Characterization and Amino Acid Composition of a Hypertrehalosaemic Neuropeptide from the Corpora cardiaca of the Cockroach, *Nauphoeta cinerea*

Gerd Gäde

Institut für Zoologie IV der Universität Düsseldorf, Universitätsstraße 1, D-4000 Düsseldorf 1, Bundesrepublik Deutschland

Z. Naturforsch. **42c,** 225–230 (1987); received October 17, 1986

Corpora cardiaca, Hypertrehalosaemic Neuropeptide, *Nauphoeta cinerea,* Blood Carbohydrates, Fat Body Glycogen Phosphorylase

Nauphoeta cinerea corpora cardiaca contain peptide material which is capable of eliciting strong hypertrehalosaemia (maximum increase: 27 mg carbohydrates/ml haemolymph) and fat body glycogen phosphorylase-activation (maximal: 70% of the total phosphorylase activity in the a-form) in the American cockroach, *Periplaneta americana.* It appears that comparable amounts of bioactive material are stored in the corpora cardiaca of *N. cinerea* and *P. americana.* Purification of a methanolic extract of corpora cardiaca from *N. cinerea* by reversed-phase HPLC revealed that the hypertrehalosaemic phosphorylase-activating activity is concentrated in a single peak. The amino acid composition of the purified neuropeptide material was determined after acid hydrolysis with HCl and methanesulfonic acid. The analyses demonstrated that the *N. cinerea* hypertrehalosaemic factor is a decapeptide which contains the following amino acid residues: Asp, Thr, Ser, Glu, Pro, Gly (2), Val, Phe, and Trp.

Introduction

Studies by Steele [1, 2] have demonstrated that injection of an aqueous extract of corpora cardiaca from the American cockroach, *Periplaneta americana,* into the haemocoel of adult American cockroaches causes an elevation of the concentration of haemolymph trehalose, a concomitant decrease in the levels of fat body glycogen and an activation of fat body glycogen phosphorylase. The presence of hypertrehalosaemic factors were subsequently demonstrated in a number of different insect species (for recent reviews, see [3, 4]). Despite many attempts by various groups to isolate and characterize the hypertrehalosaemic factor from the corpus cardiacum of the American cockroach, its chemical identity has remained elusive for many years (see, [3]). With the improvement of chromatographic techniques for peptides by reversed-phase high performance liquid chromatography (reversed-phase HPLC), progress has been made on the purification of hypertrehalosaemic neuropeptides.

Recently, two myoactive peptides (designated M I and M II) separated from cockroach corpora cardiaca were isolated [5] and sequenced using fast atom bombardment mass spectrometry [6]. These peptides proved to be identical to the hypertrehalosaemic factors isolated by Gäde [7–9] and sequenced by Scarborough *et al.* [10]. Both peptides are octapeptides belonging to the so-called adipokinetic/red pigment-concentrating hormone-family (AKH/RPCH-family) of arthropod peptides [11] and both act *via* the adenylate cyclase and glycogen phosphorylase system [12].

The other hypertrehalosaemic factor isolated [7, 13] and sequenced [14] is the peptide II from the corpus cardiacum of the stick insect, *Carausius morosus.* It is a decapeptide with an almost identical structure to the locust decapeptide adipokinetic hormone I sequenced 10 years ago [15].

Recently, we found a peptide with hypertrehalosaemic activity in the corpus cardiacum of the cockroach, *Nauphoeta cinerea* [16]. After chromatography on reversed-phase HPLC gland extracts showed a single absorbance peak at 210 nm with hypertrehalosaemic activity. The retention time was distinct from all previously described arthropod neuropeptides but very close to that of the American cockroach hypertrehalosaemic peptide I.

The purpose of the present study was to purify sufficient material from the corpora cardiaca of *N. cinerea* for the determination of its amino acid composition. It was of further interest to show in detail the action of corpus cardiacum material and of the purified peptide on hypertrehalosaemia and the

Verlag der Zeitschrift für Naturforschung, D-7400 Tübingen
0341–0382/87/0300–0225 $ 01.30/0

activation of fat body glycogen phosphorylase. Since the haemolymph of *Nauphoeta cinerea* clots rapidly the American cockroach *Periplaneta americana* was used as acceptor species (see also, [16]).

Materials and Methods

Insects

Adult cockroaches, *Nauphoeta cinerea,* were a gift from Dr. B. Lanzrein (University of Bern, Switzerland) and adult American cockroaches, *Periplaneta americana,* were supplied by Professor Dr. K. Hansen (University of Regensburg, F.R.G.) or from Thompson Company (Düsseldorf, F.R.G.). Both species were kept in our laboratory at about 25 °C with a LD, 14:10 light cycle. They were fed with bran, oat and dog flakes supplemented with apples or bananas and water *ad libitum.* Two hours prior to the bioassays American cockroaches were removed from the stock cages to minimize stress conditions and kept individually in small containers without any food.

Chemicals

Biochemicals (β-nicotinamide-adenine dinucleotide phosphate, adenosine-5′-monophosphate, glucose-1,6-diphosphate) and the enzymes phosphoglucomutase and glucose-6-phosphate dehydrogenase were obtained from Boehringer (Mannheim, F.R.G.). AMP-free glycogen was prepared according to [17]. Acetonitrile and water (both HPLC grade) were purchased from Baker Chemicals (Groß-Gerau, F.R.G.). Trifluoroacetic acid (Uvasol) and all other chemicals (analytical grade) came from Merck (Darmstadt, F.R.G.).

Peptide source and preparation of extracts

Whole corpora cardiaca from adult *Nauphoeta cinerea* were collected by dissection, and methanolic extracts prepared for bioassays and reversed-phase HPLC as described previously [18].

Bioassays

Hypertrehalosaemic activity was measured by injection of the corpus cardiacum extract of *Nauphoeta cinerea* into adult male acceptor cockroaches, *Periplaneta americana* (see [19]). Concentrations of total haemolymph carbohydrates (anthron-positive material) were analyzed 120 min after injection as outlined previously [12].

Fat body glycogen phosphorylase of female American cockroaches was assayed 20 min after injection of the corpus cardiacum material of *Nauphoeta cinerea* as outlined in detail elsewhere [12] by determining the activity in the direction of glycogen breakdown spectrophotometrically [17]. All values for active phosphorylase (in the absence of AMP) are given as the percentage of total phosphorylase activity (in the presence of AMP).

Reversed-phase HPLC

Methanolic extracts of corpora cardiaca from *Nauphoeta cinerea* were dried down at reduced pressure (Speed Vac, Savant) and dissolved in 80% methanol for application onto a Nucleosil C-18 column. Details of the equipment used and the conditions applied are given elsewhere ([13]; see also legend to Fig. 2). The collected fractions were dried down *in vacuo,* resuspended in 100 µl of double distilled water, and an aliquot used for the bioassays (see above) by injection of a 10 µl dose into at least four assay cockroaches.

Amino acid analysis and determination of tryptophan

Lyophilized fractions containing between 600 and 1400 pmol of peptide material were dissolved in 5.7 M HCl, hydrolyzed in N_2-flushed, evacuated and sealed glass tubes for 24 h or 48 h at 110 °C and analyzed with a Beckmann Model 121 M amino acid analyzer using ninhydrin detection or by HPLC using a Waters Picotag system.

For tryptophan detection, lyophilized fractions were taken up into 4 M methanesulfonic acid containing 0.2% tryptamine and hydrolyzed for 20 h at 120 °C.

Results

Dose-response relationships for hypertrehalosaemia and phosphorylase activation

Corpus cardiacum extract from *Nauphoeta cinerea* was injected into acceptor American cockroaches in order to demonstrate hypertrehalosaemic or phosphorylase-activating responses. Complete dose-response curves were determined for each bioassay system in order to ascertain the potency of the *N.*

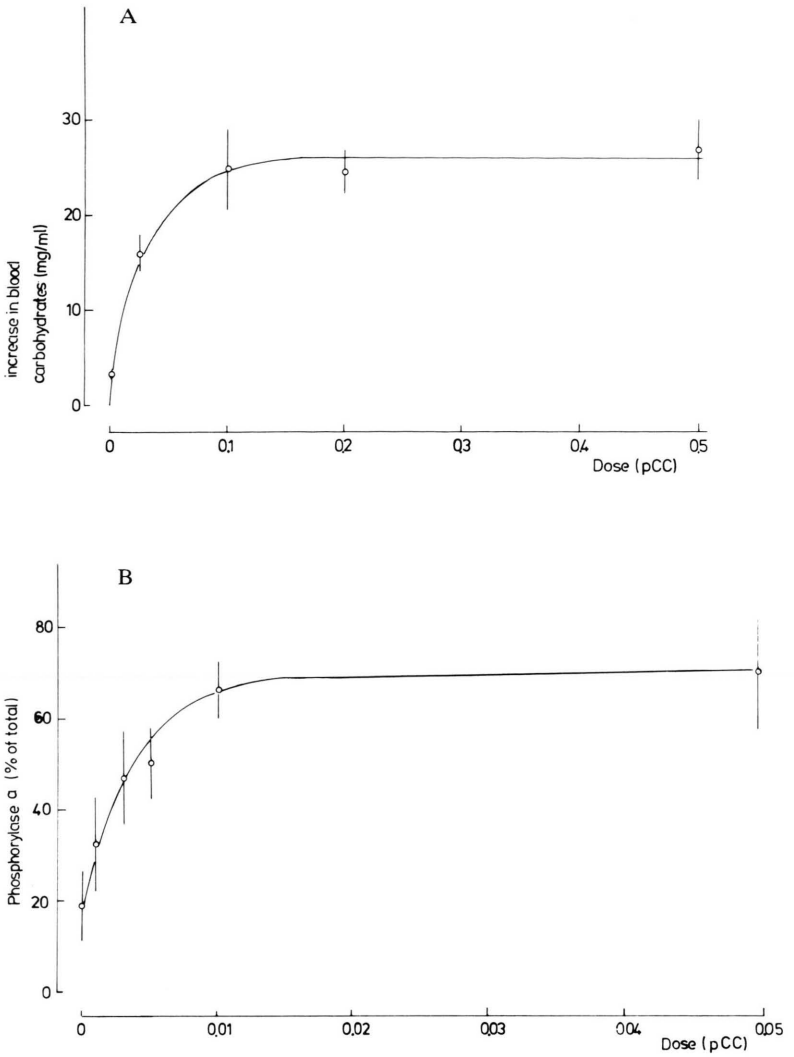

Fig. 1. Hypertrehalosaemic effect (A) and fat body glycogen phosphorylase-activation (B) in response to different doses of corpus cardiacum equivalents (pCC) from *Nauphoeta cinerea*. At least 6 individual American cockroaches were used as acceptor insects. Values are given as means ± S.D.

cinerea corpus cardiacum extract and thus the total quantity of bioactive material it contained. This procedure allowed a direct comparison of the effect of *N. cinerea* corpus cardiacum extract with that of American cockroaches in their own assay system. Furthermore, dose-response relationships for both bioassays are useful to establish the doses that can be detected easily during the isolation procedure.

The hypertrehalosaemic response to *N. cinerea* corpus cardiacum extract in American cockroaches is maximal at about 0.1 gland equivalents and 0.005 equivalents are needed to produce a significant response ($p = 0.001$, Student's t-test; Fig. 1A). The maximal increase in haemolymph carbohydrate levels is between 25 to 27 mg/ml.

Activation of fat body glycogen phosphorylase of the American cockroach by *N. cinerea* corpus cardiacum extract is achieved with smaller doses when compared to the effect on blood carbohydrates (Fig. 1B *versus* 1A). Maximal activation is observed by the injection of 0.01 corpus cardiacum equivalents and about 0.002 gland equivalents are needed to activate phosphorylase significantly ($p = 0.01$, Student's t-test; Fig. 1B). Phosphorylase is maximal activated when about 70% of the total activity is represented by the active form.

Reversed-phase HPLC

A crude methanolic extract of 50 pairs of *N. cinerea* corpora cardiaca was separated by reversed-phase HPLC on an analytical column. The major peak of absorbance with a retention time of 14.0 min exhibited hypertrehalosaemic activity in American cockroaches (Fig. 2); no other fractions were active in the bioassay. To test recovery of hormonal activities, a methanolic extract from *N. cinerea* corpora cardiaca was divided and tested for hypertrehalosaemia and phosphorylase-activation in *P. americana* before and after reversed-phase HPLC. 0.025 Gland equivalents were injected to ascertain the activity of the extract on blood carbohydrates and 0.0025 corpus cardiacum equivalents injected for the activation of phosphorylase. As shown in Table I the hypertrehalosaemic and the phosphorylase-activating activity of the corpora cardiaca of *N. cinerea* are fully recovered after chromatography.

Further purification of the hypertrehalosaemic material was carried out in duplicate using an extract from two batches each comprising 150 pairs of corpora cardiaca from *N. cinerea*. This was chromatographed by HPLC, the appropriate peak collected manually, lyophilized and then re-chromatographed to obtain a homogeneous compound. The chromatogram showed a single large absorbance peak with no hint of resolution (Fig. 3). After collection and

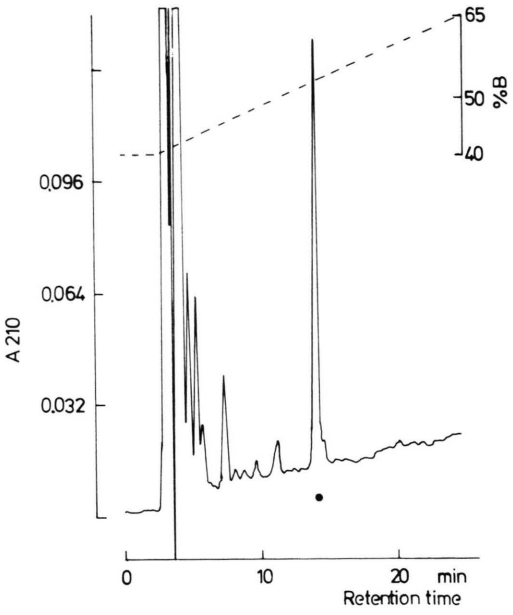

Fig. 2. The separation of the hypertrehalosaemic peptide from a methanolic extract of corpora cardiaca (40 glands) from *N. cinerea* using reversed-phase HPLC. The analysis was performed on a Nucleosil C-18 column which was eluted with a linear gradient of 0.1% trifluoroacetic acid (solvent A) and 0.1% trifluoroacetic acid in 60% acetonitrile (solvent B). The gradient ran from 40 to 65% B within 22.5 min at a flow rate of 1 ml/min. The gradient lag time after injection (0 min) was 2 min. The elution was monitored at 210 nm. The peak containing biological activity is indicated by a solid circle.

Table I. Recovery of hypertrehalosaemic and glycogen phosphorylase-activating activity of an methanolic extract of corpora cardiaca from *N. cinerea* after reversed-phase HPLC[a].

Treatment	*n*	Increase of haemolymph carbohydrate level [mg/ml]	*n*	Active glycogen phosphorylase (% of total activity)
Control				
distilled water	6	1.1 ± 1.6	4	18.8 ± 6.3
Before HPLC gland equiv.				
0.025	5	13.3 ± 2.6	–	– – –
0.0025	–	– – –	4	44.1 ± 2.0
After HPLC gland equiv.				
0.025	6	14.5 ± 3.1	–	– – –
0.0025	–	– – –	3	43.7 ± 3.8

[a] A methanolic extract of corpora cardiaca was tested either directly, after appropriate dilution, or after purification using reversed-phase HPLC. Values are given as means ± S.D.

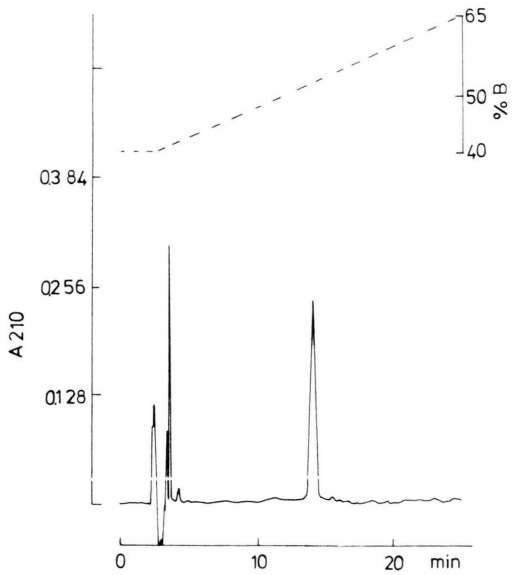

Fig. 3. Rechromatography of the *N. cinerea* hypertrehalosaemic peptide peak (see Fig. 2) from 150 glands on a Nucleosil C-18 column (for details see Fig. 2). The peak material was used for amino acid analysis.

Table II. Amino acid composition of *N. cinerea* hypertrehalosaemic peptide after reversed-phase HPLC[a].

Amino acid residue	Hydrolysis in HCl	Hydrolysis in Methanesulfonic acid	Number of residue present
Asp	0.82	0.79	1
Thr	0.84	0.87	1
Ser	0.82	0.85	1
Glu	1.00	1.00	1
Pro	0.88	0.93	1
Gly	2.21	2.30	2
Val	0.90	0.91	1
Phe	0.78	0.73	1
Trp	n.d.	0.65	1

[a] Values are given as molar ratios to Glu = 1. All other amino acid residues detected had a molar ratio <0.30. n.d. = not detectable in this system.

lyophilization aliquots were used for amino acid analyses.

Amino acid composition of N. cinerea hypertrehalosaemic peptide

The amino acid analyses were performed on peptide material eluted from the second HPLC step, in duplicate.

Samples were further analyzed by hydrolysis in HCl (24 or 48 h) or in methanesulfonic acid (20 h) in order to detect tryptophan. The results of representative analyses are summarized in Table II. All analyses gave consistent values and revealed the presence of 10 amino acid residues. Corpora cardiaca of *N. cinerea* thus contain a hypertrehalosaemic peptide with the following amino acid composition: Asp, Thr, Ser, Glu, Pro, 2 Gly, Val, Phe and Trp.

Discussion

The corpus cardiacum of the cockroach *N. cinerea* contains a potent substance which elevates blood carbohydrates and activates the fat body glycogen phosphorylase in American cockroaches. The maximum elevation of *P. americana* haemolymph carbohydrates occurs with 0.1 gland equivalents of *N. cinerea* corpora cardiaca. For comparison about the same amount of crude extract (0.05 to 0.1 gland equivalents) from *P. americana* are required to achieve the same effect [2, 20, 21]. On a molar basis 5 pmol of synthetic *P. americana* hypertrehalosaemic hormone I and II cause the same degree of hypertrehalosaemia [11].

Similarly, maximal activation of *P. americana* glycogen phosphorylase occurs with about 0.01 pairs of corpora cardiaca from *N. cinerea,* and the same dose is needed from corpus cardiacum material of *P. americana* [21]. Therefore, a 10-fold lower dose of each cockroach corpora cardiaca extract is needed to activate phosphorylase compared to that required for elevation of blood carbohydrates. Apparently, the corpora cardiaca of both species store about the same amount of bioactive material.

The American cockroach contains two hypertrehalosaemic neuropeptides in its corpora cardiaca [7, 8, 10, 21], in contrast to only one hypertrehalosaemic factor in the glands of *N. cinerea.* This was observed during the isolation of the active material from the corpora cardiaca of *N. cinerea* which was achieved using a rapid and simple method described previously for the separation of other insect neuropeptides [7, 8, 13, 16, 18]: a methanolic extract of corpora cardiaca from *N. cinerea* was chromatographed on reversed-phase HPLC and the bioactive material rechromatographed to obtain a homogeneous peptide for the amino acid analysis. No loss of bioactivity was detected during the chromatographic steps (Table I).

The hypertrehalosaemic neuropeptide of *N. cinerea* differs from the American cockroach hypertrehalosaemic peptides M I and M II in that the latter compounds are octapeptides [6, 8, 10], whereas the material from the corpora cardiaca of *N. cinerea* is a decapeptide. The decapeptide of *N. cinerea* has seven amino acid residues in common with M I and six with M II. *N. cinerea* peptide and the hypertrehalosaemic factor II from *Carausius morosus* [14] are both decapeptides and have seven amino acid residues in common.

The *N. cinerea* peptide has also seven amino acids in common with another decapeptide, the locust adipokinetic hormone I. A corpus cardiacum extract of *N. cinerea*, however, has no pronounced adipokinetic effect in locusts [16]. Previous studies on the structure-activity relationship of different peptides belonging to the AKH/RPCH-family [22] or of AKH I analogues [23] have already revealed that, for a full adipokinetic response, the precise sequence of AKH I is needed. This, then, may explain the poor response of the *N. cinerea* corpus cardiacum extract in the locust.

The present study has shown that

1) *N. cinerea* corpora cardiaca contain a novel hypertrehalosaemic peptide as originally suggested [16] and
2) reversed-phase HPLC purification of *N. cinerea* hypertrehalosaemic neuropeptide is sufficient to enable the structure of the peptide to be elucidated.

The amino acid composition data indicates that the *N. cinerea* peptide belongs to the AKH/RPCH-family and therefore it is likely that this compound has blocked N- and C-termini. Therefore, the technique of fast atom bombardment mass spectrometry could be employed as the method of choice for assigning the sequence.

Acknowledgements

The author wishes to thank Miss Iris Göbel for excellent technical assistance, Prof. Dr. K. Beyreuther (Institut für Genetik, Universität Köln) and the staff of Millipore, Waters Chromatography (Leverkusen) for performing amino acid analyses, Dr. H. G. E. Lloyd (Physiologisches Institut I der Medizinischen Einrichtungen, Universität Düsseldorf) for correcting the English manuscript, and the Deutsche Forschungsgemeinschaft for financial support (Ga 241/6-1). The author was supported by a Heisenberg Fellowship awarded from the Deutsche Forschungsgemeinschaft (Ga 241/5-1).

[1] J. E. Steele, Nature **192,** 680–681 (1961).
[2] J. E. Steele, Gen. Comp. Endocr. **3,** 46–52 (1963).
[3] G. J. Goldsworthy and G. Gäde, in: Invertebrate Endocrinology, **Vol. 1,** Endocrinology of Insects (H. Laufer and R. G. H. Downer, eds.), pp. 109–119, Alan Liss, New York 1983.
[4] J. E. Steele, in: Invertebrate Endocrinology, **Vol. 1,** Endocrinology of Insects (H. Laufer and R. G. H. Downer, eds.), pp. 427–439, Alan Liss, New York 1983.
[5] M. O'Shea, J. Witten, and M. Schaffer, J. Neuroscience **4,** 521–529 (1984).
[6] J. L. Witten, M. H. Schaffer, M. O'Shea, J. C. Cook, M. E. Hemling, and K. L. Rinehart, Biochem. Biophys. Res. Commun. **124,** 350–358 (1984).
[7] G. Gäde, J. Insect Physiol. **30,** 729–736 (1984).
[8] G. Gäde, Z. Naturforsch. **40c,** 42–46 (1985).
[9] G. Gäde, Naturwissenschaften **72,** 95–96 (1985).
[10] R. M. Scarborough, G. C. Jamieson, F. Kalish, S. J. Kramer, G. A. McEnroe, C. A. Miller, and D. A. Schooley, Proc. Nat. Acad. Sci. U.S.A. **81,** 5575–5579 (1984).
[11] G. Gäde, Z. Naturforsch. **41c,** 315–320 (1986).
[12] G. Gäde, Z. Naturforsch. **40c,** 670–676 (1985).
[13] G. Gäde, Biol. Chem. Hoppe-Seyler **366,** 175–179 (1985).
[14] G. Gäde and K. L. Rinehart, Jr., Biol. Chem. Hoppe-Seyler, in press.
[15] J. V. Stone, W. Mordue, K. E. Batley, and H. R. Morris, Nature **263,** 207–211 (1976).
[16] G. Gäde and M. Scheid, Physiol. Entomol. **11,** 145–157 (1986).
[17] R. Ziegler, M. Ashida, A. M. Fallon, L. T. Wimer, S. S. Wyatt, and G. R. Wyatt, J. Comp. Physiol. **131,** 321–332 (1979).
[18] G. Gäde, G. J. Goldsworthy, G. Kegel, and R. Keller, Hoppe-Seyler's Z. Physiol. Chem. **365,** 391–398 (1984).
[19] G. Gäde, J. Insect Physiol. **26,** 351–360 (1980).
[20] K. Hanaoka and S. Y. Takahashi, Insect Biochem. **6,** 621–625 (1976).
[21] K. J. Siegert, P. J. Morgan, and W. Mordue, Insect Biochem. **16,** 365–371 (1986).
[22] G. J. Goldsworthy, K. Mallison, C. H. Wheeler, and G. Gäde, J. Insect Physiol. **32,** 433–438 (1986).
[23] J. V. Stone, W. Mordue, C. E. Broomfield, and P. Hardy, Eur. J. Biochem. **89,** 195–202 (1978).