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Ultrastructure of Cytoplasmic Matrix

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Abstract. Because of the limitations inherent in conventional transmission electron microscopy (TEM) using epoxy ultrathin sections for a clear recognition of biological entities having electron densities similar to or lower than that of epoxy resin, embedment-free sectioning for TEM has been developed. Embedment-free section TEM is reliably performed using water-soluble polyethylene glycol (PEG) as a transient embedding media, with subsequent de-embedment of PEG by immersion into water, followed by critical point-drying (CPD) of the embedment-free section. The present author has stressed that this approach clearly discloses structures whose contours and/or appearance are accordingly vague and/or fuzzy in conventional TEM, but that it does not reveal any new artefactual structures. Based on embedment-free section transmission electron microscopy (PEG-TEM), this paper presents five major findings regarding strand- or microtrabecular lattices which have been clearly revealed to occur in the cytoplasmic matrix—an impossibility with conventional TEM. These are 1) the appearance of lattices of different compactness in various cells and in intracellular domains of a given cell; 2) the faithful reproduction from an albumin solution in vitro of strand-lattices with correspondingly increasing compactness following increasing concentrations; 3) the appearance of more compact lattices from gelated gelatin than from solated gelatin at a given concentration in vitro; 4) the appearance of either greater or less lattice-compactness by hyper- or hypo-tonic pre-treatments of cells; and 5) the appearance of certain intracellular proteins confined to the centripetal demilune-domain of centrifuged ganglion cells which is occupied with strand-lattices of a substantial compactness, whereas certain other proteins remained evenly throughout the centripetal and centrifugal domains of the cells. From these findings, questions now arise as to the biological significance of the individual strands themselves in the microtrabecular lattices in PEG-TEM. In contrast, it may be plausible that the appearance of strand-lattices as a whole in a given biological domain represents the presence of soluble proteins; the lattice-compactness indicates the concentration of soluble proteins in the domain, which are in favor of the solution-idea for the aqueous cytoplasm. Furthermore, the appearance of two contiguous domains exhibiting differing degrees of lattice-compactness in a given cell indicates that cytoplasmic proteins are solated in a domain with more compact lattices, whereas they are gelated in the other domain, which is in favor of the non-solution/structure-association or structure-forming idea of the aqueous cytoplasm. Therefore, it is appropriate to understand that the aqueous cytoplasm behaves as a dynamic combination of the solution- and the structured situations. These proposed interpretations need to be confirmed by further studies. If confirmed, the control mechanisms of the localization and movement of intracellular organelles could then be understood on the

basis not only of information about the cytoskeletons but also of cell ultrastructure-related information on the concentration and sol/gel states of intracellular proteins, representing a resurrection of the cytoplasmic sol/gel doctrine.

Keywords: cytoplasmic matrix, sol/gel, protein concentration, embedment-free TEM

1. Introduction

Since the cell (*cella*: "inner chamber" or "small room" in the Greek) was established to be a morphological unit by Robert Hooke in the 17th century, and later by Matthias Schleiden and Theodor Schwann et al in the 19th century (Welch and Clegg, 2010), protoplasm (*prote*: "first, original"; *plasma*: "something formed or molded" in the Greek)– the material basis of life based on the principle that there is a fundamental substance underlying form and function in the world around us and "hylozoism" (*hyle*: "matter"; *zoe*: "life") – became the symbol of the functionality of life at the most basic level, and the protoplasm was known to be a gelatinous dispersion composed mostly of proteins. In an early description by M'Kendrick in the late 19th century, protoplasm was defined commonly as a diaphanous semiliquid, viscous mass, extensible but not elastic, homogenous without structure, without visible organization, having in it numerous granules, and endowed with irritability and contractility. Such early consideration of the cellular composition led to realize that protoplasm must have two primary phases: "fluid" and "solid" (Hardy, 1899; Porter, 1984). By the turn of the 20th century, the expression "cytoplasm" was becoming a popular descriptor of the cellular makeup. This word was coined by Albrecht von Koelliker in the 1860s to designate the fraction of the cell outside the nucleus, but it gradually replaced "protoplasm" in the course of the 20th century. On the other hand, with the rise of biochemistry in the latter part of the 19th century and thereafter, enzymes, the fundamental mechanistic elements executing cellular processes, became the center of attention. The view has been taken as a matter of course that most metabolic activity of the cell results from the superposition of the action of individual enzymes dissolved in an aqueous phase, that is, the cell is merely a homogenous "bag of enzymes" in weak electrolyte solution. In contrast to the former view of "fluid and solid" phases in mixture "non-solution/structured idea", this view of "solution idea" was steadily accelerated by development of a wide variety of purification methods including selective protein precipitation, ion-exchange chromatography, gel filtration, electrophoresis and ultracentrifugation, further in synchrony with the prosperity of molecular biology in late 20th century up to now. As a result, few attentions have recently been paid to the nature of cytoplasmic domains, the cytoplasmic matrix, occupying among such visible structures as organelles and cytoskeletons , (Porter, 1984; Satir, 1984), although the signal transduction pathways are theoretically included in the cytoplasmic domains.

It was KR Porter who challenged the "bag of enzymes" view for the cell in mid and late 20th century (Porter, 1945; Buckley and Porter, 1975; Wolosewick and Porter, 1979; Porter, 1987). Using a high voltage (1000 kV) transmission electron microscope (HV-TEM) on fixed, unembedded, processed in critical point-drying (CPD) and whole-mounted cultured cells, he described the presence of an extensive network of strands (~7 nm in diameter and of variable length) which ramified throughout the cell, seemingly connecting all the formed elements including the three cytoskeletons. He proposed that the strands termed "microtrabeculae or microtrabecular strands" represent the solid phase of the cytoplasm while the intertrabecular spaces represent the fluid phase of it. As Porter stated (Wolosewick and Porter, 1979), the question of what is artifact and what is, is a persistent one in electron microscopy, especially where micrographs depict what are essentially new or unexplored structures. This was the case for the microtrabeculae. In the strictest sense, of course, the content of the micrograph images is all artifact where the usual procedures are employed, due to the unavaidable dehydration. Empirically, several criteria for reality are pointed out such as the constant, consistent and universal appearance, some regularities in appearance/alignment, and stimulus-induced reasonable

changes which can be understood by related scientific knowledges (Kondo, 2006; 2008). In this regard, the constant occurrence of the microtrabecular lattice (MTL) was confirmed by various fixation methods including freeze-drying (FD) or freeze-substitution (FS), although the variety in the diameter and length of the microtrabecular strands is not compatible with the regularity in appearance/alignment. However, the reality of MTL has hardly been accepted, believing that MTL was an artifact of fixation or critical point-drying resulting in the deposition of previously "soluble" proteins on other cell components, as well as the formation of artificial protein-protein aggregates.

2. Polyethylene glycol (PEG)-embedded and postsectioning de-embedded TEM

In order to examine whether or not MTL occur also in any tissue cells in the same way as cultured cells, a novel method of prototype was developed by Porter and his group (Wolosewick, 1980), in which fixed tissue blocks are embedded in polyethylene glycol (PEG) having high water-solubility and their sections are de-embedded by dipping into water, processed in CPD, and observed in ordinary TEM under 100 kV. This is termed PEG-TEM in this article. Details of preparation procedures for PEG-TEM can be found in previous reports by the present author (Kondo, 1984a; 1984b) and a brief schematic drawing of this method is shown in figure 1. With this method, the universal occurrence of MTL, which is another criterion for reality of newly visible formed-elements, has been confirmed in any tissue cells (Kondo, 1984a; 1984b; 1985; 1987; 1987b; 1995; 2003; 2006; 2008; 2011; Kondo et al., 1980; 1982; 1983). Furthermore, since MTL was confirmed to occur in the same pattern as PEG-TEM in CPD-processed sections on a cryostat of several fixed tissue cells immersed in sucrose-solutions, that is, water-embedded and subsequently –de-embedded and critical-point dried sections (Buckley 1975; Buckley and Porter, 1975; Kondo, 2006), the possibility was ruled out that PEG-embedding and subsequent de-embedding themselves could produce MTL.

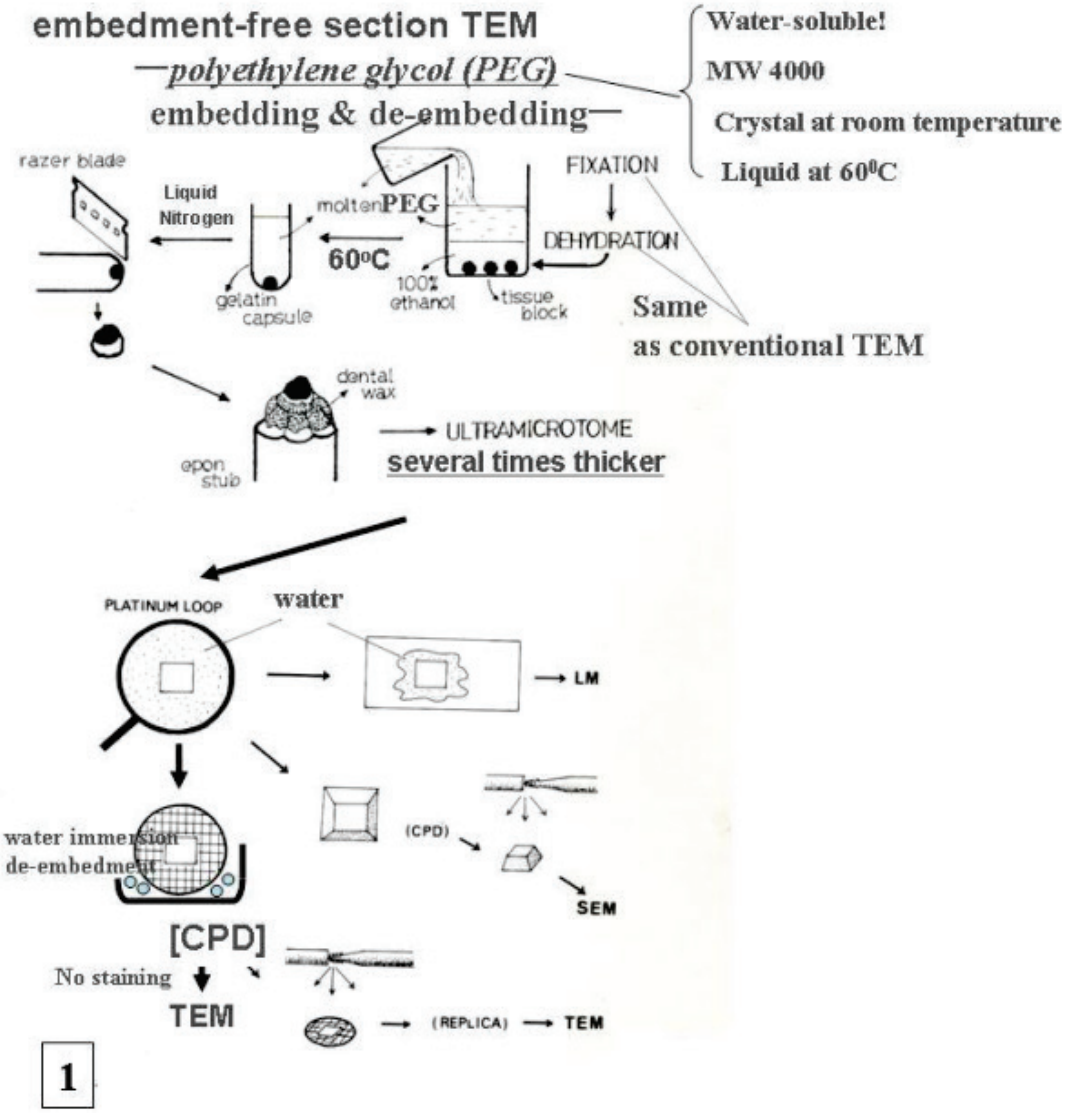

Figure 1. Schematic drawing of embedment-free section TEM. Water-soluble polyethylene glycol (PEG) of molecular weight (MW) 4000 is used as embedding & subsequently de-embedding media. Specimens are fixed with aldehyde and OsO4 in the same way as conventional epoxy TEM and are infiltrated in molten PEG at 60oC and embedded in PEG contained in gelatin capsules at liquid nitrogen temperature. PEG-embedded specimens are trimmed with razer blades and mounted on epon stubs with molten dental wax or PEG. Sections, several times thicker than conventional epoxy sections, are made on an ultramicrotome with dry glass knifes and are picked up with water-filled platinum loops and transferred on formvar film-covered mesh grids. Sections on the grids are dipped in water to be de-embedded, are dehydrated on critical point-drying (CPD) and are observed in TEM under 100 kV without uranium and lead staining. In addition, PEG-sections, when mounted on glasses slides, are observed with regular staining in light microscopy. PEG-tissue blocks, after de-embedded by dipping as a whole in water, are observed in scanning electron microscopy (SEM), while de-embedded sections on grids, after shadowed with platinum and carbon and replicated, are observed in TEM**.**

3. Authenticity and reliability of methodology PEG-TEM

A key question in the skepticism on MTL, in spite of the confirmation of its constant and universal appearance, was how much images in PEG-TEM correspond to those in TEM by other methodologies.

3.1. Comparison with conventional epon-embedded TEM

Before comparison, it is meaningful first to review briefly about how formed elements of cells/tissues are recognized as biologically meaningful entities in the conventional TEM using epoxy resins as embedding media. The extent of the electron scattering of observation targets — after binding with osmium in the fixation and with heavy metals such as uranium and lead in TEM staining — has to surpass sufficiently that of the epoxy embedding resin for clear view of the contours of observation-targets. On the other hand, observation-targets with an electron scattering property similar to or below that of the embedment are not clearly discernible and have thus unavoidably been not worth attention and neglected in conventional TEM. A good example for these targets is the description of images showing vague networks or meshworks of fuzzy or flocculent strands between neurofilaments and neurotubules in the axoplasm in conventional epon-embedded TEM (Metuzals, 1969; Tani and Ametani, 1970; Yamada et al., 1971; Burton et al., 1973). Although those authors struggled to enhance by employing various stainings the appearance of vague networks and to relate them to the microscopic appearance of possible mechanisms for axoplasmic transport, it cannot be said that the enhancement was successful and most of their contemporaries were convinced or even interested in the networks. Repeated to say, the cytoplasmic domain occupied by the vague networks in the axoplasm represents the cytoplasmic matrix of axons. Considering that the clear visibility of observation-targets requires for their electron scattering extent to sufficiently surpass that of epon, it was reasonable to expect a way of epon-deletion. Although sodium hydroxide was already used by some to partially delete epoxy embedding media from epon sections for certain histochemical stainings in light microscopy, the chemical simultaneously dissolves biological materials more or less and it is clear that the treatment of epoxy sections with the chemical is not appropriate for the present purpose to enhance the appearance of observation-targets. It is thus clear that such embedment-free TEMs developed by Porter as HV-TEM of non-embedded and whole-mounted cultured cells and 100 kV-TEM of PEG-processed tissue-cells, fits well the purpose in terms of the eventual absence of epon in observation-specimens. As expected, all formed components in specimens exhibit sufficiently high contrasts to delineate clearly their contours even without staining by uranium or lead. When clearly viewed strand-networks (MTL) in axons processed through PEG- TEM are thus compared with the vague networks of flocculent strands in conventional epoxy TEM, it is easy to judge that both of the images correspond faithfully to each other (figure 2).

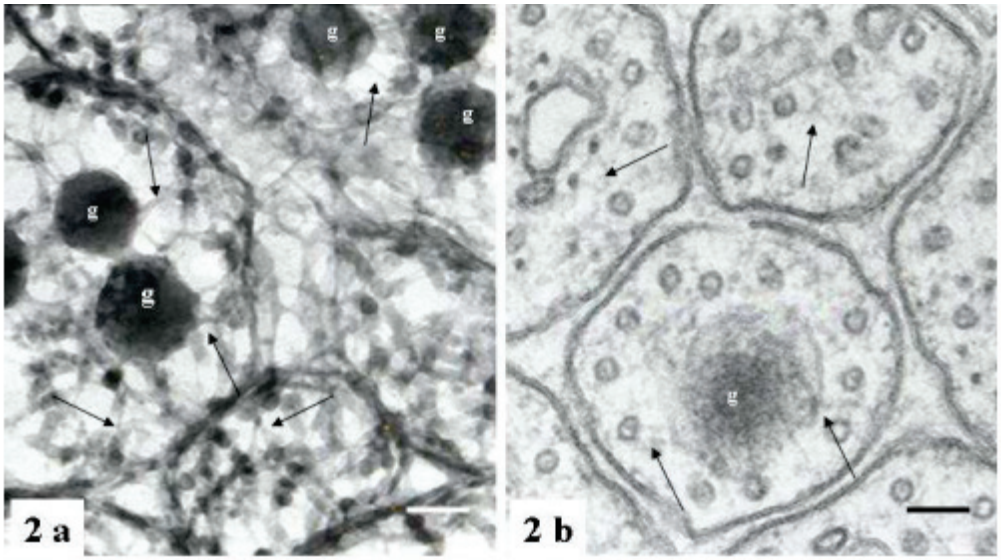

Figure 2. Pituitary neurosecretory axons of rats in PEG-TEM (Figure 2a) and conventional epoxy TEM (Figure 2b).
Distinct strands with lower electron density form microtrabecular lattices (MTL) of strands (arrows) occupying the cytoplasmic matrix between membranous organe including neurosecretory granules (g) and neurotubules and neurofilaments in PEG-TEM, while flocculent or fuzzy and ill-defined strands (arrows) are present in the cytoplasmic matrix in epoxy sections. Note well correspondence of strands between the two micrographs. Bar:0.1um.

Another unique methodological step in the embedment-free TEMs different from the conventional TEM is that specimens are treated with CPD. The CPD is widely used for scanning electron microscopy (SEM). Resulting high-resolution SEM images of cells and tissues are well established with acceptance of the fact that changes in the dimension of any observation-targets occur in an isotropic way (= evenly in all direction without any distortions) when compared with corresponding images in conventional TEM and even with hydrated targets (Boyde et al., 1977; Gusnard and Kirshner, 1977; Billings-Gagliardi et al., 1978; Wollwber et al., 1981; Nordestgaard and Rostgaard, 1985; Eskelinen and Saukko, 1983; Lindroth et al., 1988; Weiss, 1988). Such isotropic changes were noticed to occur also in PEG-TEM due to the employment of CPD. In this regard, it should be noted that such isotropic dimensional changes also occur in the rapid-freezing (RF) and deep-etched (DE) replica (RF-DE-R) TEM. This is rather ironical because a strong skeptism on MTL was expressed by researchers familiar with RF-DE-R mainly based on its original freezing as the fixation but not on any chemical fixatives whose basis is the cross-linking of proteins (Heuser and Kirshner, 1980; Heuser, 2003). Therefore, more details are discussed in the next section.

3.2. Comparison with images in RF-DE-R-TEM

As examples for the isotropic dimensional change by RF-DE-R-TEM, please note that the intracellular synaptic vesicles and the extracellular synaptic clefts appear larger in size and the postsynaptic processes of muscle cells decrease in width correspondingly in the neuromuscular junction processed for RF-DE-R (see figures 1, 2 in Hirokawa and Heuser, 1982). This result leads us to conclude as follows: 1) There presently is no objection to the superiority of RF to any other fixation methods for stop of the living activity of biological specimens. 2) Whenever biological specimens after CPD, FD or DE, all of which are essentially equivalent to dehydration, are eventually exposed to the pneumatic phase, it is highly possible that the occurrence of the isotropic changes in the dimension is unavoidable, regardless of high or low pressure circumstances in cases of CPD or FD/DE, respectively. This

situation is different from the exposure to or immersion in such liquid or solid phases as liquid-mounting media in conventional light microscopy or epoxy embedment in conventional EM, respectively, In spite of this feature, there have been very few studies pointing out the isotropic changes in specimens prepared by RF-DE-R. Therefore, the strongly negative and dominant criticism concerning MTL appears to have been due to the excessively high evaluation of RF-DE-R as a way to represent the living state — though actually overlooking such a feature as the isotropic dimensional changes common to PEG-TEM. It should also be noted that RF after conventional chemical fixation and subsequent immersion in anti-freezing substrates, instead of original direct RF without chemical fixation, has often been employed in a considerable number of studies employing RF-DE-R. It is because of the difficulty in obtaining preservation at adequate levels of biological specimens when RF without anti-freezing treatment after chemical fixation is employed. However, such a procedure without original direct freezing is regarded as the abandonment of the sole merit, the excellent stop/fixation of living activities, of RF-DE-R over PEG-TEM, resulting in ironical and unconscious equality of PEG-TEM images to those by RF-DE-R.

Additional example of the enhanced appearance of observation-targets in PEG-TEM as compared with the conventional TEM is the clear appreciation of en-face images of the diaphragms of endothelial fenestrae which are composed of radially arranged fibrils and sheets converging in a central knob (Stan, 2007; Ioannidou et al., 2006). This feature was originally observed distinctly by RF-DE-R (Bearer and Orci, 1985), but hardly been appreciated in conventional TEM due to the interference of visibility by epoxy embedding media (figure 3). This clear viewing gives an additional support for the authenticity of PEG-TEM. Needless to say, the intracellular as well as intercellular organizations of any tissue cells in PEG-TEM at lower magnification are well maintained in the same way as in conventional TEM (see figures 1-4 in Kondo, 2008).

All in all, consequently, it is reasonable to conclude that the ultrastructure of biological specimens in embedment-free TEM including PEG-EM is acceptable as comparable to those in conventional TEM and RF-DE-R-TEM. It is clear that the methodological criticism on PEG-TEM and the way to observe non-embedded whole-mounted culture cells in HV-TEM is meaningless until the methodological evolution makes it possible to permit TEM observation of biological specimens in the hydrated state like the *in vivo* situation.

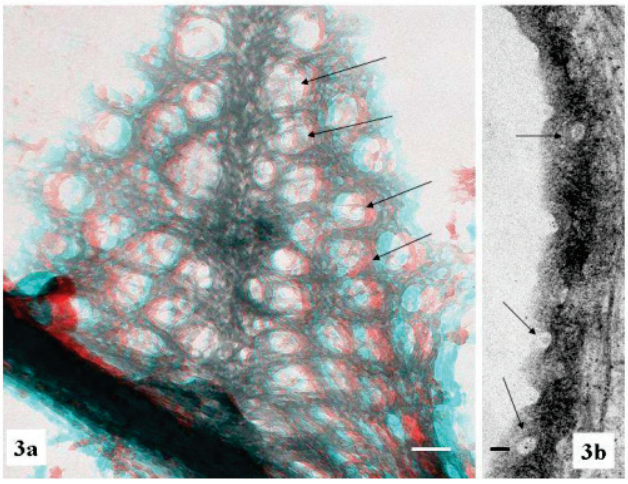

Figure 3. En-face view of endothelial fenestrae in tangential section in PEG-TEM 3a; see with a red/ blue glass for 3D viewing) and in conventional TEM (3b).
Note clear viewing of fenestral diaphragms (arrows) composed of radially arranged fibrils and sheets converging in a central knob in PEG-TEM, in contrast to vague appearance of fenestrae (arrows) in conventional TEM. Bar:0.1um.

4. Disparate lattice compactness in different cells and in domains of a given cell

Different tissue cells exhibit a wide variety in the compactness of the strand-lattices, depending on the combination of varying lengths and thicknesses of the strands. Cells exhibiting loose lattices include neuronal axons, absorptive epithelial cells of large intestines; those exhibiting moderately compact lattices include Schwann cells and hepatocytes those exhibiting highly compact lattices include splenocytes, and adipocytes. Erythrocytes exhibit no lattices of strands but take on a homogenous appearance, which may be regarded as representing an extreme case of highly compact lattices (figures 4 - 6).

The lattice-compactness may differ even within one and the same cell. For example, strand-lattices in cytoplasmic domains occupied by massive glycogen granules in hepatocytes (figures 5, 6, also see figure 12 in Kondo, 1984b) are much looser than those in remaining major domains. In neuronal perikarya, the lattices in cytoplasmic domains occupied by Nissl bodies are more compact than those in their non-Nissl peripheral domains which are similar to those in axons (figure 7).

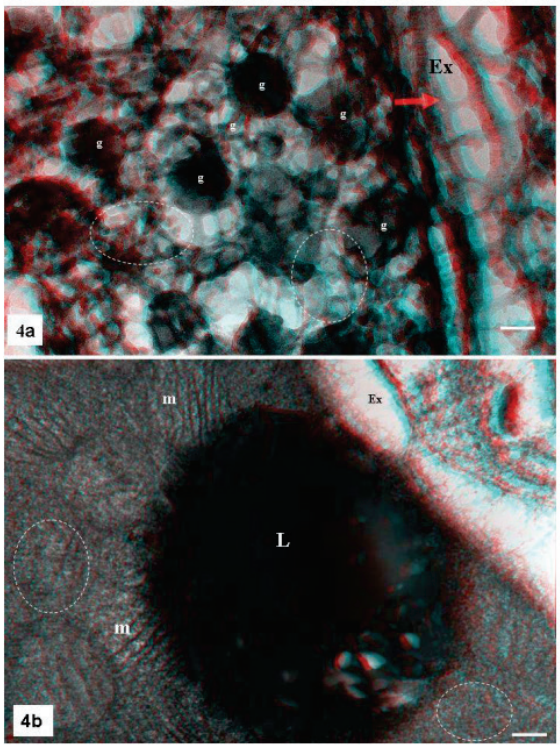

Figure 4. Loose (4a) and highly dense (4b) compactnesses of MTL in hypophyseal neurosecretory axon (4a) and adipocyte (4b) in PEG-TEM (see with a red/ blue glass for 3D viewing).
Compare encircled areas in terms of the compactness between the two micrographs. Ex: extracellular space containing collagen fibrils (arrows); g: neurosecretory granules; L:lipid droplet; m:mitochondria. Bar:0.1um.

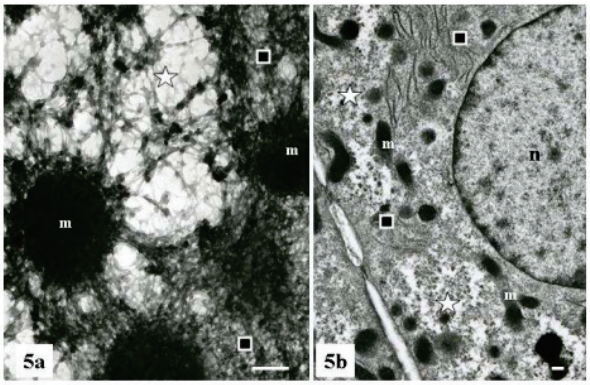

Figure 5. Loose MTL in glycogen (asterisks) areas in contrast to relatively dense MTL (black rectangles) in non-glycogen areas of hepatocytes (5a) and their lowermagnification view (5b) in PEG-TEM (5a). Bar:0.1um.

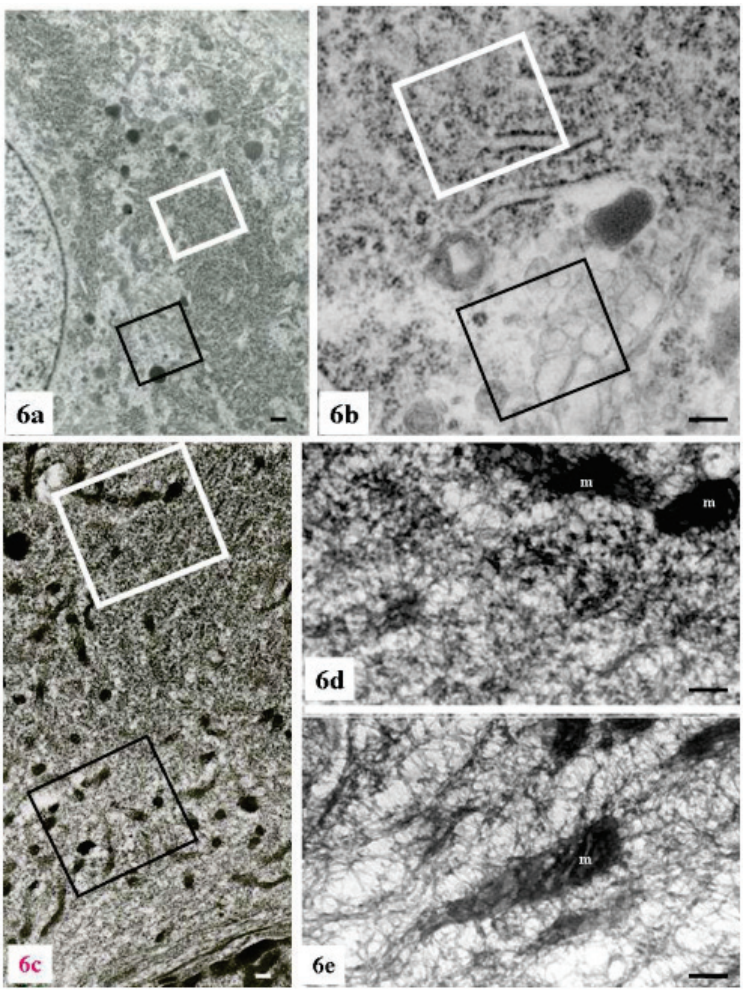

Figure 6. Neuronal perikarya in conventional TEM (6a, 6b) and in PEG-TEM (6c, 6d, 6e). Areas enclosed in white and black r ectangles represent Nissl and non-Nissl areas, respectively. In higher magnification micrographs of Nissl and non-Nissl areas(6b, 6d, 6e), dense and loose compactnesses of MTL are noted, respectively. Bar:0.1um

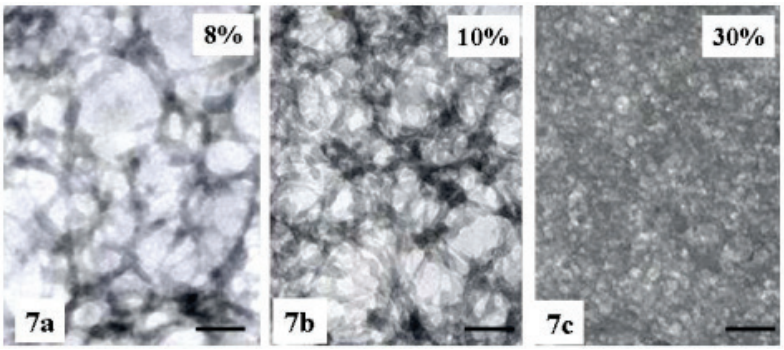

Figure 7. Bovine serum albumin solutions at 8% (7a), 10% (7b) and 30% (7c) in PEG-TEM. Note more compact lattices at higher concentrations. Bar:0.1um.

5. Putative strand lattices in artificial protein solutions

As the next step, on the simulation to the "bag of enzymes/solution" idea, solutions of bovine serum albumin (BSA) at various concentrations as well as residual blood plasma are fixed by gentle superimposition of glutaraldehyde fixative to make a niveau at the interface between the two solutions, and resulting fixed BSA are processed for PEG-TEM as well as conventional TEM. Results of this experiment revealed strand lattices of increasing compactness to occur in proportion to the increases in concentration of the BSA solution in PEG-EM (figure 7). The compactness of lattices at 7-8 % BSA, for example, is similar to that of residual blood plasma whose concentration has been described to be about 7-8% (Wintrobe et al., 1981), and similar to that of the cytoplasmic matrix of neuronal axons that are gouped into cells exhibiting loose lattices. On the other hand, highly compact lattices, which are similar to those of hepatocytes and splenocytes that are grouped into cells exhibiting compact lattices, are seen in fixed BSAs at 10-15%. At more than 40% of the concentration, the BSA solution appears homogenously electron-dense without any signs of lattices in PEG-TEM, whose appearance is quite similar to that of erythrocytes in PEG-TEM. In accord with these similarities, the concentration of hemoglobin in erythrocytes has been reported to be more than 30% (Wintrobe et al., 1981).

The concentration-dependent differences in the compactness of lattices also hold true for another protein, gelatin, which is well known to change its states of sol and gel by varying its temperature. When a solution of gelatin at a given concentration is fixed at 60 ^{o}C and observed in PEG-TEM, its resulting compactness corresponds well to that of BSA at the given concentration. On the other hand, when a solution of gelatin at a given concentration is changed into a gelated state by cooling and subsequently fixed and observed in PEG-TEM, the resulting lattices are more compact than those of the solated gelatin at the same concentration (figure 8).

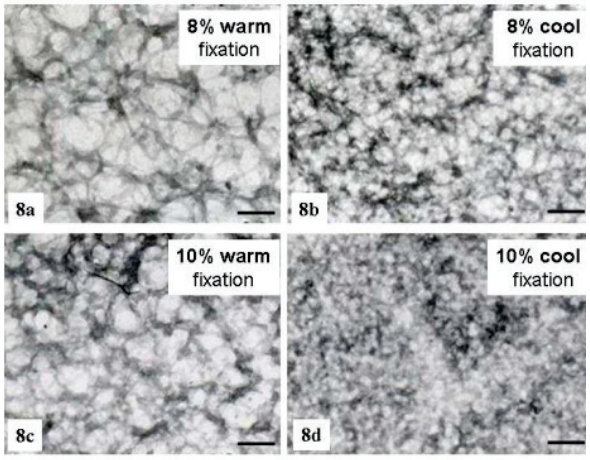

Figure 8. PEG-TEM of gelatin at 8% (8a, 8b) and 10%(8c, 8d) fixed in a state of sol at warm (8a, 8c) and in a state of gel at cold (8b, 8d) temperatures.
Note marked increase in compactness of lattices from sol to gel at both 8% and 10%. Also note similarity in lattice-compactness between gelatin sol and BSA (Fig 7) at a given concentrations. Bar:0.1um.

6. Effects of extracellular condition change on microtrabecular lattices

6.1. Osmolarity-dependent Changes in lattice compactness

The similarity of lattices in PEG-TEM between BSA solutions and the cytoplasmic matrices of some cells, and the changes in the lattice-compactness depending on the BSA concentration led us to examine the ultrastructure in PEG-TEM of cells under different osmolarities and detergent immersion before fixation, because the pretreatments are known to change the concentration of intracellular soluble proteins. Erythrocytes, when briefly (<30 seconds) immersed in hypotonic solutions and subsequently fixed and processed for PEG-TEM, clearly exhibit lattice structures with degrees of compactness roughly corresponding to those of BSA solutions at concentrations from 30 % to 10 % dependent on the duration of immersion and decreasing degrees of the hypotonicity (figures 9a,b). This change from a homogenous to a lattice-form appearance is reversible, and the erythrocytes appear homogenously dense again after being returned to the isotonic solution. Similar changes occur in peritoneal free cells as well as tissue cells, and the brief pretreatment with hypotonic solutions induces the appearance of looser lattices than those of given cells incubated in the isotonic solution. In contrast, the cytoplasmic lattices become more compact even to homogenously dense when cells have been briefly incubated in hypertonic solutions in PEG-TEM (figures 9c,d; also see figures 8, 10, 11 in Kondo, 1995). Those cells with looser or compact lattices after the osmolarity changes in PEG-TEM appear either less or more electron-dense, respectively, than the original cells in conventional TEM. On the other hand, when peritoneal free cells and tissue cells are pretreated with detergents, the compactness of the lattices becomes much looser as the duration of the pretreatment is longer, as already reported by Schliwa et al. (Schliwa, 1981).

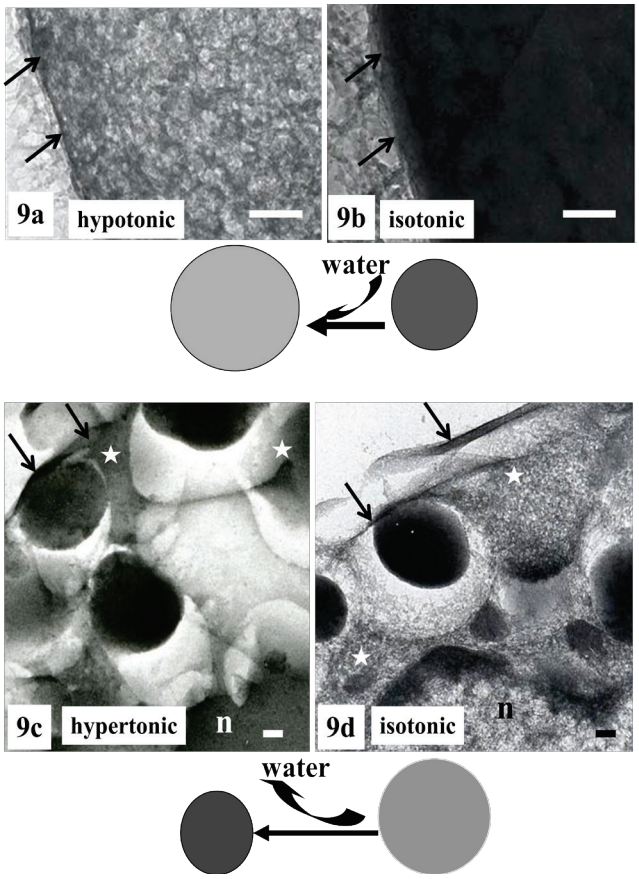

Figure 9. Changes in compactness of MTL in erythrocyte (9a, 9b) and peritoneal mast cell (9c, 9d) under different osmolarity-circumstances in PEG-TEM.
According to general idea, hypotonic treatment induces water infiltration into cells, resulting in enlargement of cell size and decrease of protein concentration, while hypertonic treatment induces water leakage from cell, resulting in shrinkage of cell size and increase of protein concentration as schematically shown individually under Figs 9ab, and under 9c d, respectively. In accord with the general idea, isotonic erythrocyte exhibits homogenously dense interior without lattices, whereas lattice, though relatively dense, appears in hypotonic erythrocyte. On the other hand, isotonic mast cell exhibits relatively dense lattice in cytoplasmic matrix (asterisks), whereas hypertonic mast cell exhibits homogenously dense cytoplasmic matrix (asterisks). Arrows indicate plasma membranes. n: nucleus. Bar:0.1um.

6.2. No distinct data for changes of lattices by various external physiological stimuli to alter cell activities

In order for any newly seen formed components to be identified as authentic entities, in addition to their constant, consistent and universal occurrence discussed in sections 1 and 2, it is also important to examine whether or not the formed components, under appropriate cell biological manipulations, respond in such a reasonable way as expected from our present understanding of cell biology.

In this regard, Porter and his colleagues examined the effects of low temperature, Mg2+, and some chemical reagents including thyrotropin on the ultrastructure of the microtrabecular strands in whole-mounted and cultured cells by CPD and high-voltage TEM (Porter, 1978; 1987; Luby and Porter, 1980; Westermark and Porter, 1982). Although various changes were reported by Porter and his group to occur in terms of contour of lattice-strands, especially in melanophores under pigment migration stimuli, no confirmative data by others were available. In contrast, the present author has examined in PEG-TEM adrenal chromaffin cells under various experimental conditions which are well known to induce marked changes in secretory activities--such as exposure to perfusates at low temperature or containing high or low Ca, high acetylcholine, or electrical stimulation of the splanchnic nerve, and no distinct evidence of significant changes in the morphology of the lattices has so far been obtained with certainty (Kondo, 1989; 1995; 2003). Only an example which has so far been obtained by the present author is a more compact MTL in some, if not all, of the neurohypophyseal axons of mice under severe dehydration as compared with the axon of normal mice (see figure 8 in Kondo, 2010).

7. New interpretations of compactness of MTL

7.1. Protein concentration in cytoplasmic matrix

First, because BSA solutions exhibit lattices in PEG-TEM, individual microtrabecular strands themselves may be regarded as having no biological significances, different from the original view by Porter as cited in Section 1.

Second, in spite of the negative conclusion on the significance of individual strands themselves, because of the concentration-dependent compactness of lattices from BSA solutions, and because of the close correspondence of the BSA-lattice compactness to that of MTL of various cells under pretreatment by different osmolarities or detergents which are supposed to change the intracellular protein concentration, MTL as a whole seems to be regarded as representing the protein concentration in the cytoplasmic matrix of a given cell. Based on this interpretation, the variety of compactness of MTL in different tissue-cells can be interpreted as representing the difference in the cytoplasmic protein concentration among the cells. In other words, a more compact lattice in the cytoplasmic matrix of one cell than in others represents a higher protein concentration of cytoplasmic proteins in that cell, whereas a homogenous appearance without a clear recognition of lattices of the cytoplasmic matrix of such cells as erythrocytes represents a protein concentration of more than 40 %. It is known that the incubation of cells in a hypertonic or hypotonic solution induces an increase or a decrease, respectively, of the cytoplasmic protein concentration, and that the pretreatment of cells with detergents decreases the cytoplasmic protein concentration by a release of the proteins through the membrane perforation. Therefore, the proposed interpretation is compatible with all the changes in the compactness of MTL by immersion in incubation media of different osmolarities and in detergents as demonstrated in Section 6.

7.2. Sol/gel states in cytoplasmic matrix

The third interpretation of the strand-lattices is that two MTLs with different compactnesses being directly contagious to each other without intercalating membranous boundaries within a given cell may represent the co-existence of proteins in sol and gel states in contiguity in the cytoplasmic matrix (Kondo, 2010). Examples of this interpretation are the findings of differences in the lattice compactness between Nissl body domains and non-Nissl domains in neuronal perikarya, and between

domains of glycogen granules and remainders in hepatocytes described in section 4 (figures 5, 6). This third interpretation is introduced because it is difficult to suppose that two cytoplasmic domains with different protein concentrations are directly apposed to each other in a state of solution without membranous partitions. Since the non-Nissl domain and the central major domain of axoplasm exhibit lattices of a similar compactness, the major axoplasmic domain may also represent the state of sol, which may be served to expedite active axoplasmic flow. Some of the cells exhibiting highly compact lattices may have gelated cytoplasm. The change in the compactness of MTL in the neurohypophyseal axon of dehydrated mice described in Section 6-2 may represent a sol to gel phase-change which may possibly occur as a cause or result of dehydration-induced hypersecretion in the axon (Kondo, 2010).

8. Nature of aqueous cytoplasm

The interpretation that MTL indicates the concentration and the sol/gel states of heterogenous cytoplasmic proteins, leads us to consider a chimeric combination of "solution idea" and "structured idea" for the nature of aqueous cytoplasm. Such a combined state of the two is supported by the finding after ultracentrifugation of viable cells (Kondo et al., 1992), which is described in details in the next section.

8.1. Centrifugation of viable mammalian cells

According to the biochemical definition, the 'cytosol' is the supernatant fraction of cell homogenates in the centripetal pole of a centrifuge tube after ultra-centrifugation at 105,000 g for 1h (Clegg 1983. Therefore, in order to see whether or not the aqueous cytoplasm represents the aqueous solution, it is important to examine whether or not the intracellular stratification by ultra-centrifugation is possible in viable cells, and whether or not some cellular proteins appear confined to the most centripetal zone of the stratification, based on the simulation of the cell to the centrifuge tube. There have been two studies using plant cells, Neurospora and Euglena, in line with this viewpoint (Zaloker, 1960; Kempner and Miller, 1968). Both of the studies reported the occurrence of intracellular stratification with the most centripetal zone containing no structural elements in light microscopy. They implied that, because of the negative reaction of the most centripetal zone by enzyme histochemistry of their day, the cytoplasmic proteins were structure-bound and/or structure-forming, allowing their co-sedimentation from the organelle-free and protein-free layer as though water was being squeezed from a sponge. However, the possibility cannot be ruled out that the enzyme histochemistry in their day was not sufficiently sensitive to detect cellular proteins in the centripetal zone.

The present author has applied ultra-centrifugation for intracellular stratification to various mammalian tissue-cells. Probably because of the absence in mammalian cells of a tough cell wall unlike plant cells such as Neurospora and Euglena, most mammalian cells do not withstand the extremely high pressure generated during the ultracentrifuge and easily burst. However, the autonomic ganglion cells and adrenal chromaffin cells are found to be the only exceptions as they are prevented from bursting, probably because of the presence of enclosing satellite cells functioning as a 'tough cell wall' as it were (Kondo et al., 1992).

In the centrifuged ganglion cells and chromaffin cells in conventional TEM as well as light microscopy, a minor centripetal cytoplasmic domain free of organelles can be discerned and is contiguous with a major centrifugal domain rich in organelles. The centripetal domain is of a demilune shape with a linear interface perpendicular to the centrifuge direction intercalating (see figures 2-6, 10-12 in Kondo et al., 1992). The minor centripetal domain appears flocculent or finely granular, while the major centrifugal domain contains regular cell organelles and microtubules without any stratification, unlike the centrifuged cell homogenates. However, no structured neurofilaments are discerned in the major centrifugal domain, although neurotubules are clearly observed in the domain. In PEG-TEM of the centrifuged cells, the centripetal domain is occupied solely by strand-lattices, suggesting the presence of proteins at a certain concentration based on the interpretation in Section 7-1, different from the previous reports cited above. The centrifugal domain is occupied by all the

organelles which are held in place by strand-lattices with a compactness similar to that of the centrifugal domain.

In immuno-light microscopy, in accord with the disappearance of structured neurofilaments in the major centrifugal domain of the centrifuged cells, no immunoreactivity for neurofilament proteins (NFPs) is found in the domain, but it is confined to the minor centripetal domain, unlike the cells before ultra-centrifugation (figure 10a, also see figures 3-6, 10 in Kondo et al., 1992). On the other hand, the immunoreactivity for tubulin and tyrosine hydroxylase is seen evenly in both of the cytoplasmic domains in the same way as in the cells before ultra-centrifugation (figure 10b, also see Ffgures 11, 12 in Kondo et al., 1992). In immuno-electron microscopy, gold particles representing NFPs are randomly deposited on potions of the microtrabecular strands without long linear alignments in the centripetal domain in immuno-PEG-TEM (see figures 14, 15 in Kondo et al., 1992).

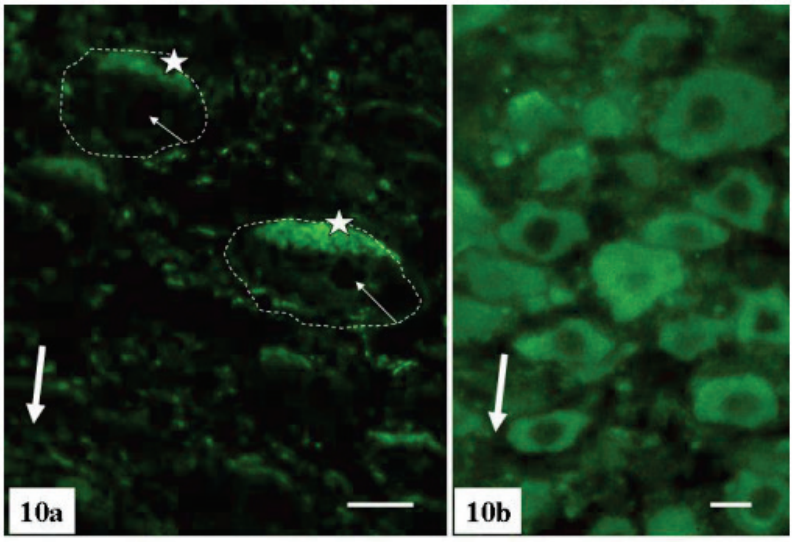

Figure 10. Immuno-light micrographs of ultracentrifuged autonomic ganglionimmune-stained for neurofilament protein (NFP) (10a) and for tyrosine hydroxylase (TH, soluble protein) (10b). Note localization of NFP-immunoreaction confined to a centripetal demilune domain (asterisks) and evenly homogenouse appearance of TH-immunoreaction both in centripetal and centrifugal domains. The contours of neurons are indicated by white broken lines. Thin arrow indicate nuclei and thick arrows indicate the direction of centrifugation. N: nucleus. Bar:1um.

The appearance of a centripetal organelle-free domain and the occurrence of displaced NFPs confined to the centripetal domain by the ultra-centrifugation are reversible: after overnight incubation of the centrifuged cells in an aeronated culture medium *in vitro* at room temperature or in the anterior eye chamber of animals, no such a centripetal organelle-free domain can be found, and the immunoreactivity for NFPs as well as tubulin and tyrosine hydroxylase is evenly distributed throughout the cells in the same pattern as normal cells in immune-light microscopy (see figures 16-18 in Kondo et al., 1992). This reversible change favors the view that the cells remain viable during the ultra-centrifugation.

The finding that immuno-gold particles for NFP in the centripetal domain of the centrifuged cells are deposited randomly but not in long linear lines on microtrabecular strands there can be interpreted as follows: the ultracentrifugation induces the depolymerization of NFPs and resulting disintegration of neurofilaments by yet unknown molecular mechanisms. Subsequently, the depolymeraized NFPs

are displaced as "free in solution" toward the centripetal pole from their original locations owing to the gravity of NFPs relative to the remaining cytoplasm in a way similar to the centrifugation of cell homogenates. Thereafter, “freely displaced NFPs” are randomly mixed with many other cytosolic proteins, and strand-lattices are then formed from the mixture of all the proteins in the centripetal domain by the specimen procedure for PEG-TEM. This interpretation seems to support the “solution” view of the aqueous cytoplasm.

In further favor of the view, the following finding is newly presented as an extreme case: When the 8% BSA solution containing polystylene beads of 100 nm in diameter (Cat# 00875, Polysciences, Inc., Warrington, PA 18976, USA) at a concentration of 1% is processed in PEG-EM, the resulting strand lattices in relation to the beads are highly similar to those of PEG-TEM- processed amine-proteinaceous endocrine cells such as the adrenal chromaffin cells and the neurohypophyseal axonal terminals of rodents, with the beads imitating the secretory granules (see figure 42 in Kondo 2008). Admittedly, and different from the model, the real secretory granules in those cells are closely related to detergent-resistant filamentous elements termed the cytoskeletons and their associated proteins, all of which appear together with MTL in PEG-TEM.

On the other hand, the ubiquitous and homogenous appearance of immunoreactivity for tyrosine hydroxylase, classified as a soluble protein according to the biochemical criteria, in the centripetal as well as centrifugal domains of the centrifuged cells is remained after the ultracentrifugation. This feature seems to be incompatible with the aqueous solution idea and it is even suggestive of “structure-associated” or “structure-forming” situation of the protein. In this regard, it should be noticed that tyrosine hydroxylase is known to be present at least partially in intimate association with catecholamine-containing granules (Nagatsu et al.,1983). In addition, recent molecular biological studies have revealed the occurrence of molecular domains characteristic of the protein-protein interaction in cytosolic proteins such as scaffold proteins represented by 14-3-3 proteins, regulatory and catalytic subunits of certain kinases, and PDZ domain-containing molecules (Taylor et al., 1999; Downes et al., 2001; Harris and Lim, 2001; Tzivion et al., 2001). Therefore, a simple question of which one, the solution idea and the structured idea, is correct does not make a sense. Judging from the temporal absence of cytoskeletal neurofilaments in the major centrifugal domain by *in situ* centrifugation, together with associated intracellular displacement of NFP in a layered way following the solution idea, and nevertheless the absence of differential stratification of various cell organelles in the major centrifugal domain, different from the cell-homogenate centrifugation, at least the presently dominated structured idea mainly based on the cytoskeleton is not satisfactorily complete. Instead, it is suggested that some mechanisms other than the cytoskeleton are also involved in the maintenance of the spatial organization and dynamics of intracellular organelles. The classical sol/gel phase transition is one of the mechanisms, and it is possible to consider that some, if not all , of cytoplasmic soluble proteins are involved in the sol/gel phase transition.

8.2. Correlation between microtrabecular strands and cross-linkers in RF-DE-R-TEM

Finally, judging from the homologous appearance in ultrastructure between PEG-TEM and RF-DE-R-TEM as discussed in Section 3-2, it is clear that microtrabecular strands themselves in PEG-TEM and fibrous structures often named as cross-linkers in RF-DE-R-TEM are equivalent to each other. Therefore, it should be borne in mind that it is risky to regard cross-linkers by RF-DE-R-TEM as real unless they survive after pretreatment with detergents, the same as for the lattice-strands in PEG-TEM. It should be noted that the cross-linkers bridging neurotubules represent one of a few examples surviving after detergent-pretreatment and chemically defined (Hirokawa et al., 1985).

Based on this homologous nature, it may also be questionable to accept that all the cross-linkers between parallel-aligned contractile filaments, actin and myosin filaments, in striated muscle fibers are real. There was a report by others that the cross-linkers remain in the A-band after strong muscle-stretch by which the thin filaments are withdrawed from the A-band (Pollack, 2001). It seems unlikely that the pre-existing cross-linkers between a given thick filament and its adjacent thin filament newly bridge between the given thick filament and a newly apposed thick filament in the extremely stretched

muscle sample. Instead, it seems more likely that certain soluble proteins existing in the slits between filaments regardless of thick or thin ones exhibit forms of cross-linkers with a certain compactness, that is, their interval, depending on the conditions of concentration and sol/gel states of the proteins. Such a question may be induced by so far published micrographs showing highly variable/random intervals between the cross-linkers because the criteria for reality of newly visible formed-elements include the regular appearance. Although the reality of the cross-linkers in striated muscles is almost well accepted, it may be meaningful to re-examine the nature of individual cross-linkers by immune-electron microscopy.

9. Concluding remarks and future prospectives

From a series of his study since 1980, the present author can now propose that microtrabecular strand-lattices in the cytoplasmic matrix as a whole, which are revealed by PEG-TEM as well as whole cell-mounted/non-embedded high-voltage TEM, represent the concentration and sol/gel states of soluble proteins although their individual strands themselves are unlikely to represent authentic in vivo structures. In other words, the cytoplasmic matrix represents a sophisticated and dynamic spatial combination of solution- and sol/gel- states of highly heterogenous proteins. The same is the case in the nucleoplasm/ extra-heterochromatin regions, details of which should be referred to a comprehensive review article by the present author (Kondo, 2008).

How advantageous biologically would the information on the spatial intracellular concentration of soluble proteins and their sol/gel states be, if obtained in association with well-established cellular ultrastructural entities as proposed in the present PEG-EM study? Needless to say, the discovery and characterization of the complex system of microtubules and filaments and their constituent proteins, collectively termed the cytoskeleton, have much advanced our understanding of the dynamic properties of the cellular ultrastructural elements in living cells. In this regard, it should be noted that such understanding of intracellular dynamics is made by neglecting the presence of intracellular soluble proteins regardless of their sol/gel states. Before development of such knowledges about the cytoskeleton, in other words, before establishment of electron microscopy in bio-medical fields, the predominant explanation of the dynamic properties of intracellular movement involved the transition between the sol/gel states of intracellular soluble proteins (Buchsbaum et al., 1987; Taylor and Condeelis 1979). However, no methods to estimate the sol/gel states of proteins as well as their concentration in association with the cellular ultrastructural entities have so far been available. Thus, only physic-chemical consideration of the involvement of the sol/gel states without any association with the ultrastructural information has so far failed to attract much attention, resulting in little progress in or even neglect of understanding about the biological significance in the intracellular dynamics of the sol/gel states of cytosolic proteins as well as their concentration at present. This is a rather irony, that is, the pursuit for observation at higher resolution in cytology results in the full discard of the afore-dominant understanding of the mechanisms on the intracellular dynamics. However, if the proposed interpretation on the cytoplasmic matrix in this study is confirmed to be correct by further studies including physic-chemical ones, it will be possible to understand the dynamic properties of the cytoplasm not only by the cytoskeleton but also by the sol/gel states of cytosolic proteins and their concentration in association with cellular ultrastructural entities. The same essentially holds true for the nucleoplasm and the intranuclear dynamics (Nalepa, 2004; Nickerson, 2001). This would be a resurrection of the cytological sol/gel.

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