Levels of 5′-nucleotidase and 3′-nucleotidase activities in healthy (H) and spiked (S) sandal leaves

Description of the leaves	Nucleotidase activity* (μg Pi liberated/40 mg tissue/2 h)							
	5′ Nucleotidase activity							
	5′-AMP		5′-GMP		5′-CMP		5′-UMP	
	H	S	H	S	H	S	H	S
Young	33.5	35.0	55.6	70.0	42.5	54.5	43.3	60.4
	(1.0)	(1.4)	(2.0)	(2.3)	(1.8)	(2.2)	(1.1)	(2.4)
Mature	37.0	31.3	68.5	55.4	63.0	44.0	65.2	48.5
	(1.7)	(1.4)	(2.5)	(2.0)	(2.1)	(2.1)	(2.5)	(1.6)
	3′-Nucleotidase activity							
	3′-AMP		3′-GMP		3′-CMP		3′-UMP	
	H	S	H	S	H	S	H	S
Young	62.3	86.5	32.5	35.0	46.4	50.2	15.0	15.2
	(1.5)	(1.4)	(1.2)	(1.7)	(1.5)	(2.2)	(1.0)	(0.8)
Mature	42.5	70.2	30.8	32.0	41.5	45.6	10.7	9.5
	(1.8)	(2.3)	(1.4)	(1.2)	(1.4)	(1.4)	(0.9)	(0.8)

* Average of 6 replications. Figures within brackets show SD.

pathogen occurs[3], may adversely affect the level of ribonucleoside triphosphates serving in the energy transfer mechanism of the cell.

Compared with the mature leaves, the relatively higher level of 3′-Ntdase activity in the young leaves of both healthy and diseased plants could possibly be correlated with the high level of ribonuclease activity noticed in them[3], since the 3′-ribonucleotides released from RNA by the plant ribonuclease are hydrolyzed to ribonucleosides, which, either directly or after conversion to the bases, are retrieved for recycling for RNA synthesis[8].

1 E. Kovacs and V. Kovacs, Experientia *13*, 481 (1957).
2 J. Stachura, Z. Porwit-Bobr and W. Jaszez, Acta med. pol. *7*, 289 (1966); Acta med. pol. chem. Abstr. *66*, 27277 (1967).
3 K. Parthasarathi, C.R. Rangaswamy, K. Jayaraman and P.S. Rao, Proc. Indian Acad. Sci. *77B*, 131 (1973).
4 K. Parthasarathi, C.R. Rangaswamy and D.R.C. Babu, Experientia *33*, 999 (1977).
5 Yee Foong Lai, J.E. Thompson and T.W. Barrel, Phytochemistry *10*, 41 (1971).
6 P. Fritzson, Eur. J. Biochem. *1*, 12 (1967).
7 P.L. Ipata, Biochemistry *7*, 507 (1968).
8 Gerhard Richter, Plant Metabolism. Georg Thieme, Stuttgart 1978.

Enhancement of lipid peroxidation in rat brain mitochondria by polyamines

P. Thyagarajan[1]

Biochemistry and Food Technology Division, Bhabha Atomic Research Centre, Trombay, Bombay 400 085 (India), 2 September 1980

Summary. Ascorbic acid-induced lipid peroxidation in rat brain mitochondria is enhanced by the addition of spermine and spermidine.

Ascorbic acid at low concentrations induces nonenzymatic lipid peroxidation of membrane lipids in mitochondria of rat liver[2] and brain[3]. Mitochondria are membraneous organelles rich in polyunsaturated fatty acids (PUFA) and the normal functions of mitochondria depend on highly integrated membrane structures. In vitro peroxidation of PUFA in cell membranes leads to the formation of a wide variety of toxic products, destruction of lipid-requiring functions and deterioration of the membrane structure[4]. The peroxidation of lipids is inhibited in various tissues by ascorbic acid at high concentrations, but the physiological significance has not been adequately stressed[5]. Since polyamines are present in relatively high concentrations in mammalian nervous tissues[6], the effects of spermine and spermidine on ascorbic acid-induced lipid peroxidation in rat brain mitochondria were examined.

Materials and methods. Spermine tetrahydrochloride, spermidine phosphate and 2-thiobarbituric acid were purchased from Sigma Chemical Co., USA. Other reagents were of analytical grade. Male Wistar rats, weighing 150–200 g and fed on stock laboratory diet, were used in this study. The animals were killed by decapitation and the brains excised, homogenized in 10 vol. of chilled 0.25 M sucrose, and centrifuged at $800 \times g$ for 10 min. The supernatant was centrifuged at $18{,}000 \times g$ for 20 min. The mitochondrial pellet was washed with 0.15 M KCl and suspended in 25 mM Tris-HCl buffer (pH 7.4) containing 150 mM KCl to a final protein concentration of 3.0 mg/ml. Mitochondria were incubated with 0–5 mM ascorbic acid at 37 °C for 75 min and aliquots removed at different time intervals for estimations. In some experiments, spermine and spermidine (1–40 mM) were added to the incubation mixture. The reaction was stopped by the addition of 1 ml 10% TCA which contained 0.02% by volume of an ethanolic solution of butylated hydroxytoluene (BHT), and centrifuged. The resultant supernatant was mixed with 1.0 ml. 0.67% thiobarbituric acid (TBA) and placed in a boiling water bath for 10 min. The absorbance at 535 nm was read and the amount of malonyldialdehyde (MDA) formed[7] calculated by using the extinction coefficient $\varepsilon = 1.56 \times 10^5\ M^{-1}\ cm^{-1}$. Protein was determined by the procedure of Miller[8]. Values are expressed as means ± SE.

Results and discussion. Incubation of a mitochondrial suspension with low concentrations (0.1–1.0 mM) of ascorbic acid resulted in the formation of MDA, an index of lipid peroxidation (fig. 1). The maximum acceleration was observed at 1 mM ascorbic acid and the reaction rate slowed down and nearly reached a plateau after about 30 min. Further increase of ascorbic acid in the medium inhibited the formation of TBA reactive material and at 5.0 µmole/ascorbic acid/ml, negligible peroxidation occurred, even after 75 min of incubation. In order to study the optimum concentration of ascorbic acid required for maximum peroxidation, ascorbic acid was added during the incubation reaction. Addition of 1 mM ascorbic acid after 30 min incubation time increased MDA formation only if the initial concentration of ascorbate was low (0.1 mM), but was slightly inhibitory if the initial concentration was already 1 mM (fig. 2). This indicates that the critical concentration above which ascorbate begins to behave as an anti-oxidant is 1.0 mM. This observation is in accordance with that reported for a variety of species[2,5,9]. It is known that with low concentrations of ascorbic acid, the reaction is autocatalytic, and the inhibition observed at high concentrations has been attributed to a lowering of the oxidation-reduction potential[10].

Addition of the polyamines spermine and spermidine at concentrations of 0.5 mM–40 mM enhanced lipid peroxidation as seen in the table. Above 10 mM, spermidine did not increase the production of lipid peroxides, whereas lipid peroxidation was still concentration-dependant at 40 mM spermine. Also, it was observed that the addition of spermine gave rise to a profuse output of peroxides as compared to spermidine. Stimulation of lipid peroxidation by polyamines could be observed only at lower concentrations of ascorbic acid, namely with 100 µM ascorbic acid. At 1.0 mM ascorbic acid, addition of spermine at 0 time or at 15 min did not have any stimulating effect (fig. 3). Moreover, the characteristics of the time course of this peroxidation stimulated by spermine revealed a rapid initial rate of peroxidation which subsided after about 15 min. Also, spermine by itself is unable to stimulate peroxidation of brain mitochondria. Thus the maximum acceleration of the peroxidation reaction was observed with 1.0 mM ascorbic acid in either of the systems employed.

It is known that polyamines behave as polycations in biological systems, have a high affinity for nucleic acids and stabilize their secondary structure[6]. They have been shown to have a high affinity for cellular polyanions[11] and

Effect of spermine and spermidine on lipid peroxidation of rat brain mitochondria

Addition	Concentration (mM)	Malonyldialdehyde formed (nmole/mg protein)
None	-	3.6±0.009
Spermine	0.5	3.9±0.008
	1.0	4.6±0.01*
	10.0	11.0±0.01*
	20.0	16.8±0.01*
	40.0	21.2±0.02*
Spermidine	0.5	3.8±0.008
	1.0	6.3±0.009*
	10.0	11.5±0.01*
	20.0	11.8±0.02*
	40.0	11.7±0.01*

Mitochondria (800 µg protein/ml) were incubated with 100 µM ascorbic acid and different concentrations of spermine and spermidine. The reaction was carried out for 90 min at 37 °C and the amount of MDA formed was estimated. Values are expressed as mean ± SE. * $p < 0.001$.

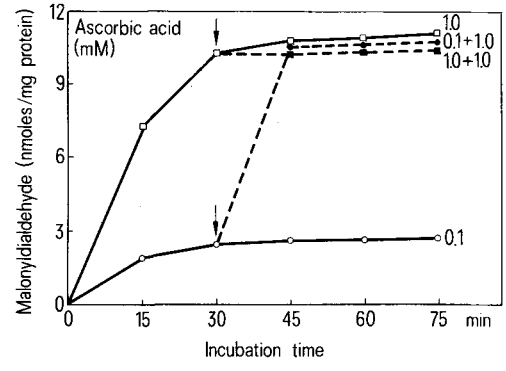

Fig. 2. Resumption of malonyldialdehyde formation by further addition of ascorbic acid. Mitochondria (800 µg protein) were incubated with 0.1 (○) or 1.0 mM (□) ascorbic acid in a final volume of 1.0 ml. At 30 min, 1.0 mM ascorbic acid was added to each reaction mixture (●, ■).

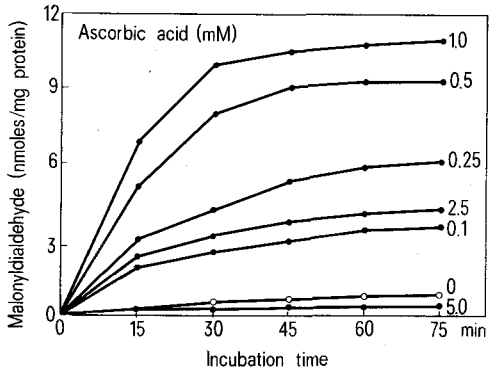

Fig. 1. Ascorbic acid-induced malonyldialdehyde formation in rat brain mitochondria. The reaction mixture consisted of 25 mM Tris-HCl buffer (pH 7.4), 150 mM KCl, various concentrations of ascorbic acid as indicated and 800 µg protein of mitochondria in a final volume of 1 ml. Incubations were carried out at 37 °C.

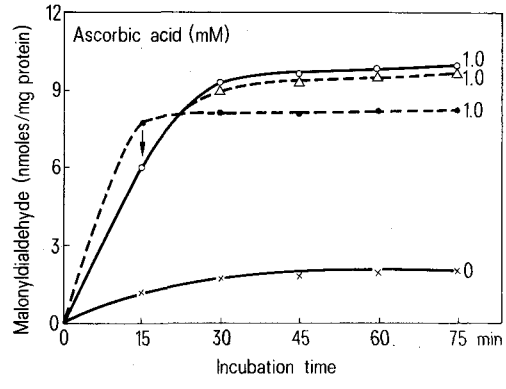

Fig. 3. Effect of spermine on ascorbic acid induced malonyldialdehyde formation. Mitochondria (600 µg protein) were incubated with 1.0 mM ascorbic acid (○) and 10 mM spermine was added at 0 time (●) or at 15 min (△). A corresponding blank was run (x) which contained mitochondria (600 µg protein) and 10 mM spermine.

the respiratory control ratio has been shown to be affected in rat liver mitochondria[12]. However, it has been recently reported that polyamines inhibited both NADPH-dependant and ascorbate-dependant lipid peroxidation in rat liver microsomes, possibly through binding to phospholipids[13]. The discrepancy with the present observations may be due to the different reaction conditions. The nonenzymic ascorbate-induced lipid peroxidation is widely distributed in the various subcellular fractions of the rat brain, whereas the enzymic NADPH linked peroxidation in brain microsomes is capable of forming only small amounts of lipid peroxides[3]. Also, in our studies peroxidation was initiated by the addition of ascorbic acid alone and nonspecific peroxidation, if any, was eliminated by the use of BHT. In addition, the phospholipid content of rat brain mitochondria is higher (530 nmoles/mg protein) than the reported values of 322 nmoles/mg protein for rat liver microsomes[14]. Moreover, MDA production during the peroxidation of membranes varies among the different types of tissues, chiefly due to the different amounts of PUFA present in the different tissues. In fact, Iwata and coworkers[15] have recently shown stimulation of thiamine diphosphatase activity in rat brain microsomes by ascorbic acid induced peroxidation in membrane lipids. Thus, further studies appear to be warranted to explain the present findings.

1 Acknowledgment. The author is deeply grateful to Dr U.K. Vakil for her kind interest and encouragement during the course of this work.
2 E.D. Wills, Biochem. J. *113*, 315 (1969).
3 S. Bishayee and A.S. Balasubramanian, J. Neurochem. *18*, 909 (1971).
4 G.L. Plea and H. Witschi, A. Rev. Pharmac. Tox. *16*, 125 (1976).
5 E.D. Wills, Biochem. J. *123*, 983 (1971).
6 C.W. Tabor and H. Tabor, A. Rev. Biochem. *45*, 285 (1976).
7 R.O. Sinnhuber, T.C. Yu and T.C. Yu, Food Res. *23*, 626 (1958).
8 G.L. Miller, Analyt. Chem. *31*, 964 (1959).
9 B.P.F. Adlard, Biochem. Soc. Trans. *2*, 281 (1974).
10 G. Haase and W.L. Dunkley, J. Lipid Res. *10*, 561 (1969).
11 B. Tadolini, Biochem. biophys. Res. Commun. *92*, 598 (1980).
12 R.J. Chafee, R. M. Arina and R.H. Rochelle, Biochem. biophys. Res. Commun. *86*, 293 (1979).
13 M. Kitada, K. Igarshi, S. Hirose and H. Kitagawa, Biochem. biophys. Res. Commun. *87*, 388 (1979).
14 A. Colbeau, J. Nachbaur and P.M. Vignais, Biochim. biophys. Acta *249*, 462 (1971).
15 H. Iwata, T. Matsuda, S. Maeda and A. Baba, Biochim. biophys. Acta *583*, 159 (1979).

On the production of 12α-hydroxysteroid dehydrogenase from *Clostridium group P*, strain C48-50 ATCC 29733

I.A. Macdonald

Departments of Medicine and Biochemistry, Dalhousie University, Halifax (Nova Scotia, Canada B3H 4H7), 9 July 1980

Summary. The production of 12α-hydroxysteroid dehydrogenase of *Clostridium group P* strain C48-50 was optimized when the organism was grown in the presence of 2% fructose and 0.1% dithiothreitol. It appears that an initial redox potential of less than −160 mV (achieved by autoclaving in the presence of dithiothreitol, dithioerythritol or cysteine) is important in the production of this enzyme.

Nicotinamide adenine denucleotide phosphate dependent (12α-HSDH) active against bile acids can be demonstrated in mixed fecal cultures[1], in *Clostridium leptum*[2] and in a number of non-fermentative clostridia[3]. Two of the above organisms, a *Clostridium leptum*[2] and a *Clostridium group P* organism[3,4] have been isolated, which contain 12α-HSDH in the total absence of 3α- or 7α-HSDH. Cell-free preparations of the latter clostridium have been used in the quantification of 12α-OH groups in bile[5] and fecal[6] extracts.

Recent failures to obtain cell-free preparations of high specific activity as reported earlier[4] have prompted us to investigate the effect of various reducing agents on the production of 12α-HSDH from *Clostridium group P*. In this communication, we report the effect of various reducing agents in the medium on the growth of *Clostridium group P*, the initial redox potential (Eh) value and the yield of 12α-HSDH.

Materials and methods. Clostridium group P strain C48-50 was grown for 96 h at 37 °C in 10 ml volumes of brain heart infusion (BHI) broth in 15 ml culture tubes as described earlier (except with reducing agent included). Glucose or fructose were added to the medium to give a final concentration of 2.0%; and a reducing agent was added to give a final concentration of 0.1% before autoclaving the medium for 20 min. Final Eh values of the medium (of duplicate tubes) were measured using an Orion platinum redox electrode (mold 96-78) attached to an Orion pH/Eh meter. A reference standard of 0.5% cysteine giving an Eh value of +25 mV was employed[7].

Cell-free peparation of 12α-HSDH were prepared as before[4] and 12α-HSDH was assayed as before[4] except

Table 1. Effect of reducing agents and sugars on the growth of *Clostridium group P* strain C48-50, production of 12α-HSDH and Eh value of the medium

Addition to medium	Absorbance of culture at 660 nm*	Units of 12α-HSDH per 10 ml culture	Initial Eh value of medium (mV)
1 No additions	0.20	0.6	−100
2 TG alone	0.22	0.6	−140
3 DTT alone	0.28	0.8	−250
4 Fructose alone	1.3	1.0	−100
5 TG + fructose	1.4	10.5	−145
6 TG + glucose	1.4	8.5	−147
7 Cyst + fructose	1.3	14.5	−165
8 Cyst + glucose	1.3	11.1	−170
9 DTE + fructose	1.8	12.5	−240
10 DTE + glucose	1.8	7.0	−240
11 DTT + fructose	1.9	15.0	−255
12 DTT + glucose	1.8	7.0	−255
13 'Aged' TG + fructose	1.4	1.0	−135
14 'Aged' TG + glucose	1.4	0.81	−137

* Growth measured after 96 h.