# Mode of Action of the Hypertrehalosaemic Peptides from the American Cockroach

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*Dedicated to my Father* Bringfried Gäde *on the Occasion of His 70th Birthday*

Cockroach Hypertrehalosaemic Hormones, Cyclic AMP, Activation of Glycogen Phosphorylase, Breakdown of Glycogen, Increase of Haemolymph Carbohydrates

Although crude extracts of cockroach *(Periplaneta americana)* corpora cardiaca have been shown previously to affect the activity of adenylate cyclase and phosphorylase, we demonstrate in the present study for the first time that low concentrations (0.5 to 5 pmol) of the synthetic myoactive peptides, M I and M II, also affect these systems; these myoactive peptides are identical to the hypertrehalosaemic hormones I and II, and cause an increase in the concentration of the second messenger cyclic AMP in the fat body. In addition, both octapeptides activate fat body glycogen phosphorylase and promote breakdown of fat body glycogen. Both peptides increase the levels of haemolymph carbohydrate in a dose-dependent manner.

The insect corpora cardiaca produce, store and release a number of neurosecretory factors involved in the regulation of a diversity of physiological processes. Many of those factors have been shown to be peptides (for reviews, see [1–3]). Until recently, only the adipokinetic hormone I (AKH I) from locusts, which mobilises diacylglycerols from triacylglycerol stores in the fat body, had been fully characterized [4] and synthesized [5]. More than 20 years ago, the presence of a factor in the corpora cardiaca of the American cockroach, *Periplaneta americana,* capable of elevating haemolymph trehalose levels in that species was demonstrated [6]. Despite many attempts by various groups to isolate and characterise the hypertrehalosaemic factor, its chemical identity had remained elusive [7]. Recently, however, two myoactive peptides, M I and M II, separated from cockroach corpora cardiaca were isolated [8] and sequenced using fast atom bombardment mass spectrometry [9], and proved to be the same compounds as the hypertrehalosaemic factors isolated by Gäde [10, 11].

Both, M I and M II, are octapeptides with the following sequences: M I: pGlu-Val-Asn-Phe-Ser-Pro-Asn-Trp-$NH_2$; M II: pGlu-Leu-Thr-Phe-Thr-Pro-Asn-Trp-$NH_2$ [9]. Subsequently, synthetic M I and M II have been assayed for hypertrehalosaemic activity in cockroaches, and they have the same biological activity as the indigenous hypertrehalosaemic hormones I and II [12]. Simultaneously, but independently from these studies, the structure of neurohormone D, which increases the frequency of the heart-beat in the cockroach, was shown to be identical to that of M I [13] and the structures of two peptides with heart-acceleratory and "hyperglycaemic" activity, isolated from cockroach corpora cardiaca, were shown to be identical to M I and M II [14].

Up to now, all studies on the mode of action of cockroach hypertrehalosaemic hormone have used crude extracts of corpora cardiaca or, at best, partially purified material [15–18]. With the availability of the synthetic compounds, M I and M II, it is feasible now to re-investigate the effects of those peptides and study the ability of small amounts of these compounds to

(a) cause an accumulation of the second messenger cyclic AMP in the fat body,

(b) activate the enzyme glycogen phosphorylase in the fat body,

(c) cause the breakdown of glycogen in the fat body, and

(d) elicit hypertrehalosaemia in the haemolymph.

## Materials and Methods

### *Insects*

Adult (male and female) cockroaches, *Periplaneta americana,* were supplied by Professor Dr. Hansen (Universität Regensburg) and Fa. Thompson (Düsseldorf) and kept as described previously [11].

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*Preparation of peptides*

Synthetic myoactive factors, M I and M II, were a gift from Professor Dr. O'Shea (University of Geneva, Switzerland), or were obtained from Peninsula Laboratories (Belmont, CA, U.S.A.). For some experiments we also used natural hypertrehalosaemic hormones I and II (identical to M I and M II) isolated by high-performance liquid-chromatography from cockroach corpora cardiaca as outlined previously [11]. The peptide material was dissolved in absolute methanol to make stock solutions. For injection into assay cockroaches, an aliquot was taken, dried under a gentle stream of nitrogen, and the residue dissolved in bidistilled water. Before injection, further dilutions were made to give appropriate concentrations in a 10 µl dose.

*Determination of fat body cyclic AMP concentration*

The abdominal fat bodies of two donor cockroaches were removed at different times after injection of either peptide material or bidistilled water (as a control). Samples were further treated and analysed as described elsewhere [19].

*Extraction and determination of fat body glycogen content*

Abdominal fat bodies were prepared from individual cockroaches 60 min after injection of 5 pmol of either M I or M II. Determination of dry weight, extraction and analysis of glycogen were as reported previously [20].

*Measurement of fat body glycogen phosphorylase activity*

Cockroaches were separated 60 min before the experiment and kept in individual containers to minimize stress conditions. They were injected with different doses of either M I and M II, and individual fat bodies removed 20 min after injection (if not otherwise stated). For this, the head of the cockroach was immersed in liquid nitrogen for 3 sec to immobilize the animal, and the fat body removed from the unfrozen abdomen as rapidly as possible. Homogenisation of the fat bodies and determination of phosphorylase activity was as outlined elsewhere [21].

*Determination of protein concentration in the fat body*

The protein content of the fat body was quantified by the method of Bradford [22].

*Measurement of hypertrehalosaemia*

Cockroaches were handled as for the phosphorylase experiments. Haemolymph carbohydrate concentrations were determined by the measurement of anthrone-positive material according to previously published methods [23, 24] 120 min after the injection of either M I or M II.

*Reversed-phase HPLC*

Corpora cardiaca, corpora allata, and brains (each from 10 adult male cockroaches) were prepared for HPLC as described previously [25]. The methanolic material of each nervous tissue was dissolved in 80% methanol and applied to a Nucleosil C-18 column. Details of the equipment used is given elsewhere [26]. A linear gradient from 40% to 65% B in 22.5 min was used with a flow rate of 1.0 ml/min (solvent A: 0.11% trifluoroacetic acid; solvent B: 0.10% trifluoroacetic acid in 60% acetonitrile). The eluent was monitored at 210 nm at a sensitivity of 0.08 or 0.16 absorbance units full scale (LKB 2151 variable wavelength detector with 10 µl HPLC flow cell; 10 mm pathlength). The fractions were lyophilised, resuspended in bidistilled water as required, and used for the carbohydrate bioassay (see above).

## Results

*1. Isolation of hypertrehalosaemic hormone activity from different parts of the nervous tissue*

When a crude methanolic extract of 10 pairs of cockroach corpora cardiaca was subjected to reversed-phase HPLC, the two major peaks of absorbance at 210 nm had an absorbance ratio of 3:1 (peak I : peak II) and corresponded with those fractions exhibiting hypertrehalosaemic activity in the bioassay (Fig. 1A and Table I). Under identical conditions, neither an extract of corpora allata nor of brains showed any distinct absorbance peaks corresponding to those obtained with extract of corpora cardiaca (Fig. 1B and C *versus* Fig. 1A). When these fractions from the HPLC runs of corpora allata or brain material were tested in the bioassay (an equiva-

| Treatment (10 µl injected) | *n* | Blood carbohydrates [mg/ml] 0 min | 120 min | Change |
|---|---|---|---|---|
| Control | | | | |
| bidistilled water | 4 | 18.7 ± 3.4 | 21.7 ± 2.9 | 3.0 ± 0.9 |
| Corpora cardiaca | | | | |
| peak I (≙ fraction 12) | 4 | 19.1 ± 1.5 | 37.9 ± 3.3 | 18.8 ± 4.6 |
| peak II (≙ fraction 17/18) | 4 | 19.5 ± 2.6 | 35.9 ± 3.5 | 16.4 ± 2.1 |
| Brain | | | | |
| fraction 12 | 4 | 18.8 ± 5.1 | 20.2 ± 5.3 | 1.4 ± 0.8 |
| fraction 17/18 | 4 | 18.4 ± 4.1 | 22.0 ± 5.6 | 3.6 ± 1.9 |
| Corpora allata | | | | |
| fraction 12 | 4 | 17.9 ± 2.3 | 18.1 ± 2.1 | 0.2 ± 1.9 |
| fraction 17/18 | 4 | 19.0 ± 2.8 | 21.6 ± 4.7 | 2.6 ± 2.0 |

Table I. Total haemolymph carbohydrate concentration in adult male and female cockroaches before and 120 min after injection of material from different fractions after HPLC of corpora cardiaca, brain and corpora allata, respectively. The values shown are the mean ± S.D.

lent of 1.4 nervous tissue material was injected), the haemolymph carbohydrates were not increased (Table I).

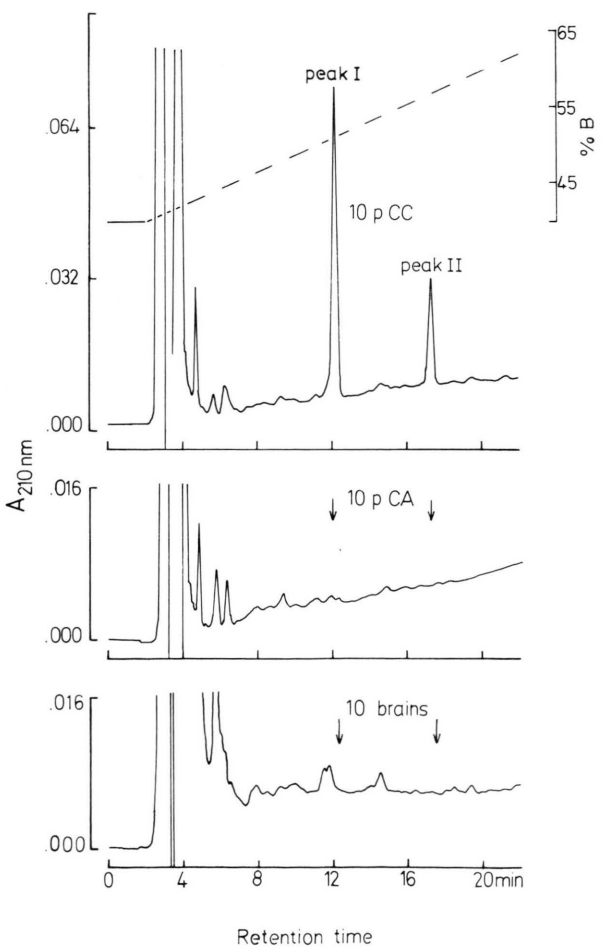


Fig. 1. The separation of hypertrehalosaemic hormones I and II from extracts of various nervous tissues from the American cockroach, *Periplaneta americana*, using reversed-phase high-performance liquid chromatography. The analyses were performed on a Nucleosil C-18 column which was eluted with a linear gradient of 0.11% trifluoroacetic acid (solvent A) and 0.1% trifluoroacetic acid in 60% acetonitrile (solvent B). The gradient ran from 40 to 65% B in 22.5 min at a flow rate of 1 ml/min. The gradient lag time after injection (time 0 min) was 2 min. The elution was monitored at 210 nm.
A. Separation of a crude methanolic extract from 10 pairs of corpora cardiaca. The fractions marked with peak I and peak II, respectively, showed hypertrehalosaemic activity in the cockroach bioassay (0.5 gland equivalents injected).
B. Separation of a crude methanolic extract from 10 pairs of corpora allata. The fractions marked with arrows were used for the bioassay (1.4 gland equivalents injected).
C. Separation of a crude methanolic extract from 10 brains. The fractions marked with arrows were used for the bioassay (1.4 brain equivalents injected).

### 2. *Effects of M I and M II on the concentration of fat body cyclic AMP*

The level of cyclic AMP in the abdominal fat body of the cockroach was low (Table II). Control injections with bidistilled water caused no accumulation of this nucleotide, but a two-fold increase in fat body cyclic AMP in response to 2.5 pmol of M I was measured 5 min after injection. There was almost no difference in the elevated level of cyclic AMP when 5 pmol were injected and the incubation time prolonged to 15 or 60 min. The response to 5 pmol of M II (15 and 60 min after injection) was similar to that of M I, but 2.5 pmol of M II had no significant effect.

Table II. Concentration of cyclic AMP in the fat body of adult male and female cockroaches after injection of synthetic M I and M II. The values shown are mean ± S.D.

| Treatment (10 µl injected) | n | Fat body cyclic AMP levels [pmol/mg protein] 0 min | n | 5 min | n | 15 min | n | 60 min |
|---|---|---|---|---|---|---|---|---|
| Control | | | | | | | | |
| without injection | 4 | 2.9 ± 1.0 | | | | | | |
| bidistilled water | | | 2 | 3.7 ± 0 | 2 | 3.6 ± 0.3 | | |
| M I [pmol] | | | | | | | | |
| 2.5 | | | 2 | 7.0 ± 1.8[a] | | | | |
| 5.0 | | | | | 6 | 5.4 ± 1.5[a] | 4 | 5.8 ± 1.3[a] |
| M II [pmol] | | | | | | | | |
| 2.5 | | | 2 | 4.7 ± 2.5[b] | | | | |
| 5.0 | | | | | 6 | 5.6 ± 1.5[a] | 4 | 4.7 ± 0.8[c] |

[a] These values are significantly different ($p \leqq 0.01$; student's t-test) compared to the mean of all control values (3.3 ± 0.8, $n = 8$).
[b] This value is not significantly different to the controls.
[c] This value is significantly different ($p \leqq 0.05$) to the controls.

### 3. *Activation of fat body glycogen phosphorylase by M I and M II*

Phosphorylase activity was determined as a percentage of the total enzyme in the active form (%phosphorylase a from total phosphorylase a + b activity). The effects on glycogen phosphorylase in the fat body of injecting low doses of M I and M II are shown in Table III. As we found no significant differences in response between sexes in pilot experiments, combined data are shown. When as little as 0.5 pmol was injected, both compounds activated the enzyme significantly compared with controls. There was no difference in activation by M I and M II. Injection of known amounts of natural hypertrehalosaemic hormone material produced the same effect as that of synthetic M I and M II; for example, 63.6 ± 11.5 (Mean ± S.D., $n = 9$) and 66.4 ± 8.9 ($n = 5$) for 1 pmol hormone I and II, respectively.

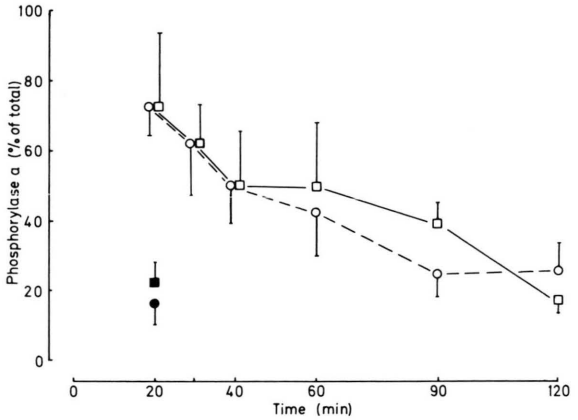


Fig. 2. Time-course for the activation state of cockroach (adult males) fat body glycogen phosphorylase after injection of 5 pmol of M I (open squares) and M II (open circles). The closed symbols represent the values for the bidistilled water injected controls. The values shown are the mean ± S.D. with $n = 4$.

Table III. Effect of synthetic M I and M II on fat body glycogen phosphorylase activity. Cockroaches were adult males and females.

| Treatment (10 µl injected) | n | Phosphorylase activity % active enzyme (mean ± S.D.) |
|---|---|---|
| Control | | |
| bidistilled water | 11 | 22.4 ± 5.7 |
| M I [pmol] | | |
| 0.5 | 6 | 43.0 ± 16.8 |
| 1.0 | 7 | 57.3 ± 17.7 |
| 5.0 | 4 | 72.8 ± 20.5 |
| M II [pmol] | | |
| 0.5 | 5 | 42.0 ± 3.5 |
| 1.0 | 4 | 68.8 ± 11.7 |

The time-course of the activation by M I and M II is depicted in Fig. 2. The high levels of phosphorylase activity had already begun to decrease within 40 min after injection and reached the control value after *c.* 120 min. The specific activity of total phosphorylase was 67.9 ± 24.8 mU/mg protein (Mean ± S.D., $n = 4$) in controls and 73.9 ± 21.9 mU/mg protein ($n = 4$) in cockroaches injected with 5 pmol of M I.

### 4. *Effect of M I and M II on the concentration of fat body glycogen*

The change in the glycogen content 60 min after injection of 5 pmol of M I and M II compared to

| Treatment (10 µl injected) | *n* | Glycogen content mg/g dry weight (mean ± S.D.) | Significance |
|---|---|---|---|
| Control | | | |
| bidistilled water | 11 | 286 ± 94 | |
| M I [pmol] | | | |
| 5.0 | 9 | 198 ± 87 | not significant from control |
| M II [pmol] | | | |
| 5.0 | 10 | 187 ± 58 | $p \leqq 0.05$ compared to control |

Table IV. Fat body glycogen concentration of adult male and female cockroaches 60 min after injection of synthetic M I and M II.

control injections of bidistilled water is shown in Table IV. As there were no significant differences in the response between sexes, combined data are given. Only M II elicited a significant decrease in the level of glycogen during the incubation; the decreased levels of glycogen in the fat body of cockroaches injected with M I were only significantly different from control values at the 10% level.

### 5. *The ability of M I and M II to cause hypertrehalosaemia*

When as little as 0.8 pmol was injected, both, synthetic M I and M II, caused a marked increase in haemolymph carbohydrates (Table V). However, the response to M II was already significant after injection of 0.5 pmol and, additionally, was at all doses tested slightly, but not significantly, higher than that of M I.

## Discussion

The results of the present study show clearly that the hypertrehalosaemic hormones I and II of the cockroach are entirely located in the corpora cardiaca; the HPLC-chromatograms of brain or corpora allata extracts do not show any absorbance peaks coincident with the hypertrehalosaemic hormones I and II. In addition, when the HPLC fractions of brain or corpora allata extracts corresponding to the bioactive fractions from corpora cardiaca are tested for hypertrehalosaemic activity, no changes in total blood carbohydrates are found. It seems obvious, therefore, that the two peptides, hypertrehalosaemic hormone I and II, are either produced in the corpora cardiaca of the cockroach or synthesized as different compounds in the neurosecretory cells of the brain and transported to the corpora cardiaca for processing and storage.

The results on the mode of action of the synthetic hormones make it very likely that phosphorylase is activated by the mediation of cyclic AMP, which is produced by the stimulation of adenylate cyclase. A three-fold increase in the activity of this enzyme upon injection of crude corpora cardiaca extract has been shown previously [17]. In addition, crude extracts of corpora cardiaca are potent in stimulating the accumulation of cyclic AMP in the fat body [16, 17]. Now we have demonstrated that low doses of both synthetic hormones produce this response (Table II).

| Treatment (10 µl injected) | *n* | Blood carbohydrates [mg/ml] 0 min | 120 min | Change |
|---|---|---|---|---|
| Control | | | | |
| bidistilled water | 10 | 14.0 ± 4.4 | 16.4 ± 5.4 | 2.4 ± 4.8 |
| M I [pmol] | | | | |
| 0.5 | 8 | 12.8 ± 2.7 | 15.7 ± 3.9 | 2.9 ± 2.7 |
| 0.8 | 5 | 17.4 ± 2.4 | 25.8 ± 2.8 | 8.4 ± 1.9 |
| 1.0 | 8 | 14.1 ± 2.6 | 21.7 ± 2.8 | 7.6 ± 3.2 |
| M II [pmol] | | | | |
| 0.5 | 8 | 11.2 ± 2.6 | 19.4 ± 4.7 | 8.2 ± 4.2 |
| 0.8 | 5 | 16.2 ± 2.6 | 25.4 ± 1.7 | 9.2 ± 2.3 |
| 1.0 | 8 | 12.9 ± 2.8 | 24.8 ± 4.2 | 11.9 ± 4.5 |

Table V. Total haemolymph carbohydrate concentration in adult male cockroaches before and 120 min after injection of synthetic M I and M II. The values shown are the mean ± S.D.

In all probability, cyclic AMP activates a protein kinase. It was found previously that two cyclic AMP-dependent protein kinases exist in the cockroach fat body [27]; only protein kinase II (but not I) was activated by crude corpus cardiacum extract [18], and it was concluded that corpus cardiacum extracts promote the conversion of the inactive holoenzyme to an active catalytic unit and, thus, activate protein kinases [18]. In theory, protein kinase catalyzes the activation of phosphorylase kinase, but this reaction has not been demonstrated so far (see, [28]). Phosphorylase kinase, in turn, catalyzes the transformation of phosphorylase b into phosphorylase a, thus, activating the enzyme. This activation has been demonstrated with crude corpora cardiaca extract *in vivo* and *in vitro* [15, 29, 30]. Various authors, however, found quite different activation states for phosphorylase in resting cockroaches: 0.7% [29] to 45% [31]. We have measured rather variable resting levels of phosphorylase, which are most likely due to the well-known short-term octopamine-mediated EXIT response [29, 32]. Therefore, we decided to measure control levels of phosphorylase 20 min after injection of bidistilled water. The state of activation, *c.* 22% in the a-form, is similar to that found for glycogen phosphorylase from the fat body of the migratory locust [33–35]. The activation by the synthetic hypertrehalosaemic hormones is dose-dependent and low doses are already effective. The activation is entirely due to the conversion of the inactive form to the active form, because the specific activity of the total enzyme is unaffected. In a previous study [31] phosphorylase was maximally activated (to 95% in the a-form) 1 h after injection of crude corpus cardiacum extract and were back to resting levels or lower (20% in the a-form) after 5 h. In contrast, we find a much faster time-course of activation after injection of synthetic hormone (Fig. 2), and the resting state is re-established 120 min after injection. A similar time-course was found for locust fat body glycogen phosphorylase [33, 34].

Utilization of glycogen depends on the phosphorylytic cleavage of terminal glucosyl residues catalyzed by phosphorylase. Therefore, we measured the concentration of the glycogen stores in the fat body after injection of the synthetic hypertrehalosaemic hormones. Under the conditions employed, only the injection of M II showed a significant effect. Previously, crude corpus cardiacum extract was shown to effect glycogen breakdown [15, 36].

The product of phosphorylase action is glucose-1-phosphate which is converted via several steps to trehalose, the main haemolymph sugar in cockroaches. It was known since 1961 [6] that crude extracts of corpora cardiaca increase the haemolymph concentration of trehalose, and this has been confirmed several times (see, [7]). Low concentrations of the synthetic hypertrehalosaemic hormones elicit a significant change in the blood sugar concentration. However, as with the action of glycogen breakdown, M II has a slightly greater effect. As little as 0.5 pmol of this hormone increases the blood sugar concentration significantly, whereas 0.8 pmol of M I is required.

In conclusion, the present study shows that, when injected in low concentrations, the hypertrehalosaemic hormones of the American cockroach are capable of causing cyclic AMP accumulation and glycogen phosphorylase activation, resulting in the breakdown of fat body glycogen and the synthesis and release of blood carbohydrates. The evidence suggest that the mode of action of the hypertrehalosaemic hormones in the fat body is analogous to the action of glucagon on the mammalian liver (see, [7, 37]). It is interesting in this context to note that there is some sequence homology between cockroach hypertrehalosaemic hormone II and the first 10 residues (counted from the amino terminal of the molecule) of glucagon as outlined in detail elsewhere [14], but, of course, the molecules are of very different size.

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