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MORPHOLOGICAL AND BIOCHEMICAL CHARACTERIZATION OF LIGHT AND HEAVY MYELIN ISOLATED FROM DEVELOPING RAT BRAIN

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SUMMARY

Myelin from developing rat brains was separated on a discontinuous sucrose gradient into subfractions of two different densities, i.e. light and heavy myelin. Electron photomicrographs showed that heavy myelin consisted primarily of large compacted multilamellar structures with a distinct intraperiod line characteristic of myelin in situ. Light myelin, on the other hand, was composed of small vesicles having a unilamellar structure. Similar to whole myelin, both membrane subfractions were highly enriched in 2′,3′-cyclic nucleotide-3′-phosphohydrolase. The specific activity of the enzyme, however, showed no developmental trend. Both subfractions contained all of the four major proteins characteristic of the whole myelin membrane. There were, however, quantitative differences in the relative distribution of these proteins between light and heavy myelin. Basic protein accounted for 55 % and proteolipid protein for 46 % of the total myelin proteins of light and heavy myelin, respectively. DM-20 (Agrawal, H. C., Burton, R. M., Fishman, M. A., Mitchell, R. F. and Prensky, A. L. (1972) J. Neurochem. 19, 2083–2089) exhibited a developmental "switch" between light and heavy myelin. Light myelin appeared to contain more DM-20 in 15- to 20-day-old rat brain, whereas the concentration of this protein was higher in heavy myelin at subsequent ages studied.

INTRODUCTION

Norton and coworkers [1] first demonstrated that purified myelin isolated from bovine white matter can be separated into two myelin subfractions, namely "light" and "heavy" myelin. Davison and coworkers [2] isolated four myelin subfractions from developing rat brain using a discontinuous sucrose gradient and found differences in the molar ratios of cholesterol, phospholipid and cerebroside. Recently, myelin subfractions from both mature and developing rat brain have been isolated

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on a sucrose or sucrose/ficoll density gradient [3–6]. The lipid to protein ratio, as well as the composition of various lipid classes (cholesterol, phospholipids and cerebrosides) in the myelin subfractions, appears to be different regardless of the isolation procedure employed. Myelin subfractions isolated from 12- to 18-day-old rat brain show some differences in the lipid composition and the activity of the myelin marker enzyme 2′,3′-cyclic nucleotide-3′-phosphohydrolase [7, 8] and pronounced differences in the ratios of the major myelin proteins, i.e. basic proteins, proteolipid protein and DM-20 [6, 9]. Light and heavy myelin exhibit distinct biochemical differences, and thereby present the possibility of different metabolic activities [6, 9].

In this communication we will present evidence that we have isolated relatively pure preparations of light and heavy myelin from both developing and adult rat brain. We found a sustained developmental difference in their morphology and in the ratios between the basic proteins and proteolipid protein. A preliminary report of this work was recently presented [10].

EXPERIMENTAL METHODS

Animals

Sprague-Dawley albino rats bred in our animal colony were used throughout this study. Litters were reduced to eight animals at birth and the animals weaned at 21 days of age. Both male and female rats were used indiscriminately.

Chemicals

All reagents used were of the highest purity available. The sources of chemicals have been described in detail previously [9].

Isolation of light and heavy myelin

The animals were killed in a cold-room maintained at 0–4 °C and the brains removed into a tared pre-cooled beaker. The tissue was finely chopped and rinsed three times with 10 ml of 0.88 M sucrose to remove adhering blood, and then homogenized by hand in 0.88 M sucrose (5 %, w/v, brain homogenate) using a Dounce glass homogenizer. The entire procedure was carried out at 0–4 °C. Light and heavy myelin were isolated as described previously [9] with the following modifications.

(1) Both crude myelin and light and heavy myelin were isolated by centrifugation at 93 000 $\times g$ for 30 min and 60 min, respectively.

(2) At 15 and 20 days of age, myelin from eight rat brains was placed on 6 discontinuous gradients. To compensate for the greater yield of myelin in 30-, 40-, and 60-day-old rats, myelin was isolated from 3–4 rat brains and similarly layered over 6 gradients. This was necessary to avoid overloading the gradient and to insure a clear separation of the light from the heavy myelin.

(3) The cross contamination of light myelin and heavy myelin was checked by relayering the isolated subfractions on the discontinuous sucrose gradient as described previously [9]. After centrifugation for 18 h at 93 000 $\times g$, both subfractions equilibrated at their characteristic position.

All fractions were suspended in double-distilled water and stored at −75 °C for the subsequent measurement of enzymatic activity, or immediately lyophilized for the analysis of proteins.

Enzyme and protein

2',3'-cyclic nucleotide-3'-phosphohydrolase was assayed according to the technique of Kurihara and Tsukada [8], with modifications introduced by Banik and Davison [13] and Agrawal et al. [9]. Total proteins of myelin subfractions were determined according to the procedure of Lowry et al. [14], as modified by Hess and Lewin [15].

Polyacrylamide gel electrophoresis in sodium dodecyl sulfate

Polyacrylamide gel electrophoresis in sodium dodecyl sulfate of light and heavy myelin proteins was carried out after removal of lipids with ether/ethanol (3 : 2, v/v) followed by solubilization in a sample buffer containing 5 mM sodium phosphate, 8 % (w/v) sucrose and 1 % (w/v) sodium dodecyl sulfate (pH 7.2) [9, 12]. The concentration of the solubilized proteins was determined by the technique of Lowry et al. [14] using bovine serum albumin dissolved in sample buffer as the standard. Electrophoresis, staining and destaining were accomplished as described previously [9, 12]. Relative ratios of proteolipid, Wolfgram, the two basic proteins and DM-20 of both light and heavy myelin were calculated from the area under the peak of each protein, which was measured by the traditional height-width calculation. Three to six gels at each age, containing 20 μg of protein, were electrophoresed and scanned at 570 nm in a Gilford Spectrophotometer model 2400 equipped with a linear transport system. The concentration of the small and large basic protein in light and heavy myelin was derived from a calibration curve prepared from homogeneous preparations of these proteins isolated from rat brains. The curve was linear between 1.5 and 10 μg of basic proteins when the gels were stained with 0.25 % (w/v) Coomassie Brilliant Blue (Fig. 1).

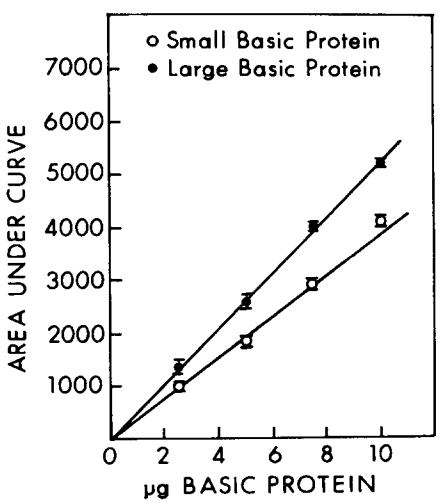

Fig. 1. Calibration curve of rat brain myelin basic proteins. 1.5–10 μg of purified large and small basic protein were electrophoresed in triplicate on 10 % polyacrylamide gels in 0.1 % (w/v) sodium dodecyl sulfate in 25 mM sodium phosphate buffer (pH 7.2). Gels were stained in 0.25 % (w/v) Coomassie Brilliant Blue and destained, and the absorbance was measured at 570 nm. Details of the methods are described in the text.

Electron microscopy

The purity of the light and heavy myelin was monitored by electron microscopy. Samples were taken from the final pellets, fixed in 2.5 % (w/v) glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4) for 12 h at 0–4 °C, and stained with 1 % OsO_4 (w/v) in 0.1 M sodium phosphate buffer for 1 h at 0–4 °C. The pellets were then dehydrated in increasing concentrations of ethanol (25–100 %, v/v). After staining in uranyl acetate (1 %, w/v in 100 % ethanol) for 90 min at room temperature, particles were embedded in Araldite. Ultra-thin sections were cut and examined with a Hitachi HU12 electron microscope.

RESULTS

Maturation of rats was reflected by an increase in body and brain weights (Table I). These changes are similar to the findings of Agrawal et al. [16], Cuzner and Davison [2] and Norton and coworkers [17]. Similarly, there was an increase in the amount of both light and heavy myelin. However, the dry weight of these two subfractions was not monitored for two reasons: (a) the uncertainty involved in quantitatively removing these fractions from the sucrose gradient as would be required for an accurate determination and (b) the possibility of the incomplete removal of sucrose, the presence of which would have given an erroneously high dry weight. However, the amount of protein recovered in both light and heavy myelin was monitored at each age and gives us an index of the relative amount of each fraction present at each stage of development. Light and heavy myelin exhibited a 5- and a 10-fold increase, respectively, in their protein concentrations between 15 and 60 days of age (Table I). It is apparent from Table I that the bulk of total myelin protein is associated with heavy myelin. The amount of protein recovered (μg protein/brain) in the myelin subfractions does not change significantly after 60 days of age (Fujimoto, K. and Agrawal, H. C., unpublished observations).

TABLE I

CHANGE IN PROTEIN CONTENT AND ENZYME ACTIVITY IN MYELIN SUBFRACTION OF DEVELOPING RAT BRAIN

Four or eight rat brains were used, depending on the age, in duplicate at each age, for the isolation of myelin subfractions. Total number of animals used at each age is given in parenthesis. Enzyme activity is given in μmol 2′-AMP produced/mg protein per h. Owing to the paucity of material, enzyme assays were carried out on one sample only, but each determination was done in triplicate.

Age (days)	Body weight (g ± S.D.)	Brain weight (g ± S.D.)	Total Protein (μg/brain ± S.D.)		2′,3′-Cyclic nucleotide-3′-phosphohydrolase	
			Light myelin	Heavy myelin	Light myelin	Heavy myelin
15 (15)	33 ± 5	1.23 ± 0.04	162 ± 24	929 ± 70	1287	930
20 (11)	40 ± 1	1.35 ± 0.07	398 ± 20	2822 ± 234	899	778
30 (9)	85 ± 9	1.38 ± 0.05	463*	8121 ± 723	1380	858
40 (9)	142 ± 8	1.51 ± 0.07	570 ± 120	9555 ± 525	925	861
60 (8)	194 ± 22	1.59 ± 0.14	756 ± 30	9259 ± 54	1283	956

* Part of the sample leaked during water washings.

Morphological characteristics of light and heavy myelin

Both light and heavy myelin fractions from rats of all ages showed a very high degree of purity. The only contaminants were a very few mitochondria. The light myelin subfraction at all ages consisted predominantly of small vesicles having a unilamellate structure. These vesicles were from 0.25 to 0.5 μm in diameter. A few larger whorls and a few composed of two or three lamellae were seen. The heavy myelin subfraction from 15-day-old rats consisted mostly of unilamellate vesicles from 0.5 to 1 μm in diameter. A few profiles having two or three lamellae were seen. Thus the difference between the light and heavy myelin at 15 days of age is the diameter of the vesicles. At 20, 30, 40 and 60 days of age, the heavy myelin consisted mostly of large whorls, although some small vesicles were always present. The large whorls were multilamellate and the number of lamellae remained surprisingly constant with age. At 20 days, the number of lamellae ranged between two and six. For all other ages the range was from six to eight lamellae. These characteristics of light and heavy myelin fractions are illustrated in Fig. 2, in which representative samples of light and heavy myelin from 15- and 40-day-old rats are shown. No difference in the periodicity of light and heavy myelin was detected.

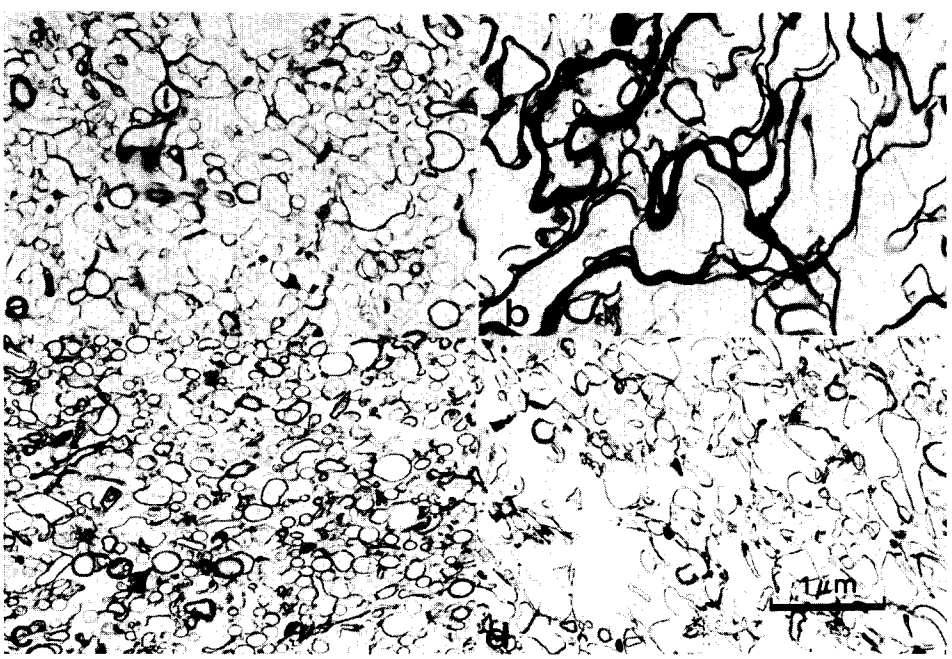

Fig. 2. Electron micrographs showing representative samples of (a) light myelin from 40-day-old rats, (b) heavy myelin from 40-day-old rats, (c) light myelin from 15-day-old rats and (d) heavy myelin from 15-day-old rats. All are at the same magnification.

Enzyme activity

Because 2′,3′-cyclic nucleotide-3′-phosphohydrolase is highly enriched in myelin when compared to other subcellular fractions [7–9, 18, 19], we monitored the activity of this enzyme in both light and heavy myelin. The activity of this enzyme was

much higher in these myelin subfractions than has been reported for microsomes, mitochondria and especially synaptosomes [6–9, 18–20]. It is apparent from Table I that the specific activity of this enzyme did not exhibit a developmental trend in either light or heavy myelin.

Protein profile by polyacrylamide gel electrophoresis in sodium dodecyl sulfate

The four major proteins of rat brain myelin, namely the two basic, proteolipid and Wolfgram proteins, and DM-20 [9, 12, 21–30] were present in light and heavy myelin at all ages studied (Fig. 3). We found a pronounced difference in the relative proportions of proteolipid protein and basic proteins in light and heavy myelin at all ages as shown by the densitometric scans of the gels (Fig. 3). Since an equal amount of the total myelin proteins (20 μg) was applied to the gel at each age, we can compare the ratios of the areas under the curve of each protein for light and heavy myelin

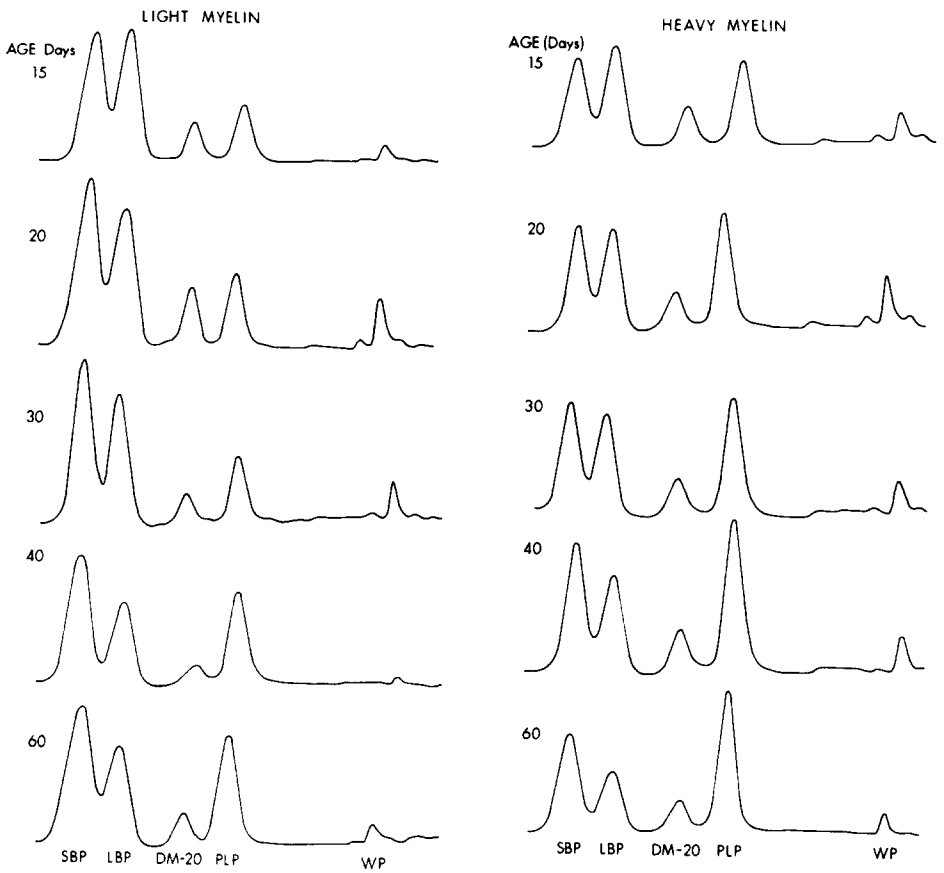

Fig. 3. Densitomeric scan of light and heavy myelin proteins of developing rat brain. 3–4 gels containing 20 μg of proteins at each age were subjected to electrophoresis in sodium dodecyl sulfate. The gels were stained with Coomassie Brilliant Blue and destained as described in the text. The gels were scanned at 570 nm and the area under the peak was measured. SBP, small basic protein; LBP, large basic protein; DM-20; PLP, proteolipid protein; WP, Wolfgram protein.

TABLE II

RATIOS OF AREAS UNDER THE CURVE: LIGHT TO HEAVY MYELIN PROTEINS OF DEVELOPING RAT BRAIN

Results are means ±S.D. The ratios were derived by dividing the area under the peak of each protein of light myelin by the corresponding protein in heavy myelin at each age (see Fig. 3).

Age (days)	Small basic protein	Large basic protein	DM-20	Proteolipid protein	Wolfgram protein
15	1.90±0.23	1.66±0.31	1.12±0.20	0.63±0.07	0.57±0.14
20	1.77±0.22	1.58±0.26	1.23±0.37	0.63±0.15	1.06±0.23
30	1.43±0.20	1.30±0.1	0.59±0.12	0.42±0.056	0.87±0.41
40	1.14±0.04	1.05±0.02	0.49±0.05	0.58±0.08	0.33±0.09
60	1.68±0.14	1.56±0.29	0.5 ±0.06	0.4 ±0.06	0.84±0.11

(Table II). It is apparent from Table II that light myelin is highly enriched in both small and large basic protein, whereas proteolipid protein accounts for a large percent of the total proteins of heavy myelin. For example, the ratios of the small and large basic proteins in light and heavy myelin at fifteen days of age are 1.90 and 1.66, respectively, and 0.63 for proteolipid protein (Table II). In 60-day-old rat brain the ratio for both proteolipid protein and for the small and large basic protein decreased (Table II). These results are in close agreement with the recent findings of Zgorzaleivich et al. [30]. The ratios for the basic proteins and proteolipid protein in light and heavy myelin are always more than one and less than one, respectively, at all stages of development (Table II). It is interesting that DM-20 is present in smaller amounts in light myelin than in heavy myelin for rat brains 30 days or older. However, because of the greater variability, the amount of DM-20 may be the same or slightly greater in light myelin at 15 and 20 days (Table II). Unlike these proteins, Wolfgram protein did not exhibit a definite trend and there was a considerable degree of variabliity from age to age in both myelin subfractions (Table II).

Since encephalitogenic basic protein has been shown to be localized exclusively in central nervous system myelin [9, 31], we also studied the quantitative changes in both small and large basic protein (characteristic of this species) in light and heavy myelin. The developmental changes in the concentration of these two proteins are shown in Table III. It is apparent that the concentration of small basic protein in both light and heavy myelin increases and that large basic protein decreases with development. This is reflected in the decrease and increase of the peak heights of the small and large basic protein as shown in Fig. 3. The two basic proteins constitute more than 55 % of the total protein in light myelin (Table III). Since proteolipid and basic proteins form approximately 80 % of the total myelin proteins, and the concentration of the two basic proteins in heavy myelin is only 35 % of the total myelin proteins, the concentration of proteolipid protein in this fraction has to be much greater than that in light myelin. These assumptions are confirmed by the results of densitometric scanning (Fig. 3) and ratios of the areas under the curve for proteolipid protein (Table II). Although a homogeneous preparation of myelin proteolipid protein isolated by preparative gel electrophoresis was available, we have not been able to prepare a calibration curve for the following reasons.

(1) Storage of this protein in concentrations greater than 3–5 $\mu g/\mu l$ leads to

TABLE III

QUANTITATIVE CHANGES IN THE SMALL AND LARGE BASIC PROTEINS IN DEVELOPING RAT BRAIN

Results are means ±S.D. Results are expressed as μg basic protein/20 μg of total myelin proteins. For the quantitation of the small and large basic proteins, sodium dodecyl sulfate gels were run in triplicate from light and heavy myelin isolated from 2–3 separate fractionations at each age. Number of animals used at each age is given in parentheses. The concentrations of the basic proteins were derived from the calibration curve prepared from small and large basic proteins purified from rat brain myelin (see Fig. 1)

Age (days)	Fraction	Small basic protein	Large basic protein	Basic proteins (% of total myelin proteins)
15 (15)	Light myelin	6.0±0.2	4.0±0.2	50
	Heavy myelin	3.1±0.1	2.6±0.1	28
20 (11)	Light myelin	7.1±0.3	4.4±0.3	57
	Heavy myelin	3.9±0.2	3.0±0.2	34
30 (8)	Light myelin	7.1±0.4	3.7±0.1	54
	Heavy myelin	4.5±0.3	2.8±0.1	36
40 (9)	Light myelin	6.8±0.4	3.1±0.2	49
	Heavy myelin	5.5±0.5	2.8±0.2	39
60 (8)	Light myelin	7.6±0.3	3.4±0.1	55
	Heavy myelin	4.6±0.1	2.4±0.2	35

aggregation as shown by all sodium dodecyl sulfate gel systems used.

(2) If concentration of reducing reagent, e.g. dithiothreitol or 2 mercaptoethanol, exceeds 3 %, aggregation results.

(3) Repeated freezing and thawing of the isolated protein stored in sodium dodecyl sulfate (1 %, w/v) results in aggregation.

Even when these three conditions were controlled, the isolated preparation of proteolipid protein aggregated when 15 μg was applied to the gel, which was subsequently stained with Coomassie Brilliant Blue. The dye binding capacity of this protein is at least six times lower than basic protein. Therefore, we were limited to a calibration curve with a concentration range of 5–10 μg, quantities too low to determine accurately the amount of proteolipid protein in heavy myelin.

DISCUSSION

Because of their distinct morphological differences (Fig. 2) and the definite differences in the concentration of basic and proteolipid proteins (Tables II and III), it is highly likely that the light and heavy myelin subfractions are biological entities and not an artifact of the sucrose density gradient centrifugation. Since the proteins and lipids are found as protein-lipid complexes in the membrane, the selective accumulation, movement or transfer of any one protein into a particular myelin subfraction during isolation is unlikely. Another possibility is that we are isolating myelin subfractions from developing rats where various parts of the brain and different tracts are at different stages of myelination. This is ruled out by the fact that both light and heavy myelin are present in 60-day-old rat brains, when myelination has been

almost completed. In addition, preliminary results obtained in this laboratory indicate that light myelin was essentially absent in the cerebellum and cerebral cortex of 12- to 15-day-old rats. We found, however, that light myelin was present in these same regions at 20 days of age. In contrast, both myelin subfractions were present in the pons-medulla at each age studied (Fujimoto, K. and Agrawal, H. C., unpublished observations). These observations clearly indicate that there may be a continuum of myelin membrane of different biochemical characteristics in the brain. Similarly, these subfractions may reflect different stages of myelination.

We have found that both light and heavy myelin exhibit the typical multilamellar structure characteristic of myelin in situ and that both fractions contain all four of the major myelin proteins. However, differences in protein patterns do exist. The concentration of basic protein in light myelin at all stages of development is much greater than that in heavy myelin. In contrast, proteolipid protein forms the bulk of the protein in the heavy myelin subfractions. The biochemical significance of a differential distribution of these two proteins in light and heavy myelin is unknown.

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