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***Review Article***

# Rear Mirror View of Some Biological Tools and Processes

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It is indeed remarkable how many developments in biological sciences have their origin in earlier ideas which were nearly forgotten and were buried. This review illustrates this with a few examples. Free solution capillary electrophoresis is a refined version of moving boundary electrophoresis. Immobilised metal affinity chromatography rests on the early work on protein precipitation with metal ions. The concept of proteins as templates was rejected in early times but has resurfaced in the context of prions and intrinsically disordered protein regions. The approach of using enzymes in organic solvents is credited to papers published in late 1980s; the idea though is as old at least as the beginning of the 20th century.

**Keywords: Capillary Electrophoresis; Cold Denaturation; Enzymes In Organic Synthesis; Idiotype Network Theory; Immobilized Metal Affinity Chromatography; Intrinsically Disordered Proteins; Moonlighting Proteins; Prions**

*Regular minds find similarities in stories [and situations]; finer minds detect differences .... Many are so original they study history to find mistakes to repeat* (Nassim Nicholas Taleb; 'The Bed of Procrustes').

Last year, there was a post by the Boston Consulting Group in LinkedIn which made an interesting statement: "There are no old roads to new directions". This set us thinking whether this applied to science. The Latin phrase *nanos gigantum humeris insidentes* was restated by Sir Isaac Newton: "If I have seen further, it is by standing on the shoulders of giants". The sentiment is to look backwards, examine the past wisdom, and see whether something new or a better version emerges. This short review discusses a few instances when doing exactly that has profited researchers working in the area of biological sciences.

## Moving Boundary Electrophoresis and Capillary Electrophoresis

Arne Tiselius, as a part of his PhD thesis, developed the first electrophoretic method in 1930s (Tiselius, 1937). This method is known as moving boundary electrophoresis and Tiselius was awarded the Nobel Prize for this in 1948. It used Schlieren optics developed by Foucault in 1859 (Tobin, 2003). This particular optics measured the change in refractive index of the solution as the protein band moved in free solution under the influence of an applied electric field. An early model of analytical centrifuge sold by Beckman also used the same optics. This was phased -out with the introduction of UV absorbance-based optics. Probably as an aside, change in refractive index is always a more versatile property; in HPLC, refractive index (RI) detector is called a universal detector! So, old concepts never fade away totally, they are often worth reviving, albeit in a different context.

Reverting to Tiselius's method, convection and diffusion in free solutions resulted in broad bands. For a long time, scientists searched for a material through which proteins could move without band broadening.

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It was the introduction of polyacrylamide gel electrophoresis by Ornstein and Davis (1964) which made gel electrophoresis much more accessible and hence popular. One of the next major advances in electrophoretic techniques was capillary electrophoresis (CE) (Jorgenson and Lukacs, 1981) which has emerged today as a powerful method. CE is actually an "umbrella term" as many variants of CE exist. The earliest version of CE (which is still used) is capillary zone electrophoresis (CZE). CZE is also called free solution capillary electrophoresis (FSCE). Rightly so, because in CZE, electrophoresis is carried out in free solution! How does then CZE differ from Tiselius's moving boundary electrophoresis? The capillary provides a high surface to volume ratio. Hence, heat produced during electrophoresis is dissipated faster. Use of capillaries also makes electro-osmotic force important. The overall result is a very fast separation. Convection current and diffusion become insignificant. Other variants of CE use matrix-filled capillaries, but those were developed much later. CZE resulted from scientific workers never forgetting Tiselius's work. Today CE is a part of quite a few hyphenated techniques like CE-MS. Re-purposing of mevalonate pathway blockers like lovastatin was shown to have dose-dependent anti-cancer activity (Kobayashi *et al*. 2017). The metabolome of an ovarian cancer cell line was monitored using capillary electrophoresis coupled with mass spectrometry. Glutathione and metabolites associated with glycolysis were found to be reduced upon drug treatment while those associated with the tricarboxylic acid cycle (TCA) were increased (Kobayashi *et al*. 2017). It is interesting to note that the work manipulated 'Warburg effect', first described by Otto Warburg in 1920 and so christened by Ephraim Racker much later (Racker, 1972). The phenomenon essentially refers to the adaptive mechanism by cancer cells in switching over to glycolysis predominantly for deriving energy followed by lactic acid fermentation even in the presence of abundant oxygen (Liberti and Locasale, 2016). Normal cells divert pyruvate to mitochondria for oxidation under oxygen rich conditions. This metabolome analysis (Kobayashi *et al*. 2017) suggested that the statin disrupted the "Warburg effect" seen in tumour cells, explaining its anti-cancer activity, and activated the TCA cycle by reducing the ratio of NAD+/NADH. Differences in glycosylation pattern/s are associated with changes in physiological and pathophysiological conditions like malignancy. CE-MS has also been employed for the differential analysis of $\alpha$2,3- and $\alpha$2,6-sialylated glycopeptides as potential biomarkers in various diseased conditions (Kammeijer *et al*. 2017). These linkage isomers derived from glycopeptides obtained following tryptic digestion of prostate-specific antigen had identical fragmentation patterns but showed differential electrophoretic mobility (Fig. 1) due to differences in acidity. The analytical method had the additional advantage that it did not require pre-treatment of the sample.

**World War II and a Technique Called Immobilized Metal Ion Affinity Chromatography (IMAC)**

Science funding and consequently the rate of advancement of science increases when wars and epidemics happen. During World War II, USA invested a very large amount of money in science. This led to the development of work on protein purification in an intensive manner (Cohn *et al*., 1946; Farrugia and Robert, 2006). Understandably, the focus was on purification of blood proteins. Protein chromatography developed much later. In those early days, the idea was to see whether purification methods generally used by organic chemists could also be used for proteins. Precipitation with water-miscible organic solvents like alcohols and acetone was one such approach (Green and Hughes, 1955; Curling, 1980). Precipitation with metal ions like Cu2+ and Zn2+ was also tried (Cohn *et al*., 1950). While the approach can still be used successfully (Iyer and Przybycien, 1995; Zimmerman *et al*., 2016), precipitation with organic solvents or ammonium sulphate, especially the latter, is more common (Arakawa and Timasheff, 1985; Scopes, 1994). Such steps are used more for concentrating dilute solutions; any serious purification inevitably requires chromatography. Let us shift from a view of the past to the present scenario. The most commonly used protein purification technique today is immobilized metal ion affinity chromatography (IMAC) (Sulkowski, 1983; Kuo and Chase, 2011). The technique was developed by Jerker Porath (Porath *et al*., 1975), whose earlier notable contributions were (i) inventing the technique of gel filtration and developing Sephadex$^{TM}$ as the first

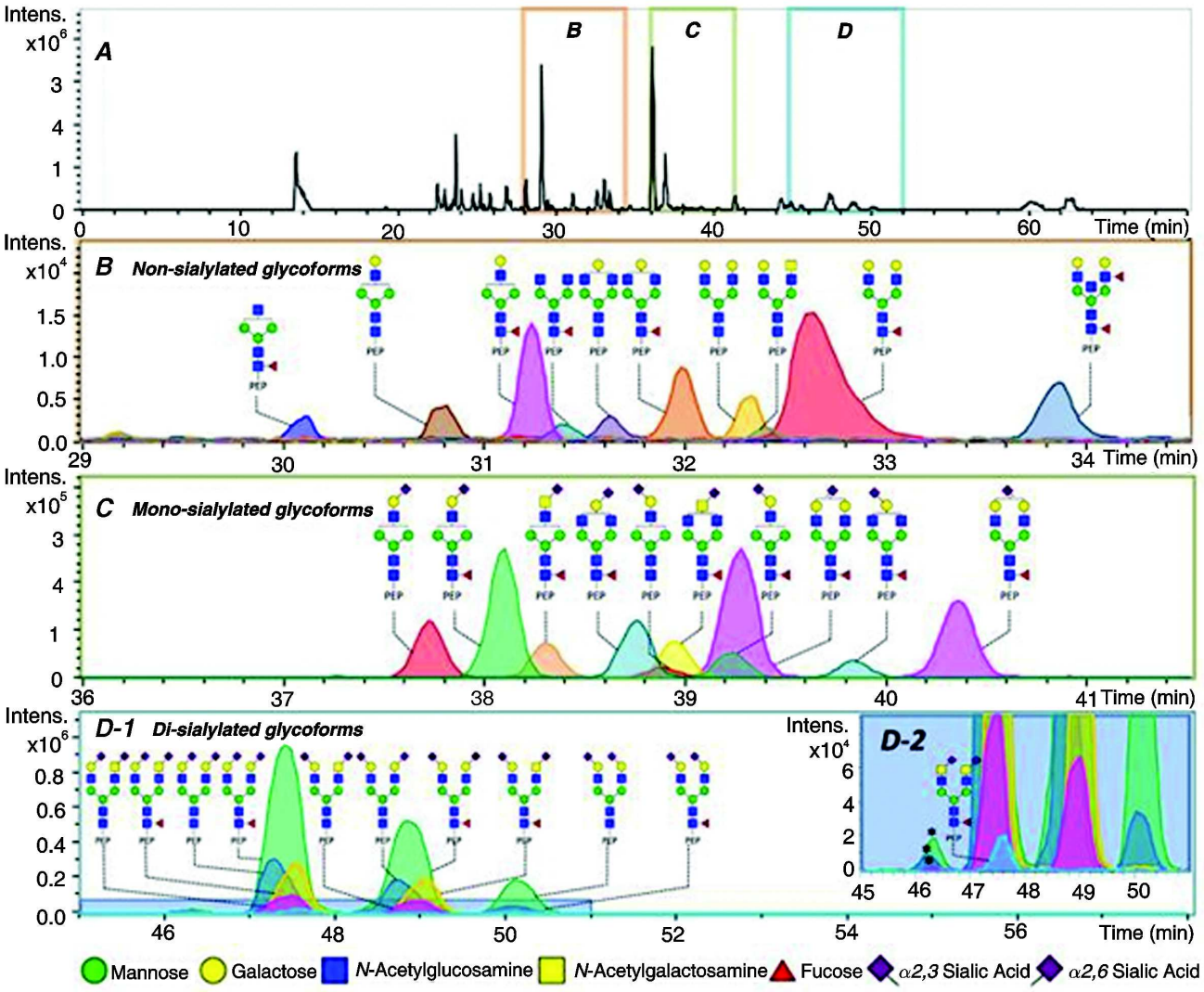


**Fig. 1: CE-ESI-MS analysis of prostate specific antigen (PSA) tryptic (glyco) peptides. (A) Representative base peak electropherogram observed for a tryptic digest of PSA. Based on the electrophoretic separation, three distinct clusters were defined. (B) Cluster with only non- sialylated glycopeptides of PSA. (C) Cluster containing mono-sialylated glycopeptides of PSA. (D) Cluster with di-sialylated glycopeptides of PSA. A total of 75 different glycopeptides were identified. Peaks with a star (*) were not assigned. The "PEP" label illustrates the tryptic peptide sequence N 69K to which the glycan is attached. Reproduced from Kammeijer *et al.* (2017) under creative common license**

commercial medium for gel filtration (Porath and Flodin, 1959). Today, these media are sold globally by GE Healthcare Life Sciences, (ii) the chemical coupling method based on cyanogen bromide (CNBr). The availability of CNBr coupling method contributed significantly to the twin areas of affinity chromatography and protein immobilization (Axén *et al*., 1967; March *et al*., 1974; Hermanson *et al*., 2013). With these tremendous successes behind him, Porath had been looking at metal chelation with proteins. The question was if a metal chelate (fixed to a solid matrix like agarose) were to bind to a protein, could this lead to a new separation method for proteins? The idea was not without its challenges. The most important side-chain of proteins, which was found to interact with $Cu^{2+}$, $Ni^{2+}$ and $Zn^{2+}$, was the imidazole group of histidine amino acid. How does one elute bound the protein in such a manner that the chelated metal ion does not come off along with the eluted protein? That will not do as many subsequent applications of proteins may not be compatible with the presence of such metal ions. The solution was to

search for appropriate chelating agents with appropriate binding constants. Out of the many which Porath's group tried, iminodiacetic acid (IDA) and nitrilotriacetic acid (NTA) were found to be the most suitable (Porath *et al*., 1975). It surely helped that Porath was quite comfortable with coordination chemistry! In the recombinant DNA era, it became easy to insert multiple histidine residues in the protein being cloned. Such inserts are called "fusion tags". IMAC developed by Porath took off as a favourite tool of molecular biologists (Sulkowski, 1985; Porath, 1992; Kuo and Chase, 2011). Porath's development of IMAC was facilitated by (i) the early work on protein precipitation by metal ions, (ii) a large amount of quantitative data on metal ion-amino acid interaction, (iii) his early work on affinity chromatography starting with affinity matrices based on agarose and coupling methods like CNBr. Porath looked at all this holistically and this development of IMAC came about as one of the most popular techniques of our times (Fig. 2). It pays to look back.

## Concept of Template, Prion and Intrinsic Disorder

In the beginning, we had RNA world. DNA and proteins came later. As cells evolved to become more complex (to fulfill their role in multicellular organisms), a variety of catalysts were needed. RNA could no longer cope up with this role. Enzymes evolved; their synthesis was governed like other proteins by the "central dogma": DNA → RNA → proteins. As the RNA world was replaced and both DNA and proteins evolved; there was a division of labour and proteins (unlike RNA in the ancient world) were not expected to function as templates. In fact, the central dogma treats proteins as a dead-end on the path of information flow. The general impression among biochemists is that proteins cannot act as templates. Some people, however, believe that the issue is far from settled (Vitek and Jackson, 2008). Historically, an early mention of the template concept occurs in the discussion on Fischer's lock-and-key hypothesis, which was also called template hypothesis (Lichtenthaler, 1995). The active site of the enzyme was viewed as a template in which reactions occurred.

### *Immune System and Network Theory*

However, the more controversial discussion involving template was in the context of nature of antibodies. Pauling, the leader of "instruction theory" group believed that antigens act as templates around which antibodies fold to form specific binding sites. Ultimately, the school of thought who believed in "selection theory" won the debate. The "clonal selection theory" is a widely accepted theory to explain the functioning of immune system (Hodgkin *et al*., 2007; Neuberger, 2008). Associated with the debate about instruction vis-à-vis selection was the debate about genetic versus somatic nature of the way antibodies acquire specificity. If selection takes place from pre-existing clones of lymphocytes, the basis of specificity is obviously genetic in nature. However, we do know now that subsequent experiments showed that this was not the whole story. Affinity maturation and hypermutation are somatic in nature (Muramatsu *et al*., 2000; Di Noia and Neuberger, 2002; Teng and Papavasiliou, 2007; Neuberger, 2008). So, after the initial "clonal selection", there is an element of "instruction" by the antigen. Also, while the "clonal selection" theory is now accepted as the correct view of the way immune system functions, there is another theory which is still not totally discarded. Niels Jerne was also awarded the Nobel Prize for "theories concerning the specificity and development and control of the immune system". Jerne is credited with the development of idiotype network theory (INT) (Jerne, 1974). Jerne argued that if the antibody is the mirror image (complementary shape) of the antigen, the antibody to antibody would be similar to the antigen! According to INT, such a network does exist and the antigens modulate the network (Fig. 3). In fact, the term 'epitope' (normally called 'antigenic determinant' now) was coined by Jerne (Jerne, 1985). The epitope on antigens combine with 'paratopes' (binding sites) on the antibody. Jerne viewed the relationship between epitope and paratope akin to lock and key! Contrary to the impression created by most of the text books, Jerne's ideas continue to attract attention (Mitra-Kaushik *et al*., 2001; Behn, 2007; Gorczynski and Hoffmann, 2017). The role of antigen, thus, is not limited to selection. It continues to 'instruct' how the immune system functions. This may not be like a classical template but it still validates some of

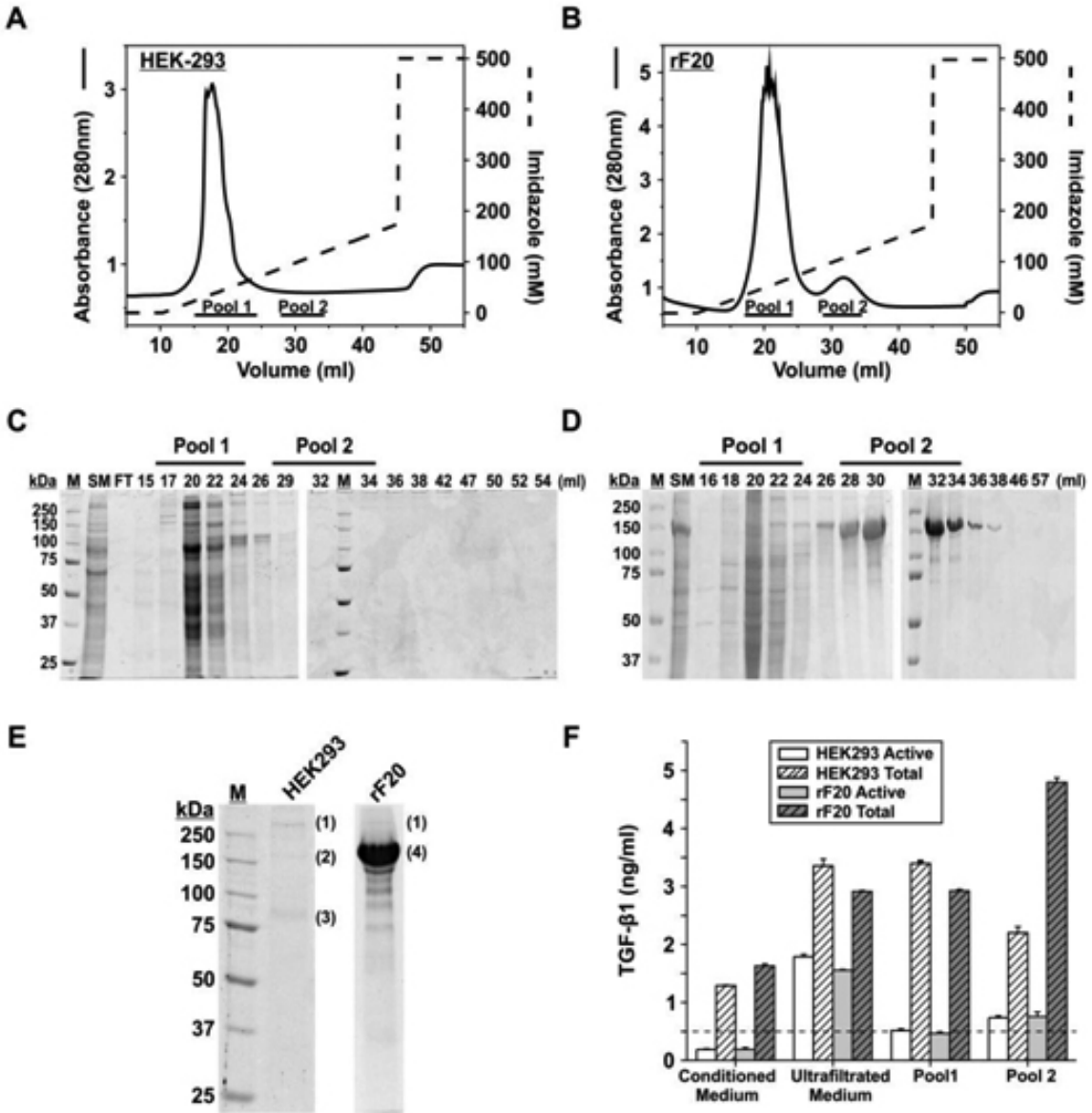


**Fig. 2: Mock and rF20 purification by IMAC. A: Chromatogram illustrating the elution profile of concentrated medium from non-transfected HEK293 cells on the IMAC column. The elution volume is indicated on the x-axis and absorbance at 280 nm is indicated on the left-axis (black line). Protein bound to the column was displaced by an imidazole gradient as indicated (dashed line). B: Chromatogram illustrating the elution profile of rF20 on an IMAC column. C: Aliquots (20 µl) of relevant peak fractions from the mock purification were analyzed on a 7.5% SDS-PAGE and Coomassie staining (SM = Start Material, FT = flow-through). Pool 1 corresponds to weakly/non-specifically bound proteins. Pool 2 corresponds to the elution volume correlating with the elution volume for rF20 (see D, Pool 2). As the medium was from non-transfected HEK293 cells no protein peak at OD280 nm occurred in the absence of a strongly expressed recombinant protein. D: Aliquots (20 µl) from relevant peak fractions were analyzed from the rF20 purification on a 7.5% SDS-PAGE and Coomassie staining. E: 20 µl of Pool 2 from the mock and the rF20 purification visualized by colloidal Coomassie staining on a 7.5% gel under reducing conditions. Pool 2 rF20 was overloaded in order to identify minor bands. Four bands labeled (1), (2), (3) and (4) were excised and analyzed by MS/MS. They are identified as tenascin C (1), keratin-1 (2) keratin-9 (3) and fibrillin-1 (4). M indicates globular marker proteins in kDa. F: The concentrations of TGF-β1 in ng/ml present at every step of the purification scheme was determined by an ELISA (r2 = 0.982). Conditioned medium refers to medium collected from confluent cell layers. Concentrated medium is the concentrate of the ultrafiltration that is loaded onto the IMAC column. Plain bars represent active TGF-β1 present in the mock (white) and rF20 (light grey) purification at different stages. The dashed bars illustrate total TGF-β1 (active and latent) present in the mock (white) and rF20 (dark grey) purifications. *The threshold level for reliable readouts is indicated by a dashed line. Reproduced from Kaur J and Reinhardt DP (2012) under creative common license**

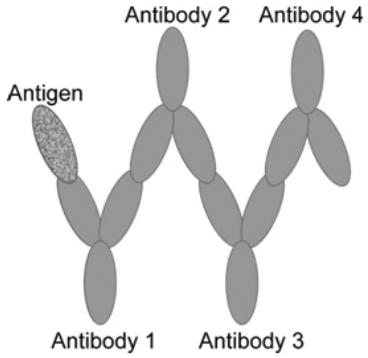


**Fig. 3: Schematic diagram of idiotypic network**

the ideas of Pauling. While on Pauling, his (Pauling, 1948) and Jencks's (Jencks, 1969) prediction that an antibody raised against a transition state intermediate will be catalytic did turn out to be correct after several decades (Lerner *et al*. 1991). All early efforts to verify Pauling's prediction were carried out with polyclonal antibodies which are obtained by experimental immunization of laboratory animals. These preparations contain multiple antibodies directed against many antigenic determinants on the injected antigen used during immunization. With the availability of monoclonal antibodies produced by hybridoma technology based upon mammalian tissue culture techniques (Kohler and Milstein, 1975), one could obtain a more specific and single antibody preparation. With the availability of monoclonal antibodies generated against transition intermediates of chemical reactions, catalytic antibodies were obtained which actually acted as a biocatalyst for the corresponding chemical reactions (Green, 1989). Sometimes, when one is ahead of one's times, the original idea may not turn out to be completely true but it does pave the way for discovering truth.

### *Intrinsically Disordered Proteins*

What about Fischer's view of enzyme-substrate interaction? Koshland's induced fit theory merely talked of minor conformational adjustments in the enzyme structure. This subsequently led him to propose the sequential model for allosteric enzymes (Koshland *et al*., 1966). It seems that while Anfinsen was correct about spontaneous formation of protein conformations; for proteins to function, some instruction from the substrate/ligand was very much relevant (Koshland, 1987). Now we know the importance of intrinsically disordered protein regions (IDPRs) in biology (Cumberworth *et al*., 2013; Oldfield and Dunker, 2014). A polypeptide chain containing 20-30 amino acid residues (may be even more) which has no ordered structure constitutes an IDPR. Many proteins with IDPRs are being increasingly reported. There are also a large number of reports of complete proteins having no tertiary structures; such proteins are called 'intrinsically disordered proteins'. It turns out that this disorder is essential for their biological function. For example, many regulatory proteins which bind to multiple ligands happen to be IDPs. In these proteins, it is the ligand which acts as a sort of template around which the protein assumes functional form of the structure (Gupta and Mukherjee, 2013; Oldfield and Dunker, 2014).

### *Prions*

Let us now look at prions. However, even before that, let us briefly discuss the concept of chameleon sequences. Since around 1996, proteins have been known to contain short stretches of sequences which can assume different secondary structures depending upon the overall protein fold containing them. Such sequences are called chameleon sequences (Minor and Kim, 1996; Tidow *et al*., 2004; Li *et al*., 2015). These sequences indicate the metastable nature of the protein structure. Petsko and Ringo have written that "studies of synthetic peptides derived from the prion sequence indicate that a stretch of up to 55 residues in the middle of the protein has the propensity to adopt both alpha-helical and beta-sheet conformations. Presumbly, the infectious form arises spontaneously in a small number of molecules, as a result of this inherent plasticity" (Petsko and Ringo, 2009). Yet, at least in the case of scrapie (see below), conversion of prion to the scrapie form is reported to involve a high activation energy barrier (Morrissey and Shakhnovich, 1999). In 1968, Adams and Field described the infectious disease called scrapie in sheep (Adams and Field, 1968), although it had been previously hypothesized that the infectious agent responsible for many TSEs was a protein (Alpers *et al*., 1967; Griffith, 1967; Soto, 2011). In 1982, Prusiner called the causative agent as *prion* (Prusiner, 1982). Even before scrapie, another encephalopathy called Kuru was reported in 1950s. The Fore tribe in Papua New Guinea had a funeral ritual in which the brain of the dead person was cooked and eaten (Alpers, 1987;

Meads *et al*., 2003). It was however the scare about the 'mad cow disease' which focussed wider attention to TSEs. In 2001, an epidemic called BSE (bovine spongiform encephalopathy, colloquially called mad cow disease) gripped the UK (Colchester and Colchester, 2005). It was believed that the bovine prions crossed the species barrier as consumption of infected beef led to the development of a variant of Creutzfeld- Jakob Disease (CJD) in humans. Now the list of TSE is longer and continues to grow. In recent years, several neurodegenerative diseases like Parkinson's, Alzheimer's, Huntington's, etc. have been speculated to have prion-like character (Aguzzi and Rajendran, 2009; Stopschinski and Diamond, 2017). Prions occur in different species of higher animals as well as in yeast (Fig. 4). The normal prion protein PrPC is anchored to the membrane. The protein has an alternate form PrPSc which is more prone to aggregation. They also differ in their glycosylation patterns. It is not clear what triggers the conversion of PrPC to PrPSc!

More relevant to our current discussion is the process of transmission of the infection in prion diseases (Victoria and Zurzolo, 2017). It is increasingly believed that prions act as a *template* to convert PrPC to PrPSc although everyone is not equally convinced that prions can pass from one cell to another. So, contrary to what many believed, it seems that proteins (at least prions) can act as templates. Chao *et al*. have recently (Chao *et al*., 2017) reported that polyethylene glycol (PEG) achieves a metastable organised structure on the protein surface. Using molecular dynamics simulations, they observed that PEG formed both H-bonds and hydrophobic bonds with the surface of a protein lambda 6-85. Intrinsic disorder (Uversky, 2013) is involved in many biological phenomena like moonlighting proteins (Tompa *et al*., 2005), catalytic promiscuity (Gupta and Mukherjee, 2013) and host-pathogen relationships (Franzosa and Xia, 2011). It is curious how the concept of templates has played out in both *in vivo* and *in vitro* contexts over the years.

### *Enzymes in Organic Synthesis and Low Water Enzymology*

The two papers from Massachusetts Institute of Technology (USA) by Zaks and Klibanov (Zaks and Klibanov, 1984; 1985) have more than 2,786 citations so far (Google Scholar, accessed on 05.02.2018). The work described in these is considered to be a starting point of using enzymes in organic solvents. The importance of this approach lies in the fact that

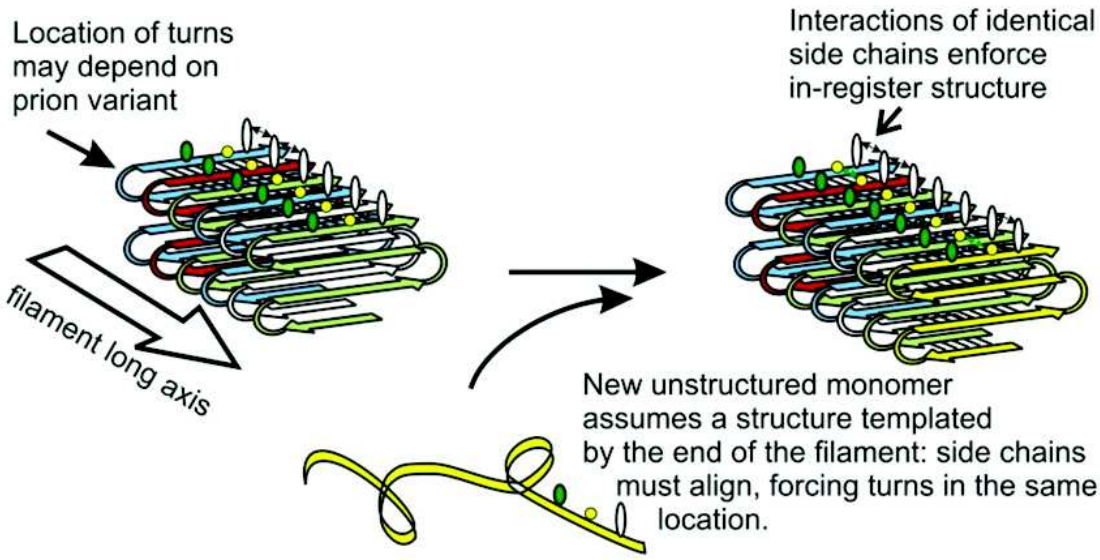


**Fig. 4: Protein conformation templating mechanism. The in-register parallel amyloid architecture naturally suggests a mechanism for transfer of conformation information from molecules in the filament to those joining the filament. H-bonding or hydrophobic favorable interactions among identical side chains require in-register alignment for the interactions. This directs the monomer joining the end of the filament to have its folds/turns at the same residues as previous molecules in the filament. Different prion variants have folds/turns at different locations, but each is faithfully propagated by this mechanism. Reproduced from Wickner *et al*. (2015) under creative common license**

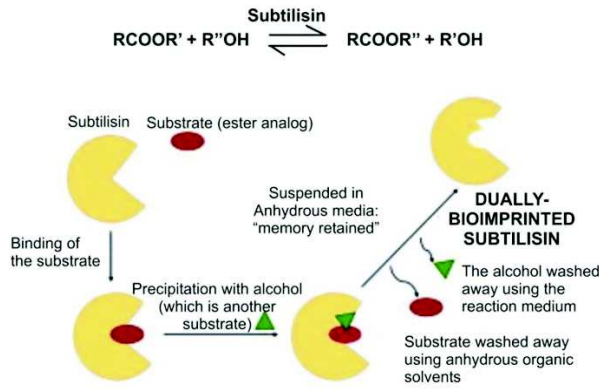


**Fig. 5: Bioimprinting. Reproduced from Mukherjee and Gupta (2015b), with permission from the publisher**

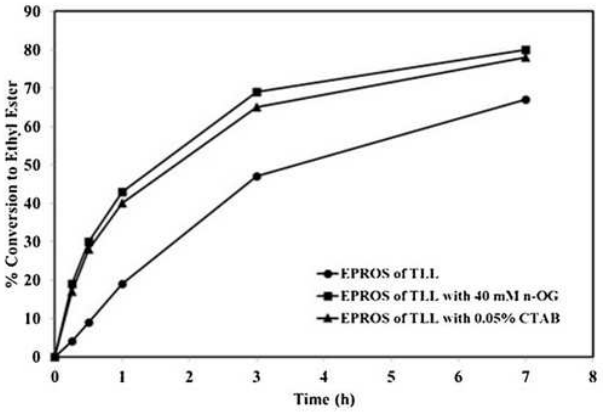


**Fig. 6: Synthesis of biodiesel from soybean oil and ethanol, using different preparations of *Thermomyces lanuginosus* lipase (TLL) (28 U/ g of oil) in solvent free medium. 0.5 g oil was used for the reaction and 140 ìL ethanol was added so that oil: ethanol ratio is 1:4. The reaction was carried out at 40°C at 200 rpm. Aliquots were taken at different intervals and analysed by gas chromatography. EPROS: enzyme precipitated and rinsed with organic solvents, OG: *N*-octyl-β-D-glucopyranoside, CTAB: Hexadecyl trimethyl ammonium bromide. The reaction was carried out in duplicates and the error within each set was within ±3%. Reproduced from Mukherjee and Gupta (2016), under creative common license**

inexpensive hydrolases (enzymes which hydrolyze their substrates) can carry out reverse reaction, i.e. synthesis, under such low water conditions. Today, chiral purity of molecules and materials has become an absolute necessity. Judicious choice of the organic media and level of low water activity allow both asymmetric synthesis and resolution of racemates (Gupta, 1992; Gupta and Roy, 2004; Drauz *et al*., 2012; Khorsand *et al*., 2017). Halling and Kvittingen (1999) raised an important question: "Why did biocatalysis in organic solvents not take off in the 1930s?" Their interesting article refers to the work of a Polish scientist Ernest Aleksander Sym who published his work on lipases in organic media in fairly reputed journals between 1933-1936. As a rejoinder to Halling and Kvittingen's article, Klibanov (2000) has argued that probably Sym's work did not lead to the area taking off as his work was limited to lipases. Also, argued Klibanov, that subsequent applications in obtaining chirally pure compounds made all the difference. In fact, as early as 1913, Bourquelet and Bridel (1912) reported stereospecific synthesis of various alkyl glycosides. During 1966-1967, Dastoli *et al*. reported the use of chymotrypsin (Dastoli *et al*., 1966) and xanthine oxidase (Dastoli and Price, 1967) in dry organic solvents. So, what made Zaks and Klibanov's work so important? Klibanov had earlier worked at the great school of Berezin at Moscow which was working on the use of aqueous-organic cosolvent mixtures to carry out organic synthesis with enzymes (Klibanov *et al*., 1977). The highly cited papers by Zaks and Klibanov describe the first extensive and systematic investigation of enzyme behaviour under low water conditions using two well characterized enzymes, viz. subtilisin and chymotrypsin (Zaks and Klibanov, 1984; 1985). Halling and Kvittingen (1999) point out that while Sym was known in Poland, his work was not recognized outside the national boundary. He probably also did not have the resources to go around and talk about his work all over the world. The important point is that those who look back at the literature certainly reap the benefits. Zaks and Klibanv are rightly credited as the force behind the renaissance in the area of enzyme catalysis in organic solvents (Mattiasson and Adlercreutz, 1991).

## Cold Denaturation

If one is to look at any current edition of a standard text in biochemistry, it is unlikely that cold denaturation will find a mention. The phenomenon, which refers to loss of biological activity of a protein upon cooling, may appear to be counterintuitive to an undergraduate/postgraduate researcher with a working knowledge of enzymes. It is high temperature which is more commonly known to cause protein denaturation (Gupta, 1993). Yet, cold

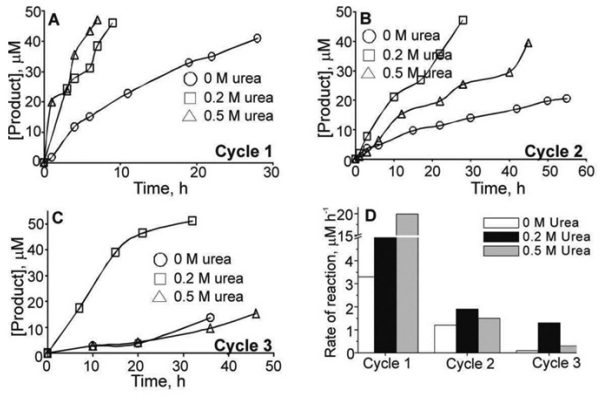


**Fig. 7: Effect of co-lyophilization with urea on enzymatic activity. Subtilisin Carlsberg (5 mg) was dissolved in 20 mM Tris HCl, pH 7.5 in the absence and presence of 0.2 M and 0.5 M urea and lyophilized. The formation of N-acetyl-L-phenylalanine propyl ester (N-OAc-Phe-OPr) from N-acetyl-L-phenylalanine ethyl ester (N-OAc-Phe-OEt) was monitored over time by RP-HPLC after (A) first, (B) second and (C) third cycle of trans-esterification. The enzyme was recovered after each cycle of incubation and used for the next round. (D) Comparison of initial reaction rates after each cycle. Reproduced from Prasad *et al.* (2016), with permission of the publisher**

denturation goes on turning up in literature in different contexts. For example, a review in 1991 lists residual activity of enzymes upon undercooling/freezing (Mathias, 1991). In this study, only one enzyme, alkaline phosphatase, lost 50% activity upon storage at cold temperature after 20 weeks. On the other hand, freezing at subzero temperature made three proteins lose a large fraction of their activities after different periods of time. Freezing invokes additional mechanical stress and we will not discuss this further. What is worth mentioning is that the commonly believed notion that "colder is better" when it comes to storage of enzymes/proteins can turn out to be wrong in many cases. In fact, an alert to this effect was sounded by Murphy *et al*. that shipping enzymes on dry ice may not always be safe (Murphy *et al*., 2013).

Let us go back to 1930 to see some buried information (Hopkins, 1930). Knowing that would have been less surprising to several later workers including those of the previously cited reference (Murphy *et al*., 2013). Hopkins (1930) and Simpson and Kauzmann (1953) observed that coefficient of denaturation of ovalbumin in urea solutions is negative. Interesting enough, it is positive at low concentrations of urea. Similar results are reported with â-lactoglobulin (Griko and Privalov, 1992), so ovalbumin is not unique in this aspect.

Somewhere down the line, the impression grew that cold denaturation (without the presence of any chemical denaturant) is a property of multimeric proteins (Clark, 1945). The inter- subunit interaction being largely hydrophobic, which decreases with decrease in temperature, cold denaturation was thought to be merely dissociation of subunits. Somewhere, enzymologists missed out looking backwards and did not realize that ovalbumin is a monomer; so dissociation of subunits could not have been the sole cause of cold denaturation. Privalov has listed dozens of proteins which undergo cold denaturation (Privalov, 1990). He has also discussed several thermodynamic factors implicated for cold denaturation (Privalov, 1997). Our understanding of cold denaturation is less than complete and the phenomenon continues to be discussed (Lopez *et al*., 2008; Dias *et al*., 2010; Murphy *et al*., 2013; van Dijk *et al*., 2016).

Psychrophilic organisms live under colder conditions (Deming, 2002; De Maayer *et al*., 2014). The enzymes isolated from such organisms are also active at low temperatures (Van Petegem *et al*., 2003; Do *et al*., 2015). Such enzymes have attracted considerable attention in recent years as these show considerable promise in industrial applications (Margesin and Feller, 2010; Sandle and Skinner, 2013). Microbiologists have struggled to understand the catalytic activity of such cold-adapted enzymes (Feller *et al*., 1996). Not many refer to the work on cold denaturation. How do all enzymes from psychrophilic organisms avoid cold denaturation?

The general belief is that adaptation to ambient temperature by organisms is largely mediated by trade-off between stability and catalytic activity (Goldstein, 2011; Mukherjee and Gupta, 2015a). However, there is sufficient evidence to believe that that paradigm may be an oversimplification (Prasad *et al*., 2016; Nguyen *et al*., 2017). This last example on cold denaturation is to illustrate that we sometimes miss out when we do not look at the rear view.

**Table 1: Summary of cases described in this work**

| Rear view | 'Modern' view |
|---|---|
| Moving boundary electrophoresis | Capillary electrophoresis is carried out infree solution for high resolution |
| Precipitation of proteins with metal ions; cyanogen bromide coupling | Immobilized metal ion affinitychromatography: Purification of tagged proteins |
| Fischer's lock-and-key hypothesis(template hypothesis) | • Idiotype network theory (INT) views the relationship between epitope (onantigen) and paratope (on antibody) akin to lock and key<br>• Catalytic antibodies<br>• Intrinsically disordered protein regions (IDPRs) as hubs of protein-proteininteractions<br>• Chameleon sequences<br>• Prion conversion |
| Ernest Sym's work on lipases in organicmedia | Enzyme-catalyzed asymmetric synthesisand resolution of racemates in low water media |
| Coefficient of denaturation of ovalbuminin urea solutions | Cold denaturation of proteins |

## Conclusion

Decades back, one of us was travelling with Dr. Jerker Porath (who developed Sephadex$^{TM}$ gel filtration media (Porath and Flodin, 1959), cyanogen bromide coupling procedure (Axén *et al*., 1967) and immobilized metal ion affinity chromatography (Porath *et al*., 1975) among other path-breaking inventions). Dr. Porath looked out of the car window at French buildings and remarked at the domes of many buildings as an influence from early times. Upon being replied that all efforts in art, culture and science seem to follow cyclic fashion, he remarked, quite perceptively, that with each cycle, it is not an exact repeat. There are always minor tweaks and changes. Art, culture and science seem to evolve from that. It seems that new directions often emerge from old roads in scientific endeavours (Table 1).

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