CHANGES IN ASCORBIC ACID CONTENT AND ACETYLCHOLINESTERASE ACTIVITY IN THE MUSCLE OF FROG FOLLOWING SCIATECTOMY

T. SOMASEKHAR AND R. V. KRISHNAMOORTHY

Department of Zoology,
University of Agricultural Sciences,
G.K.V.K. Campus, Bangalore - 560 065

(Received on November 10, 1983)

Summary : 1. Unilateral-sciatectomy for three months in the frog, *Rana cyanophlictis* resulted in a substantial increase on unit weight basis in the ascorbic acid (ASA) and dehydroascorbic acid (DHA) contents of the sciatectomized gastrocnemius muscle. Diketogulonic acid (DKA) levels did not vary. On whole muscle-weight basis only the ASA level increased.

2. The AChE activity in sciatectomized muscle is significantly lower than that of the control.

3. Partially purified preparation of the AChE from the sciatectomized muscle showed different kinetics compared to that from innervated control.

4. *In vitro* additions of ASA in physiological concentration to the enzyme assay medium inhibited the AChE activity significantly and the inhibition was an un-competitive type.

5. Reduced activity of the enzyme has been correlated to the increased concentration of ASA in the sciatectomized muscle.

Key words : ascorbic acid | dehydroascorbic acid | sciatectomy | frog gastrocnemius muscle | AChE activity | loss of trophic influence

INTRODUCTION

Recent studies revealed that the release of transmitters in nerve terminals is under the control of ascorbic acid (1, 3, 14). However, it is not clear whether the ascorbic acid has any influence over the AChE activity in the muscle. The ascorbic acid accumulates in the muscle during denervation (8) and the AChE activity changes on time course of denervation (Somasekhar, unpublished data). The acetylcholine content decreases in the muscle after denervation (9). In order to study more precisely, the effects of ascorbic acid and its metabolites on the AChE activity of the muscle and their role in the muscular denervation-atrophy, it is desirable to investigate the *in vitro* effects of ascorbic acid on the AChE activity of the muscles. This paper describes some of the work done in order to achieve these goals.

MATERIAL AND METHOD

Male frogs, *Rana cyanophlictis* in a range of 20–25 g body weight were used for experiments. The unilateral sciatectomy (alternatively right or left) was done as described earlier (10) three months before the gastrocnemius muscles were removed under light anaesthesia with ether. The frogs were maintained in large aquarium tanks, allowing free movement and force-fed every alternate day with sliced earthworms *ad libitum.*

The excised gastrocnemii were weighed immediately and then chilled on ice-cubes before they were used for further experiments. The endogeneous levels of Ascorbic acid (ASA), Dehydroascorbic acid (DHA) and Diketogulonic acid (DKA) in the muscles were estimated colorimetrically according to Roe *et al.* (16).

The AChE of muscles was partially purified through ammonium sulphate fractionation as described by Habibulla and Newburg (5). The partially purified enzyme was dialyzed against glass distilled water. Dialysis was carried out in cellophane tubes (0.5 cm diameter, 15 cm long) at 4°C for 12 h suspended in 2 L distilled water. The dialysing fluid was changed every 3 h. The AChE was assayed colorimetrically according to Hestrin (6). The protein in the enzyme extract was estimated colorimetrically (13) using bovine serum albumin as a standard.

The AChE activity in extraneously added ASA-media was assayed in order to study the effects of ASA on the kinetics of the enzyme. 300 *μg* L-ascorbic acid (product of Sigma Co.) was added to one *ml* of the assay medium for these kinetic studies. This concentration was chosen as the endogenous level of total ASA in the muscle, as the total ASA level in the muscles of laboratory frogs vary between 160–300 *μg*/muscle (8). The Michaelis-Menten kinetics of the AChE were studied both in the presence of ASA as well as in the medium without ASA.

RESULTS

The scheme adopted for purifying the muscle AChE partially in the present study yielded 53.39% protein and 6.35 times greater enzymic activity. The fold purification was found to be about 6.35 and consistent for both the muscle preparations. The dialysis of the ammonium sulfate fractionated protein yielded nearly 1.9-fold higher enzyme activity and about 75.45% protein.

ASA in cells will be oxidized reversibly and enzymatically to unstable DHA; the latter on hydrolysis irreversibly yields DKA (2). In active tissues these three metabolites occur in varying proportions, depending on the extent of oxidations and hydrolysis to

TABLE I : Protein yield and AChE activity in the purification steps of the method adopted (see text for details) in a representative assay.

S. No.	*Step*	*Volume of fraction in ml*	*Protien concentration*		*Enzyme specific activity*	*Fold–purification*
			mg/ml	*Total amount mg*		
1.	Crude homogenate	200	0.457	91.4	10.9	1.0
2.	Ammonium sulphate fraction	100	0.324	64.8	38.8	3.56
3	Ammonium sulphate fraction after dialysis and centrifugation	50	0.244	48.8	69.3	6.35

TABLE II : Endogenous levels of Ascorbic acid metabolites in the gastrocnemius muscle of frogs sciatectomized for 3 months.

Muscle	$\bar{X}$ *µg/g muscle ± S.D. n=6*		
	ASA	*DHA*	*DKA*
Innervated	67.4±10.9 (11.79±1.9)	89.1±25.3 (15.59±4.42)	89.5±28.1 (15.66±4.91)
Sciatectomized	130.6±17.8 (16.95±2.30)	147.1±28.2 (19.12±3.66)	97.5±23.3 (12.67±3.01)
Incidence of change on sciatectomy* (% change over control)	Increase P<0.001	Increase P<0.05	No change

Numbers in parenthesis are the levels per whole muscle ($\bar{X}$ ± S.D., n=6).

*When the concentrations per unit wet weight were compared.

which the cells are subjected. All these three metabolites occur in the gastrocnemius muscle of frog. In the innervated muscle the DHA and DKA concentrations were nearly equal and greater than that of ASA (Table II). Sciatectomy increased the levels of ASA and DHA in the muscle per unit weight basis but not the DKA level. Sciatectomized muscles for 3 months showed 26±2% atrophy (loss of muscle mass) and when expressed on whole muscle basis the ASA level significantly increased (P<0.001), but the DHA and DKA levels did not vary much.

The ASA/DHA ratio in the innervated muscle is 0.753 and in sciatectomized muscle, 0.884. Greater ratios indicate more of ASA concentration existing in the muscle relative

to that of DHA. Sciatectomized muscle showed greater ratios indicating probably poor oxidation of ASA in the muscle. DHA/DKA ratio in the innervated muscle is 0.994 and in sciatectomized muscle, 1.509. Higher ratios indicate, more DHA concentration relative to that of DKA; hence poor hydrolysis of DHA into DKA would have resulted in the muscle.

Table III presents the data on *in vitro* additions of ASA on the AChE activity. It is clear from this table that the sciatectomy decreases the specific activity of the enzyme. Sciatectomy broughtforth about 16% reduction in the enzyme activity. In the presence of ASA, this reduction was more (i.e., about 18%) due to sciatectomy. *In vitro* addition of ASA inhibited the AChE activity considerably irrespective of the nature of the muscle. There was about 20% inhibition of activity in the innervated muscle enzyme and about 24% in the sciatectomized muscle enzyme.

When ASA concentration was varied (from 0 to 300 *μg/ml*) in the assay medium, the degree of inhibition did not vary much ($P > 0.01$). As ASA is known to be a powerful reducing agent (2), it is felt that the inhibition caused here, could be due to the reducing nature of the ASA. But when tested it was found that AChE specific activity was not altered by the additions of other reducing agents like KCN, NADH, NADPH, strontium chloride and mercaptoethanol.

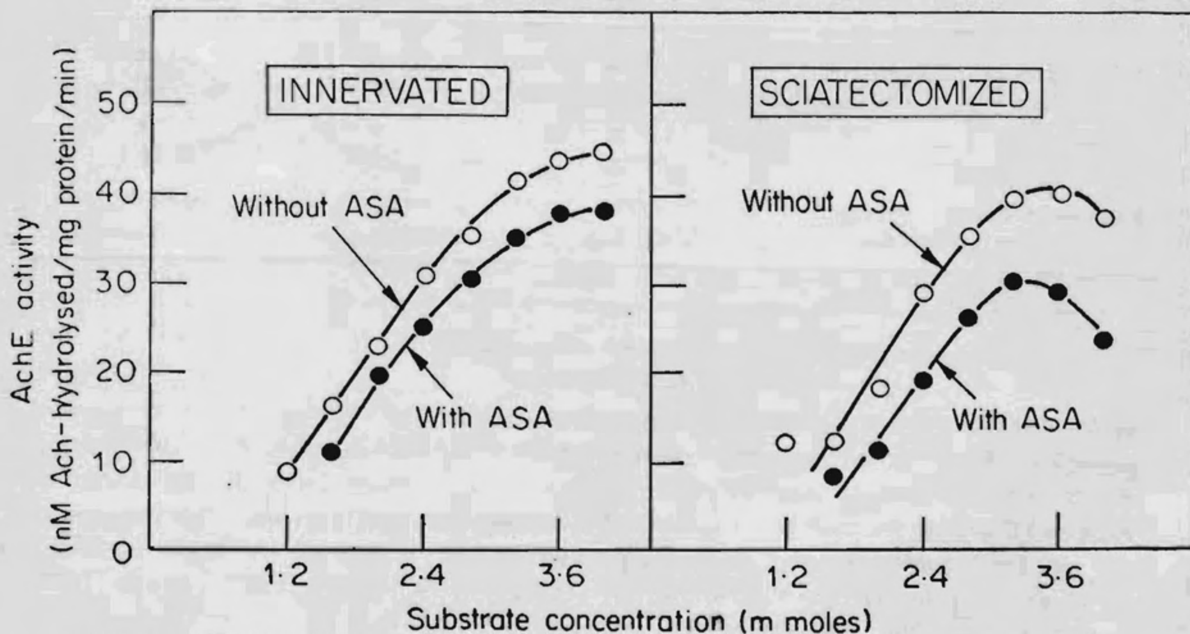

Fig. 1 : Substrate versus rate of enzyme activity profiles for AChE purified from gastrocnemius muscle of frog sciatectomised for 3 months. 300 μg of Ascorbic acid (ASA) was added to whole assay medium to study the influence of ASA on the enzyme activity. The enzyme was assayed at 4 m M Ach, 26°C and pH 7.1.

Figure 1 demonstrates the effect of ACh (substrate) concentration on the muscle AChE activity. Both the enzyme preparations i.e., from innervated as well as from sciatectomized muscles showed a significant inhibition, when the physiological concentration of ASA (300 μg) was added to the whole assay medium. Sciatectomy broughtforth more ASA influence on the enzyme activity. Fig. 1 also illustrates that the sciatectomized muscle-enzyme is substrate inhibited and has a lower substrate optimum than those of the innervated control muscle.

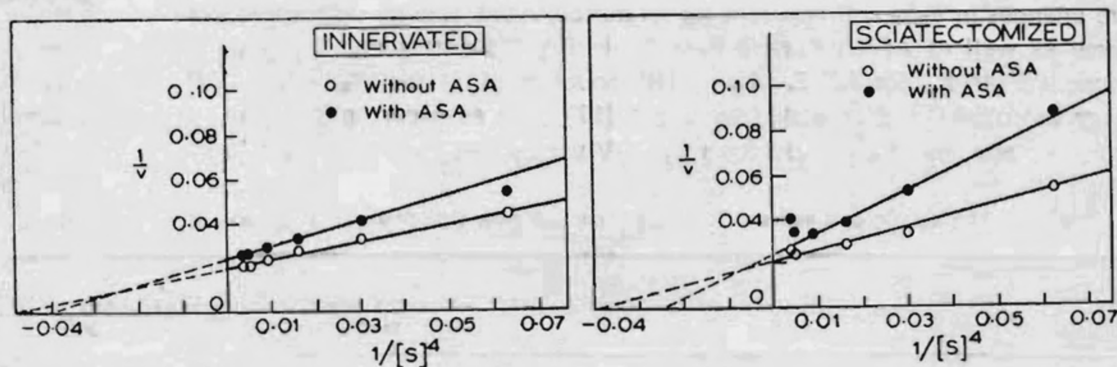

Fig. 2 : 1/v Vs $1/(S)^4$ plots of the data presented in Fig. 1.

TABLE III : Influence of *in vitro* additions of Ascorbic acid on the gastrocnemius AChE* activity of frogs, sciatectomized for 3 months.

Mucle	*AChE activity : nano mole ACh hydrolysed mg protein/minute* ($\overline{x}$ ± S.D.. n=6)		*Incidence of chenge on ASA in vitro additons.*
	Without ASA	With ASA (300 μg/ml)	
Innervated	46.0±2	35.6±1.7	Decrease P<0.001
Sciatectomized	38.8±1.7	29.3±1.6	Decrease P<0.001
Incidence of change on sciatectomy	Decrease P<0.001	Decrease P<0.001	

*assayed at 4 mM ACh, 26°C and pH 7.1.

An attempt to establish Lineweaver-Burk plots and study the kinetics of enzyme for both the muscle enzymes has become futile, because the enzyme is not obeying the Michaelis-Menten rules. The double reciprocal plots were not at all getting linearized. The data in Fig. 1 point out to a clear sigmoid response rather than a linear or regular hyperbolic response of S versus V profile. Therefore the sigmoid kinetics were applied to the data by establishing $1/V$ versus $1/[S]^4$ plots (Fig. 2) and also through Hill equation (17) (Table III). $1/V$ versus $1/[S]^4$ plot was the best linear fit obtained (Fig. 2) to arrive at V_{max} and apparent K_m. As is clear from the linear plots of $1/V$ versus $1/[S]^4$, there are changes in V_{max} and apparent K_m of muscle AChE activity with reference to sciatectomy as well as ASA-influence (see Table IV). Sciatectomy lowered the V_{max} and increased the K_m for AChE. Using Hill equation (17) the K_m values (K′ in Table IV) were calculated and as stated by Segal (17), and as expected they are slightly higher than the apparent K_m derived from the $1/V$ versus $1/[S]^4$ plot (Table IV).

TABLE IV : Kinetic parameters of the AChE purified from the control and sciatectomized frog muscles.

Enzyme	*Vmax**	*K′ calculated from Hill's equation*	*K′ (graphical) (see fig. 2)*
Innervated			
Without ASA	51.28	2.13	1.8
With ASA	42.55	2.23	1.7
Sciatectomized			
Without ASA	47·62	2.17	2.0
With ASA	42.55	2.32	2.0

*read from $1/[S]^4$ vs $1/V$ plot and bear the units: nM ACh hydrolysed/min^{-1} 1/*mg* protein.

K′ is the apparent K_m in mm ACh.

In general, the ASA addition to the assay medium reduced the V_{max} and increased the K_m in both the muscle preparations (Table IV). As high K_m represent the low affinity of the enzyme to the substrate, the ASA seems to lower the affinity of the enzyme. The $1/V$ versus $(1/[S]^4$ plots for both the muscles, show varying kinetic parameters with reference to ASA-inhibition (Fig. 2). Hence the type of inhibition of AChE on control and sciatectomized muscle, in the presence of ASA may be an un-competitive one (17).

DISCUSSION

Dialysis of the partially purified enzyme preparation seems to accelerate the enzymic activity (Table I). Obviously, this illustrates the occurrence of possible dializable inhibitors

in the enzyme obtained after ammonium sulphate fractionation. When examined, we noticed that the ASA is dializable and the dialized enzyme preparation is free from ASA contamination. Studies on such ASA-free preparation, have ruled out the interferences by enzyme activations either due to endogenous levels of the ASA or of its cumulative levels on account of extraneous additions.

The data on ASA/DHA and DHA/DKA ratios suggest the catabolic patterns of the ASA in the sciatectomized muscles. Poor oxidation of ASA in the sciatectomized muscle may have several implications. Poor oxidation of ASA would increase the endogenous level of ASA in the muscle. As ASA is not synthesized in the muscle (12), all the ASA content of the muscle must have its origin from tissue stores like liver, adrenals etc. Krishnamoorthy and Satyam (8) found no changes in the catabolic patterns of ASA in the denervated muscle. The observed differences in the muscle following sciatectomy, therefore show that the latter alters the permeability of the muscle membrane.

Earlier studies in this laboratory substantiated that long-term sciatectomy in frogs results in the atrophy of gastrocnemius muscle (11) and the atrophied musle is characterized by a significant decrease in ACh content and lowered AChE activity as a function of time of sciatectomy (Somasekhar, unpublished data). The results presented in the present study indicated that the AChE of sciatectomized muscle differs in enzyme kinetics; particularly with reference to un-competitive inhibition by ASA. The cause for these changes must be sought in the configurational changes following sciatectomy occurring in AChE molecules. There is evidence now that AChE exists in multimolecular forms which are in turn regulated by innervation patterns (19). Loss of trophic influence (4) effected through sciatectomy might have been responsible for these changes in the kinetics of AChE.

Another feature found in the results is the importance of ASA in the maintenance of ACh activity in the muscle. It is clearly shown that ASA inhibits AChE activity and alter its kinetics. This is particularly interesting to note as ASA is known to promote the activities of many enzyme systems like the phosphatases (12), alkaline phosphatase (7) and inhibit hepatic catalase (18). However, how these inhibition and promotion of enzyme activities by the ASA are effected are not clear.

Chen-Hui and Yoshida (1) reported that ASA is an endogenous factor in releasing ACh from synaptic vesicles. It is thus interesting to note that while the endogenous ASA releases the ACh from synaptic vesicles (*op. cit.*), it inhibits the AChE activity of the muscle (present results).

How the ASA accumulates in the atrophied muscle is not clear. Krishnamoorthy and Satyam (8) suggested that permeability changes occurring at muscle membrane following denervation permit the blood ASA to accumulate inside the sarcoplasm. While this is the only suggestion found to explain the occurrence of higher concentration of

ASA in the atrophic muscle, the increased endogenous synthesis of ASA in the frog muscle tissue following denervation cannot be totally ruled out. It is not known whether the frog can synthesize ASA in its tissue like any other vertebrate or is dependent on dietary resources like rat, and primates (15). But presence of ASA metabolites like DHA and DKA in the muscles shows the existence of active ASA metabolism in frog muscles which are at the fulcrum of trophic influences of the nerve, innervating the gastrocnemius muscle.

ACKNOWLEDGEMENTS

We thank the CSIR for the award of a Senior Research Fellowship to one of us (TS). Our thanks are also due to Dr. R. Narayana, DI BSH, University of Agricultural Sciences, Bangalore for facilities and encouragement.

REFERENCES

1. Chi-Hui, K. and H. Yoshida. Ascorbic acid as an endogeneous factor required for acetylcholine release from the synaptic vesicles. *Japan J. Pharmacol.*, **: 30** 481–492, 1980.
2. Fruton, J.S. and S. Simmonds. Text Book of General Biochemistry. Asia Pub. House, Madras 362, 1973.
3. Greig, M.E. The effect of ascorbic acid in reducting the inhibition of brain metabolism produced by pentabarbitol *in vitro. J. Pharmacol. Exp. Ther.*, **91** : 317–323, 1947.
4. Guth, L. 'Trophic influences of nerve on muscle. *Physiol. Rev.*, **48** : 645–687, 1968.
5. Habibulla, M. and R.W. Newburg. Studies of acetylcholinesterase of the central nervous system of *Galleria mellonella. Insect Biochem.* **3** : 232–242, 1973.
6. Hestrin, S. The reaction of acetylthiochcline and other carboxylic acid derivatives with hydroxylamine and its analytical application. *J. Biol. Chem.*, **180** : 249, 1949.
7. Krishnamoorthy, R.V. Alkaline phosphatase activity during the fatigue of an isolated frog muscle. *Proc. Indian Acad. Scis.* LXX : 1–8, 1969
8. Krishnamoorthy, R.V. and P Satyam. Changes in ascorbic acid content in denervated frog gastrocnemius muscle. *Experentia*, **25** : 264–265, 1969.
9. Krishnamoorthy, R.V. Increased histaminase activity in the atrophic muscle of denervated frog. *Enzyme*, **22** : 73–79, 1977.
10. Krishnamoorthy, R.V., T. Somasekhar and J. Begum. Calcium bindng capacity and calcium sensitive protein content in denervated frog muscles. *Cell Calcium*, **5** : 21–27, 1984.
11. Krishnamoorthy, R.V., M.V.V. Subrahmanyam and T. Somasekhar Degenerative changes in the frog gastrocnemius muscle after long term sciatectomy. *Proc. Indian Acad. Sci.*, 1984 (In Press).
12. Lloyd, B.B. and H.M. Sinclair "Vitamin C In Biochemistry and Physiotogy of Nutrition, Vol. I. (Editors: G.H. Bourne and G.W Kidder), Ch. 11, Academic Press, N.Y., pp. 369–471, 1953.
13. Lowry, O.H., N.J. Rosenbrough, A.L. Farr and R.J. Randall. Protein measurement with the folin phenol reagent. *J. Biol. Chem.*, **193** : 265–275, 1951.
14. Patterson, J.W. and D.W. Mastin. Some effects of dehydro-ascorbic acid on the central nervous system. *Am. J. Physiol.*, **167** : 119–126, 1951.
15. Prosser, C.L. Comparative Animal Physiology. 3rd Ed. W.B. Saunders, Philadelphia, p 362, 1973.
16. Roe, J.H., M.B. Mills, M.J. Osterling and C.M. Damron. Differential method for determination of ascorbic acid, dehydroascorbic acid and diketogulonic acid. In : Methods of Biochemical Analysis. Ed. D. Glick. Vol I. pp. 132–134, 1948.
17. Segal, I. Enzyme Kinetics. Behaviour and Analysis of Rapid Equilibrium and Steady State Enzyme Systems. John Wiley & Sons, New York, pp. 346–464 1975
18. Sridhara, S. and R.V. Krishnamoorthy. Inverse adaptation of ascorbic acid senstive hepatic catalase in the frog *Rana cyanophlictis* during cold adaptation. *Indian J. Exp. Biol.*, **18** : 1123–1126, 1980.
19. Wilson, B.W., M.A. Kaplan, W.C. Merhoff and S.S. Mori. Innervation and the regulation of acetylcholinesterase actvity during the development of normal and dystrophic chick muscles *J. Exp. Zool.*, **174** : 39–54, 1970.