# Glycogen Phosphorylase in the Fat Body of Two Cockroach Species, *Periplaneta americana* and *Nauphoeta cinerea:* Isolation, Partial Characterization of Three Forms and Activation by Hypertrehalosaemic Hormones*

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*Dedicated to my father* Bringfried Gäde, *who made my scientific career possible, on the occasion of his 75th birthday*

Glycogen Phosphorylase, Fat Body, *Nauphoeta cinerea, Periplaneta americana,* Phosphorylase Kinase

The presence of endogenous phosphorylase kinase and phosphorylase phosphatase in crude extracts of fat bodies from the cockroaches *Nauphoeta cinerea* and *Periplaneta americana* is demonstrated *in vitro* by activation/inactivation of glycogen phosphorylase under appropriate conditions. Fractionation of fat body extracts of both cockroach species on an anion-exchange medium results in the elution of three peaks with phosphorylase activity. According to their AMP dependency these activity peaks are designated as phosphorylase b (inactive without AMP), phosphorylase ab (active without AMP, but several times stimulated with AMP) and phosphorylase a (active without AMP). It is shown chromatographically that incubating crude extracts of fat bodies from both cockroaches, under conditions where the phosphorylase kinase is active, results in all phosphorylase b being converted to the ab- or a-form, whereas under conditions where the phosphorylase phosphatase is active all phophorylase a is converted to the ab- or b-form. Endogenous phosphorylase kinase of *N. cinerea* crude fat body extract can convert vertebrate phosphorylase b into the a-form, and, conversely, vertebrate muscle phosphorylase kinase and phosphorylase phosphatase, respectively, are able to convert partially purified *N. cinerea* phosphorylase ab or b and the ab- und a-form, respectively. In resting cockroaches most of the phosphorylase activity resides in the b-form and only a small fraction (10%) in the a-form, whereas between 26% *(N. cinerea)* and 35% *(P. americana)* occurs in the ab-form. Injection of endogenous hypertrehalosaemic peptides into *N. cinerea* (the decapeptide Bld-HrTH) or *P. americana* (the two octapeptides Pea-CAH-I and II) causes interconversion of phosphorylase; after injection, mainly (60%) phosphorylase a is present, while 25% and 15% exists in the ab- und b-form, respectively. Purification of the three phosphorylase forms from *N. cinerea* is achieved by anion-exchange chromatography on DEAE-Sephacel followed by affinity chromatography on AMP-Sepharose. The final specific activities are 2.1, 6.9 and 27.2 U/mg protein for the a-, ab- und b-form. The molecular mass of the active molecules on gel filtration is between 173,000 and 177,000, and SDS gel electrophoresis reveals a subunit mass of 87,100, suggesting a homodimeric structure for all three forms. Kinetic studies show hyperbolic saturation curves for the substrates glycogen and $P_i$, respectively, with $K_M$-values of 0.021, 0.019 and 0.073% for glycogen and 8.3, 6.3 and 17.9 mM for $P_i$ (a-, ab- and b-form). Phosphorylase a exhibits a more or less hyperbolic response to AMP and needs 70 μM AMP for maximal stimulation. The kinetics for the ab- and b-forms are sigmoidal and maximal activities are displayed at about 3 mM (half-maximum activation as calculated from Hill plots are 55 and 280 μM for the ab- und b-form, respectively). Caffeine is a strong inhibitor of the b-form, but has only a slight inhibiting effect (10–20%) on the ab- and a-form in the presence of AMP.

* The physiological studies reported here were described in brief in Verh. Dtsch. Zool. Ges. **81,** 309 (1988) and at the 14th Conference of European Comparative Endocrinologists in Salzburg/Austria (September 1988).

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## Introduction

Cockroaches such as *Periplaneta americana* are thought to rely exclusively upon carbohydrate metabolism for flight activity [1]. Glycogen stores in the fat body are the main carbohydrate reserves. Utilization of fat body glycogen depends upon the activity of glycogen phosphorylase. This enzyme

catalyzes the degradation of glycogen by the cleavage of the terminal glycosylunit from the parent molecule and thus makes available the building blocks for the synthesis of the predominant haemolymph sugar, trehalose (*cf.* [2]).

In muscle and fat body tissues of various insects it has been reported that glycogen phosphorylase exists in two interconvertible forms: a and b [2–4], and in the fruit fly, *Drosophila melanogaster* [5], it has been shown that reversible phosphorylation-dephosphorylation occurs during the interconversion between the a- and b-form: the non-phosphorylated b-form is inactive and exhibits catalytic activity only in the presence of high levels of AMP, while the phosphorylated a-form is largely independent of AMP and active by itself. In the fat body and flight muscle tissue of the migratory locust, *Locusta migratoria,* a third form, designated ab and supposedly a partially phosphorylated intermediate, has been demonstrated [3, 6].

In cockroaches as well as in locusts fat body glycogen phosphorylase is activated by hormonal stimulation [2]. In both species the endogenous neuropeptides found in the corpus cardiacum have been shown to exert their effect on the enzyme when injected as synthetic compounds [7, 8, 9].

In the present study we report the presence of phosphorylase kinase and phosphorylase phosphatase in the fat body of two cockroach species, show the existence of three forms of glycogen phosphorylase and investigate the influence of synthetic hormone injection on the relative amounts of the three forms. In addition, the three forms have been isolated and partially purified from the fat body of *Nauphoeta cinerea* and some structural and kinetic properties have been studied.

## Materials and Methods

### *Insects*

Adult cockroaches, *Nauphoeta cinerea,* of both sexes were a gift from Dr. B. Lanzrein (University of Bern, Switzerland). Adult American cockroaches, *Periplaneta americana,* of both sexes were supplied by Professor Dr. K. Hansen (University of Regensburg, F.R.G.) or Thompson Company (Düsseldorf, F.R.G.) and male adult migratory locusts, *Locusta migratoria,* were purchased from a commercial dealer. All animals were kept in our insectary under crowded conditions at about 25 °C with an LD of 14:10 h light cycle and were maintained as described previously [10, 11].

### *Chemicals*

Biochemicals and enzymes were obtained from Boehringer GmbH (Mannheim, F.R.G.), except rabbit muscle phosphorylase kinase, which was purchased from Sigma Chemical Company (Deisenhofen, F.R.G.). Sephadex G-25 M (prepacked PD-10 columns), DEAE-Sephacel, 5′-AMP-Sepharose 4 B and Sephacryl S 300 came from Pharmacia GmbH (Freiburg, F.R.G.).

Chemicals for electrophoresis and the kit for protein determination were obtained from Bio-Rad Laboratories (München, F.R.G.) and the $Na_2$-β-glycerophosphate came from Merck (Darmstadt, F.R.G.). Protein phosphatase 1 c (the catalytic subunit) from rabbit skeletal muscle was a gift from Dr. Mieskes (Abteilung für Klinische Biochemie, Zentrum Innere Medizin, Göttingen, F.R.G.). All other chemicals (analytical grade) were purchased from Merck (Darmstadt, F.R.G.).

The *P. americana* hypertrehalosaemic octapeptides, (Pea-CAH-I and II according to the new nomenclature by Raina and Gäde [12]), as well as the *N. cinerea* hypertrehalosaemic decapeptide (acronym: Bld-HrTH), were obtained from Peninsula Laboratories (Belmont, C.A., U.S.A.).

### *Preparation of fat body extracts for incubation experiments*

Cockroaches were separated 3 h before the experiment and kept in individual containers to minimize stress conditions. For the preparation of fat body, the cockroach was briefly immersed in liquid nitrogen to immobilize the insect, and the fat body was removed from the unfrozen abdomen as rapidly as possible and collected in liquid nitrogen. Pooled fat bodies (8 to 30 insects) were homogenized in 2–4 ml of ice-cold 50 mM Tris-HCl buffer (pH 7.0). Using a glass homogenizer with a motor-driven teflon pestle, the buffer contained 5 mM NaF and 1 mM EDTA to prevent any conversion of the phosphorylase due to the action of putative endogenous phosphorylase phosphatase and/or phosphorylase kinase. After centrifugation for 10 min at 10,000 × *g* in the cold, the infranatant was pressed through a Spartan filter (Schleicher &

Schüll, Dassel, F.R.G.) and the clear solution centrifuged for 30 min at 35,000 × *g* at 4 °C. The supernatant was diluted 1:10 (v/v) with the above Tris-HCl buffer (without NaF and EDTA; to abolish the inhibitions of these substances) and used for 4 different incubation experiments. Incubations were performed at 25 °C in a final volume of 1 ml, containing 0.8 ml of diluted fat body extract and (as final concentrations) 1 mM ATP and 1 mM magnesium acetate (incubation A); 1 mM ATP, 1 mM magnesium acetate and 5 mM NaF (incubation B); 5 mM EDTA (incubation D); 50 mM potassium phosphate (pH 7.0) and 1 mM magnesium acetate (incubation C). At various times 30 to 50 µl aliquots were taken from the different incubation media and the phosphorylase activity assayed (see below).

*Isolation and fractionation of phosphorylase on DEAE-Sephacel*

For locust phosphorylase we followed the procedure described in [3]. The only modifications (number of animals, column used, size, flow rate, application of gradient) are given in the legend to Fig. 3. For cockroaches a slightly modified protocol was used.

Fat bodies from 8 cockroaches were prepared as above with the exception that the homogenizing buffer was 50 mM Tris-maleate (pH 7.5), containing 25 mM NaF and 5 mM EDTA. After the centrifugation steps (see above), the supernatant containing the phosphorylase activity was diluted to the proper ionic strength with 4 vol 1 mM dithiothreitol (DTT), and applied to a DEAE-Sephacel column (for dimensions and flow rate, see Fig.), previously equilibrated with elution buffer (20 mM Tris-HCl, pH 7.0, containing 5 mM NaF, 1 mM EDTA and 1 mM DTT). After application, phosphorylase was eluted with a linear gradient of 0–200 mM or 50–250 mM (see Fig.) $Na_2$-β-glycerophosphate (200 ml total volume) in elution buffer. Fractions were collected every 15 min and assayed for phosphorylase activity (see below).

Fractionation of phosphorylase from cockroaches was also performed after *in vitro* incubation (see above) of the crude homogenate with either ATP, magnesium acetate and NaF or with EDTA only. In another series of experiments cockroaches were injected 15 min prior to isolation and fractionation of phosphorylase with 10 pmoles of Pea-CAH-I or II *(P. americana)* or with 10 pmoles of Bld-HrTH *(N. cinerea)*.

*Incubation with phosphorylase kinase or phosphorylase phosphatase*

Phosphorylase b and the phosphorylase ab-form obtained by chromatography of *N. cinerea* fat bodies on DEAE-Sephacel (see above) were incubated separately *in vitro* in the presence of rabbit muscle phosphorylase kinase as described previously [3]. We used about 4–20 mU of purified phosphorylase b or ab and about 0.2 units of phosphorylase kinase in a final volume of 1 ml. The reaction was started with phosphorylase kinase, aliquots were taken after several times and assayed for phosphorylase activity (see below). DEAE-purified phosphorylase a from fat body of *N. cinerea* (about 1 mU) was incubated *in vitro* with 0.2 µg rabbit phosphorylase phosphatase in a final volume of 20 mM Tris/HCl buffer, pH 7.0, containing 5 mM EDTA. The reaction was started with the phosphatase, aliquots were taken after several times, and assayed for phosphorylase activity (see below).

*Determination of phosphorylase activity*

The activity of glycogen phosphorylase was measured spectrophotometrically in a final volume of 1 ml at 25 °C in the direction of glycogen breakdown according to the method in [13]. Active phosphorylase was assayed in the absence of 5′-AMP, whereas total phosphorylase was determined in the presence of 1.3 mM 5′-AMP. Enzyme activity is either expressed as Δ absorbance per min and ml or as mU per ml (formation of 1 nmol glucose-1-phosphate per min).

*Purification of phosphorylase*

Fat body glycogen phosphorylase from the cockroach *N. cinerea* was purified using about 40 insects per preparation. The first steps of purification (crude extract, centrifugation, DEAE-Sephacel) were as described above. Subsequently, peak fraktions of the three forms obtained were further purified (separately) on a column (5.2 cm high × 1.6 cm diameter) packed with 5′-AMP-Sepharose 4B essentially according to the method described

in [14]. Peak fractions with phosphorylase activity were pooled and, to eliminate 5'-AMP, passed through a PD-10 column of Sephadex G-25 M material equilibrated and eluted with Tris-HCl buffer (pH 7.0) as above.

The eluate was concentrated by ultrafiltration (Amicon cell with a YM 10 membrane) and used for kinetic studies or electrophoresis.

*Electrophoresis*

Polyacrylamide gel electrophoresis (PAGE) of the native enzyme using 4 µg enzyme protein (a-form), 3.2 µg (b-form) and 1.5 µg (ab-form) was performed according to [15] and 7.5% polyacrylamide used. After the run, the gel was cut into two halves. One half was stained for general protein with Coomassie Brilliant Blue R250 (Serva GmbH, Heidelberg, F.R.G.) and the other half was stained for phosphorylase activity according to the method in [16]. Molecular weights of the native phosphorylase were determined using analytical gel filtration on Sephacryl S-300 (column dimensions: 1.6 cm diameter, 100 cm height) in an identical buffer system as described previously [4]. The flow rate during elution was 7.5 ml/h and the column was calibrated with the following standard proteins: thyroglobulin (molecular weight: 660,000), ferritin (440,000), catalase (210,000), aldolase (158,000), bovine serum albumin (67,000) and cytochrome *c* (12,500). Discontinuous SDS-PAGE according to [17] was used to determine the subunit molecular weight of phosphorylase. The samples containing about 2 µg enzyme protein (a-form), 1.6 µg (b-form) and 0.74 µg (ab-form) (assayed as outlined in [18] using bovine serum albumin as a standard) were denatured by boiling for 5 min in Tris-HCl buffer (50 mM, pH 6.8) containing 5% β-mercaptoethanol and 2.5% SDS. The following molecular mass standards were used: carboanhydrase (30,000), lactate dehydrogenase (36,000), ovalbumin 45,000), catalase (60,000), bovine serum albumin (67,000) and rabbit muscle phosphorylase b (94,000). Proteins were visualized by staining with Coomassie Brilliant Blue R250.

*Kinetic measurements*

Phosphorylase activity was determined as outlined above. Substrate affinities and enzyme activity rates were determined at saturating concentrations of cosubstrate and AMP when not stated otherwise. Apparent Michaelis constants ($K_M$) were calculated from double-reciprocal plots and construction of regression lines. Values for glycogen are given as percentages since the exact molecular weight of glycogen is not known. In case of sigmoidal kinetics, Hill plots were used to calculate values for $S_{0.5}$ and the Hill coefficient ($n_H$).

## Results

*Existence of phosphorylase kinase and phosphorylase phosphatase in cockroach fat body*

The effect of endogenous interconverting enzymes on cockroach fat body phosphorylase was studied in crude extracts. In the presence of ATP and magnesium ions ($Mg^{2+}$), crude extracts from the fat body of *P. americana* and *N. cinerea*, respectively, showed a time-dependent increase in

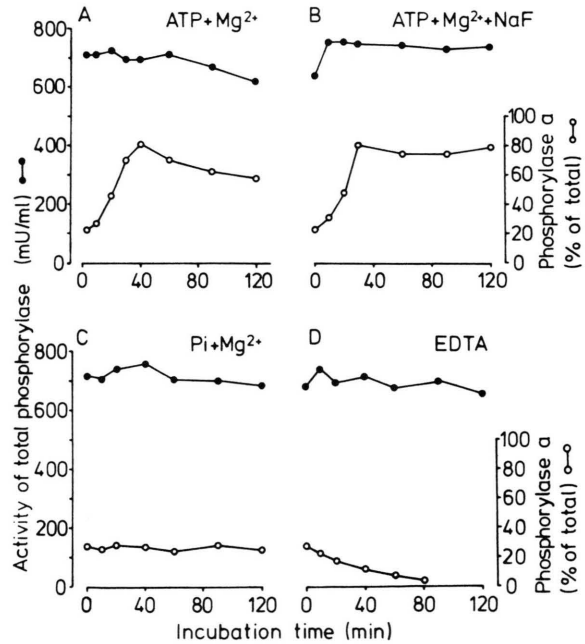


Fig. 1. Interconversion of glycogen phosphorylase by endogenous phosphorylase kinase and phosphorylase phosphatase in crude extracts of *Periplaneta americana* fat body. The incubations contained: **A** 1 mM ATP and 1 mM magnesium acetate; **B** as A plus 5 mM NaF; **C** 50 mM potassium phosphate (pH 7.0) and 1 mM magnesium acetate and **D** 5 mM EDTA. The total phosphorylase activity (●–●) and the activity without AMP (○–○) as percentage of the total activity is given.

the activity of phosphorylase a (independent of AMP): 80–95% of the total phosphorylase (a + b-form) activity was in the active form after about 20 min *(N. cinerea)* or 40 min *(P. americana)*, whereas the activity of the total phosphorylase was virtually unchanged *(N. cinerea)* or decreased only slightly after prolonged incubation as was the case with the a-form *(P. americana)* (Fig. 1 A and 2 A). The activation was dependent on the presence of ATP, since substitution of this substrate by $P_i$ resulted in no activation; in both cockroaches the activation state of phosphorylase of 20% *(P. americana)* and about 40% in the a-form *(N. cinerea)* remained constant under such conditions (Fig. 1 C and 2 C). Thus, no interconversion took place and the results of both experiments strongly indicate the existence of a phosphorylase kinase in crude extracts of cockroach fat body. This statement is supported by experiments where commercially available rabbit muscle phosphorylase b is converted time-dependently to phosphorylase a during incubation with cockroach fat body extract in the presence of ATP and $Mg^{2+}$ (Table I).

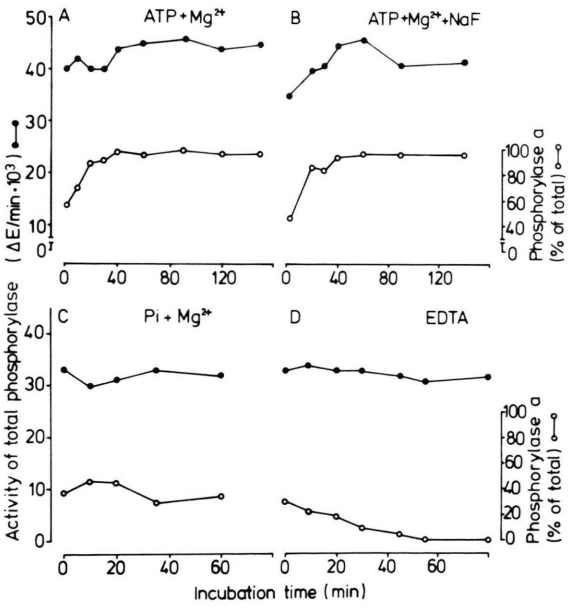


Fig. 2. Interconversion of phosphorylase by endogenous phosphorylase kinase and phosphorylase phosphatase in crude extracts of *Nauphoeta cinerea* fat body. The incubations contained: **A** 1 mM ATP and 1 mM magnesium acetate; **B** as A plus 5 mM NaF; **C** 50 mM potassium phosphate (pH 7.0) and 1 mM magnesium acetate and **D** 5 mM EDTA. The total phosphorylase activity (●–●) and the activity without AMP (○–○) as percentage of the total activity is given.

The slight inactivation of the a-form during prolonged incubation in *P. americana* (Fig. 1 A) is interpreted as a dephosphorylation by the action of endogenous phosphorylase phosphatase. A known inhibitor of the latter enzyme, NaF, prevented the inactivation of phosphorylase a when added to the incubation medium (Fig. 1 B). As such inactivation was not seen in the experiments with fat body extracts of *N. cinerea*, probably due to the low activity of the phosphorylase phosphatase, the results of incubation with and without NaF in the presence of ATP and $Mg^{2+}$ are almost identical (Fig. 2 A *versus* 2 B). However, the presence of endogenous phosphorylase phosphatase in the fat body extracts of both cockroaches was also demonstrated by another series of incubations. Inhibition of phosphorylase kinase activity by addition of EDTA to the incubation medium (without ATP and $Mg^{2+}$) resulted in a complete inactivation of the existing phosphorylase a activity in about 60 to 80 min (Fig. 1 D and 2 D).

### *Existence of three forms of fat body phosphorylase in cockroaches*

In 1985 van Marrewijk *et al.* [3] had shown the occurrence of three forms, designated a, b and ab, in the fat body of the migratory locust when partially purified on DEAE-Sephacel. In a first series of experiments we repeated this study to check the methodology, and fractionated a fat body extract from *L. migratoria* by ion-exchange chromatography. As depicted in Fig. 3 such a chromatogram

Table I. Effect of endogenous phosphorylase kinase of a cockroach *(N. cinerea)* fat body extract on vertebrate phosphorylase b.*

| Incubation time | Phosphorylase a activity (% of total phosphorylase) |
|---|---|
| 0 | 15 ± 3.2 |
| 20 | 50 ± 5.8 |
| 40 | 90 ± 9.4 |

* An aliquot of *N. cinerea* crude extract was incubated with rabbit muscle phosphorylase b (2 U; Boehringer) in the presence of ATP, $Mg^{2+}$ and $Ca^{2+}$ and the phosphorylase activity assayed with and without AMP.

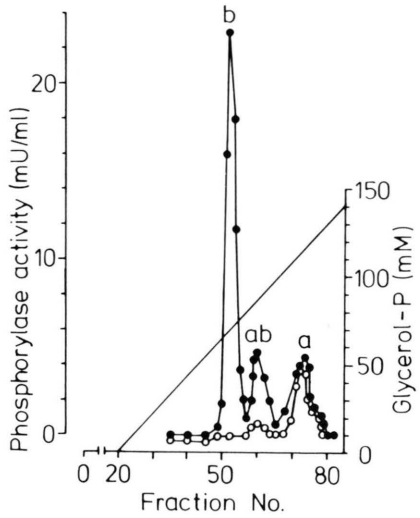


Fig. 3. Elution profile of fat body phosphorylase from resting *Locusta migratoria* on DEAE-Sephacel. Fat body tissue from 8 adult male locusts was pooled. Chromatography was carried out on a Pharmacia-column (30 cm high, 1.8 cm diameter) with a flow rate of 9.2 ml/h. Fractions were collected every 15 min; after 20 fractions a linear gradient of $Na_2$-β-glycerophosphate (0–200 mM; total volume: 280 ml) in Tris-HCl buffer was started. Fractions were assayed for phosphorylase activity in the absence (○–○) and in the presence (●–●) of AMP.

revealed three peaks with phosphorylase activity. The first peak that eluted after applying the $Na_2$-β-glycerophosphate gradient was active only by the addition of AMP and hence designated phosphorylase b. The second peak showed slight activity without AMP and was several times activated by this nucleotide. This intermediate is called phosphorylase ab according to the terminology of [3]. A third activity peak exhibited almost full activity without AMP and was only marginally further stimulated by this nucleotide and, hence, is the a-form of phosphorylase.

In the second series of experiments fat body extracts from resting cockroaches *P. americana* (Fig. 4) and *N. cinerea* (Fig. 5) were separated on DEAE-Sephacel. As shown in Fig. 4A and 5A cockroach phosphorylase also existed in three activity forms; most of the enzyme of both species occurred in the b-form (inactive without AMP) and only a small fraction (about 10%) in the a-form (active without AMP), whereas the intermediate ab-form was proportionally higher in *P. americana* (about 35%) than in *N. cinerea* (about 26%).

*Interconversion of phosphorylase*

In our first experiments we showed that upon incubation of crude extracts of cockroach fat body with ATP, $Mg^{2+}$ and NaF, phosphorylase b was converted to phosphorylase a by endogenous phosphorylase kinase (Fig. 1B and 2B). Such experiments were repeated and, after 1 h of incuba-

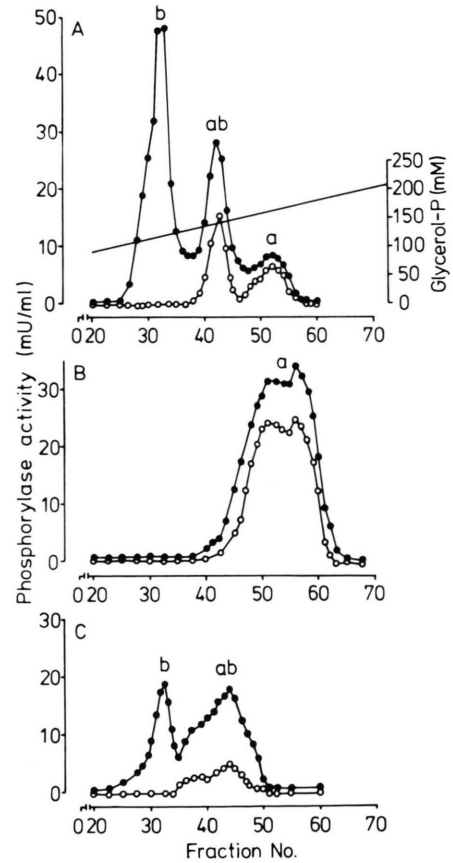


Fig. 4. Elution profile of fat body glycogen phosphorylase from *Periplaneta americana:* **A** pooled tissues from 8 adult female cockroaches; **B** a crude extract of pooled fat bodies from 8 female cockroaches was incubated with ATP, $Mg^{2+}$ and NaF prior to chromatography for 1 h; **C** as in B, but 1 h incubation with EDTA. Column as in Fig. 3; flow rate: 10 ml/h; fractions every 15 min; gradient (50–250 mM; total volume: 250 ml) started immediately. Activity labelled as in Fig. 3.

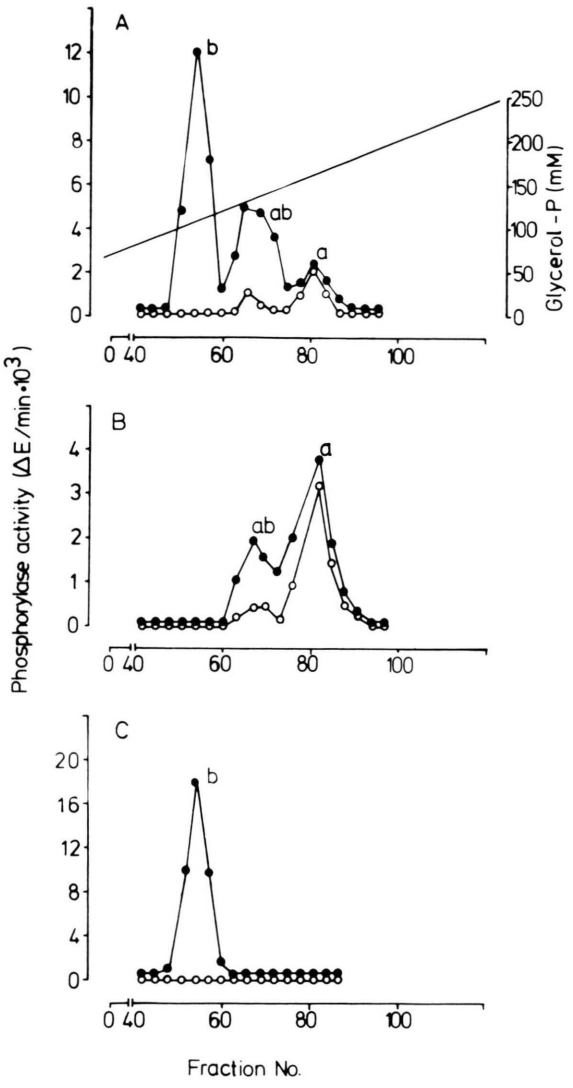


Fig. 5. Elution profile of fat body phosphorylase from *Nauphoeta cinerea:* **A** pooled tissues from 6 adult cockroaches; **B** a crude extract of pooled fat bodies from 4 cockroaches was incubated with ATP, $Mg^{2+}$ and NaF prior to chromatography for 1 h; **C** as in B, but 16 fat bodies were used and incubation was with EDTA for 1 h. Column as in Fig. 3; flow rate: 8 ml/h; fractions every 15 min; gradient (0–250 mM; total volume: 250 ml) started immediately. Activity labelled as in Fig. 3.

tion, the extracts subsequently separated on DEAE-Sephacel. In both cases (Fig. 4B for *P. americana;* Fig. 5B for *N. cinerea*) phosphorylase b was completely absent. In *P. americana* all the activity was now in the a-form (Fig. 4B), whereas in *N. cinerea* there was still a minor peak of the ab-form present, but the majority of the activity resided in the a-form (Fig. 5B).

On the other hand, when crude fat body extracts were incubated under conditions when the endogenous phosphorylase phosphatase was active and the phosphorylase kinase inhibited (in the presence of EDTA), such extracts exhibited no peak of the a-form on DEAE-Sephacel (Fig. 4C for *P. americana;* Fig. 5C for *N. cinerea*). The conversion to phosphorylase b was complete in the case of *N. cinerea* (Fig. 5C), but in *P. americana* a large proportion of the phosphorylase still occurred in the ab-form (Fig. 4C).

Conversion of the b- and ab-form isolated by ion-exchange chromatography from fat body of *N. cinerea* (see Fig. 5A) can also be shown by incubation with vertebrate (rabbit) muscle phosphorylase kinase. Time-dependently the percentage of activity in the a-form increased due to the action of the added enzyme (Fig. 6A, B), whereas the total activity remained fairly constant. After 30 min of incubation about 40% of the phosphorylase existed in the a-form. It became clear from the control experiment that vertebrate phosphorylase b was converted to the a-form (about 80%) by vertebrate phosphorylase kinase in 5 min (Fig. 6C). Conversion of phosphorylase a partially purified from *N. cinerea* fat body is shown by incubation with vertebrate phosphorylase phosphatase (Fig. 7A). With time phosphorylase a activity is diminished. However, this process takes rather a long time compared to the conversion of vertebrate phosphorylase a by phosphatase (Fig. 7B). Partially purified phosphorylase ab from *N. cinerea* was also subjected to dephosphorylation by vertebrate phosphatase. Due to the low activity of such a preparation in the absence of AMP (see Fig. 5) activity was measured only before and 90 min after the addition of phosphatase. Clearly, a decrease of the a-activity to about 50% of its initial value could be measured without any change in the total activity (results not shown).

*Interconversion of phosphorylase upon injection of hypertrehalosaemic hormones*

When cockroaches had been injected with their respective hypertrehalosaemic hormones (10 pmol of Pea-CAH-I or II into *P. americana* and 10 pmol

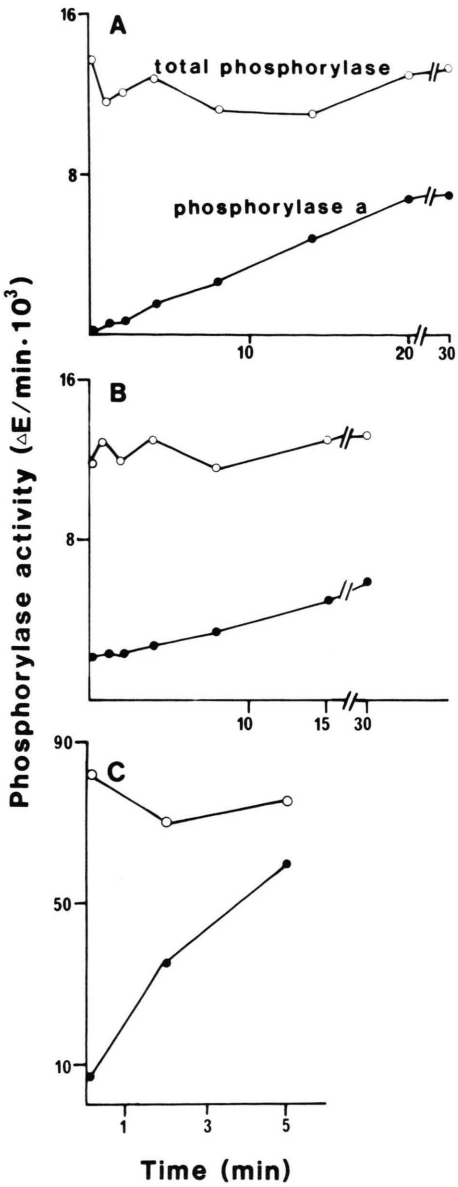


Fig. 6. Conversion of partially purified phosphorylase b (A) and ab (B) from *N. cinerea* fat body by rabbit muscle phosphorylase kinase. The cockroach enzymes were purified by DEAE-Sephacel chromatography: about 4–20 mU phosphorylase b or ab were used in the incubation (with ATP, $Mg^{2+}$ and $Ca^{2+}$) and 0.2 U rabbit phosphorylase kinase. In Fig. 6C, 100 mU vertebrate phosphorylase b were incubated with 1 U of phosphorylase kinase.

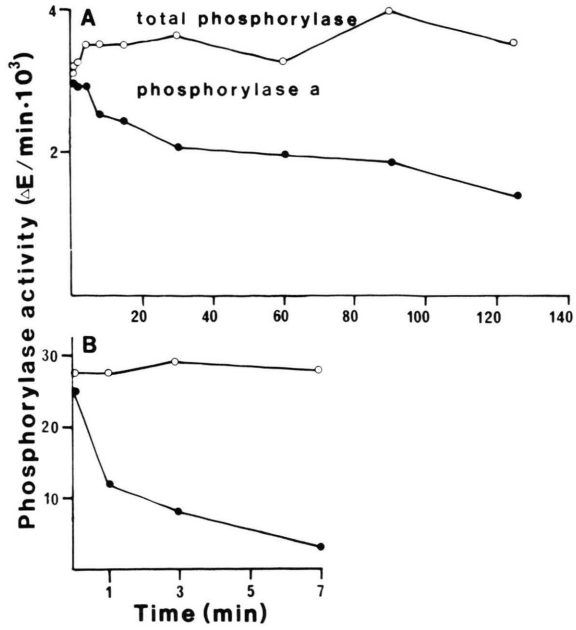


Fig. 7. Conversion of partially purified phosphorylase a (A) from *N. cinerea* fat body by rabbit phosphorylase phosphatase. The cockroach enzyme was purified by DEAE-Sephacel chromatography; about 1 mU phosphorylase a was used in the incubation medium (20 mM Tris/HCl buffer, pH 7.0; 5 mM EDTA) and 0.2 µg phosphatase protein. In Fig. 7B, 10 mU vertebrate phosphorylase a were incubated with 0.2 µg phosphatase protein.

Bld-HrTH into *N. cinerea*) 15 min prior to the preparation of crude extracts of the fat body and these were subsequently separated on DEAE-Sephacel, it is evident from Fig. 8A, B *(P. americana)* and Fig. 9 *(N. cinerea)* that the relative amounts of the three phosphorylase forms are present in different ratios when compared to resting cockroaches (see Fig. 4A and 5A). Hormone injection resulted in all cases in the presence of mainly (*ca.* 60%) phosphorylase a, while the ab-form represented about 25% and the b-form only *ca.* 15%. Injections of control solutions (distilled water, saline) had no effect on the different phosphorylase forms compared to resting insects (results not shown). Smaller quantities of hormone (2 pmol of Bld-HrTH) injected into *N. cinerea* had the same qualitative effect as described above for 10 pmol, resulting in a shift to the active phosphorylase form (results not shown).

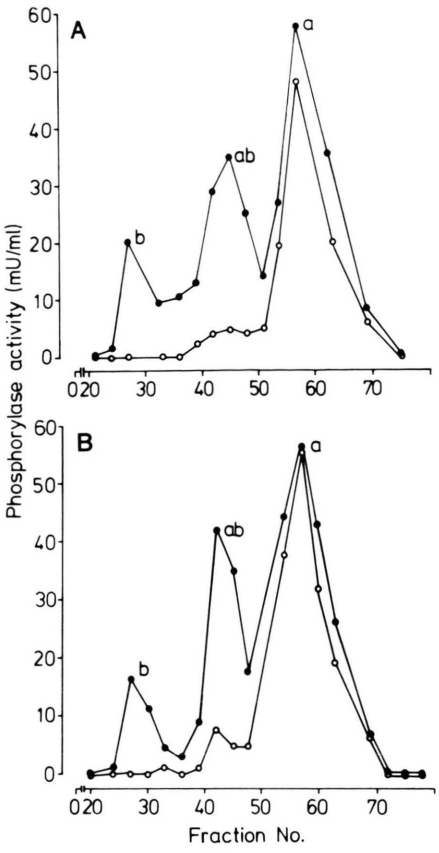


Fig. 8. Elution profile of fat body phosphorylase from *Periplaneta americana* injected 15 min previously with 10 pmol of Pea-CAH-I (A) and Pea-CAH-II (B). Pooled tissues from 5 adult female cockroaches; all other conditions as given in legend to Fig. 4.

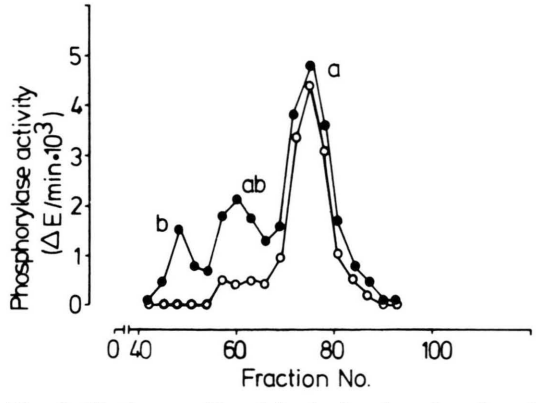


Fig. 9. Elution profile of fat body phosphorylase from *Nauphoeta cinerea* injected 15 min previously with 10 pmol of Bld-HrTH. Pooled tissues from 5 adult cockroaches; all other conditions as given in legend to Fig. 5.

*Purification of phosphorylases and physicochemical properties*

Crude extract of fat bodies was applied to a DEAE-Sephacel column and the three forms separated and isolated as described above. Complete purification of the pooled fractions of each form was achieved by affinity chromatography on an AMP-Sepharose column eluting the phosphorylase under investigation with 10 mM AMP after the unbound protein was removed by washing (see Fig. 10 as an example). Between 40 to 55% of the activity applied was recovered. After concentration the final specific activity (U/mg protein) of the three forms was 2.1 (a-form), 27.2 (b-form), 6.9 (ab-form). As judged by PAGE of the native purified forms each preparation showed one major band that was active when stained for activity (results not shown). The native molecular masses for the different phosphorylase forms as judged by analytical gel filtration on Sephacryl S-300 were 177,000 (a-form), 173,000 (b-form) and 175,000 (ab-form) whereas commercial rabbit phosphorylase a had a molecular mass of 350,000 in our system (results not shown). The molecular mass of the phosphorylase subunits was calculated to be

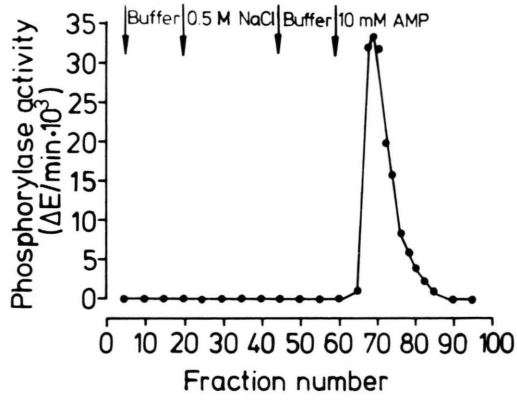


Fig. 10. Affinity chromatography (5′-AMP-Sepharose 4B) of phosphorylase b from fat body of *Nauphoeta cinerea* previously purified on DEAE-Sephacel. The enzyme was applied to the column (11 cm high, 1.0 cm in diameter) and the column washed with 2-fold volume of 20 mM Tris-HCl buffer (pH 7.0, containing 5 mM NaF, 1 mM EDTA and 1 mM DTT), 3-fold volume of 0.5 M NaCl in that buffer, equilibrated with 2-fold volume of Tris-buffer, and the enzyme eluted with 10 mM AMP in Tris-buffer. The flow rate was 34 ml/h and fractions of 1.4 ml were taken and assayed for phosphorylase activity.

87,100 after SDS-PAGE for all three forms (results not shown).

*Catalytic properties of the different phosphorylase forms*

Kinetic analyses of the three enzyme forms indicated a normal hyperbolic saturation with respect to glycogen at saturating conditions of AMP and $P_i$. The double-reciprocal plots (Fig. 11) were linear and revealed $K_M$ values of 0.021, 0.019 and 0.073% for glycogen for phosphorylase a, ab, and b, respectively. The saturation curves obtained with $P_i$ for the enzyme forms (a, ab and b) were hyperbolic, too (results not shown), and $K_M$ values were 8.3, 6.3 and 17.9 mM, respectively.

The effect of AMP on the activity of the different enzyme forms is shown in Fig. 12. Whereas phosphorylase a is almost completely independent of AMP (more than 70% of the maximal possible activity without AMP), phosphorylase b is completely inactive in the absence of AMP; the intermediate ab-form exhibits *ca.* 12% of its maximal activity without AMP. Large differences were also found in the affinity for AMP of the different forms. Phosphorylase a exerted a more or less hyperbolic response and maximal stimulation of this enzyme form was achieved with about 70 µM AMP. Both the b- and ab-forms followed sigmoidal kinetics with maximal activities displayed at about 3 mM AMP. All three forms were substantially inhibited at AMP concentrations above

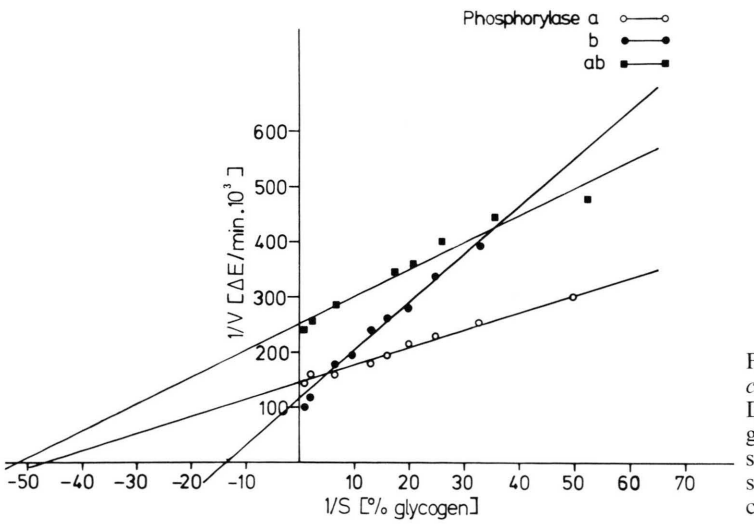


Fig. 11. Kinetic properties of purified *N. cinerea* fat body phosphorylase forms. Double-reciprocal plots with respect to glycogen as the variable substrate are shown. The fixed concentrations of substrate and activator were at saturating concentrations.

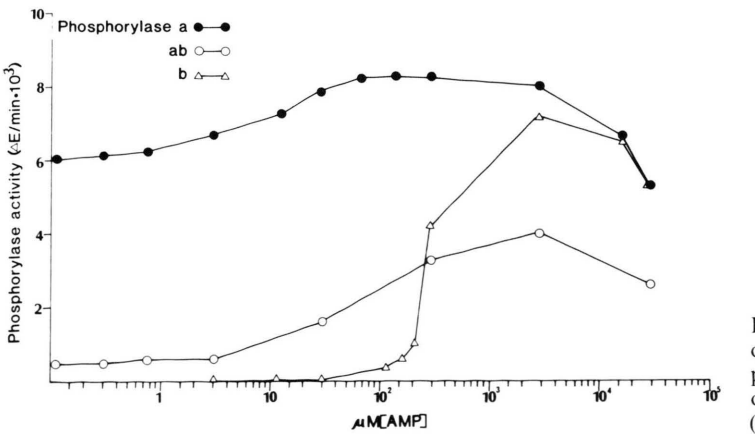


Fig. 12. Effect of increasing concentrations of AMP on the activity of the three phosphorylase forms purified from the fat body of *N. cinerea*. Substrate concentrations (glycogen, $P_i$) were at saturating levels.

Table II. Effect of caffeine (5 mM) on the activity of isolated phosphorylase a, ab and b from fat body of *N. cinerea* in the absence (phosphorylase a) and in the presence (all forms) of AMP (1.5 mM).

| Treatment | Relative activity [%] | | | |
|---|---|---|---|---|
| | − AMP | + AMP | | |
| | Phosphorylase a | Phosphorylase a | ab | b |
| Control | 100 | 100 | 100 | 100 |
| Caffeine | 21 | 91 | 81 | 0 |

about 3 mM. From Hill plots a half-maximum activation of phosphorylase ab and b by AMP is calculated as 55 and 280 μM, respectively.

The effect of caffeine on the activity of the different enzyme forms is given in Table II. Whereas phosphorylase b was completely inhibited by 5 mM caffeine, phosphorylase a and ab activity (in the presence of AMP) was reduced only by about 10 and 20%, respectively. However, in the absence of AMP phosphorylase a activity was strongly suppressed to about 20% of the activity without caffeine.

## Discussion

*The phosphorylase system in cockroach fat body and its regulation by endogenous hormones*

The present study shows the existence of both endogenous phosphorylase kinase and phosphorylase phosphatase in crude extracts of fat body from the two cockroach species investigated. The evidence is twofold. First, phosphorylase a production occurred after the addition of ATP and $Mg^{2+}$ and was not shown when $P_i$ instead of ATP was used. On the other hand, the inhibition of phosphorylase inactivation when the phosphatase inhibitor NaF was used (with *P. americana* crude extract) and the complete conversion to phosphorylase b when the phosphorylase kinase inhibitor EDTA was introduced (for both species) argue for the existence of an endogenous phosphorylase phosphatase. These observations were substantiated when the crude extracts were partially purified on DEAE-Sephadex: under conditions when endogenous phosphorylase kinase was active the phosphorylase b peak has completely abolished and, conversely, under conditions when endogenous phosphorylase phosphatase was active no phosphorylase a peak could be detected. Secondly, rabbit muscle phosphorylase b was readily converted to phosphorylase a by incubation with *N. cinerea* crude extract. Existence of phosphorylase kinase in the fat body of other insects (Lepidoptera and Orthoptera) or whole insects (Diptera) shown by the activation of phosphorylase in the presence of ATP and $Mg^{2+}$ has been reported previously [3, 13, 19–22].

As in locust fat body ([3], this study) phosphorylase in the fat body of the two cockroaches investigated here exists in three forms as shown by chromatography on an ion-exchange support: the b-form which is totally dependent on AMP for activation, the a-form being almost exclusively active without AMP and an additional ab-form that is slightly active without AMP, but can be activated 2- to 7-fold in the presence of AMP. In locusts evidence was presented that the hybrid ab-form is only partially phosphorylated (one of the two subunits) [23]. Hybrid forms of phosphorylase have been reported previously from some vertebrate muscle tissues [24–27] but appear to be lacking in *Drosophila melanogaster* [5, 22] and the muscle tissues of the shrimp *Crangon crangon* (Crustacea) [28], whereas some circumstantial evidence may argue for a hybrid form in the lugworm *Arenicola marina* (Annelida) [29]. There are good indications that the ab-form in cockroaches is also partially phosphorylated. Firstly, complete conversion of this form in *P. americana* crude extract under conditions when endogenous phosphorylase is active and in *N. cinerea* crude extract under conditions when endogenous phosphorylase phosphatase is active. Secondly, conversion of the partially purified form from *N. cinerea* by vertebrate phosphorylase kinase and phosphatase, respectively.

Our experiments on the phosphorylation of *N. cinerea* partially purified phosphorylase b or on the dephosphorylation of partially purified phosphorylase a with vertebrate kinase or phosphatase, respectively, show a much more protracted time course when compared with the vertebrate substrates. One interpretation may be a species specificity of the vertebrate enzymes. It was reported that *D. melanogaster* phosphorylase b was phosphorylated with rabbit muscle phosphorylase kinase at the same rate as the rabbit muscle phosphorylase b, but that dephosphorylation of fruit fly phosphorylase a required much more rabbit phos-

phatase than that of rabbit phosphorylase a [22]. Later these authors demonstrated that *D. melanogaster* phosphorylase a was also a poor substrate of fruit fly's own phosphatase (whereas rabbit muscle phosphorylase a was dephosphorylated much faster) and concluded that species specificity of the phosphatase was not the reason, but that the structures around the phosphorylation sites of fruit fly and rabbit phosphorylase a are different [5]. Supportive evidence provided by this group included distinctive differences for *D. melanogaster* and rabbit muscle phosphorylase with respect to amino acid composition, one-dimensional peptide maps and distribution of reactive SH groups.

It is well known that carbohydrate homeostasis in cockroaches is regulated by so-called hypertrehalosaemic factors originating in the corpus cardiacum [see reviews 2, 30]. Isolation and sequencing of these neuropeptides in *P. americana* [31–35] and *N. cinerea* [11, 36] was achieved recently. In *P. americana* the two synthetic octapeptides (Pea-CAH-I: pGlu–Val–Asn–Phe–Ser–Pro–Asn–Trp–$NH_2$ and Pea-CAH-II: pGlu–Leu–Thr–Phe–Thr–Pro–Asn–Trp–$NH_2$) were shown to activate fat body glycogen phosphorylase when injected *in vitro* in low doses of 0.5 pmol [8]; in *N. cinerea* it is a decapeptide (Bld-HrTH: pGlu–Val–Asn–Phe–Ser–Pro–Gly–Trp–Gly–Thr–$NH_2$) that activates fat body phosphorylase fully upon injection *in vivo* of 0.5 pmol [37]. In the present study it was shown conclusively that injection of the species-specific peptides in *P. americana* and *N. cinerea,* respectively, results in an increase of phosphorylase a at the expense of phosphorylase b. There seems to be no contribution of the ab-form. This statement, however, may be misleading since a static situation is recorded in our experimental protocol. It may well be that phosphorylase b is first partially phosphorylated to the ab-form prior to its total phosphorylation to the a-form. Such a kinetic event can only, if at all, be seen when the time-course of incorporation of [$\gamma$-$^{32}$P] ATP upon neuropeptide activation is quantitatively followed.

Nevertheless, our results that cockroach fat body phosphorylase is to a large extent activated upon injection of endogenous hypertrehalosaemic peptides by conversion of the b- to the a-form has a strong physiological relevance when one accepts that these peptides are released from the corpus cardiacum upon stimuli of heavy activity such as fast running or flight. Unfortunately, no titre determinations have been published; it is, however, demonstrated *in vitro* that the decapeptide Bld-HrTH as well as the octapeptides Pea-CAH-I and II, respectively, are released from the corpora cardiaca of *N. cinerea* and *P. americana,* respectively, during depolarization with an elevated potassium saline [37, 38]. In *L. migratoria* flight-induced activation of fat body phosphorylase was supposedly due to the action of adipokinetic hormone [39], whereas the phosphorylase from the fat body of the moth *Manduca sexta* is activated not only in intact moths during flight, but also in cardiaectomized moths [40]. We tried to perform flight experiments with *P. americana,* but did not get unequivocal results. First, the insects were very reluctant to fly on the flight mill. Secondly, although the percentage of the phosphorylase a in crude extracts of flown cockroaches was higher than in resting ones and, after DEAE-chromatography, the a-form was increased, we got almost the same results (not statistically different) with control insects that had hung on the flight mill without flying, although struggling quite vigorously. Thus, no specific flight-induced activation of phosphorylase could be established.

### *Similarities between physico-chemical and catalytic properties of N. cinerea fat body phosphorylase forms and phosphorylases from other sources*

Isolation of the three forms of phosphorylase was achieved by anion-exchange chromatography and final purification reached by specific affinity chromatography on AMP-Sepharose. The final specific activity of the purified forms is within the range given for insect [4, 41–43] or other invertebrate phosphorylases [28, 29]. The native molecular weights of 173,000 to 177,000 found by gel filtration for the enzyme forms from *N. cinerea* fat body and the subunit molecular weight of 87,100 estimated from SDS gels for all three forms strongly suggests a dimer consisting of two identical subunits for all forms. These results are in agreement with those of other invertebrate [4, 28, 29, 42] and most vertebrate phosphorylases [see review 44]. A tetrameric form, known from rabbit muscle phosphorylase a [see review 45] was not found in our preparations. Two insect phosphorylases, however, were reported to contain monomeric forms

[41, 43]. As pointed out earlier [5, 42] the interpretation of the results on blowfly phosphorylase is questionable.

With respect to catalytic properties *N. cinerea* phosphorylase closely resembles those of phosphorylases from other insects/invertebrates. The $K_M$ value for glycogen and $P_i$ are at the lower end of the range for a variety of insect [see 2] and other invertebrate enzymes [28, 29]. In general, the $K_M$ values of the *N. cinerea* ab-form resemble more closely the a-form than the b-form and, a- and ab-form have higher affinities to glycogen and $P_i$ than the b-form, a phenomenon also found in most other phosphorylases (citations as above). In common with vertebrate and other invertebrate phosphorylases the a-form of *N. cinerea* was only slightly activated by AMP, whereas the ab- and b-forms needed a 50-fold higher concentration. Also the effect of caffeine on cockroach phosphorylases is similar to the effect on the respective enzymes from vertebrates and other insects.

What conclusion about the physiological action can be drawn from these data? Unfortunately, to our knowledge no data on intracellular AMP and $P_i$-concentrations are available for the fat bodies of cockroaches or other insects. In *P. americana* we measured about 1.34 µMol ATP per g fresh weight (unpublished observation), thus roughly 1 mM, AMP concentration should not be more than 10%, hence roughly 100 µM. However, these levels may be much too high since it has been shown that in mammalian muscle levels of free phosphate and AMP are much lower when measured *in vivo* by nuclear magnetic resonance compared to determination in tissue extracts [46]. For lugworm and shrimp muscle [28, 29, 47] values of 1–5 mM $P_i$ and 2–4 µM AMP are assumed. If these concentrations are true for cockroach fat body, too, non-covalent activation of phosphorylase b is not possible. Even if the AMP concentration is increased by hypertrehalosaemic peptides to the same extent as it is elevated by working muscle (*e.g.* shrimps to 80 µM; [29]), cockroach phosphorylase b would still be inactive. Thus, for an adequate rate of glycogenolysis it is obvious to convert phosphorylase b to a. Phosphorylase a (and part of phosphorylase ab) is active without effector and mainly dependent on the substrate concentrations. One of the substrates, glycogen, is present in excess and the enzyme should work under saturating conditions. Free phosphate is low in most tissues [46]. In order to obtain a maximum phosphorylase activity an increase in $P_i$ concentration in cockroach fat body has to be postulated. It would be interesting, indeed, to show such an elevation upon administration of hypertrehalosaemic peptides. In future, studies using the non-invasive technique of $^{31}P$-nuclear magnetic resonance spectroscopy may help to solve this problem.

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[1] R. G. H. Downer and J. R. Matthews, Amer. Zool. **16,** 733–745 (1976).
[2] J. E. Steele, Insect Biochem. **12,** 131–147 (1982).
[3] W. J. A. van Marrewijk, A. Th. M. van den Broek, and A. M. Th. Beenakkers, Insect Biochem. **15,** 341–347 (1985).
[4] W. J. A. van Marrewijk, A. Th. M. van den Broek, and A. M. Th. Beenakkers, Insect Biochem. **18,** 37–44 (1988).
[5] V. Dombrady, J. Matko, Z. Kiss, L. Kiss, P. Friedrich, and G. Bot, Comp. Biochem. Physiol. **84B,** 537–543 (1986).
[6] S. H. Vaandrager, W. J. A. van Marrewijk, and A. M. Th. Beenakkers, Insect Biochem. **16,** 749–756 (1986).
[7] G. Gäde, J. Insect Physiol. **27,** 155–161 (1981).
[8] G. Gäde, Z. Naturforsch. **40c,** 670–676 (1985).
[9] W. J. A. van Marrewijk, A. Th. M. van den Broek, and A. M. Th. Beenakkers, Gen. Comp. Endocrinol. **50,** 226–234 (1983).
[10] G. Gäde and M. Scheid, Physiol. Entomol. **11,** 145–157 (1986).
[11] G. Gäde, Z. Naturforsch. **42c,** 225–230 (1987).
[12] A. K. Raina and G. Gäde, Insect Biochem. **18,** 785–787 (1988).
[13] R. Ziegler, M. Ashida, A. M. Fallon, L. T. Wimer, S. S. Wyatt, and G. R. Wyatt, J. Comp. Physiol. **131,** 321–332 (1979).
[14] S. H. Vaandrager, W. J. A. van Marrewijk, and A. M. Th. Beenakkers, Insect Biochem. **17,** 695–700 (1987).
[15] L. Ornstein and H. J. Davis, Disc Gel Electrophoresis, Distillation Products Industries, Rochester, N.Y. (1963).
[16] K. Takeo and S. Nakamura, Archs. Biochem. Biophys. **153,** 1–7 (1972).
[17] U. K. Lämmli, Nature **227,** 680–685 (1970).
[18] M. M. Bradford, Analyt. Biochem. **72,** 248–254 (1976).
[19] E. Stevenson and G. R. Wyatt, Archs. Biochem. Biophys. **108,** 420–429 (1964).
[20] A. W. Wiens and L. I. Gilbert, Comp. Biochem. Physiol. **21,** 145–159 (1967).
[21] H. A. Yanagawa and Y. Horie, Insect Biochem. **8,** 155–158 (1978).
[22] V. Dombradi, P. Devay, P. Friedrich, and G. Bot, Insect Biochem. **16,** 557–565 (1986).
[23] W. J. A. van Marrewijk, A. Th. M. van den Broek, P. N. E. de Graan, and A. M. Th. Beenakkers, Insect Biochem. **18,** 821–827 (1988).
[24] S. S. Hurd, D. Teller, and E. H. Fischer, Biochem. Biophys. Res. Commun. **24,** 79–84 (1966).
[25] G. Bot, E. F. Kovacs, and P. Gergely, Biochem. Biophys. Acta **370,** 70–77 (1974).
[26] G. Vereb, A. Fodor, and G. Bot, Biochem. Biophys. Acta **915,** 19–27 (1987).
[27] G. Vereb, K. Szücs, J. Szabo, M. Belanova, and G. Bot, Molec. Cell. Biochem. **69,** 139–146 (1986).
[28] G. Kamp, Biol. Chem. Hoppe-Seyler **370,** 565–573 (1989).
[29] G. Kamp, Biol. Chem. Hoppe-Seyler **367,** 109–117 (1986).
[30] G. J. Goldsworthy and G. Gäde, in: Invertebrate Endocrinology, Vol. 1, Endocrinology of Insects (H. Laufer and R. G. H. Downer, eds.), pp. 427–439, Alan Liss, N.Y. (1983).
[31] R. M. Scarborough, G. C. Jamieson, F. Kalish, S. J. Kramer, G. A. McEnroe, C. A. Miller, and D. A. Schooley, Proc. Natl. Acad. Sci. U.S.A. **81,** 5575–5579 (1984).
[32] 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).
[33] G. Gäde, Naturwissenschaften **72,** 95–96 (1985).
[34] G. Gäde, Z. Naturforsch. **40c,** 42–46 (1985).
[35] K. J. Siegert and W. Mordue, Physiol. Entomol. **11,** 205–211 (1986).
[36] G. Gäde and K. L. Rinehart, Biochem. Biophys. Res. Commun. **141,** 774–781 (1986).
[37] G. Gäde, Z. Naturforsch. **43c,** 108–116 (1988).
[38] M. O'Shea, J. Witten, and M. Schaffer, J. Neurosci. **4,** 521–529 (1984).
[39] W. J. A. van Marrewijk, A. Th. M. van den Broek, and A. M. Th. Beenakkers, Gen. Comp. Endocrinol. **64,** 136–142 (1986).
[40] R. Ziegler and M. Schulz, J. Insect Physiol. **32,** 997–1001 (1986).
[41] C. C. Childress and B. Sacktor, J. Biol. Chem. **245,** 2927–2936 (1970).
[42] V. Dombradi, J. Hajdu, P. Friedrich, and G. Bot, Insect Biochem. **15,** 404–410 (1985).
[43] I. Morishima and S. Sakurai, Comp. Biochem. Physiol. **81B,** 453–458 (1985).
[44] D. J. Graves and J. H. Wang, in: The Enzymes (P. D. Boyer, ed.), **Vol. 7,** pp. 435–482, Acad. Press, N.Y. (1972).
[45] E. H. Fischer, L. M. G. Heilmeyer, and R. H. Haschke, Curr. Top. Cell. Regul. **4,** 211–251 (1971).
[46] D. G. Gadian, Nuclear Magnetic Resonance and its Application to Living Systems, Clarendon Press, Oxford (1982).
[47] G. Kamp and H. P. Juretschke, Biochem. Biophys. Acta **929,** 121–127 (1987).

*Note added in proof:*

During the preparation of this manuscript the following publications on phosphorylases in insects [48, 49] and in a fish [50] have been published:

[48] W. J. A. Marrewijk, A. Th. M. van den Broek, and A. M. Th. Beenakkers, Comp. Biochem. Physiol. **94B,** 165–169 (1989).
[49] I. Morishima and T. Ueno, Comp. Biochem. Physiol. **96B,** 591–595 (1990).
[50] H. Schmidt and G. Wegener, Am. J. Physiol. **258,** C 344–C 351 (1990).