for the phosphatidylethanolamine-L-serine phosphatidyl transferase is based on the extraction procedure of Weiss, Smith, and Kennedy (8). [1,2-$^{14}$C]Ethanolamine was used as the labeled substrate in this assay according to a procedure described elsewhere (7). Assays were conducted for 10 min at 37°C in a system of 0.4 ml containing 0.05 M imidazole, pH 7.8, 10 mM $CaCl_2$, 0.125 M sucrose, 0.5 mM [1,2-$^{14}$C]ethanolamine, and protein. "Zero-time" controls were subtracted from the results reported herein.

### Assay of CDP-diglyceride-L-α-glycerophosphate phosphatidyl transferase

The CDP-diglyceride-L-α-glycerophosphate phosphatidyl transferase was assayed by "Method I" of Chang and Kennedy (9), but with Patterson-Greene counting solution (10). L-[2-$^{3}$H]Glycerol-3-phosphate was prepared by the method of Chang and Kennedy (9), and CDP-dipalmitin was prepared by the method of Paulus and Kennedy (11). Assays were conducted for 30 min at 37°C in a system of 0.4 ml containing 0.05 M Tris–HCl, pH 8.0, 0.25 mM CDP-dipalmitin, 0.125 M sucrose, 1 mM L-[2-$^{3}$H]glycerol-3-phosphate, and protein. Control values were observed without added CDP-dipalmitin for each protein sample and were subtracted from the results reported herein.

## RESULTS

Fresh rat liver was separated into the following five fractions according to the procedure of Wilgram and Kennedy (3): *1*) nuclei and debris, *2*) mitochondria, *3*) intermediate fraction, *4*) microsomes, and *5*) soluble supernatant fraction. Assays for phosphatidylserine decarboxylase, phosphatidylethanolamine-L-serine phosphatidyl transferase, and CDP-diglyceride-L-α-glycerophosphate phosphatidyl transferase were then carried out on the whole homogenate and on each fraction.

### Linearity of assay and recovery of activity

When active fractions were assayed using the procedure described above for decarboxylase activity, and for the enzyme catalyzing the synthesis of phosphatidylglycerophosphate, there was a satisfactory linear response of product formed with different amounts of added fraction. With less active fractions, such as the soluble supernatant or microsomes, the response was proportional to the amount of fraction added, but it was not linear. Assays for the exchange enzyme, even with active fractions, gave a proportional but not linear response, probably for reasons discussed elsewhere (7). To avoid these difficulties, two similar fractionations of rat liver were carried out, but they were assayed in somewhat different ways. For fractionation 1, each fraction was tested (in duplicate) at a single level, chosen on the basis of preliminary experiments to yield as nearly as possible the same amounts of product as the other fractions, which were tested in the same assay. The recovery of total activity from the fractions obtained in fractionation 1 is shown in Table 1. For fractionation 2, each fraction was tested at four levels of added fraction, and the activity was calculated from the slopes of the most nearly linear portions of the response curve. Results of the two methods of assay were in good agreement as to the relative activities of the subcellular fractions. Although there may be uncertainty about the precise activity of the least active fractions, there is, fortunately, no ambiguity about the principal site of localization. The specific activities of the fractions obtained in fractionation 2 are presented in Figs. 1–3.

It should be noted that the procedure of Wilgram and Kennedy (3) was designed to bring about an effective separation of mitochondria, microsomes, and supernatant fraction, but it does not lead to a quantitative recovery of mitochondria or of microsomes, since there are large losses in the nuclear–debris and intermediate fractions. Specifically, the nuclear–debris fraction contains a large amount of unbroken cells and mitochondria; this

TABLE 1. Enzymatic activity of subcellular fractions of rat liver for fractionation 1

| Fraction | Protein | Total Activity | | |
|---|---|---|---|---|
| | | Decarboxylase | Exchange | Transferase |
| | *mg* | *nmoles/min* | | |
| Whole homogenate | 459 | 28 | 29 | 24 |
| Nuclei and debris | 103 | 17 | 4.7 | 14 |
| Mitochondria | 30 | 12 | 0.5 | 5.8 |
| Intermediate fraction | 41 | 2.1 | 5.2 | 3.0 |
| Microsomes | 104 | 1.3 | 22 | 2.9 |
| Supernatant | 200 | 0.1 | 0.0 | 0.2 |
| Total activity | 478 | 32.5 | 32.4 | 25.9 |
| Recovery | 104% | 116% | 112% | 108% |



Downloaded from www.jlr.org by guest, on June 19, 2012

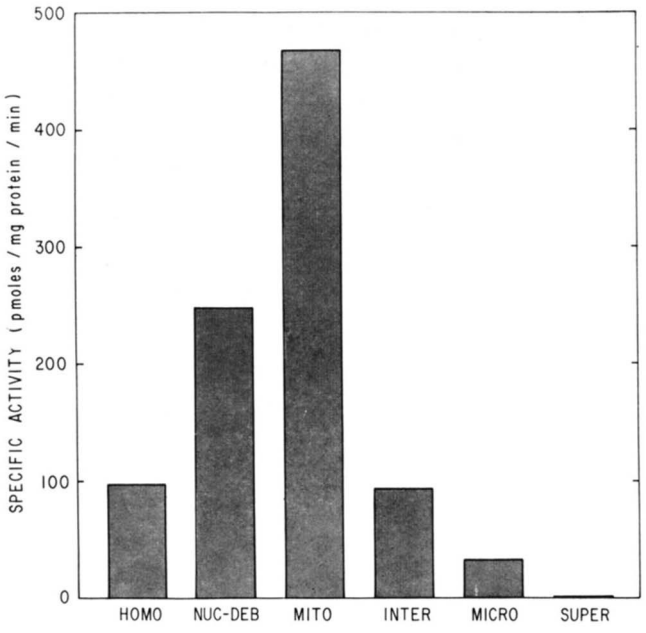


FIG. 1. Phosphatidylserine decarboxylase distribution in subcellular fractions of rat liver prepared as described under Materials and Methods. The fractions are designated as follows: *HOMO*, unfractionated homogenate; *NUC–DEB*, nuclei and debris; *MITO*, mitochondria; *INTER*, intermediate fraction, described in the text; *MICRO*, microsomes; *SUPER*, soluble supernatant fraction.

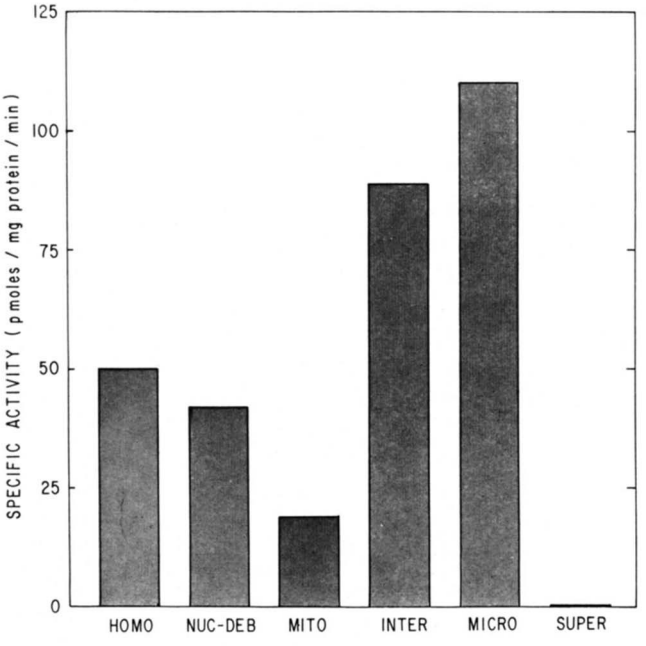


FIG. 2. Phosphatidylethanolamine-L-serine phosphatidyl transferase. The arrangement of the figure and the notation of the fractions are the same as in Fig. 1.

results in a relatively high total activity (Table 1) and specific activity (Figs. 1 and 3) for the nuclear–debris fraction, particularly for the decarboxylase and the transferase which are mitochondrial. Similarly, the intermediate fraction contains a large amount of microsomes which results in a high total activity (Table 1) and specific activity (Fig. 2) for the exchange enzyme, which is microsomal. For this reason, the data on the specific activity of each enzyme (Figs. 1–3) in the mitochondrial and microsomal fractions are more meaningful. Since the mitochondrial fraction has very little "exchange enzyme" while the microsomes have very little decarboxylase, it is clear that the separation of the two fractions was indeed effective.

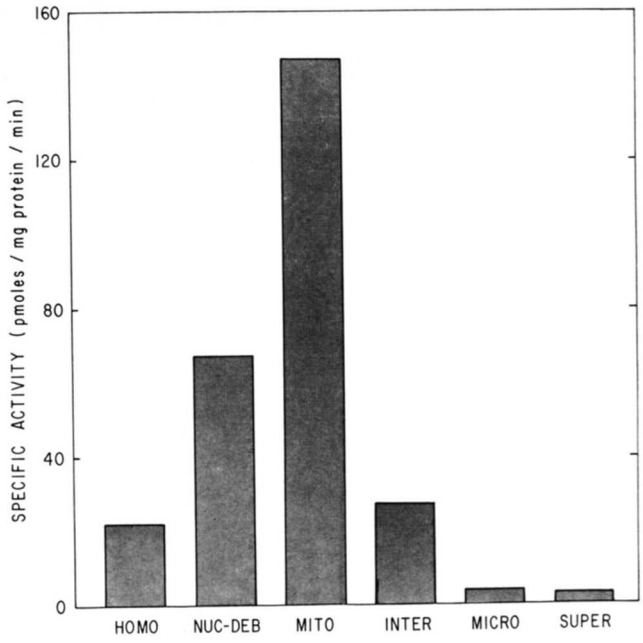


FIG. 3. CDP-diglyceride-L-α-glycerophosphate phosphatidyl transferase. The arrangement of the figure and the notation of the fractions are the same as in Fig. 1.

## DISCUSSION

Several models of mitochondrial biogenesis may be proposed, in which these subcellular organelles are postulated to have different degrees of autonomy. In the *fully autonomous* model, the protein and phospholipid building blocks of the mitochondrion would be synthesized entirely from low molecular weight precursors by enzymes localized in the mitochondrion itself. In the *nonautonomous* model, all of the proteins and phospholipids of the mitochondrion would be synthesized elsewhere in the cell and then later assembled to form the mitochondrion. Hybrid models, of different degrees of autonomy, can also be proposed, in which some of the mitochondrial proteins and phospholipids are synthesized by mitochondrial enzymes (perhaps coded by mitochondrial DNA), while others are synthesized elsewhere in the cell and then transported to the site of mitochondrial assembly. Studies of the biosynthesis of mitochondrial



Downloaded from www.jlr.org by guest, on June 19, 2012

proteins reviewed by Ashwell and Work (12), and of mitochondrial phospholipids, to be discussed below, suggest that on the basis of our present limited knowledge, a *partially autonomous* model of mitochondrial biogenesis may be preferred.

### Phosphatidylcholine

The principal pathway for the de novo biosynthesis of phosphatidylcholine involves CDP-choline as an intermediate. The final step in this pathway is catalyzed by CDP-choline–diglyceride transferase:

CDP-choline + 1,2-*sn*-diglyceride → phosphatidylcholine + CMP [I]

It was shown by Wilgram and Kennedy (3) that this enzyme is almost entirely microsomal, a finding which has been confirmed by McMurray and Dawson (13), who obtained evidence that the small activity observed with isolated mitochondria may be due to contaminating microsomes. Stoffel and Schiefer (1) reported that the activity of microsomes was about 10 times higher than that of mitochondria, but they also provided evidence that the mitochondrial activity may be associated exclusively with the outer mitochondrial membrane.

A second enzymatic pathway leading to the formation of phosphatidylcholine involves successive methylation of phosphatidylethanolamine by *S*-adenosylmethionine. This pathway appears to occur at a significant rate only in liver, where it may function at a rate of about one-fourth that of the CDP-choline pathway (14). It appears also to be catalyzed by enzymes in the microsomal fraction, so that mitochondrial phosphatidylcholine first formed by this mechanism must also have arisen largely in the microsomes.

If the bulk of the mitochondrial phosphatidylcholine is formed in the microsomes, an effective mechanism of intracellular transport must be invoked to explain the rapid labeling of mitochondrial phosphatidylcholine in vivo. The recent discovery by Wirtz and Zilversmit (15) and by McMurray and Dawson (13) of a factor in the supernatant fraction of liver that catalyzes the rapid equilibration of microsomal and mitochondrial phosphatidylcholine may in fact be part of such an intracellular transport system.

### Phosphatidylethanolamine

The de novo synthesis of this phospholipid involves CDP-ethanolamine and 1,2-*sn*-diglyceride in a reaction essentially analogous to reaction [I] (16). The enzyme catalyzing this step is also localized in the microsomal fraction, and indeed may possibly be identical with that catalyzing the formation of phosphatidylcholine, as is suggested by unpublished studies in this laboratory and by the interesting work of Chojnacki (17). In the original studies of Kennedy and Weiss (16), evidence was obtained from heat inactivation studies which suggested that the two enzymes may be distinct; later studies suggest that heat inactivation of interfering activities may have been responsible for the original observations. It is essential to extract and purify the two activities in order to settle this question. The localization of the activity responsible for the synthesis of phosphatidylethanolamine in the microsomes is consistent with the results obtained for [$^{14}$C]ethanolamine incorporation by McMurray and Dawson (13) and Bjørnstad and Bremer (14).

### Serine–ethanolamine cycle

In animal tissues, the formation of phosphatidylserine appears to take place exclusively by way of an exchange reaction with preformed phosphatidylethanolamine:

phosphatidylethanolamine + L-serine ⇌ phosphatidylserine + ethanolamine [II]

phosphatidylserine → phosphatidylethanolamine + $CO_2$ [III]

Net: L-serine → ethanolamine + $CO_2$

The subsequent decarboxylation of phosphatidylserine regenerates phosphatidylethanolamine and provides a cycle for the continuous conversion of serine to ethanolamine (18).

The present work demonstrates that the decarboxylase-catalyzing reaction [III] is almost exclusively mitochondrial (Fig. 1), opening the possibility that mitochondrial phosphatidylethanolamine may be formed by the action of this enzyme. However, the substrate, phosphatidylserine, is synthesized almost exclusively in the microsomes (Fig. 2). Thus mitochondrial phosphatidylethanolamine, whether synthesized via CDP-ethanolamine and 1,2-*sn*-diglyceride, or via phosphatidylserine, appears to have its origin in the microsomes.

Since L-serine is very rapidly decarboxylated by intact liver slices, the phosphatidylserine formed on the microsomes via reaction [II] must be readily transported to the mitochondrial sites of decarboxylation, suggesting that factors such as those described by Wirtz and Zilversmit (15) and by McMurray and Dawson (13) may play a general role in the intracellular redistribution of lipids.

### Diphosphatidylglycerol

This lipid, also known as cardiolipin, may be of special importance in the process of mitochondrial biogenesis since, unlike phosphatidylcholine and phosphatidylethanolamine, it appears to be uniquely mitochondrial, present only in small amounts in extramitochondrial



Downloaded from www.jlr.org by guest, on June 19, 2012

structures. It is, therefore, of great interest that the enzymes catalyzing the following pathway appear to be largely or entirely mitochondrial:

CDP-diglyceride + L-α-glycerophosphate → phosphatidylglycerophosphate + CMP [IV]

phosphatidylglycerophosphate → phosphatidylglycerol + $P_i$ [V]

phosphatidylglycerol → diphosphatidylglycerol [VI]

Data reported in this paper (Fig. 3) confirm the previous observation (19) that the enzyme-catalyzing reaction [IV] is almost lacking in microsomes and very active in mitochondria. Although detailed studies are not yet available, it appears from the work of Davidson and Stanacev (20), as well as from earlier studies in this laboratory, that reactions [V] and [VI] are also localized in the mitochondria.

Since some mitochondrial proteins appear also to be synthesized in the mitochondrion, the possibility may be considered that diphosphatidylglycerol may combine with such proteins to form specific lipoprotein complexes, which may then act as nuclei or matrices for the further addition of lipids and proteins synthesized elsewhere in the cell.

This work has been supported by grants from the U.S. Public Health Service, grant no. NB-02946 from the National Institute of Neurological Diseases and Blindness, and grant no. GM-13952 from the National Institute of General Medical Sciences, and the Life Insurance Medical Research Foundation. E.A.D. was a Postdoctoral Fellow of the U.S. Public Health Service, National Institute of Arthritis and Metabolic Diseases, no. 5-F2-AM-20,008.

*Manuscript received 20 August 1971; accepted 5 November 1971.*

## REFERENCES

1. Stoffel, W., and H. G. Schiefer. 1968. Biosynthesis and composition of phosphatides in outer and inner mitochondrial membranes. *Hoppe-Seyler's Z. Physiol. Chem.* **349:** 1017–1026.
2. Wooley, J. G., and W. H. Sebrell. 1945. Niacin (nicotinic acid), an essential growth factor for rabbits fed a purified diet. *J. Nutr.* **29:** 191–199.
3. Wilgram, G. F., and E. P. Kennedy. 1963. Intracellular distribution of some enzymes catalyzing reactions in the biosynthesis of complex lipids. *J. Biol. Chem.* **238:** 2615–2619.
4. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
5. Borkenhagen, L. F., E. P. Kennedy, and L. Fielding. 1961. Enzymatic formation and decarboxylation of phosphatidylserine. *J. Biol. Chem.* **236:** PC28–PC30.
6. Baer, E., and J. Maurukas. 1955. Phosphatidyl serine. *J. Biol. Chem.* **212:** 25–38.
7. Dennis, E. A., and E. P. Kennedy. 1970. Enzymatic synthesis and decarboxylation of phosphatidylserine in *Tetrahymena pyriformis*. *J. Lipid. Res.* **11:** 394–403.
8. Weiss, S. B., S. W. Smith, and E. P. Kennedy. 1958. The enzymatic formation of lecithin from cytidine diphosphate choline and D-1,2-diglyceride. *J. Biol. Chem.* **231:** 53–64.
9. Chang, Y.-Y., and E. P. Kennedy. 1967. Biosynthesis of phosphatidyl glycerophosphate in *Escherichia coli*. *J. Lipid Res.* **8:** 447–455.
10. Patterson, M. S., and R. C. Greene. 1965. Measurement of low energy beta-emitters in aqueous solution by liquid scintillation counting of emulsions. *Anal. Chem.* **37:** 854–857.
11. Paulus, H., and E. P. Kennedy. 1960. The enzymatic synthesis of inositol monophosphatide. *J. Biol. Chem.* **235:** 1303–1311.
12. Ashwell, M., and T. S. Work. 1970. The biogenesis of mitochondria. *Annu. Rev. Biochem.* **39:** 251–290.
13. McMurray, W. C., and R. M. C. Dawson. 1969. Phospholipid exchange reactions within the liver cell. *Biochem. J.* **112:** 91–108.
14. Bjørnstad, P., and J. Bremer. 1966. In vivo studies on pathways for the biosynthesis of lecithin in the rat. *J. Lipid Res.* **7:** 38–45.
15. Wirtz, K. W. A., and D. B. Zilversmit. 1968. Exchange of phospholipids between liver mitochondria and microsomes *in vitro*. *J. Biol. Chem.* **243:** 3596–3602.
16. Kennedy, E. P., and S. B. Weiss. 1956. The function of cytidine coenzymes in the biosynthesis of phospholipides. *J. Biol. Chem.* **222:** 193–214.
17. Chojnacki, T. 1964. On the *in vitro* formation of phospholipids containing unnatural bases, via the cytidine mechanism. *Acta Biochim. Pol.* **11:** 11–23.
18. Kennedy, E. P. 1962. The metabolism and function of complex lipids. The Harvey Lectures, Series 57. Academic Press, New York. 143–171.
19. Kiyasu, J. Y., R. A. Pieringer, H. Paulus, and E. P. Kennedy. 1963. The biosynthesis of phosphatidylglycerol. *J. Biol. Chem.* **238:** 2293–2298.
20. Davidson, J. B., and N. Z. Stanacev. 1971. Biosynthesis of cardiolipin in mitochondria isolated from guinea pig liver. *Biochem. Biophys. Res. Commun.* **42:** 1191–1199.



Downloaded from www.jlr.org by guest, on June 19, 2012