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Properties of Mevalonate-Activating Enzymes in Developing Chick Brain

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Phosphorylation of mevalonic acid has been studied in 1-3 day chicks. Addition of Mg^{2+} or Mn^{2+} strongly increased the formation of phosphorylated derivatives. Mevalonate phosphorylation showed an absolute nucleotide requirement, being ATP and ITP the most effective phosphate donors. Unlike other vertebrate mevalonate kinases, the enzyme from chick brain did not require thiol group protectors as activators. However, the enzyme was found to be sensitive to thiol binding reagents.

Although various studies have been reported on cholesterol metabolism to steroid hormones and cholesterol content in non-laying and laying hens, little is known about the origin of tissue cholesterol in newly hatched birds. The endogenous or exogenous origin cholesterol in the chick embryo is not clear. TSUJI *et al.* (16) concluded that there was no net cholesterol synthesis in the embryo but that cholesterol is esterified and transferred to the embryo. However other investigators have maintained that cholesterol biosynthesis does occur in the chick embryo. So, CONNOR *et al.* (3) indicated that while most of the cholesterol in the chick is originated from the egg yolk, cholesterol biosynthesis was active in brain and provided about 90 % of its cholesterol content.

Mevalonic acid (MVA) is one of the most frequently used substrates in studies on biosynthesis of different isoprenoid compounds (2). This precursor is converted to the isoprene unit through phosphomevalonic acid (MVAP) and pyrophosphomevalonic acid (MVAPP). In 1976 we presented the first communication on incorporation of MVA to phosphorylated derivatives by neonatal chick liver (4) and reported the pattern of this mevalonate phosphorylation during neonatal development. Recently we have studied the quantitative role of the brain and kidney of the chick in MVA metabolism during early steps of development (9), showing that MVA phosphorylation was always higher in brain than in both kidney and liver during the first period after hatching. These results agree with the different role of these tissues during neonatal develop-

ment, being the brain the main tissue which contributes to cholesterol synthesis in newly hatched chicks.

In the present paper we have examined the characteristics of the enzymes that catalyze MVA phosphorylation in brain of newly hatched chicks, providing an insight of mechanism of cholesterol biosynthesis during the active myelination.

Materials and Methods

[2-^{14}C]MVA lactone was supplied by the Radiochemical Centre, Amersham, England. Unlabelled MVA in the lactone form and nucleotides were purchased from Sigma. The potassium salt of MVA was prepared by treating the lactone at 36° C for 30 min with an excess of KOH. Labelled and unlabelled salts were mixed in sufficient concentration to obtain the required specific activity. All other reagents used were analytical grade.

New-born White Leghorn male chicks were obtained from a commercial hatchery and maintained on a commercial diet.

Cell-free extracts were obtained as previously described (4). Protein content was determined by the method of LOWRY *et al.* (10) using bovine albumin as standard. Unless otherwise specified, the reaction system contained 7.5 μmol of $MgCl_2$, 15 μmol of ATP, 50 nmol of [2-^{14}C]MVA (0.5 μCi), 75 μmol of Tris-maleate buffer pH 7.5, and 0.1-0.3 mg of protein in a final volume of 1.5 ml. Reactions and identification of MVAP and MVAPP were carried out as previously described (5, 6).

Results

Effect of divalent metal ions on MVA phosphorylation. The formation of phosphorylated derivatives of [2-^{14}C]MVA by cell-free extracts from chick brain was studied in the presence of Mg^{2+}, Mn^{2+}, Ca^{2+}, Zn^{2+} and Hg^{2+} at different concentrations and compared with that obtained without addition of any metal ion. The results given in table I show that only a small amount of MVAP was carried when enzymatic reactions were carried out without metal supplementation. Addition of Mg^{2+} or Mn^{2+} strongly increased MVA phosphorylation. The data presented in table I also show that at lower concentrations (0.1-1.0 mM) Mn^{2+} was a better activator than Mg^{2+}, but when Mn^{2+} concentration increased to 5.0-10.0 mM a marked inhibition in the MVAP formation was observed. As Mg^{2+} concentration was increased (5.0-10.0 mM), both MVAP and MVAPP formation increased, reaching levels higher than those

Table I. *Effect of metal ions on MVA phosphorylation by neonatal chick brain.* Incubations contained the standard radiochemical assay mixture, except for the metal ions. Results are given as means ± S.E.M. of three experiments.

Metal ion	Concentration (mM)	Phosphorylated derivatives (dpm × 10^{-4}/mg protein/30 min)	
		MVAP	MVAPP
None	—	27.9± 3.4	0.0
Mg^{2+}	0.1	19.2± 9.6	0.0
	0.5	63.8± 3.7	0.0
	1.0	70.9±21.1	12.6±6.4
	5.0	204.1±12.2	24.1±2.1
	10.0	301.9±15.2	39.7±7.4
Mn^{2+}	0.1	108.4± 7.3	0.0
	0.5	221.0±12.2	0.0
	1.0	264.0± 8.4	0.0
	5.0	194.7± 6.9	18.6±3.6
	10.0	145.8±16.3	29.3±4.5
Ca^{2+}	0.1	13.4± 1.0	0.0
	0.5	23.3± 2.1	0.0
	1.0	24.4± 3.2	0.0
	5.0	27.8± 0.5	0.0
	10.0	36.6± 1.2	0.0
Zn^{2+}	0.1	50.4± 1.8	0.0
	0.5	55.1± 1.8	0.0
	1.0	57.6± 0.6	0.0
	5.0	38.4± 4.3	0.0
	10.0	0.0	0.0

obtained in the presence of Mn^{2+} at any concentration.

The amount of MVAP formed in the presence of Ca^{2+} or Zn^{2+} was lower than that obtained in the presence of Mn^{2+}, increasing slowly with metal concentration in the case of Ca^{2+} and remaining more or less constant up to 1.0 mM in the case of Zn^{2+}. In the presence of this metal ion a clear inhibition was observed at higher concentrations. Supplementation of 0.1-10.0 mM Hg^{2+} inhibited MVA phosphorylation, which suggests that thiol groups may participate in the active site of mevalonate kinase from chick brain.

Nucleotide dependence. The MVA phosphorylation by chick brain extracts shows an absolute requirement of nucleotide. As can be seen in table II, the MVA phosphorylation did not occur without addition of any nucleotide. The total amount of phosphorylated derivatives formed from MVA was highest in the presence of ATP in comparison with other nucleotides. ITP was also an effective phosphate donor. Only low phosphorylation of MVA occurs in the presence of UTP or GTP.

Further examination of the data in table II reveals that the formation of MVAP increased with ATP or ITP concentration increasing, whereas the maximum amount of MVAPP was not reached in the same way; in this case 0.5 mM ATP or 5.0 mM ITP were the optimum nucleotide concentration. Anyhow, it is interesting to notice that in the conditions described in Materials and Methods, only MVAP and MVAPP were observed as derivatives of [2-^{14}C] MVA.

Sensitivity to thiol reagents. The results in table III show that contrary to what was to be expected mevalonate-activating enzymes from chick brain were not activated by —SH group protectors such as dithiothreitol, reduced glutathione, cysteine or β-mercaptoethanol in the range

Table II. *Nucleotide requirements of MVA phosphorylation by neonatal chick brain*
Incubations contained the standard radiochemical assay mixture, except for the nucleotide. Results are given as means ± S.E.M. of three experiments.

Nucleotide	Concentration (mM)	Phosphorylated derivatives (dpm × 10^{-4}/mg protein/30 min)	
		MVAP	MVAPP
None	—	0.0	0.0
ATP	0.1	63.1± 6.8	39.3± 4.8
	0.5	86.5± 4.5	112.4±13.2
	1.0	112.2± 2.0	96.5±13.6
	5.0	174.8±10.8	77.6±15.5
	10.0	226.5± 7.1	47.1±17.4
ITP	0.1	0.0	0.0
	0.5	47.2± 7.9	0.0
	1.0	122.2±12.9	13.8± 1.5
	5.0	149.4± 6.8	24.7± 5.8
	10.0	173.2±13.3	18.9± 8.2
UTP	0.1	3.0± 0.2	4.6± 1.7
	0.5	2.8± 0.2	7.0± 1.7
	1.0	5.9± 1.6	5.9± 0.2
	5.0	5.9± 0.2	4.9± 1.0
	10.0	0.0	0.0
GTP	0.1	2.0± 0.4	2.9± 1.1
	0.5	3.1± 0.3	6.9± 0.8
	1.0	3.9± 0.9	5.9± 0.2
	5.0	0.0	0.0
	10.0	0.0	0.0

of concentrations tested (0.1-10.0 mM). No increase in the amount of MVAP and MVAPP formed was observed when these reagents were added to the standard incubation mixture. Even more, a decrease in the amount of MVAP appeared in the presence of dithiothreitol and reduced glutathione at every concentration tested. No MVAPP was observed when reduced glutathione was added. Similarly, the amount of both phosphorylated derivatives of MVA decreased as cysteine concentration was increased from 0.1 to 10 mM.

However, mevalonate-activating enzymes from chick brain were found to be sensitive to some thiol binding reagents. Ta-

Table III. *Effect of some —SH groups protectors on MVA phosphorylation by neonatal chick brain.*
Incubations contained the standard radiochemical assay mixture. Results are given as means ± S.E.M. of three experiments.

Reagent	Concentration (mM)	Phosphorylated derivatives (dpm × 10^{-4}/mg protein/30 min) MVAP	MVAPP
None	—	193.2±20.0	47.4±12.2
Dithiothreitol	0.1	114.4± 6.7	55.5± 7.8
	1.0	114.4±11.0	68.8± 9.8
	10.0	122.6±18.8	0.0
Reduced glutathione	0.1	118.6± 7.9	0.0
	1.0	119.8±20.0	0.0
	10.0	102.0±16.4	0.0
Cysteine	0.1	174.5± 4.9	43.2± 3.9
	1.0	151.6±14.4	23.6± 7.8
	10.0	104.7±14.5	0.0
β-mercaptoethanol	0.1	169.1±20.0	53.1±13.7
	1.0	161.4±15.0	45.3± 6.0
	10.0	198.6±13.6	35.0± 3.9

Table IV. *Effect of some —SH groups binding reagents on MVA phosphorylation by neonatal chick brain.*
Incubations contained the standard radiochemical assay mixture. Results are given as means ± S.E.M. of six experiments.

Reagent	Concentration (mM)	Phosphorylated derivatives (dpm × 10^{-4}/mg protein/30 min) MVAP	MVAPP
None	—	193.0±21.4	37.8±9.6
N-ethylmaleimide	0.01	175.0± 7.0	0.0
	0.1	182.2±14.7	0.0
	1.0	189.2±14.4	0.0
	10.0	166.6± 3.0	0.0
p-hydroxymercuribenzoate	0.01	0.0	0.0
	0.1	0.0	0.0
	1.0	0.0	0.0
	10.0	0.0	0.0
5,5′-dithiobis-(2-nitrobenzoic acid)	0.01	186.7± 5.6	0.0
	0.1	170.1± 2.4	0.0
	1.0	114.9± 2.0	0.0
	10.0	0.0	0.0

ble IV shows that MVA phosphorylation was completely inhibited by p-hydroxymercuribenzoate at 0.01 mM concentration. 1.0 mM 5,5′-dithiobis (2-nitrobenzoic acid) clearly inhibited the reaction, 100% inhibition being observed at 10.0 mM concentration, whereas N-ethylmaleimide had no effect on MVAP formation. As can be seen in table IV, no MVAPP was formed when reactions were carried out in the presence of thiol binding reagents, which suggests that phosphomevalonate kinase from neonatal chick brain is extremely sensitive to these reagents, even at 0.01 mM concentrations.

Discussion

Brain cholesterol importance is undoubtful constituting about 25% of the total weight of myelin lipid components. During myelination some aged-related changes in the synthesis of cholesterol must occur in the brain. Recently we have found that MVA phosphorylation by brain changes with age in coordination with other cholesterol-synthesizing enzymes (9). With this in mind, we examined the characteristics and requirements of MVA incorporation to phosphorylated derivatives in the neonatal chick brain. Because of the pattern of these reactions, we have used 1-3 day old chicks in the present study.

It can be seen that the amounts of MVAP observed were higher than those of MVAPP observed, which suggests a higher activity for mevalonate kinase during neonatal development of the chick. However, it should be borne in mind that since the reaction catalyzed by phosphomevalonate kinase is reversible (7), the amount of MVAPP accumulated is regulated by the equilibrium constant of the kinase reaction particularly when the rate of MVAPP decarboxylation is low or null (12). This aspect may be confirmed observing the variations in the rela-

tive amounts of MVAP and MVAPP formed at different ATP concentrations. When this concentration was increased (1.0 to 10.0 mM), the MVAP/MVAPP ratio increased, suggesting that the high amount of ADP formed in the reaction catalyzed by the mevalonate kinase can pull the phosphomevalonate kinase reaction towards MVAP formation.

On the other hand, the nucleotide requirements of mevalonate activating enzymes from chick brain appear to differ from those of pig liver enzymes: in this source, mevalonate kinase can utilize ATP and ITP both equally effectively at similar concentrations (8). In plant sources, neither ITP nor UTP were effective in replacing ATP as nucleotide (11, 14), whereas mevalonate kinase from yeast autolysate can also utilize GTP, UTP or CTP instead of ATP (15).

With respect to ion requirements, the mevalonate-activating enzymes from chick brain are similar to those from chick liver (5) and hog liver (1) in which MVAP formation is greater in the presence of Mn^{2+} than Mg^{2+} at low concentration. Our results also show that at higher concentration Mn^{2+} produced a clear inhibition of MVAP formation, similar to that found in plant sources (13, 17). Inhibition of MVA phosphorylation observed in the presence of Hg^{2+} at any concentration assayed (0.1-10 mM) suggests that thiol groups may participate in the active site of mevalonate kinase from chick brain. However, the results obtained show that neither dithiothreitol, reduced glutathione, cysteine nor β-mercaptoethanol activate MVA phosphorylation by chick brain enzyme preparations. Again in these properties the chick mevalonate kinase and phosphomevalonate kinase resembled the *Hevea* latex (17) and yeast enzymes (15). Nevertheless, both chick brain enzymes were highly sensitive to reagents known to react with thiol groups, like all the mevalonate-activating enzymes so far reported.

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Resumen

Se estudia la fosforilación del ácido mevalónico en cerebro de pollos de 1-3 días. La adición de Mg^{2+} o Mn^{2+} incrementa fuertemente la formación de derivados fosforilados. La fosforilación del ácido mevalónico requiere la presencia de nucleótidos, siendo el ATP y el ITP los más efectivos. Al contrario de otras enzimas semejantes de vertebrados, la mevalonato cinasa de cerebro de pollo no requiere protectores de grupos —SH como activadores de la reacción. Sin embargo, la enzima es sensible frente a los agentes que bloquean los grupos —SH.

References

1. Beytia, E., Dorsey, J. K., Marr, J., Cleland, W. W. and Porter, J. W.: *J. Biol. Chem.*, 245, 5450-5458, 1970.
2. Beytia, E. and Porter, J. W.: *Ann. Rev. Biochem.*, 45, 113-142, 1976.
3. Connor, W. E., Johnston, R. and Lin, D. S.: *J. Lipid Res.*, 10, 388-394, 1969.
4. García-Martínez, J., Segovia, J. L., Suárez, M. D. and García-Pelegrín, E.: *Biochem. Biophys. Res. Commun.*, 72, 202-208, 1976.
5. García-Martínez, J., Segovia, J. L., Suárez, M. D. and García-Peregrín, E.: *Comp. Biochem. Physiol.*, 61B, 275-279, 1978.
6. García-Peregrín, E., Suárez, M. D., Aragón, M. C. and Mayor, F.: *Phytochemistry*, 11, 2495-2498, 1972.
7. Hellig, H. and Popjak, G.: *J. Lipid Res.*, 2, 235-243, 1961.
8. Levy, H. R. and Popjak, G.: *Biochem. J.*, 75, 417-428, 1960.
9. Linares, A., García-Martínez, J., Suárez, M. D. and García-Peregrín, E.: *Biol. Neonate*, 38, 25-29, 1980.

10. LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. and RANDALL, R. J.: *J. Biol. Chem.*, **193**, 265-275, 1951.
11. POTTY, V. H. and BRUEMMER, J. H.: *Phytochemistry*, **9**, 99-105, 1970.
12. RAMACHANDRAN, C. K. and SHAH, S. N.: *Biochem. Biophys. Res. Commun.*, **69**, 42-47, 1976.
13. SUÁREZ, M. D. and GARCÍA-PEREGRÍN, E.: *Phytochemistry*, **16**, 661-665, 1977.
14. SUÁREZ, M. D., GARCÍA-PEREGRÍN, E. and MAYOR, F.: *Phytochemistry*, **13**, 1059-1063, 1974.
15. TCHEN, T. T.: *J. Biol. Chem.*, **233**, 1100-1103, 1958.
16. TSUJI, F. I., BRIN, M. and WILLIAMS, H. H.: *Arch. Biochem. Biophys.*, **56**, 290-296, 1955.
17. WILLIAMSON, I. P. and KEKWICK, R. G. O.: *Biochem. J.*, **96**, 862-871, 1965.