

The toxic and lethal effects of the trehalase inhibitor trehazolin in locusts are caused by hypoglycaemia

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Summary

The main blood sugar of locusts is trehalose, which is hydrolysed to two glucose units by trehalase. Homogenates of locust flight muscles are rich in trehalase activity, which is bound to membranes. A minor fraction of trehalase is in an overt form while the remainder is latent, i.e. active only after impairing membrane integrity. Trehazolin, an antibiotic pseudosaccharide, inhibits locust flight muscle trehalase with apparent K_i - and EC_{50} values of 10^{-8} mol l⁻¹ and 10^{-7} mol l⁻¹, respectively. Trehazolin is insecticidal: 50 µg injected into locusts completely and selectively blocked the overt form of muscle trehalase (with little effect on latent activity) and killed 50% of the insects within 24 h. Here, it is demonstrated for the first time that trehazolin causes dramatic hypoglycaemia. Injection of 10 µg trehazolin caused glucose levels to fall by over 90% in 24 h, from 2.8 mmol l⁻¹ to 0.23 mmol l⁻¹,

while trehalose increased from 61 mmol l⁻¹ to 111 mmol l⁻¹. Feeding glucose to the locusts fully neutralized the effects of a potentially lethal dose of trehazolin. This indicates that hypertrehalosaemia is not acutely toxic, whereas lack of glucose causes organ failure (presumably of the nervous system), and that sufficient haemolymph glucose can only be generated from trehalose by trehalase. The results also suggest that overt flight muscle trehalase is located in the plasma membrane with the active site accessible to the haemolymph. Trehalase inhibitors are valuable tools for studying the molecular physiology of trehalase function and sugar metabolism in insects.

Key words: trehalose, glucose, haemolymph, insect, trehalase localisation, flight muscle, *Locusta migratoria*.

Introduction

Trehalose (α -D-glucopyranosyl- α -D-glucopyranoside) is a non-reducing disaccharide present in fungi, algae and several invertebrate phyla such as nematodes, annelids and arthropods (see Elbein, 1974). In many insects, trehalose constitutes the major haemolymph (blood) sugar, whereas glucose is often present at much lower concentrations (for a review, see Wyatt, 1967; Becker et al., 1996). Trehalose is synthesized and released into the haemolymph by the fat body, the central organ of intermediary metabolism in insects (Candy and Kilby, 1959, 1961).

Trehalose is split into two glucose units by the enzyme trehalase (EC 3.2.1.28), which is present in many insect tissues (for a review, see Becker et al., 1996). In locusts, the enzyme has been found in thoracic ganglia (Strang and Clement, 1980), brain and skeletal muscle. Trehalase activity is very low in locust haemolymph, and it is doubtful whether this activity is due to a 'true' trehalase (Vaandrager et al., 1989). High activity has been found in flight muscle (Candy, 1974; Worm, 1981; Vaandrager et al., 1989; the present study), and trehalose has been identified as an important fuel in locust flight (Mayer and Candy, 1969; Jutsum and Goldsworthy, 1976; Van der Horst et al., 1978; Candy, 1989).

In locust flight muscle, trehalase is bound to membranes that appear in the microsomal fraction upon cell fractionation, but the exact cellular location is not known. The trehalase reaction is irreversible under physiological conditions, thus the enzyme would hydrolyse all available trehalose. In resting locusts, trehalase in flight muscle shows low activity, although the concentration of its substrate in the haemolymph is very high. With the onset of flight, when ATP turnover in flight muscle increases dramatically (for a review, see Wegener, 1996), trehalose utilization rises by more than 10-fold (Van der Horst et al., 1978), and this requires a corresponding increase in trehalase activity. Hence, the activity of trehalase in locust flight muscle must be regulated, but the mechanism of control has remained obscure despite several attempts to understand this problem (Candy, 1974; Worm, 1981; Vaandrager et al., 1989; for a review, see Becker et al., 1996). Insect trehalase activity has not been found to be modulated by hormones, second messengers, allosteric effectors or reversible interconversions. It has, however, been shown that trehalase in homogenates of locust flight muscle appears in two forms, an overt form that is active without further treatment and a latent form that is inactive but can be activated *in vitro* by detergents

or other means that destroy the structural integrity of membranes (Candy, 1974; Worm, 1981; Vaandrager et al., 1989; for a review, see Becker et al., 1996). Also, Candy (1974) observed an increase in the overt form but no changes in the total activity of flight muscle trehalase after a short flight. The mechanism of this phenomenon has remained obscure.

Trehazolin is a natural pseudosaccharide (amino sugar) and a potent and specific inhibitor of trehalases (for a review, see Kobayashi, 1999). It was discovered and isolated as a product of the actinomycete *Micromonospora* by Ando et al. (1991). Trehazolin has antifungal as well as insecticidal activity but does not affect mice when injected at a dose of 100 mg kg⁻¹ (Ando et al., 1995a). The effects of trehazolin (and of related trehalase inhibitors) on trehalases have been thoroughly studied. Trehazolin is a tight-binding competitive inhibitor that seems to mimic the structure of the transition state of the substrate (Ando et al., 1995b). The effects of trehazolin and other competitive trehalase inhibitors on physiological processes such as development, metamorphosis, metabolism and flight performance in insects have also been investigated in detail in various species. Injection of the trehalase inhibitor validoxylamine in last instar silkworms (*Bombyx mori*) caused severe developmental disruption. Using ¹H- as well as ¹³C- and ³¹P-NMR spectroscopy on silkworm haemolymph, Kono et al. (1993) demonstrated a more than twofold increase in trehalose but only minor changes in other metabolites (glucose was not detected in the spectra). The mechanism of the toxic effects of trehalase inhibitors in insects, however, could not be elucidated. In the present study, the effects of trehazolin on trehalase activity of locust flight muscle *in vitro* and *in vivo* are investigated, as well as its toxicity in intact adult locusts. A hypothesis that the lethal effect of trehazolin (and similar trehalase inhibitors) is due to severe hypoglycaemia that appears to cause a failure of the nervous system is suggested and tested. Trehalase inhibitors are advocated as valuable tools in studies on insect physiology as well as cellular and molecular aspects of trehalase function and control.

Materials and methods

Animals

Adult locusts (*Locusta migratoria* L.; Orthoptera) were purchased from commercial suppliers and kept at 30±4°C and 30% relative humidity in 40 cm×40 cm×40 cm cages under crowded conditions. The cages were illuminated by 25 W bulbs for 16 h per day. The locusts were fed on grass, reed and bamboo leaves supplemented with bran and dog-flakes (Matzinger, München, Germany). They were between 20 and 30 days after the final moult when used for the experiments.

Experimental treatment of locusts

Trehazolin was dissolved in distilled water at various concentrations and 5 µl was injected into the haemolymph using a 10 µl Hamilton syringe. The needle was inserted into the thorax cavity through the soft membrane behind the base of the hind leg. Controls were injected with 5 µl distilled water.

Controls and experimental animals were kept singly in small plastic containers with access to food and water.

For collecting haemolymph, the injection puncture was widened with a needle, and the emerging fluid was absorbed in a graded capillary tube. Up to 30 µl haemolymph per insect was collected in a plastic vessel, which was incubated at 100°C for 10 min, cooled on ice and centrifuged at 10 000 g for 5 min. The supernatant was diluted with 19 parts distilled water and kept at -40°C until assayed.

Feeding glucose to locusts injected with trehazolin

Twenty male locusts were separated and fed well for 72 h. The animals were weighed and individually injected with 50 µg trehazolin in 5 µl. They were kept singly and randomly divided into two groups of 10. One group was fed glucose every 2 h while the other was fed tapwater. Glucose (in doses of 50 µl 10% glucose in tapwater) was offered to individual locusts from the tip of a pipette and was readily taken up by the animals, as was the tapwater given to the controls. Surviving experimental animals and controls were weighed after 24 h and their behaviour was further observed for up to 72 h.

Chemicals

Chemicals and enzymes were purchased from Roche Diagnostics (Mannheim, Germany), Sigma (Deisenhofen, Germany), Merck (Darmstadt, Germany), Roth (Karlsruhe, Germany) and Serva (Heidelberg, Germany). Trehazolin was prepared as described by Ando et al. (1991).

Assay of trehalase activity

Trehalase activity was followed by a spectrophotometric assay at 340 nm and 25°C. In a 500 µl total volume, the assay comprised 120 mmol l⁻¹ sodium acetate (pH 6.5), 10 mmol l⁻¹ MgCl₂, 0.6 mmol l⁻¹ NADP⁺, 1 mmol l⁻¹ ATP, 0.42 U (1 U = 1 µmol substrate transformed min⁻¹) glucose-6-phosphate dehydrogenase, 0.67 U hexokinase, trehalase sample and 20 mmol l⁻¹ trehalose (to start the reaction after a pre-incubation period of 5 min). The assays to differentiate between overt and latent trehalase activity are described below (see Tissue processing). One Unit of trehalase activity is equivalent to the hydrolysis of 1 µmol trehalose min⁻¹ at 25°C.

Assay of glucose and trehalose in haemolymph

Glucose was measured at 30°C by a specific spectrophotometric test adapted from Kunst et al. (1984). In a 500 µl total volume, the assay comprised 150 mmol l⁻¹ triethanolamine buffer (pH 7.6), 1 mmol l⁻¹ ATP, 0.6 mmol l⁻¹ NADP⁺, 8 mmol l⁻¹ MgCl₂, 0.42 U glucose-6-phosphate dehydrogenase and 0.67 U hexokinase.

The assay of trehalose was based on the trehalase assay (see above), with a haemolymph sample replacing the substrate trehalose, and 0.4 U ml⁻¹ commercial trehalase from pig kidney (Sigma) replacing the trehalase sample. Care was taken to sufficiently dilute haemolymph samples from locusts that had been injected with trehazolin because otherwise the trehazolin

in these samples may inhibit the trehalase in the assay. The extinction at 340 nm was read before starting the reaction by adding trehalase and again after 90 min incubation at 37°C. Two controls were run in which either the haemolymph sample or the trehalase was replaced with distilled water. All data are means \pm S.E.M. and were analysed for statistical differences by Student's two-sided *t*-test.

Tissue processing, membrane extraction and purification of trehalase

Locusts were immersed in liquid nitrogen and stored at -80°C until use. The thoraces were isolated and dissected while still frozen. The flight muscles were carefully freed from adhering parts of gut and fat body, collected on ice, and weighed. The tissue was homogenised for about 3 min at 600 r.p.m. in nine parts (v/w) of buffer A (50 mmol l⁻¹ maleate buffer, pH 6.5) using a Potter homogeniser (Teflon-glass; Braun, Melsungen, Germany). This crude homogenate was used to differentiate between overt, latent and total trehalase activity. Overt activity was measured in the absence of detergent, and total activity was measured after addition of 30 mmol l⁻¹ of the zwitterionic detergent CHAPS {3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate} to the homogenate. In locusts not treated with trehazolin, latent trehalase activity is the total activity minus the overt activity, according to the equation: total trehalase activity = overt trehalase activity + latent trehalase activity (see Table 1 and Discussion).

It should be mentioned that homogenates of freshly prepared flight muscles contain less overt trehalase activity than homogenates of flight muscles dissected from frozen locusts. This observation is in line with previous reports that flight muscle trehalase can be activated by repeated freezing and thawing. However, in order to process all animals used in an experiment in the same way and to determine their trehalase activity in the same series of measurements, we have routinely frozen and stored the locusts.

To extract trehalase for kinetic analysis in the absence and presence of trehazolin, the crude homogenate of locust flight muscle was centrifuged at 4°C and 40 000 g in a Sorvall RC5C for 45 min. The sediment was resuspended with four parts of a detergent buffer (buffer B: 300 mmol l⁻¹ sodium acetate, pH 6.5, containing 30 mmol l⁻¹ CHAPS) and again centrifuged as before. The supernatant (membrane extract) was stored at -40°C . Trehazolin was added before the enzyme assays were started.

Purification of trehalase was performed at 4°C. Media contained 0.1 mmol l⁻¹ phenylmethylsulfonyl fluoride (PMSF) and 0.01% sodium azide and were degassed by sonication. The crude muscle homogenate was centrifuged at 100 000 g for 1 h (Beckman L8-55 centrifuge). The sediment was resuspended in four volumes of homogenisation buffer (buffer A) in a Potter homogeniser (800 r.p.m., 3 min) and incubated with 30 mmol l⁻¹ CHAPS at room temperature for 30 min. After another centrifugation at 100 000 g, approximately 95% of the trehalase activity was found in the supernatant, which was

chromatographically separated on chelated Cu²⁺ ions (iminodiacetic acid-epoxy-activated Sepharose 6B) using a Pharmacia chromatography unit. The Cu²⁺-chelate column was equilibrated with buffer C (0.1 mol l⁻¹ sodium acetate, pH 6.5, 0.1% Triton X-100), loaded with the solubilised trehalase and washed with 0.5 mol l⁻¹ KCl in the same buffer to remove non-specifically bound proteins. Trehalase was eluted by a gradient of 0–1 mol l⁻¹ glycine in buffer C at 40 ml h⁻¹.

Fractions containing more than 2% of the initial trehalase activity were combined and subjected to affinity chromatography using a lectin concanavalin A column (Con-A-Sepharose 4B; Sigma) according to Jahagirdar et al. (1990). The column was washed with 0.25 mol l⁻¹ NaCl in Con-A buffer (0.1 mol l⁻¹ sodium acetate, pH 7.0, 1 mmol l⁻¹ CaCl₂, 1 mmol l⁻¹ MnCl₂ and 0.1% Triton X-100). Elution (at 50 ml h⁻¹) was achieved with 0.2 mol l⁻¹ α -methyl-D-mannoside in Con-A buffer. As before, trehalase fractions were combined. They were concentrated by ultrafiltration using N₂ at 200–300 kPa pressure (Amicon unit, Millipore cellulose filter, 30 kDa). The α -methyl-D-mannoside was removed by gel filtration on Sephadex G25. The preparation was finally dialysed against 80% (v/v) glycerol in Con-A buffer and stored at -40°C . Protein was determined with bovine serum albumin as standard (Bradford, 1976).

Results

Inhibition of locust flight muscle trehalase by trehazolin in vitro

Flight muscle was homogenised and centrifuged at 40 000 g, and the centrifugation sediment (containing membrane fragments and virtually all trehalase activity) was extracted with detergent buffer to solubilize trehalase (see Materials and methods). Trehalase activity in the extract was assayed at four fixed concentrations of trehalose with varied concentrations of trehazolin. The apparent inhibitor constant (K_i), determined according to Dixon (1953), was 10 nmol l⁻¹ (Fig. 1). The inhibitor concentration required for 50% inhibition (IC₅₀; at 20 mmol l⁻¹ trehalose) was 90 nmol l⁻¹.

Virtually all trehalase activity was found in the total membrane fraction of locust flight muscle after centrifugation of the muscle homogenate at 100 000 g for 1 h. Trehalase was purified from this fraction (see Materials and methods) about 700-fold, with a 24% yield, to a specific activity of 12.5 U mg⁻¹ protein. Trehalase activity followed Michaelis–Menten kinetics (not shown) with a K_m of approximately 1 mmol l⁻¹. The effects of trehazolin on purified trehalase were similar to those with membrane extracts. The apparent K_i was 8 nmol l⁻¹ and the IC₅₀ was 120 nmol l⁻¹ trehazolin.

Inhibition of locust flight muscle trehalase by trehazolin in vivo

Total trehalase activity in homogenates of flight muscle was approximately 4 $\mu\text{mol min}^{-1} \text{g}^{-1}$ at 25°C (4 U g⁻¹). Only a fraction of the total trehalase activity was active if the homogenates had not been incubated with detergent. This overt

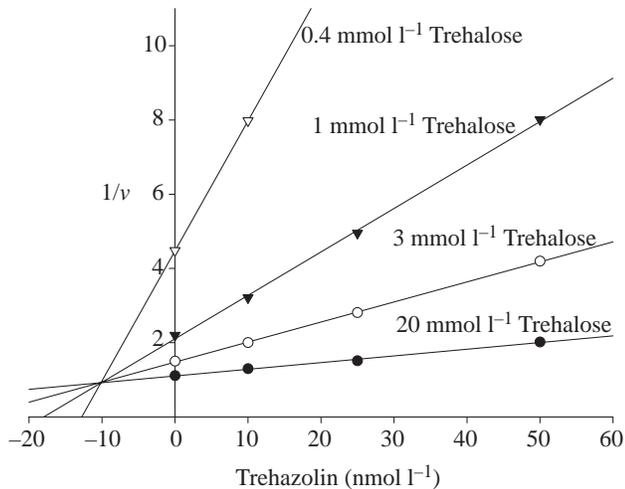


Fig. 1. Effect of trehalose on the activity of trehalase extracted from locust flight muscle membranes by detergent (see Materials and methods). The data, plotted according to Dixon (1953), show trehalose to be a competitive inhibitor with an apparent inhibitor constant (K_i) of 10 nmol l^{-1} .

trehalase activity accounted for less than 18%; hence, more than 82% was present in a latent form that needed detergent for activation (Table 1). Interestingly, trehalose injected into locusts strongly inhibited the overt form of trehalase but had much less effect on total activity. Thus, $50 \mu\text{g}$ trehalose per locust completely blocked the overt trehalase in flight muscle homogenates, while total activity was only reduced by 35%. However, total trehalase after activation by detergent is fully susceptible to inhibition by trehalose.

Toxicity of trehalose in locusts

Eleven male locusts were each injected with $50 \mu\text{g}$ trehalose. These insects showed reduced motor activity. They moved sluggishly, if at all, and failed to show the normal avoidance or flight reflexes when prodded. Six locusts had died after 24 h, indicating that the LD_{50} (24 h) is approximately $50 \mu\text{g locust}^{-1}$ (see below).

Approximately two hours before they died, the locusts started to tumble, with poor co-ordination of body and

Table 1. Effect of trehalose, injected into locusts, on the activity of overt and latent trehalase in flight muscle homogenates

Trehalose injected per locust (N)	Trehalase activity ($\mu\text{mol min}^{-1} \text{g}^{-1}$ muscle)		Overt/total (%)
	Overt	Total	
$0 \mu\text{g}$ = control (9)	0.67 ± 0.05	3.86 ± 0.18	17.4
$50 \mu\text{g}$ (4)	ND	2.51 ± 0.09	0

Although the relative effects of trehalose on overt and total trehalase activity were vastly different, they were both highly significant at $P < 0.001$. Means \pm s.e.m. are given. N , number of experiments. ND, not detectable.

extremities. The animals fell to their sides about 30–45 min before death, and, lying on their side or back, some of them showed intense contractions of their jumping legs. During the final 5–10 min, an intense tremor was observed, with rapid clonic cramps of the hind legs. We classed the locusts as dead when no movements could be elicited.

Trehalose causes severe hypoglycaemia in locusts

In order to understand the mechanisms of the toxic action of trehalose in locusts we followed changes in sugar content of locust haemolymph. Adult locusts (of both sexes) were injected with $10 \mu\text{g}$ trehalose in $5 \mu\text{l}$ distilled water (experimental animals), while controls received only $5 \mu\text{l}$ water. $20 \mu\text{l}$ of haemolymph (one sample per locust) was collected at six intervals after the injection and assayed for trehalose and glucose. Trehalose is the main blood sugar of locusts, corresponding to $21.0 \pm 1.1 \text{ g l}^{-1}$ haemolymph in controls ($N=5$), whereas glucose accounted for $0.50 \pm 0.03 \text{ g l}^{-1}$ haemolymph, i.e. only 2.4% of the trehalose content.

As was expected from work in other insect species, trehalose caused a marked increase in the trehalose concentration of locust haemolymph, which rose from 21 g l^{-1} ($=61.35 \pm 3.1 \text{ mmol l}^{-1}$) in controls by over 80% to 37.9 g l^{-1} ($=110.9 \pm 3.8 \text{ mmol l}^{-1}$) 24 h after the injection of trehalose (Fig. 2A).

The effect of trehalose on haemolymph glucose was even more dramatic than that on trehalose, although in the opposite direction (Fig. 2B). Haemolymph glucose decreased precipitously, from $0.50 \pm 0.03 \text{ g l}^{-1}$ (2.8 mmol l^{-1}) in controls, by 50% within 2 h and reached $0.041 \pm 0.07 \text{ g l}^{-1}$ (0.23 mmol l^{-1}), i.e. 8.2% of the control level, after 24 h. At this time, the glucose content in the haemolymph was only 0.11% of the trehalose content. Hypoglycaemic effects of trehalose or other trehalase inhibitors have not been reported before.

Feeding glucose to locusts neutralizes the toxic effects of trehalose

To study whether hypoglycaemia was causing the toxic and lethal effects of trehalose, we fed glucose to locusts as a possible antidote to trehalose. 20 male locusts were randomly divided into two groups of 10, and each locust was injected with $50 \mu\text{g}$ trehalose. The 10 animals of the control group (which received only water) had a mean body mass of $1.40 \pm 0.08 \text{ g}$, and the survivors had not lost mass after 24 h. However, food uptake in all control animals was markedly reduced, and four of the 10 locusts did not consume any food. However, all animals accepted tapwater when this was offered from a pipette in $50 \mu\text{l}$ portions every 2 h for 24 h. The first animal died 4 h after the injection of trehalose, the second after 10 h; after 24 h, five locusts had died, and only three were alive 36 h after the injection (Fig. 3). This is in line with the previous observation that trehalose is toxic in locusts, with an LD_{50} (24 h) of $50 \mu\text{g locust}^{-1}$. Based on body mass, the LD_{50} (24 h) was $36 \mu\text{g g}^{-1}$.

Glucose (10%), instead of water, was offered to the 10 locusts in the experimental group at 2 h intervals for 24 h. Their

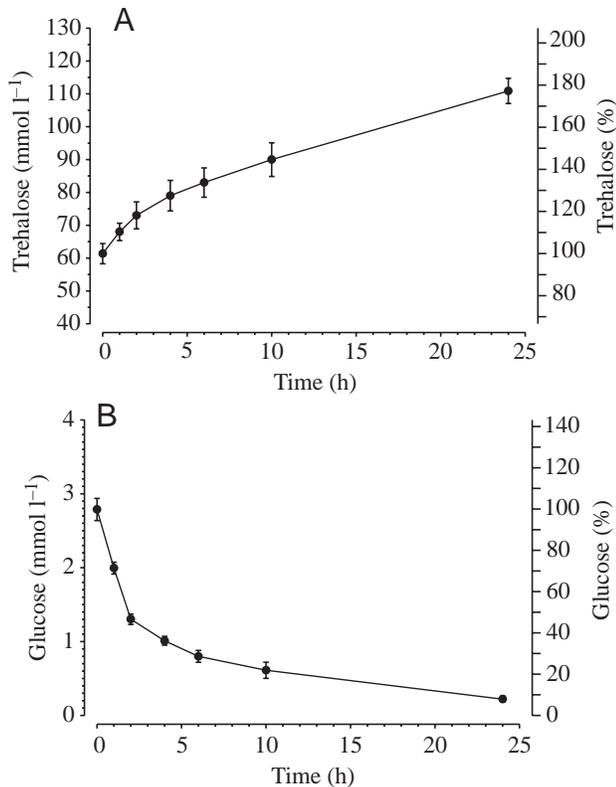


Fig. 2. (A) Hypertrehalosaemic and (B) hypoglycaemic effects of trehazolin in locusts. Locusts were injected with 10 μg trehazolin in 5 μl distilled water or with water only (control = 0 h). Haemolymph samples were collected over 24 h for assays of trehalose and glucose. The data are given in mmol l^{-1} (left scale) and % (right scale) of control and are means \pm S.E.M. ($N=5$ independent assays). The inhibitor-induced increase in haemolymph trehalose was significant at $P<0.05$ after 1 h and 2 h, at $P<0.01$ after 4 h, and at $P<0.001$ thereafter. The hypoglycaemic effect was highly significant at all times ($P<0.001$).

mean mass was 1.35 ± 0.10 g, and this was not significantly changed after 24 h. Nine locusts took the glucose eagerly and did not show any adverse effects of trehazolin in their behaviour. The animals were active and ingested food in a similar manner to untreated locusts; their movements were fully coordinated and their avoidance and flight reflexes unaffected. After 24 h, these locusts appeared completely normal, and none of them died in the following two days of the observation period. Hence, feeding glucose to locusts injected with a potentially lethal dose of trehazolin can fully relieve the toxic effects of trehazolin. This indicates that the lethal effect of trehazolin is due to its hypoglycaemic action (see Discussion). The one locust that did not accept the glucose offered behaved unusually from the start of the experiment: it hardly moved after the injection of trehazolin, did not show avoidance reflexes and died within 6 h. We assume that it must have been either diseased or injured by the injection. Hence, the unusual behaviour of this one locust does not invalidate our conclusion that glucose can neutralize the toxic effects of trehazolin.

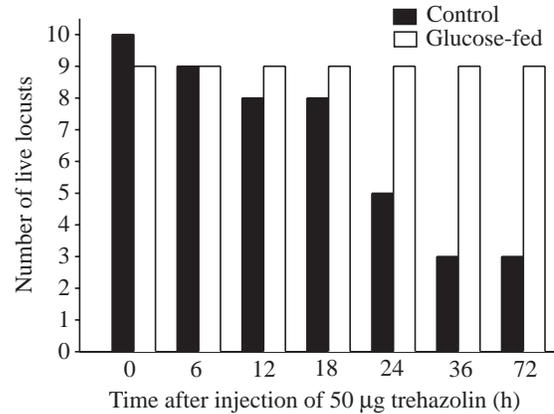


Fig. 3. The lethal effect of trehazolin in locusts is due to lack of glucose (hypoglycaemia), as it can be prevented by feeding glucose to the insects. Male locusts were injected with 50 μg trehazolin each. The animals were randomly divided into two groups, of which one was fed with tapwater (filled bars) while the other was offered 10% glucose (open bars). None of the locusts that ingested glucose ($N=9$) died during the observation period, whereas 70% of the locusts fed with water ($N=10$) died within 36 h (for details, see text)

Discussion

Trehazolin as enzyme inhibitor and experimental tool to study locust flight muscle trehalase

The apparent K_i of trehazolin was similar when tested in extracts of whole membrane fractions or in trehalase purified from locust flight muscle membranes. Trehazolin binds specifically to the active site, and the binding is very tight (Ando et al., 1995b). Because trehalase has a much higher affinity for the inhibitor than for the substrate, as indicated by the K_m/K_i ratio of approximately 10^5 , the inhibition is very strong and long-lasting at physiological trehalose concentrations.

We have studied the effect of trehazolin on trehalase from locust flight muscle, a tissue particularly rich in trehalase, but, given that trehazolin is a substrate analogue, there can be little doubt that trehalases from all locust tissues will be inhibited by trehazolin. So far, trehalases from all sources (invertebrates and vertebrates) have been found to be susceptible to trehazolin or similar competitive inhibitors.

Of interest is our observation that trehazolin (and probably other competitive trehalase inhibitors) selectively affects the overt form of flight muscle trehalase, as this offers the possibility to differentiate between overt and latent trehalase. The fact that a trehalase inhibitor differentiates between overt and latent trehalase has not been reported before. Although the activation of insect trehalases by treatments that interfere with membrane structures had been noticed early on (Zebe and McShan, 1959; Gussin and Wyatt, 1965; Gilby et al., 1967), the molecular basis of this phenomenon has remained elusive (for a review, see Becker et al., 1996).

Latent trehalase, which is catalytically inactive *in vitro*, must be derived from a trehalase form that is protected from inhibition by trehazolin in the intact locust. The fact that latent

trehalose becomes active and susceptible to trehazolin if treated with detergent suggests that enzyme latency also occurs *in vivo* and is based on a structural barrier that prevents equally the substrate and the inhibitor from reaching the active site of the enzyme (see below). The above-mentioned properties make trehazolin a useful tool in physiological experiments as it facilitates studies of location and function of trehalases in live insects as well as manipulation of glucose and trehalose in insect haemolymph.

Hypertrehalosaemic and hypoglycaemic effects of trehalase inhibitors

The main blood sugar in locusts is not glucose but trehalose, as had already been shown in the 1950s when this sugar was rediscovered in insects (for reviews, see Wyatt, 1967; Becker et al., 1996). Trehalose has two major advantages over glucose as a blood sugar: (1) as a disaccharide, trehalose is less osmotically active than glucose and can, therefore, be tolerated at higher levels (w/v) in blood than glucose and (2) trehalose is non-reducing and hence not involved in the non-enzymatic glycosylation of proteins, which is a major factor in glucose toxicity (as in diabetes mellitus; for a review, see Cohen, 1986).

Trehazolin caused a marked increase in trehalose in the haemolymph of locusts. This effect can be regarded as typical for trehalase inhibitors of this kind, as it has been demonstrated in all insects tested so far and also with trehalase inhibitors other than trehazolin. For instance, validoxylamine A was recently shown to bring about a marked and long-lasting increase in haemolymph trehalose in migratory locusts (Kono et al., 1999).

Glucose is much less prominent in locust haemolymph, accounting <2.5% of trehalose on a mass basis. The low glucose content in insect haemolymph is probably the reason why the inhibitor-induced dramatic decrease in haemolymph glucose has escaped notice for so long. In previous studies, the effects of trehalase inhibitors on haemolymph constituents were usually analysed by NMR-spectroscopy (Kono et al., 1993, 1994a,b, 1999; Takahashi et al., 1995). This powerful method can detect many compounds simultaneously, yet is not sensitive enough to follow a decrease in glucose content.

Thus, it is suggested that trehalase inhibitors, such as trehazolin and validoxylamine A, bring about a marked increase in haemolymph trehalose in locusts and cause severe hypoglycaemia, and this will hold true for other insects that are similarly susceptible to trehalase inhibitors.

Haemolymph glucose is derived from trehalose and is essential for locust survival

Our hypothesis that the toxic and lethal effects of trehazolin are due to lack of glucose has been substantiated by feeding glucose to trehazolin-injected locusts. Not only did the glucose result in the survival of the locusts (Fig. 3) but it also prevented all behavioural impairment by trehazolin. This observation leads to interesting conclusions about the metabolic physiology of locusts (and probably other insects). (1) Feeding glucose will stimulate rather than reduce the synthesis of trehalose and

yet enables the locusts to tolerate lethal doses of trehazolin. The inhibitor-induced non-physiologically high levels of haemolymph trehalose do not therefore contribute noticeably to the acute toxicity of trehazolin in locusts. (2) Although trehalose is far more prominent than glucose in haemolymph, glucose is absolutely necessary for locust survival. This would require haemolymph levels of glucose to be regulated more strictly than those of trehalose, which has indeed been reported (Mayer and Candy, 1969; Strang and Clement, 1980). (3) The fraction of trehalase *in vivo* that gives rise to the overt form *in vitro* must be intimately involved in the production of haemolymph glucose, and haemolymph trehalose appears to be the major, if not the only, source of glucose in insects. (4) Trehalose and trehazolin are both hydrophilic, and there is no indication that they are transported into locust cells. We therefore assume that trehazolin and trehalose have access to the same metabolic compartments. Thus, the observation that trehazolin in live locusts had not reached the fraction of trehalase that corresponds to the latent enzyme activity *in vivo* (after 24 h of incubation; see Table 1) would mean that this fraction of trehalase (*in vivo* latent trehalase) is separated from its substrate *in vivo* and is hence not active in live locusts (see below). (5) Unlike vertebrates, in which blood glucose is produced by the liver, locusts (and probably other insects) do not have an organ specialised for glucose production. (6) Locusts must possess vital organs (cells) that require glucose for proper functioning. This is backed up by reports that fuel other than glucose, such as lipids and amino acids, which are prominent in insect haemolymph (Mullins, 1985), is not depleted in insects injected with trehalase inhibitors (Kono et al., 1993, 1994a,b, 1999). It is not known which organ failure proves to be fatal, but the behaviour of the trehazolin-poisoned locusts preceding death suggests that the central nervous system is the most likely candidate. Failure of the nervous system can be triggered by subjecting insects to anoxia (Walter and Nelson, 1975), and this brings about behavioural responses (reviewed by Wegener, 1993) similar to those in locusts succumbing to trehazolin. Our hypothesis that failure of the nervous system brings about the lethal effect of trehazolin is in line with the observation that glucose is a much better substrate for isolated thoracic ganglia from locusts than is trehalose (Strang and Clement, 1980; for reviews, see Strang, 1981; Wegener, 1987). The results further suggest that trehazolin inhibits the trehalase of the nervous tissue, so that glucose cannot be produced locally from haemolymph trehalose.

Where is trehalase localized in locust flight muscle?

Trehalases in flight muscle of locusts and other insects with synchronous flight muscles, such as cockroaches and Lepidoptera, are membrane-bound enzymes that can be activated by treatments that interfere with the structural integrity of membranes. This has been known since the 1950s (e.g. Zebe and McShan, 1959; Gussin and Wyatt, 1965; Gilby et al., 1967; Candy, 1974; Worm, 1981; Vaandrager et al., 1989), but the important question of how and where trehalase

is bound to muscle cell membranes has remained unanswered despite many attempts at answering it (for a review, see Becker et al., 1996). Also not known are the physiological (*in vivo*) equivalents of overt and latent trehalases and their possible roles in the control of trehalase activity in flight muscle. Trehazolin could be useful for a novel approach to answering some of these questions.

To simplify the discussion of our working hypothesis, the following terms to describe the different forms of trehalase activity will be used. Trehalase that cannot be sedimented by centrifugation at 100 000g for 60 min is termed soluble trehalase (trehalase s). Trehalase s is fully active and cannot be further activated by detergents or repeated freeze-thawing. Trehalase that can be sedimented at 100 000g is called membrane-bound (or particulate) trehalase (trehalase p). The activity of trehalase p *in vitro* can be subdivided into an overt fraction (trehalase p_{overt}) and a latent fraction (trehalase p_{latent}). Trehalase p_{overt} has originated from a fraction of trehalase *in vivo* that we will hence call *in vivo*-overt trehalase. *In vivo*-overt trehalase is thought to be located in or attached to plasma membranes of muscle cells such that the active site is accessible for the substrate trehalose and the inhibitor trehazolin (if present) from the haemolymph. This would explain how, by inhibiting the *in vivo*-overt trehalase, injection of trehazolin eliminates trehalase p_{overt} and that only *in vivo*-overt trehalase can be catalytically active to produce sufficient haemolymph glucose in locusts. This view is supported by the lack of evidence for a trehalose transporter and trehalose transport in locust flight muscle.

The *in vivo*-latent trehalase would appear in the *in vitro*-latent fraction (trehalase p_{latent}) after homogenisation of flight muscle. The active site of *in vivo*-latent trehalase must be shielded from trehalose and trehazolin alike, with the effect that this fraction would be neither catalytically active nor susceptible to inhibition by trehazolin in live locusts. The observations of Candy (1974) and our own preliminary studies suggest that *in vivo*-latent trehalase can be transformed into *in vivo*-overt trehalase and that this is essential for the control of trehalase activity in locust flight muscle *in vivo*. Trehalase could therefore be a prototype of a novel mechanism for regulating enzyme activity. The exact localization of both overt and latent trehalase in locust flight muscle and the molecular mechanism of their proposed transformation will be studied with more direct methods.

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References

Ando, O., Satake, H., Itoi, K., Sato, A., Nakajima, M., Takahashi, S., Haruyama, H., Ohkuma, J., Kinoshita, I. and Enokita, R. (1991). Trehazolin, a new trehalase inhibitor. *J. Antibiot.* **4**, 1165-1168.

- Ando, O., Kifune, M. and Nakajima, M. (1995a). Effects of trehazolin, a potent trehalase inhibitor on *Bombyx mori* and plant pathogenic fungi. *Biosci. Biotech. Biochem.* **59**, 711-712.
- Ando, O., Nakajima, M., Kifune, M., Fang, H. and Tanzawa, K. (1995b). Trehazolin, a slow tight-binding inhibitor of silkworm trehalase. *Biochim. Biophys. Acta* **1244**, 295-302.
- Becker, A., Schlöder, P., Steele, J. E. and Wegener, G. (1996). The regulation of trehalose metabolism in insects. *Experientia* **52**, 433-439.
- Bradford, M. M. (1976). A rapid and sensitive method for the quantification of microgram quantities of proteins utilizing the principle of protein dye binding. *Anal. Biochem.* **72**, 248-252.
- Candy, D. J. (1974). The control of muscle trehalase activity during locust flight. *Biochem. Soc. Trans.* **2**, 1107-1109.
- Candy, D. J. (1989). Utilization of fuels by the flight muscles. In *Insect Flight* (ed. G. J. Goldsworthy and C. H. Wheeler), pp. 305-319. Boca Raton: CRC Press.
- Candy, D. J. and Kilby, B. A. (1959). Site and mode of trehalose biosynthesis in the locust. *Nature* **183**, 1594-1595.
- Candy, D. J. and Kilby, B. A. (1961). The biosynthesis of trehalose in the locust fat body. *Biochem. J.* **78**, 531-536.
- Cohen, M. P. (1986). *Diabetes and Protein Glycosylation*. New York, Berlin: Springer.
- Dixon, M. (1953). The determination of enzyme inhibitor constants. *Biochem. J.* **55**, 170-171.
- Elbein, A. D. (1974). The metabolism of α,α -trehalose. *Adv. Carbohydrate Chem.* **30**, 227-256.
- Gilby, A. R., Wyatt, S. S. and Wyatt, G. R. (1967). Trehalases from the cockroach, *Blaberus discoidalis*: activation, solubilization and properties of the muscle enzyme and some properties of the intestinal enzyme. *Acta Biochim. Pol.* **14**, 83-100.
- Gussin, A. E. S. and Wyatt, G. R. (1965). Membrane-bound trehalase from cecropia silkmoth muscle. *Arch. Biochem. Biophys.* **112**, 626-634.
- Jahagirdar, A. P., Downer, R. G. H. and Viswanatha, T. (1990). Purification and characterization of trehalase-hydrolyzing enzymes from thoracic musculature of the American cockroach, *Periplaneta americana*. *Insect Biochem.* **20**, 511-516.
- Jutsum, A. R. and Goldsworthy, G. J. (1976). Fuels for flight in *Locusta*. *J. Insect Physiol.* **22**, 243-249.
- Kobayashi, Y. (1999). Chemistry and biology of trehazolins. *Carbohydrate Res.* **315**, 3-15.
- Kono, Y., Takeda, S., Kameda, Y., Takahashi, M., Matsushita, K., Nishina, M. and Hori, E. (1993). Lethal activity of a trehalase inhibitor, validoxylamine A, and its influence on the blood sugar level in *Bombyx mori* (Lepidoptera: Bombycidae). *App. Entomol. Zool.* **28**, 379-386.
- Kono, Y., Takahashi, M., Matsushita, K., Nishina, M., Kameda, Y. and Hori, E. (1994a). Inhibition of flight in *Periplaneta americana* (Linn.) by a trehalase inhibitor, validoxylamine A. *J. Insect Physiol.* **40**, 455-461.
- Kono, Y., Takeda, S. and Kameda, Y. (1994b). Lethal activity of a trehalase inhibitor, validoxylamine A, against *Mamestra brassicae* and *Spodoptera littura*. *J. Pesticide Sci.* **19**, 39-42.
- Kono, Y., Tanaka, S., Hasegawa, E., Takahashi, M., Nishina, M. and Matsushita, K. (1999). The role of trehalose in lipid mobilization of *Locusta migratoria*: effects of a trehalase inhibitor, validoxylamine A, on flight activity and hemolymph trehalose and lipid concentrations. *Entomol. Sci.* **2**, 449-456.
- Kunst, A., Draeger, B. and Ziegenhorn, J. (1984). D-Glucose. UV-methods with hexokinase and glucose-6-phosphate dehydrogenase. In *Methods of Enzymic Analysis*, vol. 6 (ed. H. U. Bergmeyer), pp. 163-172. Weinheim: Verlag Chemie.
- Mayer, R. J. and Candy, D. J. (1969). Changes in energy reserves during flight of the desert locust *Schistocerca gregaria*. *Comp. Biochem. Physiol.* **31**, 409-418.
- Mullins, D. E. (1985). Chemistry and physiology of the hemolymph. In *Comprehensive Insect Physiology, Biochemistry and Pharmacology*, vol. 3 (ed. G. A. Kerkut and L. I. Gilbert), pp. 355-400. Oxford, New York: Pergamon Press.
- Strang, R. H. C. and Clement, E. M. (1980). The relative importance of glucose and trehalose in the nutrition of the nervous system of the locust *Schistocerca americana gregaria*. *Insect Biochem.* **10**, 155-161.
- Strang, R. H. C. (1981). Energy metabolism in the insect nervous system. In *Energy Metabolism in Insects* (ed. R. H. Downer), pp. 169-206. New York: Plenum Press.
- Takahashi, M., Kono, Y., Kurahashi, H., Matsushita, K., Nishina, M. and

- Kameda, Y.** (1995). Effect of a trehalase inhibitor, validoxylamine A, on three species of flies. *Appl. Entomol. Zool.* **30**, 231-239.
- Vaandrager, S. H., Haller, T. B., Van Marrewijk, W. J. A. and Beenackers, A. M. Th.** (1989). Fractionation and kinetic properties of trehalase from flight muscle and hemolymph of the locust, *Locusta migratoria*. *Insect Biochem.* **19**, 89-94.
- Van der Horst, D. J., Van Doorn, J. M. and Beenackers, A. M. Th.** (1978). Dynamics in the haemolymph trehalose pool during flight of the locust *Locusta migratoria*. *Insect Biochem.* **8**, 413-416.
- Walter, D. C. and Nelson, S. R.** (1975). Energy metabolism and nerve function in cockroaches (*Periplaneta americana*). *Brain Res.* **94**, 485-490.
- Wegener, G.** (1987). Insect brain metabolism under normoxic and hypoxic conditions. In *Arthropod Brain: its Evolution, Development, Structure, and Functions* (ed. A. P. Gupta), pp. 369-397. New York: John Wiley & Sons.
- Wegener, G.** (1993). Hypoxia and posthypoxic recovery in insects: physiological and metabolic aspects. In *Surviving Hypoxia. Mechanisms of Control and Adaptation* (ed. P. W. Hochachka, P. L. Lutz, T. Sick, M. Rosenthal and G. van den Thillart), pp. 417-434. Boca Raton: CRC Press.
- Wegener, G.** (1996). Flying insects: model systems in exercise physiology. *Experientia* **52**, 404-412.
- Worm, R. A. A.** (1981). Characterization of trehalase from locust flight muscle. *Comp. Biochem. Physiol. B* **70**, 509-514.
- Wyatt, G. R.** (1967). The biochemistry of sugars and polysaccharides in insects. *Adv. Insect Physiol.* **4**, 287-360.
- Zebe, E. C. and McShan, W. H.** (1959). Trehalase in thoracic muscle of the woodroach, *Leucophaea maderae*. *J. Cell. Comp. Physiol.* **53**, 21-29.