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Carbohydrate Metabolism in Drosophila: Reliance on the Disaccharide Trehalose

Alejandro Reyes-DelaTorre, María Teresa Peña-Rangel
and Juan Rafael Riesgo-Escovar

Additional information is available at the end of the chapter

1. Introduction

Work on Drosophila has been so influential that it has even impinged on human metabolism and health. The reason for this is obvious: There is a strong evolutionary conservation between biological processes of flies and humans. Also, cloning and functional analyses of genes in Drosophila allowing study of many cellular processes have greatly aided in the identification of homologous genes and processes in humans and other organisms, as exemplified by the study of homeotic genes and mutations [1]. A great number of genes required for a myriad different functions (from transcription factors, structural proteins, ion channels and signaling molecules, to those required for behavior and sleep) is common between the two species [2]; in fact, [3] showed that from a pool of 287 genes known to be implicated in humans in cancer and malformations, and neurological, cardiovascular, hematological, immune, endocrine, renal and metabolic diseases, 178 (62%) had their counterparts in flies. These findings underscore the potential impact of Drosophila as a powerful system for gene discovery, but also for study of diseases' symptoms and complications, and the discovery of therapeutic treatments.

Drosophila melanogaster was originally introduced as a research organism and genetic model at the beginning of the twentieth century by T. H. Morgan and co-workers. From their pioneering studies on heredity and the chromosome theory of inheritance [4], work with this model organism has been the source of biological insights in many areas, including genetics and development [5]. Clearly, Drosophila offers an array of advantages: its cultures in the laboratory are easy and economical, its life cycle is short (approximately ten days at room temperature), its embryonic development lasts only twenty four hours, there is no meiotic recombination in males, it has clearly defined and easily identified structures in the cytoskeleton (subject to mutational change, and, correspondingly, easy to score), has a

group of marked balancer chromosomes that impede meiotic recombination in females thus allowing the recovery and maintenance of other mutations of interest, has only four pairs of chromosomes, etc. These characteristics all greatly aid in genetic analyses [6], and since the sequencing of its genome (first released in 2000 [2, 7]), has arguably become the best characterized multicellular eukaryote model system. Critically, there are also large collections of mutant strains, with the one housed at the Bloomington Drosophila Stock Center at Indiana University [8] culturing over 49,000 different mutant stocks. Finally, since the publication of the fly genome, and its refinements with time, emphasis has also been placed on analyses and studies of physiology and signaling pathways involved, rather than just concentrating on 'gene hunts' or 'fishing expeditions' for candidate genes [9].

Since the discovery and characterization of abnormal phenotypes of insulin pathway mutants in flies [10], one of recent focuses of work in Drosophila have been metabolic studies, especially those of carbohydrates. One of the underlying reasons for this is, no doubt, the recent surge in obesity and diabetes mellitus type 2 among the world's population in both developing and developed countries [11, 12]. Just as it happens in vertebrates, *Drosophila melanogaster* regulates its levels of circulating carbohydrates, and stores excesses in the forms of glycogen and lipids, especially of triglycerides, in the fat body [13]. Another fruit of this recent surge in carbohydrate studies has been the elucidation of evolutionarily conserved signaling pathways, like the insulin/IGF/Target of Rapamycin (TOR) pathway [14].

2. The insulin pathway

Glucose homeostasis in mammals is maintained by feedback mechanisms balancing glucose cellular import and replenishment to the blood, so as to maintain a constant 5.5 mM level [15]. This level is the result of glucose cellular entry rates, glucose removal by the liver and new glucose coming from digestion of food glucose sources to the blood. Members of the Glut family of facilitated transporters, of which mammals possess several isoforms and genes, carry out glucose cellular import. It was recognized in the middle nineties that insulin stimulation in adipocytes in culture resulted in activation of a phosphatidyl-inositol-3-kinase (PI3K) isoform, and more glucose cellular import, resulting from Glut4 plasma membrane translocation [16]. Nowadays, Glut4 plasma membrane translocation from an internal vesicle pool is recognized as the rate-limiting step in glucose import by muscle (skeletal and cardiac) cells, and vertebrate adipocytes [17].

In contrast, the relation between insulin signaling and glucose cellular import in insect cells is not that clear. In Drosophila, there is a glucose transport system with similar kinetics to vertebrate Glut transporters [18]. A Drosophila Glut1 homolog cDNA has been cloned and described [19]. Nonetheless, experiments have shown that there is no increase in cellular import of 2-deoxyglucose labeled with ^{3}H in Drosophila Kc cells in culture after activation of the insulin pathway [20], and neither manipulation of activation levels of PI3K or protein kinase B (also called AKT or PKB), both molecules in the insulin pathway, in Drosophila S2 cells augments glucose cellular import [21].

These results could lead one to think that insulin pathway function in vertebrates and insects is not well conserved, and that insect insulin-like-peptides (ILPs) have no role in glucose and / or carbohydrate homeostasis, yet there is ample evidence to the contrary. The Drosophila genome possesses eight insulin-like–peptides (ILPs) [22-25] at last count, and these have been shown to interact with the Drosophila insulin receptor homolog (InR). Moreover, the Drosophila insulin receptor homolog can be functionally exchanged with the vertebrate insulin receptor [26], arguing that functional conservation must be significant.

Furthermore, ablation of the neurosecretory cells (NSC) that secrete DILPs 2, 3 and 5 in larvae gives rise to adults with developmental delays, reduced body size, and high levels of carbohydrates and lipids. Ablation of these same cells in adult flies lead to hypertrehalosemia without growth phenotypes. Knockdown of DILP2 alone by RNAi in NSC results in higher corporal trehalose levels [27, 28]. These effects, due to lower levels of circulating DILPs, causing lipid and carbohydrate accumulation, are analogous to effects observed in diabetic patients or diabetic mice when there is generalized insulin resistance, like in the insulin receptor knockout mice model [13].

Perhaps the clearest demonstration of the insulin pathway's role in carbohydrate and lipid metabolism comes from studies of Drosophila insulin pathway mutants: From the pioneering studies on Chico, a Drosophila insulin-receptor-substrate (IRS) homolog [10], it has now been demonstrated that practically all viable mutant combinations, that create partial loss-of-function or hypomorphic conditions for the insulin pathway, course with altered lipid and carbohydrate levels [29]. This is also akin to having congenital diabetes mellitus type 2, as these flies are born with a dysfunctional insulin pathway. Besides this metabolic disarray, these mutants have developmental delays, fewer and smaller cells, and concomitantly, smaller sizes, these last being cell independent [10]. In this regard, fly mutant growth phenotypes are similar to IRS1 loss-of-function mice models [30, 31] and other murine diabetic models [32] where growth phenotypes also exist; besides, in flies, the insulin and IGF (insulin growth factor) pathways are mediated by a common route. In addition, fly mutants also have nervous system abnormalities [29].

The insulin and insulin growth factor (IGF) pathways, as stated above, are united in Drosophila (see figure 1). Eight ILPs function as ligands for the sole InR in the fly genome [22, 23]. ILPs 1, 2, 3, 5 and 7 are secreted from NSC in the brain, whereas ILPs 4 and 6 are expressed in the medial intestine and the fat body. Binding of any of these ILPs to the InR causes receptor oligomerization and InR auto-phosphorylation, as the InR is a receptor tyrosine kinase. The InR itself is translated as a single polypeptide, but during its maturation within the cell and before insertion in the plasma membrane, it is proteolytically cleaved, both pieces held together by disulphur bridges. Once phosphorylated, the InRs recruit the IRS-like adaptor proteins, Chico and Lnk [10, 33]. Besides Chico and Lnk, the Drosophila InR has a long cytoplasmic tail that also acts as an IRS, in effect acting as a third IRS [26] (vertebrates have four IRS genes). InR phosphorylates the IRS proteins, like Chico, generating docking sites for other proteins in the pathway.
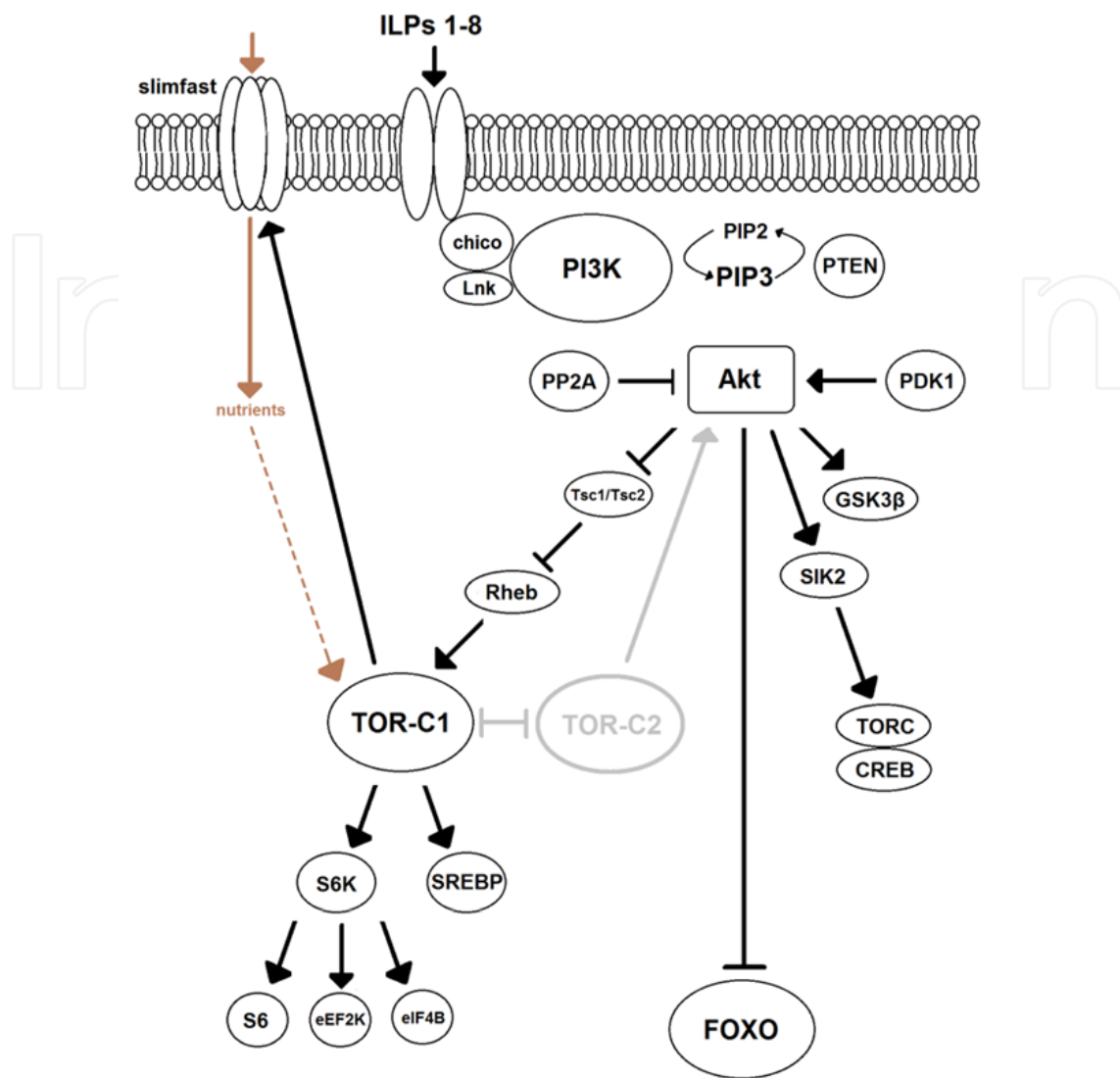
Figure 1. The insulin-signaling pathway in Drosophila. See text for details. Modified from [13].

The activated InR-IRS complex then recruits a PI3K complex (called Dp110 and Dp60, catalytic and regulatory subunits, respectively) to the membrane [14, 34]. Activated PI3K then generates phosphatidyl-inositol 3, 4, 5 trisphosphate (PIP3) from membrane phosphatidyl-inositol 4, 5 biphosphate (PIP2) and ATP. PTEN, a tumor suppressor gene, counteracts this enzymatic activity of PI3K, regulating PI3K output [35]. Plasma membrane accumulation of PIP3 then leads to membrane recruitment and activation by phosphorylation of two kinases, PDK1 and PKB (also called AKT). PKB in particular then phosphorylates and regulates several target proteins involved in metabolic control, including glycogen synthase kinase 3β (GSK-3β, or *shaggy* (*sgg*), as the gene is called in Drosophila), the transcription factor Foxo, the Tuberous sclerosis complex 2 protein, or Tsc2 (called *gigas* (*gig*) in Drosophila) and the salt-inducible kinase 2, or SIK2. PKB's activity is negatively regulated by a protein phosphatase, of class 2A (PP2A), which de-phosphorylates PKB and inactivates it. PKB can also be phosphorylated and activated by the Target of Rapamycin-complex 2 (TOR-C2) [13].

Activated *shaggy* then reduces glycogen synthesis by phosphorylation of glycogen synthase, besides participating in several other signaling networks, most notably the Wnt pathway [10, 13]. SIK2 phosphorylates and inactivates the transcriptional coactivator TORC (CRTC2 in mammals), precluding it from acting as a CREB co-activator. Foxo acts as a central catabolic regulatory point in cells; its phosphorylation by PKB enables its cytoplasmic retention, thereby inhibiting its nuclear localization, and transcriptional activity. Part of insulin's (or ILPs) anabolic effects relies on counteracting Foxo activity, which promotes energy conservation. Foxo also activates InR transcription, as part of a feedback loop.

Gigas' phosphorylation by PKB stimulates the activity of the Target of Rapamycin Complex 1 (TOR-C1) mediated by the Rheb GTPase. The Target of Rapamycin (TOR) kinase is a central anabolic kinase, regulating many aspects of general metabolism. Besides receiving input from the insulin pathway, TOR also receives input from nutrient sensors like Slimfast, an amino acid plasma membrane transporter. TOR regulates lipid, carbohydrate and autophagy levels. TOR itself forms part of two different complexes, TOR-C1 and TOR-C2. TOR-C1 is anabolic and regulates cellular growth and size, controlling cells' commitment to growth and energy utilization. Among its phosphorylation targets are the transcription factor SREBP (sterol-regulatory-element-binding-protein), and the ribosomal S6 kinase (S6K). TOR-C2 acts antagonistically to TOR-C1.

SREBP, a target of TOR-C1, regulates expression of genes involved in sterol biosynthesis. S6K, another TOR-C1 target, phosphorylates and regulates several proteins, chief of which is the ribosomal protein S6, besides the initiation and elongation factors elF4B and eEF2K. S6K promotes growth and protein synthesis [13]. In summary, Drosophila and vertebrates have great similarities in the insulin pathway, both at the structural and functional levels.

In vertebrates, glucose homeostasis also depends on another hormone that has opposite effects from those of insulin: Glucagon. Both are secreted from the pancreas. Insulin facilitates glucose translocation from the general circulation to the cell interior via plasma membrane translocation of Glut4 glucose transporters (acting as an anabolic hormone), besides activating growth. In contrast, glucagon activates glycogen hydrolysis (acting as a catabolic hormone), helping to maintain constant glucose levels in the blood. In insects, these basic homeostatic mechanisms are evolutionarily conserved, with a family of eight peptides akin to insulin acting as anabolic hormones in glucose homeostasis, as stated, and the adipokinetic peptides, a subfamily of which constitutes the so-called hypertrehalosemic hormones, playing the role of glucagon.

Despite the close homology throughout the insulin pathway and sugar metabolism, there is one clear and fundamental difference between carbohydrate metabolism of insects and vertebrates: Insects have trehalose (or α1-D-glucopyranosyl-α1-D-glucopyranoside; see figure 2). In fact, glucagon's functions are partially diverted to trehalose synthesis by means of the afore-mentioned hypertrehalosemic hormones. In having trehalose, insects are not alone: Many other species also use trehalose.
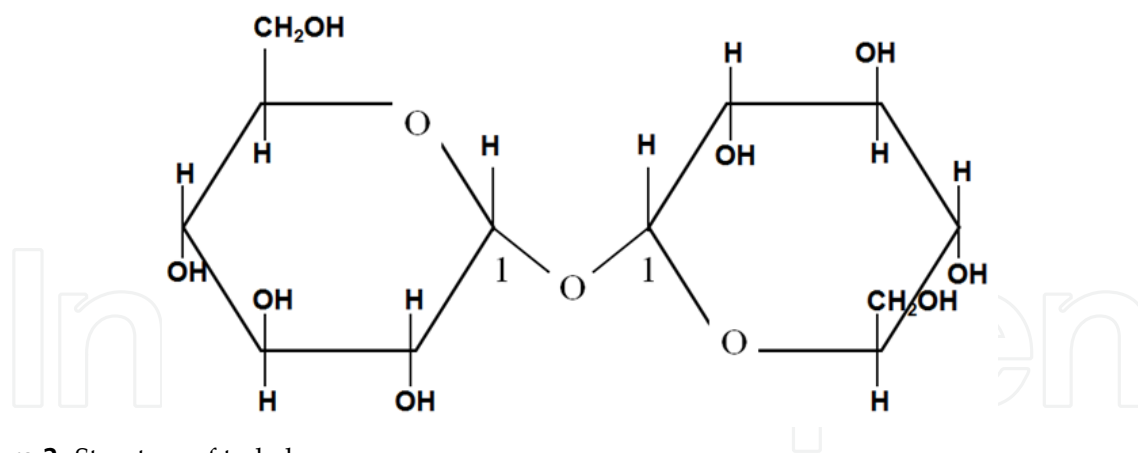
Figure 2. Structure of trehalose.

3. Why trehalose? Physicochemical properties of trehalose

Trehalose was discovered in the mid-nineteen century in rye ergot and then as a component of the cocoons of beetle species of the genus *Larinus* [36]. Many organisms use trehalose as a go-between between carbohydrate storage as glycogen and the cellular availability of glucose for energy needs (besides the other cellular roles trehalose plays in the cells' economy as an anti-stress factor): Why is this the case? Vertebrates have foregone altogether the use of trehalose, but still possess unique trehalose-degrading enzymes (trehalases; for example, human trehalase, used to digest trehalose from the diet and garner the glucose moieties within [37]). What advantages do trehalose imparts that has assured its selection in many diverse organisms (bacteria, algae, plants, fungi, invertebrates including insects), yet its loss in the vertebrate lineage? Part of the reason lies in the unique physicochemical properties of trehalose.

Trehalose is a disaccharide formed from two glucose moieties: It is synthesized by the union of two glucopyranose rings at the reducing end of the glycosyl residues. As a consequence, it is a non-reducing sugar. It demonstrates exceptional stability (with a high melting temperature, above 200°C, a high glass transition temperature, above 100°C, a slow rate of hydrolysis, and very high stability in extreme pHs; more than 99% of trehalose is still present in a pH range from 3.5 to 10 after 24 h at 100°C). It is not easily hydrolysed by acid (less so than sucrose, another non-reducing disaccharide found mainly in plant tissues), and its glycosidic bond is not cleaved by α-glycosidase, either. It also has low hygroscopicity, normally appearing as a dihydrated form. This form forms hydrogen bonds with the two water molecules, albeit at relatively short distances, making it an unusually strong interaction. In solution, all of the trehalose hydroxide groups make hydrogen bonds with water molecules. These bonds are easier to form than those with sucrose, leading to trehalose solutions with higher viscosity at equivalent concentrations, and less diffusion [36, 38].

The fact that trehalose is so resistant to acid hydrolysis may be key to understanding the use of trehalose in insect blood or hemolymph: Insect hemolymph has high amounts of peptides, free amino acids, and proteins, all of which have α amine and amino functional groups. A less resistant sugar might then react with these and be subject to condensation

reactions resulting in imines and enamines, altering the structure of proteins and peptides. Also, it is less likely, from the foregoing, to cause 'browning', where the sugar's reactivity ultimately leads to the formation of pigments, and protein degradation, something sucrose is more prone to do. Sucrose circulates in plant sap without these problems mainly because these liquids have much lower levels of proteins, amino acids, and lipids. The slow rate of hydrolysis of trehalose also makes it a very stable compound, and one that is likely to aid in stabilizing other components of mixtures where trehalose is an ingredient [38].

Trehalose is highly soluble in water (up to about 50 g/100 g water at 20°C), and even if it is less water-soluble than sucrose (200 g/100g water), solubility is still high enough to allow it to accumulate to the 100 mM range in bodily fluids, like insect hemolymph. This allows trehalose to be readily transported and to accumulate in various extracellular and intracellular milieus. Also, it is a sweet compound, although, again, less so than sucrose. In solution, trehalose does not normally form intra-molecular hydrogen bonds [38].

On the other hand, trehalose also functions as a stabilizer in solutions for other organic molecules, like lipids and proteins. It seems to do so stemming from some of the physicochemical properties discussed: higher hydration capacity, higher viscosity and hydrogen bonding with proteins and other organic molecules, displacing water, than the disaccharide sucrose.

3.1. Hemolymph trehalose concentrations: Enantiostasis

Contrary to what happens with glucose in insect hemolymph (or for that matter, with glucose in vertebrates in their blood), where human glucose concentration is held constant at about 0.1% independently of nutritional status, ambient temperature, water availability, and / or oxidative stress, among other factors), trehalose levels vary normally within the hemolymph by an order of magnitude [15]. Since 1956 it was known that trehalose is a very abundant component of insect hemolymph [39], and also since then, various reports have shown that trehalose levels can vary greatly [36, 39]. As stated before, there are several reasons for this: Trehalose levels respond to a variety of environmental conditions including changes in ambient and concomitant bodily temperature, nutritional status, developmental stage, oxidative stress, salinity, etc., such that the term 'enantiostasis' was coined to describe this condition. Enantiostasis signifies that organisms' adaptations to various environmental variables require corresponding co-variance of some cellular and organismal parameters in order for the organisms to cope with the environmental challenge. In other words, trehalose variations are not only normal, but are actually adaptive, and a way of addressing changing environmental conditions. Trehalose levels can rise as much as up to 2% in hemolymph [36].

One condition that allows this compound to be used as enantiostatic is that it is non-toxic, even when in large amounts and / or high concentration. Again, the physical and chemical properties of trehalose are favorable: it is a very non-reactive sugar, much less likely than glucose or other reactive sugars to form adducts or derived metabolites. Also, a given concentration of trehalose exerts only half the osmotic effect than an equivalent concentration of glucose. Besides, as stated before, it is exceptionally stable within a large range of pH, salinity, temperature and osmotic values.

3.2. Trehalose functions

These properties lead to some of trehalose's functions in eukaryotes in general: a) as an energy source, b) as a cryoprotector, allowing hemolymph to remain liquid at normally freezing temperatures in insects, c) as a participant in a mechanism for anoxia tolerance, d) as a protein stabilizer during stress (osmotic, oxidative, thermal), and e) as a structural component for plant and bacterial cell wall polysaccharides as well as in chitin synthesis. In general, trehalose's roles can be viewed as double: on one side, as a source of energy and carbon, and on the other as a preservative and protectant against various forms of cellular and organismal stresses. These characteristics may explain in part why trehalose has been evolutionarily retained in so many organisms. Besides these roles, modern pharmacology and cosmetology makes use of trehalose in many formulations as a protein stabilizer, and for pill fabrication as a general preservative and excipient [38].

3.3. Trehalose's role as energy source

One of trehalose's most important functions, as stated before, is as a source of energy and carbon. This has been extensively reviewed in studies of metabolism in insect flight, since insect indirect flight muscles are the most energy-demanding in the animal kingdom, and are known to sustain the highest metabolic rates of all animal tissues (oxygen consumption when starting from the resting state to flight increases up to 70-fold, compared to a maximum of 20-fold increase in human muscles due to exercise). Besides, flight muscles account for about 20% of a flying insect's body mass (18% in the locust) [40, 41]. It becomes obvious that insects need "high octane" fuels to maintain these powerful motors running.

In flying insect species, flight muscles make use and oxidize different energy-rich molecules (glucose directly, glycogen, trehalose, proline, phosphoarginine and lipids) with a species-specific characteristic pattern of consumption and with time of intensive use, and according to its availability in hemolymph. The cockroach (*Periplaneta americana*) which uses glucose as its primary flying fuel [42], opts for trehalose as a high energy substrate for prolonged flights. Administration of an inhibitor of trehalase (the enzyme involved in trehalose breakdown) significantly decreases cockroach flight time [43]. Many other long distance fliers, like locusts, also use trehalose [36].

3.4. Trehalose anabolism

The ultimate source of trehalose comes from diet carbohydrates, but their use for trehalose synthesis is not necessarily direct. Under conditions where carbohydrates are abundant in the diet, hexoses are transported in the intestine by means of facilitated diffusion. A Drosophila Glut1 homolog was cloned and sequenced, with a 68% homology to human Glut1 [19]. Glut1 transporters are not specific for glucose, being able to transport other carbohydrates, like mannose, galactose and glucosamine. Insects with protein-rich diets utilize amino acids to synthesize *de novo* glucose, amino acids being also an indirect source for trehalose synthesis. When not required for energy, glucose from the diet can be stored as

glycogen in the fat body. Glycogen is generally a more proximate source of glucose for trehalose synthesis.

Trehalose is synthesized in a condensation reaction of two glucose molecules, generally derived from glycogen (in effect, one of the constituent glucose substrates is in the form of UDP-glucose, derived from glucose-1P, whereas the other glucose substrate is glucose-6P). Since glucose is at the crux of trehalose anabolism, glucose regulation in the fat body impinges directly on trehalose regulation. Both vertebrates and invertebrates, in general, have very similar glucose regulatory mechanisms: insulin signaling and glucagon / hypertrehalosemic (HTH) signaling peptides, as stated above. Hypertrehalosemic hormones in particular are a family of adipokinetic (AKH) neuropeptides whose function is to derive glucose into trehalose synthesis in the fat body.

3.4.1. Insect fat body

Hemolymph circulating trehalose is synthesized in insects' fat body, a very important and extended organ analogous to both vertebrate liver and adipose tissue in insects [44]. Fat body is distributed throughout the insect body. It is also the principal organ for hemolymph circulating proteins and metabolites syntheses. It performs key roles in energy metabolism, as it both stores lipids and carbohydrates (these last in the form of glycogen), and responds to energy demands liberating both glucose and trehalose to the hemolymph. A *Drosophila melanogaster* fat body transcriptome analysis has shown that over 2200 genes are expressed in fat body cells, 290 of which attain high levels of expression. Among these are genes required for the insulin pathway, and lipid regulators. Many of the genes identified remain uncharacterized, and so, more work is required to define in greater detail the fat body transcriptome [45].

Several cell types constitute the fat body, the main one being the adipocyte (see figure 3). The adipocyte is a very dynamic cell type, with an active metabolism, regulating organismal carbohydrates and lipids, and also being an important endocrine cell [44].

The fat body is the main insect organ storing energy reserves. Most insect reserves are in the form of lipids, and these, mainly as triacylglycerols (TAGs). TAGs are stored in lipid droplets within adipocytes (figure 3). Lipid droplets have an outer coat of a monolayer of phospholipids and cholesterol molecules enclosing a core of TAGs and cholesterol molecules. In this outer coat some proteins are embedded, especially PATs (perilipin and adipocyte differentiation-related proteins). In insects two PAT proteins have been identified: Lsd1 and Lsd2 [46]. Together, they control synthesis and lipolisis of lipid stores.

Trehalose synthesis in response to energy needs normally courses through glycogen breakdown by glycogen phosphorylases in the fat body (see figure 4). Since the glucose thus obtained can also vie for glycolysis, this last path has to be inhibited. This is achieved in the fat body of cockroaches, where it has been studied, by reduction of fructose-2, 6-bisphosphate, an allosteric regulator of phosphofructokinase, the key regulatory enzyme in glycolysis [15, 47]. The adipokinetic hormones in turn, stimulate this decrease.
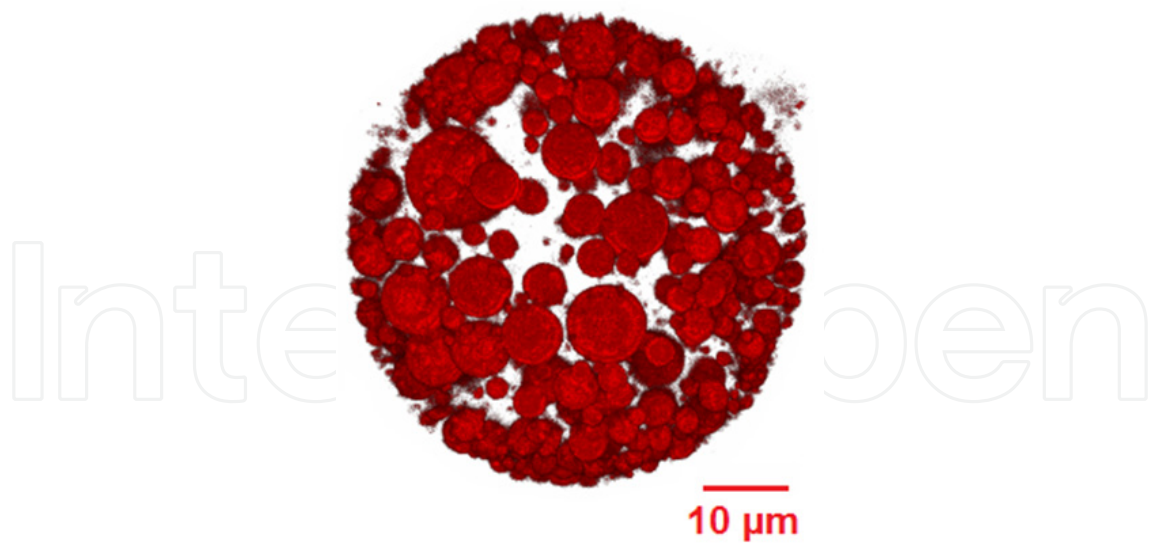
Figure 3. 2-D projection of a confocal stack of an abdominal adipocyte isolated from an Akt (PKB) *Drosophila melanogaster* mutant. The adipocyte was stained with Nile Red to image neutral lipids in order to evidence and quantify lipid droplets within the cell.
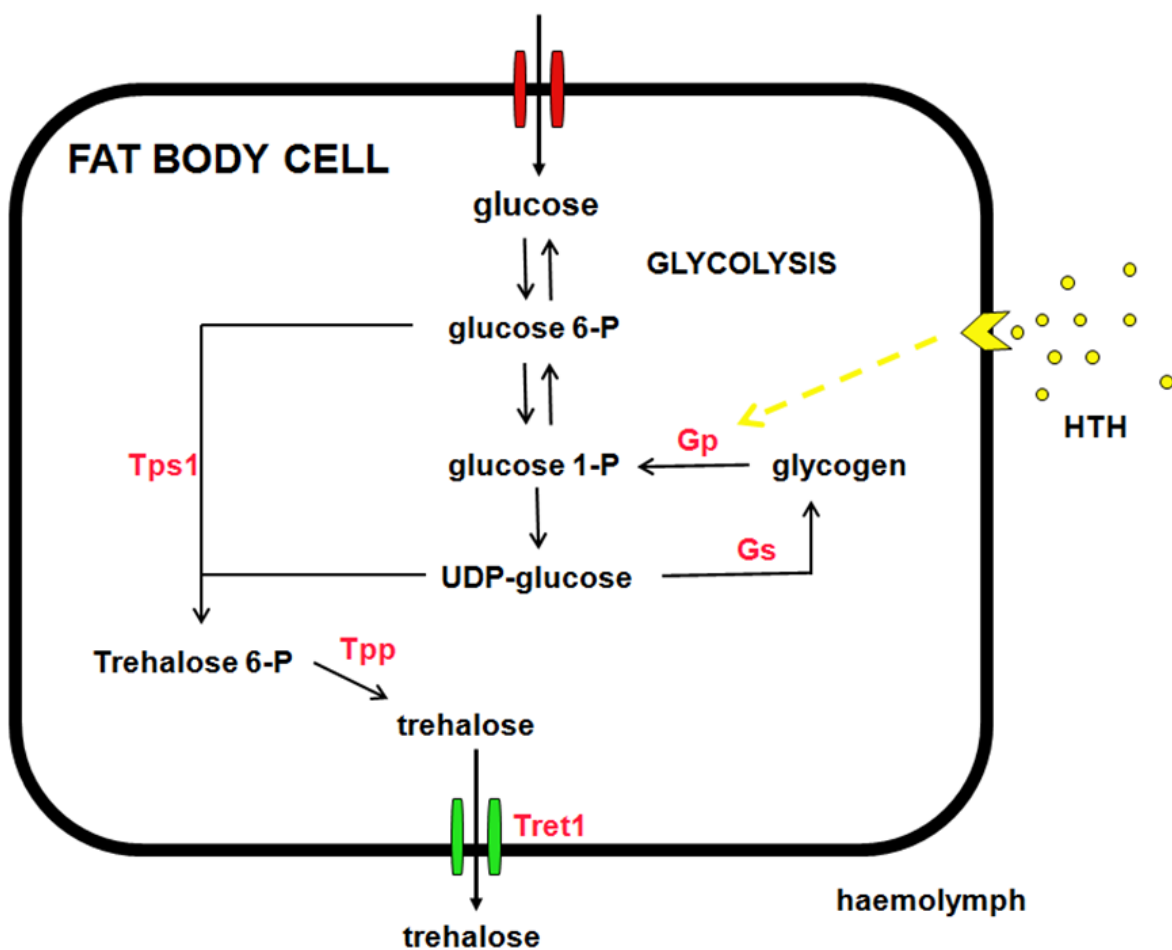

Figure 4. Trehalose synthesis in an insect fat body cell. See text for details. Abbreviations: HTH hypertrehalosemic hormones; Tret1, trehalose transporter 1; Tps1, trehalose phosphate synthase 1; Tpp, trehalose phosphate phosphatase; Gs, glycogen synthase; Gp, glycogen phosphorylase.

3.4.2. Adipokinetic hormones (Hypertrehalosemic hormones)

AKH were originally shown to induce lipid catabolism. In insects, as stated, one subgroup of these hormones produces an increase in circulating trehalose in the hemolymph [48]. About fifty HTH have been reported to date, and it is known that they participate in different functions. They are produced by neurosecretory cells of the *corpora cardiaca*, part of the ring gland of insects together with the prothoracic gland and the *corpus allatum*. The ring gland is located surrounding the anterior proventriculus in the thorax [49].

HTHs are synthesized from longer precursor peptides that are proteolitically processed to 8-10 amino acid long peptides. When the HTH neurosecretory cells are ablated, there is a significant drop in circulating trehalose levels in Drosophila larvae; over-expression of these HTHs leads to higher trehalose levels [48]. These hormones are secreted in response to various stimuli that would necessitate high circulating levels of trehalose, like lack of food, dehydration or thermal stress, or during flight. Some insects, like locusts, initiate flight utilizing trehalose, but switch to lipids later, whereas others, like some cockroaches, depend solely on trehalose supply. Still others use proline as an energy source, like some beetle species [44].

HTHs have membrane receptors. The Drosophila and homologous *Bombyx mori* HTH receptors have been cloned; they are G-protein-coupled-receptors (GPCRs) and are sequence-wise related to the gonadotrophin releasing hormone receptors of mammals [50]. Mutations in the Drosophila receptor show that it indeed mediates the HTH response: Mutants had glycogen and lipid accumulation in the fat body, irrespective of nutritional status, whereas rescue experiments returned glycogen and lipids to wild type levels [51].

There are scant data on the signaling pathways activated by HTHs. In cockroaches, HTH signaling increases inositol trisphosphate levels, leading to intracellular high Ca^{+2} concentrations and higher activity of protein kinase C (PKC). This produces an increase in the activity of glycogen phosphorylase, and trehalose synthesis. Inhibitors of PKC reduce trehalose production [52]. On the other hand, in locusts, AKH stimulation leads to cAMP production [53].
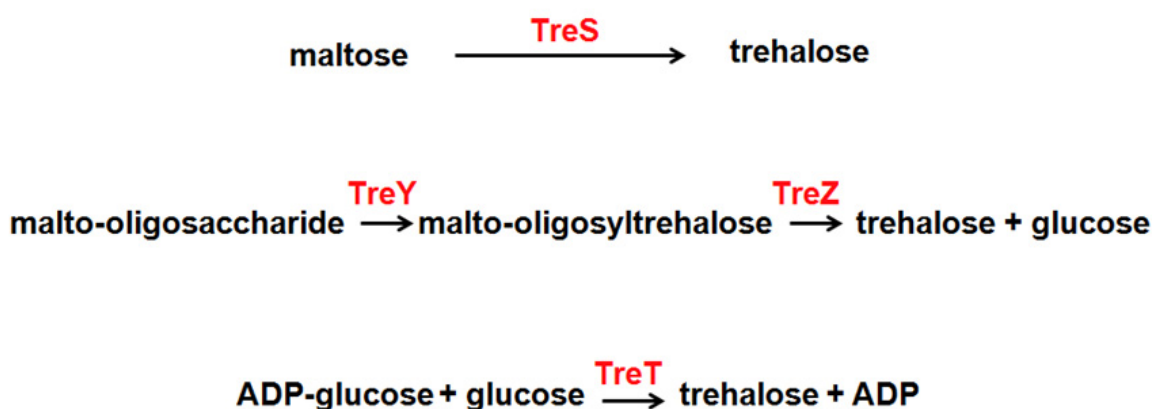
3.4.3. Final reactions of trehalose synthesis

Trehalose phosphate synthase catalyses condensation of glucose 6-P and UDP-glucose to generate trehalose 6-P in the fat body. Trehalose 6-P is then de-phosphorylated by the protein phosphatase coded by the *trehalose phosphate phosphatase (tpp)* gene to generate trehalose [54]. Both trehalose phosphate synthase and glycogen synthase use UDP-glucose as substrate, so besides competition for glucose between trehalose synthesis and glycolysis, the opposing trehalose phosphate synthase and glycogen synthase enzymes must be regulated too in order to shunt glucose to one or the other metabolic outcome [55]. Km values for trehalose phosphate synthase vary in different species where it has been studied (the moth *Hyalophora cecropia*, the cockroach *Periplaneta americana*, the blowfly *Phormia regina*, the sleeping chironomid *Polypedilum vanderplanki*, and the fruit fly *Drosophila melanogaster*). *trehalose phosphate synthase 1 (tps1)* overexpression in flies results in elevated trehalose levels,

whereas mutations in this gene are lethal in the first larval instar. Trehalose phosphate synthases have also been studied in other organisms, like *Escherichia coli*, the yeast *Saccharomyces cerevisiae*, and plants like *Selaginella lepidophylla*, *Myrothamus spp.* and *Arabipdosis thaliana*. In *A. thaliana*, whose genome has been sequenced, there are eleven *tps* and ten *tpp* genes, suggesting that trehalose metabolism in plants is also very important [55, 56].

The aforementioned synthesis pathway is the one most commonly found; but there are, nevertheless, four other biosynthetic routes, three of them only present in prokaryotes. Some prokaryotes can use up to three different synthesis routes. The genes encoding trehalose synthase (*treS*), maltooligosyl-trehalose synthase (*treY*), maltooligosyl-trehalohydrolase (*treZ*) and trehalose glycosyltransfering synthase (*treT*) are prokaryotic, whereas trehalose phosphorylase (*treP*) is present in both [56] (see figure 5).

PROKARYOTES

$$\text{maltose} \xrightarrow{\text{TreS}} \text{trehalose}$$

$$\text{malto-oligosaccharide} \xrightarrow{\text{TreY}} \text{malto-oligosyltrehalose} \xrightarrow{\text{TreZ}} \text{trehalose + glucose}$$

$$\text{ADP-glucose + glucose} \xrightarrow{\text{TreT}} \text{trehalose + ADP}$$

EUKARYOTES AND PROKARYOTES

$$\text{glucose 1-P + glucose} \xrightarrow{\text{TreP}} \text{trehalose + Pi}$$

Figure 5. Alternative pathways of trehalose synthesis in prokaryotes and eukaryotes.

3.5. Trehalose in circulation

Once synthesized, trehalose is secreted to the hemolymph. A trehalose transporter cloned from several species, called Tret1, accomplishes this. It has been described in the mosquito *Anopheles gambiae*, the fruit fly *Drosophila melanogaster*, the moth *Bombyx mori* and the bee *Apis mellifera*, besides the sleeping chironomid *Polypedilum vanderplanki*. The *P. vanderplanki*

trehalose transporter gets activated by dehydration conditions and that leads to trehalose accumulation such that up to 20% of total body weight can be composed of trehalose [57]. Besides Tardigrades, this last species is the pluricellular eukaryote that can withstand the harshest environmental conditions known, due in no small part to its trehalose accumulation strategy [58, 59].

The Tret1 transporter has twelve predicted membrane-spanning segments, similar to Glut family sugar transporters. Cloned Tret1 transporters from the aforementioned species were expressed in *Xenopus* oocytes. All cloned genes were functional, although kinetic parameters differed [60]. In *Drosophila melanogaster* there are two genes coding for trehalose transporters: Tret1-1 and Tret1-2, with Tret1-1 having several isoforms. Tret 1-2 shows no trehalose uptake and might be a pseudogene [60]. High expression has been noted in the fat body according to FlyAtlas for both genes [61]. Also, expression of both appears to be coincidental in many tissues, besides the fat body [62]. Transport of trehalose appears to be bi-directional for these transporters, such that not only secretion from the fat body cells goes through them, but also trehalose import to other cell types as well. In accordance with this, in *Bombyx mori* expression is also seen in muscle, testis and head, besides the fat body [60].

3.6. Trehalose catabolism

The enzyme responsible for trehalose breakdown is trehalase. This reaction produces two glucose molecules. The *treh* genes encode trehalase. *treh* has been cloned from several insect species, including the fruit fly *Drosophila melanogaster*. There are both membrane attached and soluble forms. In fruit flies it is expressed in many tissues, as expected. In many other insect species expression is seen in hemocytes (hemolymph cells).

There are trehalases in many other organisms, like yeast, thale cress and humans. Trehalase is regulated both at the transcriptional level (depending on nutritional status), and at the post-transcriptional level, by phosphorylation and by the presence of a trehalase inhibitor (this last in the cockroach *P. americana*) [36]. Altogether, trehalase appears to be finely regulated.

3.7. Trehalose and stress

Besides its role as an energy reserve, especially in response to high demands (flight, reproductive efforts like ovogenesis, etc.), trehalose functions as a molecule that aids organisms to cope with stresses from the environment. Organisms are frequently exposed to adverse environmental conditions like lack of water or food, lack of oxygen, oxidative stress and thermal variations, all of which conspire against the organism's survival. In response to these conditions living beings have evolved different strategies, involving the production of anti-freeze, chaperone and heat shock proteins, changes in membrane phospholipid composition, and cryoprotector metabolites such as glycerol, sorbitol, manitol and proline, besides trehalose. In extreme cases, organisms may even enter states of suspended animation, or cryptobiosis, where metabolism is stopped, or all but stopped. Depending on

what causes it, cryptobiosis is called differently: anydrobiosis when induced by lack of water, cryobiosis when induced by cold temperatures, chemobiosis when induced by the presence of toxic chemicals, and anoxybiosis when induced by lack of oxygen.

Studies of trehalose have shown it to be a sort of jack-of-all-trades, employed in a variety of stress-provoking situations. This may explain its widespread occurrence and production in nature, despite its metabolic cost. Part of this is, of course, explained by the unusual physicochemical properties of trehalose.

3.7.1. Anhydrobiosis

Some organisms have evolved adaptations to cope with environments of transient very low humidity (mosses, famously the Tadigrades), where vital functions are just maintained or even stopped altogether. The chironomid *Polypedilum vanderplanki* can withstand up to 106°C while dessicated. Experiments with *P. vanderplanki* larvae show that gradual dehydration is accompanied by production and accumulation of trehalose, up to about 18% of total dry weight [59]. In another series of experiments, activity of the synthesis enzymes for trehalose, trehalose phosphate synthase and trehalose phosphate phosphatase was measured. These enzyme's activities increased during dehydration, whereas that of trehalase decreased. This was not only due to altered enzymatic activity, but also to transcriptional control, as the levels of the synthesis enzymes cDNAs increased at the beginning of dehydration, whereas that of trehalase increased only after forty eight hours since inititation of dehydration, perhaps as preparation for subsequent rehydration [63]. Other species also use trehalose as a protectant during anhydrobiosis: the crustacean *Artemia salina* and the nematode *Avelenchus avenae*. It has been suggested that this increase in trehalose is driven by higher concentrations of ions in bodily fluids, rather than stemming from hormonal action.

Fruit flies can also withstand dehydration: 60% of fruit fly larvae subjected to conditions of less than 5% of relative humidity survived after subsequent rehydration. During dehydration and rehydration trehalose levels were measured, and went from 2 to 10 μg per larvae during the eight hours the gradual dehydration procedure lasted in the experiment. This correlated with a decrease in trehalase activity and an increase in trehalose phosphate synthase activity. Conditions reversed upon rehydration [64].

3.7.2. Cryobiosis

Bacteria like *Escherichia coli* use trehalose as a cryoprotectant. Changes in temperature from 37°C to 16°C drive an increase in trehalose levels of about an order of magnitude, and even though this does not affect growth, it does favorably affect survival when cells are subsequently transferred to a medium at 4°C. Bacteria mutant for *otsA* and *otsB*, the homologs of trehalose phosphate synthase and trehalose phosphate phosphatase die faster when transferred to 4°C; this lethality was rescued when transfected with a plasmid

carrying these two genes, demonstrating the reliance on trehalose synthesis for survival for survival [65].

The lepidopteran *Cydia pomonella*, whose larvae are the common apple worm pest, also use trehalose as cryoprotectant. In a study that measured trehalose levels throughout the year, and correlated those measurements with temperature changes, they observed higher levels of trehalose (up to 18.4 mg/g of fresh weight) in January, where the temperature dipped to 0.4°C, compared to August (4.8 mg/g), where the temperature reached 23°C [66].

3.7.3. Thermal shock tolerance

Yeast cells (*Saccharomyces cerevisiae*) use trehalose as protection against heat shocks. In a microarray-based study, yeast cells subjected to a 30°C to 40°C heat shock survived better if trehalose accumulated within them. Especially successful were yeast cells with forced trehalose accumulation made by over-expressing *tps1*, the gene that codes for the biosynthetic enzyme for trehalose, together with mutations on the genes *nth1*, *nth2* and *ath1*, that code for trehalose degrading enzymes. The same set of heat shock-responsive genes were induced by heat treatment in both trehalose overexpressing and not overexpressing cells, showing that these heat shock genes are not responsible for increased survival (rather, trehalose is). Hexose transport genes were induced by heat shock, and heat shocked yeast cells transport more glucose into the cell, leading to subsequent increases in trehalose [67].

3.7.4. Oxidative stress

Metabolic activity generates, as a byproduct, reactive oxygen species (ROS) like hydrogen peroxide, superoxide ions, or hydroxyl radicals, among others, that can oxidize several types of biomolecules (like protein oxidation and crosslinking, nucleic acid modification and lipid peroxidation). Due to its prevalence, cells have evolved several different mechanisms to counter these toxic effects: Enzymes like superoxide dismutase or catalase, or molecules like thioredoxin and gluthatione, all counteract ROS toxic effects. Trehalose is also one of these protective mechanisms.

Yeast cells subjected first to a 28°C to 38°C heat shock to induce trehalose accumulation (see paragraph above), and then subjected to oxidative stress with a combination of hydrogen peroxide and ferric chloride incubation, fared much better (survival close to 100%) compared to other yeast cells not subjected to a heat shock first, whose measured trehalose levels were lower. Mutations in the trehalose synthesis genes (*tps1* and *tps2*, coding for trehalose phosphate synthase and trehalose phosphate phosphatase, respectively), did not fare better than control cells without heat shock even though they were subjected to a heat shock before incubation with hydrogen peroxide / ferric chloride. Under these conditions, mutant cells survived better if trehalose was also included in the medium, proving that trehalose accumulation is key for survival under these conditions. In these experiments *tps2* induction required YAP1 (stress response element binding protein), a protein required for catalase and thioredoxin expression, suggesting an integral response to oxidative stress [68].

3.7.5. Anoxybiosis

There is a very large body of literature concerning cellular and organismal responses to oxygen deprivation: cells are generally very sensitive to decreases of oxygen in the medium, especially nervous tissue. Yet some cells and organisms have trehalose-based anoxia tolerance mechanisms. Drosophila kept in a 100% nitrogen atmosphere for five minutes constitutively over-expressing *tps1* (trehalose phosphate synthase) compared to flies not over-expressing *tps1* fared better after the anoxic treatment. *tps1* over-expression lead, of course, to higher trehalose levels. Loss-of-function mutations in *tps1*, conversely, stimulated anoxia sensibility [55].

There are two basic ideas that try to explain in general why trehalose is so helpful in these stressful environments, and they both rely on the lack of reactivity of trehalose and its capacity to form hydrogen bonds with other biomolecules and / or its vitrification at high temperature. Trehalose hydrogen bonding with other biomolecules could substitute for water, preventing or reducing protein aggregation and denaturation, while not otherwise chemically reacting with them. Likewise, trehalose vitrification could lead to formation of trehalose "crystals" that would allow for stabilization of other biomolecules.

3.8. Trehalose in chitin synthesis

In insects, trehalose is also used as a chitin precursor. Chitin is an N-acetyl-β-D-glucosamine polymer. Chitin synthesis is not perfectly understood in detail but is known that it initiates with trehalose being metabolized to glucose in cells, and these glucose molecules, in turn, metabolized to glucosamine sugars, which are then used in chitin synthesis. The biosynthetic pathway is complex, involving at least eight different reactions [69].

4. Conclusion

Why has trehalose become an evolutionary 'winner' molecule? And this, despite the energetic costs associated with its reasonably convoluted metabolism, as a central go-between between the diet and other storage carbohydrates. The reasons seem to stem from the fact that trehalose is not only a high energy storage molecule, with low reactivity and high stability, but also, and precisely because of this stability and lack of reactivity, because it can stabilize and preserve other biomolecules, like proteins and lipids subjected to stressful environments, besides environments; besides, it can be transported it can be transported and accumulated to high concentrations without toxic effects.

Its prevalence in many different organisms of varied evolutionary origins and relatedness strongly suggest that trehalose has been retained in most lineages, rather than introduced, and that it is only in the vertebrate lineage where it is conspicuously missing. Why this is so in vertebrates, is difficult to explain.

Nowadays, trehalose has been subject to a revival in interest, since many of the same characteristics of non reactivity, stability and no toxicity that have propelled it to such a

prominent role in many species in the natural world are now being newly appreciated by the pharmaceutical and cosmetic industries. Trehalose is being featured more and more in the excipients of drugs and pills, in creams, and as a sugar substitute in processed food formulas, as it is an energetic, non-toxic, stable meal ingredient, and has a sugary taste. Especially in Japan, where a procedure for industrial synthesis of trehalose was invented and is now being commercially applied, the allure of trehalose is proving to be irresistible, with promises in the near future to likewise take on the world as a whole [38].

Author details

Alejandro Reyes-DelaTorre, María Teresa Peña-Rangel and Juan Rafael Riesgo-Escovar[*]
Developmental Neurobiology and Neurophysiology Dept., Instituto de Neurobiología, Universidad Nacional Autónoma de México Querétaro, México

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[*] Corresponding Author

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