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The regulation of trehalose metabolism in insects

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Abstract. Trehalose is a non-reducing disaccharide comprising two glucose molecules. It is present in high concentration as the main haemolymph (blood) sugar in insects. The synthesis of trehalose in the fat body (an organ analogous in function to a combination of liver and adipose tissue in vertebrates) is stimulated by neuropeptides (hypertrehalosaemic hormones), released from the corpora cardiaca, a neurohaemal organ associated with the brain. The peptides cause a decrease in the content of fructose 2,6-bisphosphate in fat body cells. Fructose 2,6-bisphosphate, acting synergistically with AMP, is a potent activator of the glycolytic enzyme 6-phosphofructokinase-1 and a strong inhibitor of the gluconeogenic enzyme fructose 1,6-bisphosphatase. This indicates that fructose 2,6-bisphosphate is a key metabolic signal in the regulation of trehalose synthesis in insects. Trehalose is hydrolysed by trehalase (E.C. 3.2.1.28). The activity of this enzyme is regulated in flight muscle, but the mechanism by which this is achieved is unknown. Trehalase from locust flight muscle is a glycoprotein bound to membranes of the microsomal fraction. The enzyme can be activated by detergents in vitro and by short flight intervals in vivo, which indicates that changes in the membrane environment modulate trehalase activity under physiological conditions.

Key words. Trehalose; insect haemolymph; neuropeptides; insect fat body; fructose 2,6-bisphosphate; insect flight muscle; regulation of trehalase.

Introduction

Trehalose was given its name by the French chemist Berthelot in 1858[4] who found this sugar in *trehala*, a desert manna from Asia minor that is produced by the weevil *Larinus nidificans*. Trehalose is also present in fungi, algae and in several invertebrate phyla but is not known to be produced by flowering plants or vertebrates. The relation between trehalose and insects was ignored until the substance was rediscovered in insects in the mid fifties[52] and its metabolism was extensively studied in the following years. The reactions involved in the synthesis and degradation of trehalose in insects are now well understood; however, despite much effort over almost 40 years, it is still poorly understood how these processes are regulated. Trehalose is present in all insects (at least in the adults) studied in this respect, and in many insects it is present in high concentrations and constitutes the major haemolymph (blood) sugar.

Trehalose (α-D-glucopyranosyl-α-D-glucopyranoside) is a disaccharide in which two glucose molecules are linked by an α-1-1-bond. This structure has the important consequence that the reducing properties of the anomeric carbons of both glucose molecules are eliminated. Unlike glucose, which is quite toxic at high concentrations due to its reducing power, trehalose can be stored in relatively high concentration in body fluids. This is important because insects have an open circulatory system and lack capillaries in their organs, which are bathed by the haemolymph instead. Trehalose concentrations in insect haemolymph are usually between 1 and 2%, whereas blood glucose in humans is kept around 0.1% (5.5 mM).

Insect fat body and the homoeostasis of haemolymph trehalose

Trehalose is exclusively synthesised by the fat body[9,10], a conspicuous organ which in many insects extends from the head to the abdomen and consists of a meshwork of tissue lobes attached to organs and bathed by the haemolymph. The fat body is the principle organ of intermediary metabolism in insects and it combines functions that in vertebrates are executed by the liver and adipose tissue. Trehalose is synthesised from glucose phosphates and UTP (which is energetically equivalent to ATP), and hence is an energy consuming process. Two enzymes are directly involved in the synthesis of trehalose as outlined in figure 1. Trehalose 6-phosphate synthase forms trehalose 6-phosphate from glucose 6-phosphate and UDP-glucose, following which trehalose 6-phosphatase removes the phosphate to release trehalose into the haemolymph[7,9,10].

The synthesis of trehalose in the fat body is under hormonal control. Steele[37] discovered that injection of aqueous extracts of corpora cardiaca into adult cockroaches (*Periplaneta americana*) caused trehalose levels

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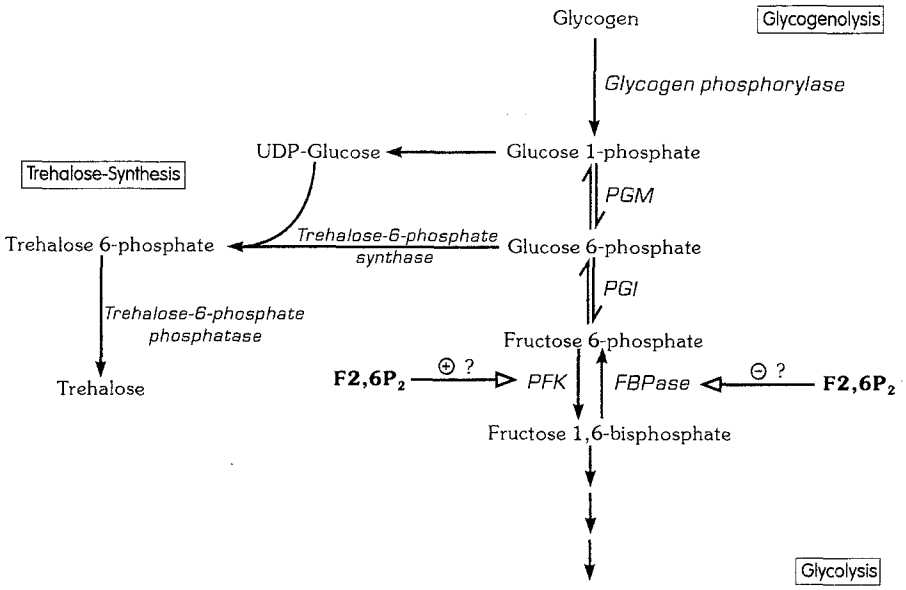

Figure 1. Synthesis of trehalose in insect fat body. Glucose derived from the breakdown of glycogen can serve as a substrate for the synthesis of trehalose as well as for the glycolytic pathway. Abbreviations used: PGM, phosphoglucomutase; PGI, phosphoglucoisomerase; PFK, 6-phosphofructo-1-kinase; FBPase, fructose 1,6-bisphosphatase; UDP-glucose, uridine diphosphoglucose.

in the haemolymph to rise. This observation was confirmed in another cockroach, *Blaberus discoidalis*, by Bowers and Friedman[5]. Under physiological conditions, neuropeptides (hypertrehalosaemic hormones) are released from the corpora cardiaca (a neurohaemal organ linked by nerves to the brain) into the haemolymph where they stimulate the synthesis of trehalose in the fat body and its export into the haemolymph. Two hypertrehalosaemic octapeptides named periplanetin CC1 and CC2, were identified in the corpora cardiaca of *Periplaneta*[35]. A similar neuropeptide (HTH, hypertrehalosaemic hormone), consisting of 10 amino acids, was later found in *Blaberus discoidalis* and *Nauphoeta cinerea*[16,21] (see table 1). The octapeptides are characteristic of the cockroach family Blattidae while HTH is the naturally occurring peptide in the families Blaberidae and Blattelidae[14,15].

A major source of haemolymph trehalose is glycogen in the fat body. It has been observed that haemolymph trehalose is homoeostatically regulated at the expense of tissue glycogen during starvation or exercise-related oxidation of trehalose. The hypertrehalosaemic neuropeptides activate glycogen phosphorylase in the fat body to break down glycogen, thus increasing glucose 1-phosphate, glucose 6-phosphate and fructose 6-phosphate which are maintained near-equilibrium by phosphoglucomutase and hexose phosphate isomerase, respectively. Both glucose phosphates are precursors for the synthesis of trehalose (see fig. 1). The energy-rich phosphate required for the synthesis of trehalose is produced mainly from the oxidation of fat, which is accompanied by a decrease in the rate of glycolytic flux in the fat body despite the increase in the levels of hexose phosphates. Inhibition of glycolysis due to the action of the hypertrehalosaemic hormones[30,49,50] is crucial because glycolysis and trehalose synthesis compete for the same substrate (see fig. 1). However, the mechanism by which the hypertrehalosaemic hormones decrease glycolytic flux is not fully understood. In the liver, where a similar situation exists, the reduction of glycolytic flux is achieved mainly by phosphorylation of a bifunctional enzyme (see below) that catalyses the synthesis as well as the degradation of fructose 2,6-bisphosphate ($F2,6P_2$). $F2,6P_2$ (not to be confused with fructose 1,6-bisphosphate) is not an intermediate of glycolysis or of any other metabolic pathway but is a signal molecule that was discovered as late as 1980 in rat liver[32,46]. $F2,6P_2$ is a potent activator of the glycolytic key enzyme phosphofructo 1-kinase (PFK-1) and an inhibitor of the gluconeogenic enzyme fructose 1,6-bisphosphatase (FBPase-1). In liver, $F2,6P_2$

Table 1. Sequence of cockroach hypertrehalosaemic peptides.

Peptide	Source	Sequence
HTH (=Bld HrTH)	*Blaberus discoidalis*	pGlu-Val-Asn-Phe-Ser-Pro-Gly-Trp-Gly-$ThrNH_2$
Periplanetin CC1 (=Pea CAH-I)	*Periplaneta americana*	pGlu-Val-Asn-Phe-Ser-Pro-Asn-$TrpNH_2$
Periplanetin CC2 (=Pea CAH-II)	*Periplaneta americana*	pGlu-Leu-Thr-Phe-Thr-Pro-Asn-$TrpNH_2$

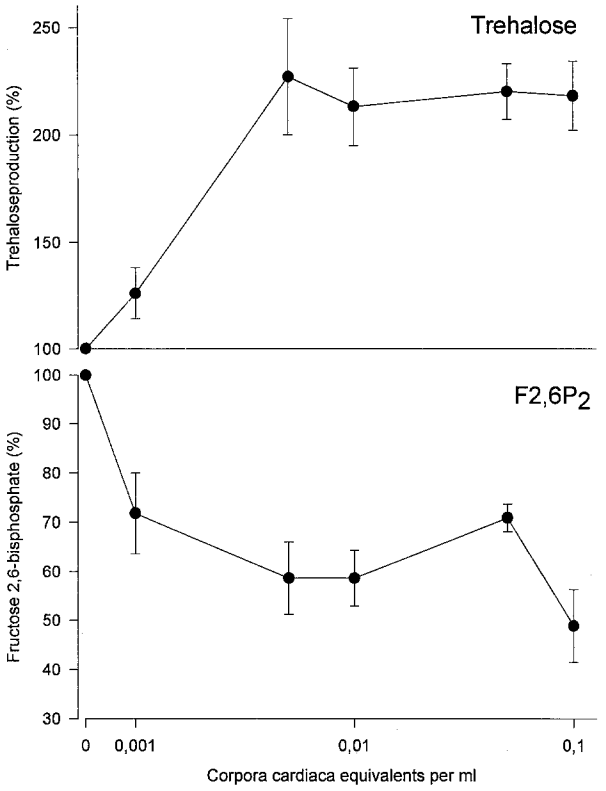

Figure 2. Dose-dependent effects of aqueous extracts of the corpora cardiaca on trehalose production and $F2,6P_2$-content of the fat body of *Blaptica dubia*. Fat body lobes were incubated for 30 min in 2 ml of physiological saline containing the indicated dose of corpora cardiaca extract. Data are expressed in relation to controls which were incubated in physiological saline. As little as 0.005 corpora cardiaca equivalents per ml of incubation medium were sufficient to stimulate the trehalose release from the fat body by more than 100% while the $F2,6P_2$-content was significantly reduced. Corpus cardiacum extract (0.1 equivalents per ml) caused a maximal decrease in $F2,6P_2$ of 51%.

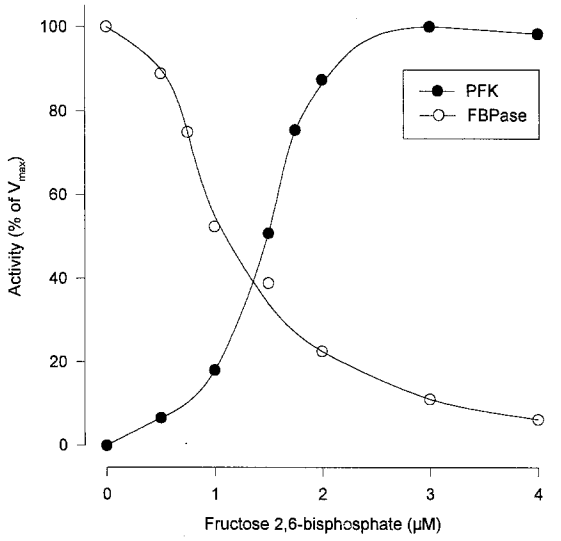

Figure 3. Inverse effects of fructose 2,6-bisphosphate on the regulatory key enzymes of glycolysis (6-phosphofructo-1-kinase) and gluconeogenesis (fructose 1,6-bisphophatase) at concentrations of substrates and effectors in the physiological range (for details see text). *Enzyme assays*. PFK: 1.7 mM ATP, 1 mM P_i, 15 µM Fructose 6-phosphate, 3.8 µM AMP, pH 7.2. FBPase: 50 µM Fructose 1,6-bisphosphate, 5 µM AMP, pH 7.5.

plays a decisive role in the co-ordinated control of glycolysis and gluconeogenesis. The synthesis and degradation of $F2,6P_2$ in the liver is catalysed by the bifunctional enzyme phosphofructo 2-kinase/fructose 2,6-bisphophatase (PFK-2/FBPase-2). Both enzyme activities are localised on a single polypeptide chain and are inversely regulated by reversible phosphorylation and by allosteric effectors. In the unphosphorylated state, kinase activity is favoured, whereas the phosphorylated enzyme acts as a phosphatase thus reducing the content of $F2,6P_2$ in the liver. This leads to an inhibition of PFK-1 and to an activation of FBPase-1 and hence to a shift from glucose consumption (glycolysis) to glucose production (gluconeogenesis).

We have studied the possible role of $F2,6P_2$ in the regulation of glycolysis in the fat body of the Argentine cockroach *Blaptica dubia* (Blaberidae) during hormone-induced trehalose synthesis using isolated fat body lobes. These were incubated in vitro such that one lobe served as the control for the paired lobe which wasincubated with the hypertrehalosaemic agents ('paired tissue technique'[38]). Aqueous extracts of the corpora cardiaca stimulate trehalose release from the isolated fat body and decrease the $F2,6P_2$-content in the fat body (fig. 2). Similar increases in trehalose efflux are produced using synthetic hypertrehalosaemic peptides instead of corpora cardiaca extracts, but HTH proved more effective than either CC1 or CC2 (Becker and Wegener, unpubl. results) which is in agreement with reports that HTH is the naturally occurring neuropeptide in the cockroach family Blaberidae. The effective dose of 10 ng of HTH per ml is equivalent to a concentration of about 10^{-8} molar.

Phosphofructo 1-kinase and fructose 1,6-bisphosphatase purified from the fat body of *Blaptica* are inversely affected by $F2,6P_2$ which proved to be an activator of phosphofructo 1-kinase but a potent inhibitor of fructose 1,6-bisphosphatase (see fig. 3).

Our results suggest that $F2,6P_2$ is part of the mechanism by which glycolysis is inhibited by the hypertrehalosaemic peptides from the corpora cardiaca. How the signal generated by the hypertrehalosaemic peptides is transduced by the cell and how the enzymes PFK-2 and FBPase-2 are regulated in cockroach fat body remains to be studied.

Hypertrehalosaemic hormones have often been compared with glucagon which in vertebrates stimulates the synthesis of blood glucose by the liver. Glucagon also decreases glycolytic flux when glucose is being produced[45]. The hormone acts through a receptor coupled via a stimulatory G-protein to adenylate cyclase to produce the second messenger cAMP. This then acti-

vates the cAMP dependent protein kinase (PKA) which phosphorylates several target enzymes. One of these is glycogen phosphorylase which is thus activated (via phosphorylase kinase) and mobilises glycogen as a source of blood glucose. At first glance it would appear that a similar interpretation could explain the stimulation of trehalose synthesis from glycogen by the hypertrehalosaemic hormones. The data, however, do not support the hypothesis since it has been shown that synthetic hypertrehalosaemic hormones do not stimulate the production of cAMP[26,31]. The evidence therefore favours the idea that hormonal activation of phosphorylase, the principal cause of the increase in trehalose synthesis, is initiated by an agent other than cAMP. The inability of both hypertrehalosaemic hormones to stimulate cAMP synthesis suggests also that any control of glycolysis by PFK-2/FBPase-2 as a result of a phosphorylation-dephosphorylation reaction is unlikely to be associated with cAMP.

The regulation of trehalose synthesis is complex, involving not only control of carbohydrate metabolism but that of lipid metabolism also. Treatment of isolated fat body of *Periplaneta americana* with corpus cardiacum extract which decreases glycolytic flux as mentioned previously, concomitantly increases the oxidation of fatty acids[30]. That this increase in fatty acid oxidation is essential to the synthesis of the trehalose is shown by the severe curtailment of hormone-stimulated trehalose production induced by pent-4-enoic-acid, an inhibitor of β-oxidation. These findings show that hormone stimulated trehalose synthesis is intimately related to the metabolism of lipid in ways that are not yet understood. Activation of phosphorylase is generally considered to be the initial and essential first step in stimulation of trehalose synthesis by the hypertrehalosaemic hormones. Apart from the glycolytic control site associated with trehalose production and already discussed, some paradoxical observations suggest that other important regulatory sites may be present in the pathway between glycogen phosphorylase and the final efflux of trehalose from the fat body cell. Although an increase in trehalose efflux initiated by hypertrehalosaemic hormone is dependent on the activation of phosphorylase, an increase in phosphorylase activation does not necessarily produce an increase in trehalose efflux. Each of cAMP[29], methyl xanthines[38] and the calcium ionophore A23187[29] result in greater activation of phosphorylase than that due to hypertrehalosaemic hormone yet there is no significant increase in trehalose production. These results strongly suggest that there are one or more regulatory sites in the pathway between glycogen phosphorylase and the release of trehalose from the cell other than that associated with phosphorylase itself. Clearly, there is much to be learned about hormone-regulated trehalose synthesis in the fat body.

Trehalose utilisation and the regulation of trehalose activity

Some insect organs, particularly working flight muscles, may use blood-borne substrates at high rates. Because there appears to be no active transport of substrates from haemolymph into the tissues, the concentration of substrates in the haemolymph must be kept sufficiently high in order to provide an adequate fuel supply. Glucose, because of its reactivity, would not be well suited for this function although it plays a paramount role in the cellular metabolism of all animals. Using the non-reducing disaccharide trehalose as the main blood sugar has the additional advantage that the osmotic effect is only half that produced by an equivalent amount of glucose.

Before trehalose can be used in cell metabolism it must be reconverted into glucose. This is achieved by the enzyme trehalase (α-glucoside-1-glucohydrolase, EC 3.2.1.28) which hydrolyses trehalose to yield glucose. Glucose can then be used for syntheses (e.g. of glycogen) or catabolised via glycolysis or the pentose phosphate pathway. Although mammals are unable to synthesise trehalose, they can use this sugar if it is present in their diet. In mammals trehalase is restricted to brush border (microvilli) membranes of the intestine and the kidney. The enzyme is an ectoenzyme bound to the membrane via a phosphatidylinositol anchor[40]. There is no indication of regulation of trehalase activity in mammals.

The trehalase reaction is irreversible under physiological conditions, thus the enzyme will hydrolyse all available trehalose. For this reason trehalase activity in insect tissues must be controlled. Insect trehalase has been thoroughly studied but the mechanism(s) by which its activity is controlled is still not understood. Unlike yeast, where trehalase activity can be modulated by reversible phosphorylation[44], no interconversion of insect trehalase could be demonstrated, nor have any allosteric modulators been found. We shall briefly discuss three examples where trehalase activity in insects tissues is affected by physiological conditions.

Trehalase activity in the intestinal tract

The intestinal tract of insects, especially the midgut, contains high trehalase activity[17,54], even though in the majority of insects trehalose is not a regular constituent of the diet. For this reason Wyatt[52] suggested that the main function of trehalase in insect gut is not the digestion of trehalose from the diet but to recover the small amount of trehalose that diffuses from the haemolymph into the gut lumen[34]. According to this hypothesis trehalose in the gut lumen will be split by the intestinal trehalase and the glucose can then enter the haemolymph for reconversion into trehalose in the fat

body. Thus the concentration of glucose in the haemolymph is kept low and a concentration gradient between gut lumen and haemolymph is maintained. For this function no control of trehalase activity is required provided sufficient enzyme is present in the intestine.
Both soluble and particulate (membrane bound) forms of trehalase have been found in insect guts[41]. The exact localisation of the particulate gut trehalase is not known. Studying silkworm midguts Azuma and Yamashita[1] found trehalase associated with the basal plasma membrane of the epithelial cells but not with the microvilli-rich membranes facing the gut lumen. This led them to suggest that the enzyme might hydrolyse trehalose from the haemolymph to supply substrate for the gut epithelium during starvation. The activity of this trehalase appears to change with the nutritional status of the silkworm but the mechanism by which these changes are brought about is not known.

Trehalase activity during oogenesis

Insect eggs store carbohydrate in the form of glycogen. In silkmoths it has been shown that the source of this store is trehalose from the haemolymph[20]. Trehalase must hence be involved in the metabolism or carbohydrate during oogenesis. This enzyme has been studied in diapause eggs which are particularly rich in carbohydrate and in which the activity of trehalase is affected by diapause hormone (see below). Some insects such as the silkmoth *Bombyx mori* can produce diapause eggs, i.e. eggs in which development is arrested at a specific point during embryogenesis. These eggs thus endure the winter in a state of developmental and metabolic rest (embryonic diapause). Embryonic diapause is induced by diapause hormone, a neuropeptide (of 24 amino acids) from the suboesophagal ganglion[19,53]. The phenomenon has attracted much interest, and it has been shown that carbohydrate metabolism of the eggs is affected by embryonic diapause. As early as the 1930s it was known that diapause eggs store large amounts of glycogen which disappear within 10 days after oviposition and reappear at the end of diapause. Chino[11] demonstrated that the glycogen is converted into sorbitol and glycerol, and Mansingh[27] suggested that these compounds function as anti-freezing agents. Hydrolysis of trehalose seems to be the rate limiting step for glycogen synthesis in oocytes and follicle cells[36]. The trehalase of oocytes and follicle cells is localised in the plasma membrane[2] and could be involved in the transport of sugar into the cell. Trehalase activity in developing ovaries can be increased by diapause hormone, probably via de novo synthesis because the effect is absent if RNA- or protein synthesis is blocked[22,39]. Diapause hormone thus controls the amount of trehalase present, an effect which requires extracellular Ca^{++}. Whether there are additional mechanisms by which the activity of membrane-bound trehalase in oocytes and/or follicle cells can be modulated is not known.

Trehalase activity during insect flight

Insect flight muscle can sustain the highest metabolic rates of all animal tissues[3,24,47]. The high concentration of trehalose in the haemolymph and high activity of trehalase in the flight muscle are vital for flight in many insects. With respect to the localisation and control of trehalase activity in flight muscle, insects can be divided into two groups.
(a) Insects in which flight muscle trehalase is associated with the mitochondria which is the case in asynchronous (fibrillar) flight muscles. These muscles can be activated by stretching. The phenomenon of stretch activation is restricted to insects and can lead to wing beat frequencies of more than 1000 per second, i.e. much higher than the spike rate of the motor neurons. This type of flight muscle has a high content of fibrils and mitochondria whereas the sarcoplasmic reticulum is reduced. In these flight muscles, trehalase is associated with the outer surface of the inner mitochondrial membrane[6]. Its activity seems to depend on substrate availability which requires transport of trehalose across the cytoplasmic membrane. No regulatory properties of this particular trehalase have yet been demonstrated and the control of trehalose degradation appears to have been shifted to the site where trehalose enters the myofibres. Little is known as to how trehalose is transported across the cytoplasmic membrane and how this transport is regulated. Fibrillar flight muscles are found in various insect orders such as hymenoptera and diptera. The mitochondrial association of trehalase has been demonstrated in the honey-bee *Apis mellifera*[6] and in some flies: *Phormia regina*[33], *Calliphora erythrocephala*[13] and *Sarcophaga barbata*[12]. In all these insects carbohydrate is the main if not the only fuel for flight.
(b) Insects in which flight muscle trehalase is bound to membranes that appear in the microsomal fraction upon cell fractionation. This type of trehalase has been demonstrated in synchronous flight muscles of many species such as the silkmoth *Hyalophora cecropia*[18], the cockroach *Blaberus discoidalis*[17], the desert locust *Schistocerca gregaria*[8], and the migratory locust *Locusta migratoria*[42,51].
In synchronous flight muscles neuronal signals (spikes) and muscle contraction are strictly synchronised. Most synchronous flight muscles use carbohydrate and lipid for flight but in different proportions depending on the duration of the flight. We shall concentrate on the migratory locust as a representative of this group of insects. Locusts are able to fly for many hours in search for food. During the initial phase of flight carbohydrate is preferentially oxidised whereas lipid provides the main fuel for prolonged flight[23,28,43,48]. Trehalose in the

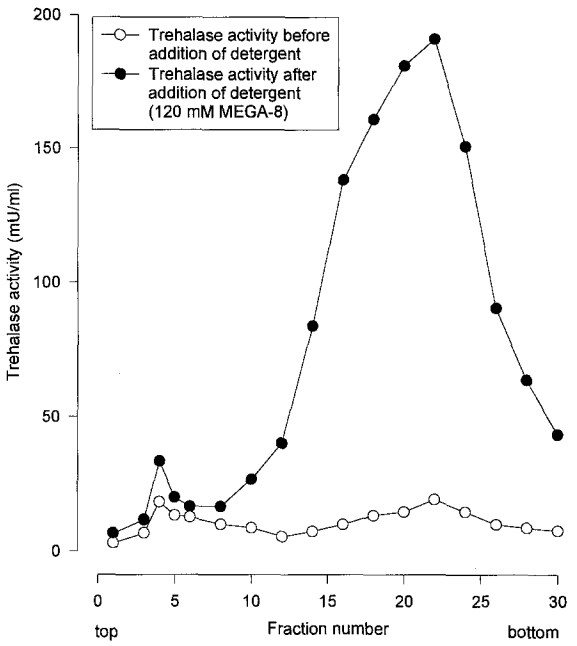

Figure 4. Total membrane fraction from locust flight muscle separated on a density gradient (25%–60% sorbitol). A crude homogenate of locust muscle was filtered through nylon gauze, centrifuged at 450 g and the supernatant was centrifuged again at 100 000 g to yield the total membrane fraction. Fractions of 1 ml were collected, trehalase activity was assayed in aliquots before and after addition of detergent (120 mM MEGA-8).

haemolymph is the main source of carbohydrate for locust flight, thus it has a key role in locust flight metabolism. The same holds good in cockroaches where injection of a competitive inhibitor of trehalase has been shown to reduce the capacity for flight while the concentration of trehalose in the haemolymph was significantly increased[25]. In resting locusts trehalase activity in the flight muscle is low although the haemolymph concentration of the substrate is high. With the onset of flight, trehalose utilisation increases more than tenfold[43]. From this observation it is clear that the hydrolysis of trehalose, i.e. trehalase activity in insect flight muscle must be controlled under physiological conditions. Trehalase from insect flight muscle has been studied for almost 40 years but as yet no hormones, second messengers or metabolites have been found that modulate its activity. How trehalase activity is regulated during flight is still not known and presents an intriguing problem for insect physiologists.

Trehalase in locust flight muscle is associated with cellular membranes, but the precise location is not known. It has, however, long been known that trehalase activity in synchronous flight muscle exists predominantly in a latent form that can be activated in vitro by detergents or other means that destroy the structural integrity of the membrane. Interestingly, a brief flight also increases the proportion of the active form of the enzyme while the latent activity decreases but the total activity is unchanged[8]. We have recently extended the characterisation of trehalase from *Locusta* by separating a total membrane fraction of locust flight muscle on a density gradient (see fig. 4). Trehalase activity is distributed over the entire gradient but different fractions differed greatly in the degree to which they could be activated. It thus became clear that the total membrane fraction, in which trehalase could be activated about fivefold by detergent, consists of different subfractions that can be activated between twofold and more than thirtyfold (fig. 4). A brief flight (5 min fixed flight) resulted in an increase in the amount of active enzyme in some subfractions while the total trehalase activity as well as the affinity for its substrate remained unchanged (Schlöder and Wegener, unpubl. results).

Our recent experiments would therefore suggest that activation of trehalase in vivo is related to changes in the membrane environment of the enzyme. The characterisation of subfractions of membrane-bound trehalase could therefore lead to a better understanding of the regulation of trehalase activity in vivo.

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