Discussion

The results of the present study indicate that there is a difference not only in the concentration of Ache, but also in the size of the neuromuscular junctions in the muscle fibres of human skeletal muscle. An *ultrastructural study on the neuromuscular junctions* of rat skeletal muscles[5] showed the presence of closely packed deeper junctional folds in the darkly stained junctions and a few, shallower folds in the case of junctions stained pale. Ache is reported to be concentrated in these folds[7] and probably the presence of fewer and shallower folds in 'P' junctions ind cating the lesser amount of Ache present, is responsible for the pale staining of these junctions. The scattered peripheral activity in some of the 'P' junctions could be due to the concentration of Ache at the lower levels of these junctional folds. The pale staining might not be due to the inadequate penetration of the substrate as the 'S' and 'P' type junctions were seen sometimes at the adjacent muscle fibres. The presence of 'P' and 'S' junctions in the same normal muscle may be either be denoting changes due to a constant process of growth and degeneration taking place as there are reports indicating such growth changes taking place in the neuromuscular junctions[8,9], or may represent the different muscle fibre types (slow and fast) present in the same muscle[10,11]. The significant difference between the mean diameter of the 'P' and 'S' junctions noticed in this study suggests that these two types of junctions differ in their intensities as well as areas of Ache activity. Further work is in progress to study the muscle fibre types of human vastus lateralis by histochemical methods, as this may throw more light in this field.

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A NEW TECHNIQUE FOR THE QUICKER SEPARATION OF UNDAMAGED MICROSOMES USING ASCORBIC ACID

S. K. JAGOTA AND H. M. DANI
Department of Biochemistry, Punjab University, Chandigarh 160 014 *(India)*

Abstract

Microsomes can be prepared by treating rat liver post-mitochondrial supernatant with 6 mM ascorbic acid within 20 minutes. Analytical studies on such microsomes have shown that the specific activities of various phosphatases and other integral components are comparable to those obtained from microsomes prepared by time-consuming ultracentrifugal technique. The attached ribosomes on rough membrane vesicles in these preparations also seem to be intact. The method can safely be used for the rapid isolation of hepatic microsomes for studies on drug metabolism, carcinogen screening by degranulatory technique and protein biosynthesis.

Introduction

MICROSOMES can be prepared by either ultracentrifugation[1] or acid precipitation[2] or gel filtration[3] or Ca^{2+} aggregation[4] techniques. But each one of these methods has its own demerits. Preparation of microsomes by ultracentrifugation and gel filtration is time-consuming and might initiate lipid peroxidation which results in degranulation of rough membrane vesicles[5]. High 'g' forces, high hydrostatic pressures

and wall effects during ultracentrifugation also degranulate rough membrane vesicles[6]. Acid precipitation inactivates certain microsomal enzymes[7] and Ca^{2++} aggregation has been reported to activate ribonuclease[8] which might damage free or bound polyribosomes.

During our studies regarding the protective effects of ascorbic acid against carcinogenic degranulation of microsomes (unpublished work), we have discovered another interesting property of ascorbic acid. Addition of ascorbic acid in very low concentrations to post-mitochondrial supernatant enables the isolation of microsomes in a cold centrifuge at low 'g' forces. Our studies on various properties of microsomes, prepared by ascorbic acid show that such preparations consist of vesicles having intact ribosomes and some membrane bound enzymes. The method described in this paper is expected to provide undamaged microsomes as compared to those obtained by previous methods and should be useful for the separation of microsomes from other organs as well.

Methods and Materials

Livers from *ad libitum* fed male albino rats of Kasaulti Strain (weighing 100 to 150 g each) were minced and homogenised in 3 volumes of ST buffer (0·225 M sucrose and 25 mM Tris at pH 7·5) in a Potter-Elvehjem apparatus at 0 to 4° C using a motor driven Teflon Pestle rotated at about 3,000 rev/min in a glass vessel. About 10 passes were used to achieve homogenisation. The homogenate was centrifuged at 10,000 g for 20 min at 4° C in a fixed angle Janetzki 6 × 26 ml rotor. The supernatant was decanted with Pasteur pipettes and centrifuged once more in a similar manner. The post 10,000 g supernatant was diluted with 2·5 volumes of 0·225 M cold ST buffer and brought to 6 mM final concentration with respect to ascorbic acid. The suspension was again centrifuged at 10,000 g for 20 min at 4° C in the same rotor. The pellet was rinsed with 0·225 M ST buffer and resuspended in it before freezing at − 20° C. Degranulation of microsomes was achieved according to a method given by Nolan and Munro[9] employing citrate plus pyrophosphate reagent.

Protein concentrations were estimated by the method of Lowry *et al.*[10] using bovine serum albumin as a standard protein. RNA concentrations were assayed according to a slightly modified method of Munro and Fleck[11] after the original method of Schmidt and Thannhauser[12]. Phospholipids were determined by extracting and purifying membrane lipids by the method of Folch *et al.*[13] followed by phosphorus estimation according to a method described by Ames[14]. Values for phospholipid phosphorus were converted to phospholipid using a factor of 25. Total lipids and cholesterol were estimated according to the methods of Frings and Dunn[15] and Niel Chiamori and Henry[16] respectively. Glucose-6-phosphatase[17], 5'-nucleotidase[18], Na^{+}–K^{+}–Mg^{++}-ATPase[19], alkaline phosphatase[20] and acid phosphatase[21] were assayed according to standard procedures.

Results and Discussion

Figure 1 depicts the microsomal yield obtained by adding different concentrations of ascorbic acid to rat liver post-mitochondrial supernatant. Addition of 1 mM ascorbic acid did not result in any membrane pellet and maximum yield was obtained at 6 mM ascorbic acid concentration which is comparable to the yield obtained by other conventional methods for preparation of microsomes[4,22,23]. Preliminary unpublished work in this laboratory also shows that 6 mM ascorbic acid separates microsomes from rat lung post-mitochondrial supernatant.

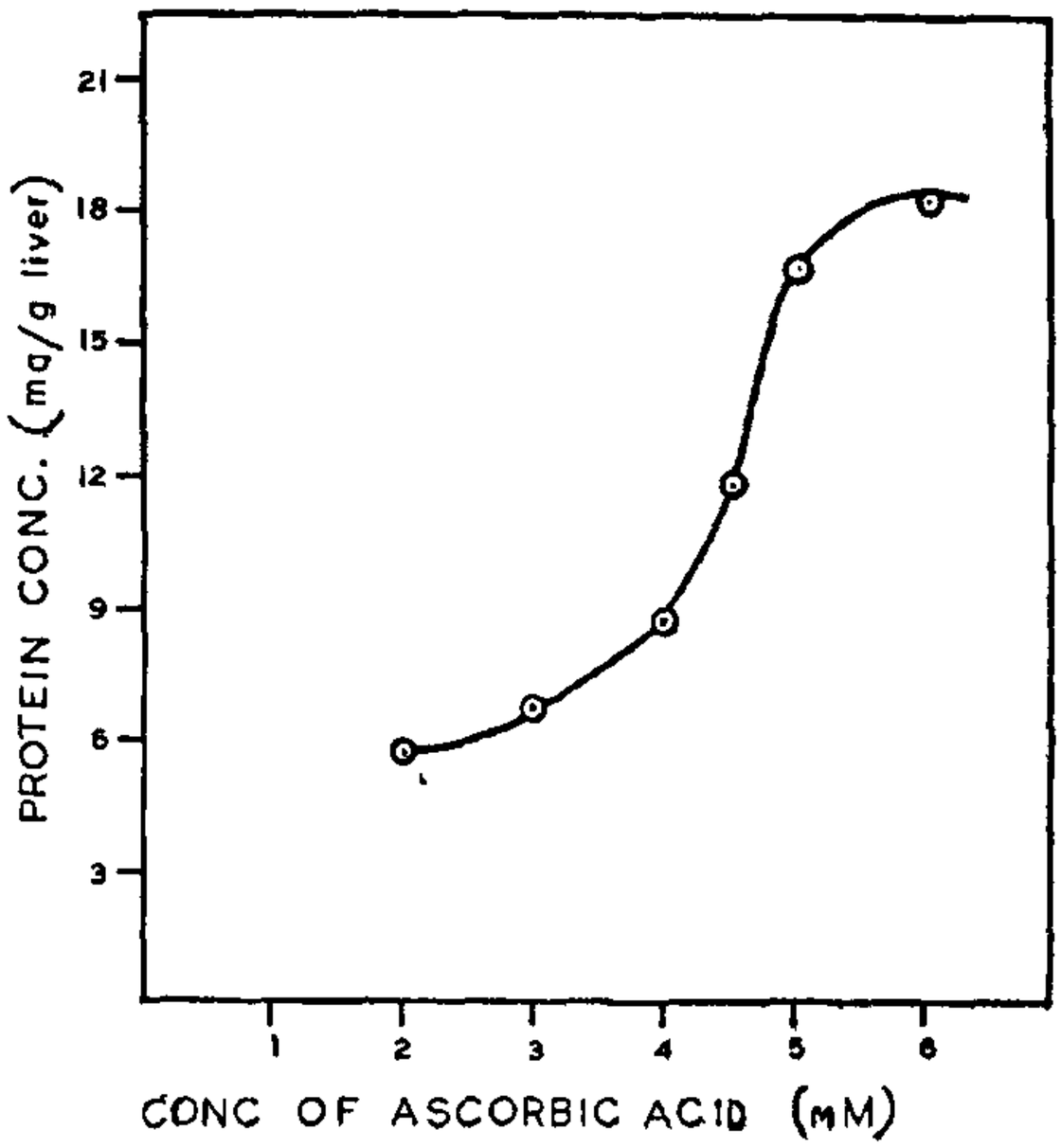

Fig. 1. Agglutination of rat liver microsomes at various concentrations of ascorbic acid.

Data reported in Table I show that RNA/protein ratio of the microsomes is close to that of pure rough membranes[24] in spite of the fact that our microsomal preparations consist of rough and smooth vesicles. Thus it is clear that these microsomes contain more ribosomes per unit area of the membrane surface. The phospholipid and total lipid contents of our microsomal preparations are slightly higher than those of microsomes prepared by Ca^{2++} aggregation method[4] which might be due to variations in the nutritional status of animals used[25]. However, the per cent values of phospholipids and cholesterol in total lipids are identical with those reported in the case of microsomes prepared by ultracentrifugation and calcium aggregation techniques[4]. Degranulation of microsomes by

citrate plus pyrophosphate reagent knocked off 60 to 70% ribosomes both on the basis of RNA/protein and RNA/phospholipid ratios. This observation illustrates that microsomes prepared by ascorbic acid have intact ribosomes. Thus microsomes obtained by ascorbic acid can be safely used for the screening of carcinogens by the degranulatory technique.

TABLE I

Analysis of rat liver microsomes prepared by ascorbic acid

1.	Yield (mg protein/g liver)	15 to 18
2.	RNA/protein ratio	$0{\cdot}156 \pm 0{\cdot}004^a$
3.	Phospholipid/protein ratio	$0{\cdot}425 \pm 0{\cdot}006^a$
4.	RNA/phospholipid ratio	$0{\cdot}365 \pm 0{\cdot}006^a$
5.	Total lipids (mg/mg protein)	$0{\cdot}460 \pm 0{\cdot}003^a$
6.	Per cent of phospholipids in total lipids	$91{\cdot}9 \pm 0{\cdot}347^a$
7.	Per cent of cholesterol in total lipids	$6{\cdot}1 \pm 0{\cdot}003^a$
8.	Glucose-6-phosphatase	$13{\cdot}55 \pm 0{\cdot}048^b$
9.	Alkaline phosphatase	$4{\cdot}10 \pm 0{\cdot}264^b$
10.	5′-nucleotidase	$5{\cdot}32 \pm 0{\cdot}276^b$
11.	Na^+–K^+–Mg^{++} ATPase	$5{\cdot}83 \pm 0{\cdot}287^b$

[a] Mean of 3 experiments ± S.D.

[b] Mean of 3 experiments measuring μ moles of inorganic phosphorus liberated/mg protein/h at 37° C ± S.D.

The possibility of contamination of our microsomes with lysosomes is ruled out from the fact that acid phosphatase activity was completely missing in all preparations. The specific activities of most of the phosphatases are identical with those reported by other authors employing conventional methods for preparation of microsomes[4] except in case of 5′-nucleotidase which is about 20% lower than the reported value[4]. It must be pointed out here that 5′-nucleotidase activity increases in case the membrane vesicles are damaged[26]. Therefore, lower specific activity of 5′-nucleotidase in our preparations further demonstrates that the microsomes are least damaged. Further studies are in progress to understand the mechanism of interaction between ascorbic acid and reticular membranes resulting in their agglutination.

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