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IN VITRO STUDIES ON HORMONE-STIMULATED LIPID MOBILIZATION FROM FAT BODY AND INTERCONVERSION OF HAEMOLYMPH LIPOPROTEINS OF *LOCUSTA MIGRATORIA*

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Abstract—Both adipokinetic hormone and octopamine have a stimulating effect on lipid release from locust fat body *in vitro*, when incubated in diluted haemolymph. The presence of adipokinetic hormone results in the formation of the flight-specific haemolymph lipoprotein A^+ accepting the increased amount of lipids released into the incubation medium. In contrast, interconversions of lipoproteins do not occur when octopamine is added to the incubation medium, which is in line with the expectations: the lipid-mobilizing effect of octopamine is a limited and short-term effect. When fat body tissue is incubated with isolated haemolymph protein fractions, the lipid-mobilizing effect of adipokinetic hormone only occurs when the incubation medium contains both lipoprotein A_y and protein fraction C, resulting in the formation of lipoprotein A^+. In similar control incubations with the hormone omitted, some lipoprotein A^+ is also formed (concomitant with a slight amount of lipid released), though significantly less than in incubations with hormone. Besides a stimulating function on lipolytic processes in the fat body, adipokinetic hormone is suggested to influence haemolymph lipoprotein rearrangement. A possible counteracting function of another factor in the haemolymph is discussed.

Key Word Index: *In vitro*, lipid mobilization, haemolymph lipoproteins, adipokinetic hormone, octopamine, *Locusta migratoria*

INTRODUCTION

A not unimportant part of *in vivo* research on flight-related metabolism in insects has been accompanied by *in vitro* research. Thus, particularly experiments involving *in vitro* incubation of fat body have been performed, in order to investigate the role of this organ in supplying lipid during flight. Tietz (1962) incubated isolated fat body of *Locusta migratoria* in saline-diluted haemolymph and showed that glycerides were released. Similar experiments with *Hyalophora cecropia* fat body demonstrated that the glycerides released were diacylglycerols (Chino and Gilbert, 1965), which was confirmed for locust fat body (Tietz, 1967). The release of diacylglycerol was found to specifically require the presence of haemolymph, suggesting haemolymph lipoproteins to be responsible for diacylglycerol uptake as well as transport from storage site to the site of energy demand.

Adipokinetic hormone, released from the corpora cardiaca after initiation of flight (Rademakers and Beenakkers, 1977), was shown to be responsible for stimulation of lipid mobilization from locust fat body by *in vivo* (Beenakkers, 1969; Mayer and Candy, 1969) and *in vitro* experiments (Spencer and Candy, 1976).

This hormone may also have an influence on the diacylglycerol-transporting lipoproteins in the haemolymph, on the lipolytic processes in the flight muscles, or both (for ref. see Beenakkers *et al.*, 1984). In the haemolymph of resting locusts, two diacylglycerol-containing lipoprotein fractions were separated by gel filtration, O and A_{yellow} (A_y), in addition to the protein fractions A_{blue} (A_{bl}), B and C (Mwangi and Goldsworthy, 1977; Van Der Horst *et al.*, 1979). During flight, a new diacylglycerol-carrying lipoprotein (A^+) appears, resulting from a rearrangement of lipoprotein fraction A_y and protein fraction C, while in addition fraction O increases in protein and lipid content (Mwangi and Goldsworthy, 1977; Van Der Horst *et al.*, 1979, 1981a, b; Wheeler and Goldsworthy, 1983a, b).

Besides adipokinetic hormone, octopamine has been shown to influence lipid release from fat body *in vitro* (Orchard *et al.*, 1982). A short-lived elevation of the octopamine level in the haemolymph during the first 10 min of flight was demonstrated in *Schistocerca gregaria* (Goosey and Candy, 1980). Octopamine has been proposed to be responsible for the initial elevation of haemolymph lipid level during locust flight (Orchard and Lange, 1983).

The present paper is concerned with the influence of adipokinetic hormone and octopamine on diacylglycerol mobilization from the locust fat body and the dynamics of haemolymph lipoproteins, in an *in vitro* system.

MATERIALS AND METHODS

Insects

Locusta migratoria migratorioides were reared in the laboratory under crowded conditions at 30°C, 40% r.h., and with a photoperiod of 12 h light per

day. The insects were fed with reed, supplemented with rolled oats. Adult male insects (12–14 days after imaginal ecdysis) were used in all experiments.

Chemicals

Synthetic adipokinetic hormone was purchased from Peninsula Laboratories (San Carlos, CA), D,L-octopamine-HCl from Sigma Chemical Company (St Louis, MO), Ultrogel AcA 22 from LKB (Bromma, Sweden).

Fat body incubations

Whole fat bodies were dissected under saline, cut into quarters, washed twice with saline and distributed between 4 incubation tubes (ϕ 13.5 mm) at 4°C, such that each tube contained tissue equivalent in amount to one fat body.

The incubation tubes contained 200 μl of incubation medium, consisting of either haemolymph (centrifuged at 12,000 g for 2 min) diluted to 30 or 50% with saline (10 mM hepes, 150 mM NaCl, 10 mM KCl, 4 mM $CaCl_2$, 2 mM $MgCl_2$, pH 7), or isolated lipoprotein fractions with final concentrations of approx. 50% of those in untreated haemolymph.

Incubations were carried out at 33°C in a shaking waterbath. After 5 min, a 10 μl sample was taken from the incubation medium with a microsyringe and lipid was determined for estimation of leakage of the fat body (in the latter case, the fat body tissue was discarded). A 10 μl sample of either hormone or saline (in experimental and control conditions respectively) was added and the incubation started. After 15, 30 and 60 min or after 1, 2 and 3 h of incubation serial samples (10 μl) were taken for lipid determination.

Isolation of haemolymph proteins

Fractionation of the different haemolymph proteins was carried out on Ultrogel AcA 22 as described earlier (Van Der Horst *et al.*, 1979). The elution buffer (pH 7.5) consisted of 130 mM NaCl, 5 mM KCl, 1.9 mM NaH_2PO_4, 1.7 mM KH_2PO_4 and 5 mM Na_2 EDTA, to which 0.2 g NaN_3/l was added.

For isolation of protein fractions to be used for incubations, 2 ml of pooled haemolymph samples, diluted with 1 ml of elution buffer, were centrifuged (2 min at 12,000 g) and the supernatant was applied to a 200 × 1.6 cm column, flow rate 8–10 ml/h. Combined fractions were concentrated by ultrafiltration using an Amicon ultrafiltration cell with a PM10 membrane (for lipoprotein A_y) or YM2 membrane (for protein fraction C) which retain molecules larger than about mol.wt 10,000 and mol.wt 1,000 respectively. During concentration, the elution buffer was completely replaced by saline, in order to prevent possible interference of buffer components.

For isolation of protein fractions from the incubation medium after 60 min or 3 h of incubation, the media of 3–6 incubation tubes were combined, centrifuged (2 min at 12,000 g) and the supernatant was applied to a 50 × 1.5 cm column, flow rate 5 ml/h, to separate the various protein fractions.

Determination of lipids

Ten μl samples of incubation medium were added to 0.5 ml concentrated sulphuric acid. Total lipids were quantified by a modified vanillin method as described by Holwerda *et al.* (1977).

After extraction of total lipids with excess chloroform–methanol, individual lipid classes were separated by thin-layer chromatography and quantified, as described earlier (Van Der Horst *et al.*, 1983).

Determination of proteins

Protein concentrations in total haemolymph or chromatography fractions were estimated by the method of Schacterle and Pollack (1973).

Immunodiffusion

Ouchterlony double diffusion was performed in 1% agarose gels in 0.05 M barbital–0.03% EDTA buffer (pH 8.5). The gels were washed in 0.1 M NaCl, stained in Amido black 10B and destained in acetic acid–ethanol–water (2:9:9, by vol).

The preparation of specific antiserum against lipoprotein A^+ was described earlier (Van Der Horst *et al.*, 1981b).

Statistical treatment of results

Differences between control and experimental results were analysed by the Wilcoxon's two-sample test.

RESULTS

Effects of adipokinetic hormone on in vitro *incubation of fat body*

Lipid release in diluted haemolymph. Figure 1 shows the time course of the effect of adipokinetic hormone on lipid release from fat body *in vitro*. Control incubation of isolated fat body in diluted haemolymph caused only a small rise in lipid level in the incubation medium. After addition of 10 pmol of hormone, the amount of lipid released by the fat body was significantly greater than in control incubations at all incubation times shown ($P \ll 0.01$).

Haemolymph dilution state influenced the rate of lipid release: after 3 h of incubation in 50% haemolymph about 4.8 mg lipid/ml incubation medium were released, whereas in 30% haemolymph a value of approx 2.8 mg/ml was reached.

In preliminary experiments, total lipids in the incubation media were extracted and separated into lipid classes. The increased amount of lipid recovered from the media after incubation in the presence of adipokinetic hormone, appeared to consist of diacylglycerols (results not shown).

Interconversions of lipoproteins in diluted haemolymph. After 3 h of incubation in 50% haemolymph, without hormone added, the elution profile remained similar to that of resting haemolymph. Addition of 10 pmol adipokinetic hormone resulted, besides a decrease in concentration of both lipoprotein A_y and protein fraction C, in the appearance of lipoprotein fraction A^+ (Fig. 2). The newly formed lipoprotein A^+ constituted about 16.5% of the total amount of protein (Table 1).

Incubation of 50% haemolymph with 10 pmol of hormone, however with fat body omitted,

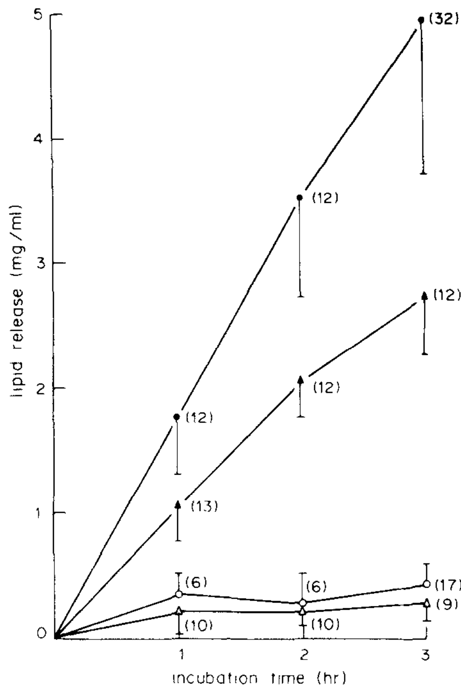

Fig. 1. Time course of the effect of adipokinetic hormone (10 pmol) and of haemolymph dilution on lipid release from fat body *in vitro*. Fat body tissue was incubated in 30% haemolymph, with hormone (▲——▲) or without hormone (△——△), and in 50% haemolymph, with hormone (●——●) or without hormone (○——○). Values of lipid release are expressed as mg lipid released/ml incubation medium/fat body. Each point is the mean ±SD for the number of incubations in parenthesis.

had no effect on the lipoprotein pattern of the haemolymph.

To assess the immunological properties of *in vitro* formed lipoprotein A^+, an Ouchterlony immunodiffusion test was performed. The immunological reaction between antiserum prepared against *in vivo* obtained lipoprotein A^+ (anti A^+) and the lipoprotein A^+ formed *in vitro*, was compared to the reaction between anti A^+ and both lipoprotein A_y and fraction C. The combination of anti A^+ and lipoprotein A^+ resulted in the formation of two precipitin lines. The outer precipitin line corresponds to that formed upon the reaction between anti A^+ and lipoprotein A_y whereas the inner one corresponds to that obtained between anti A^+ and fraction C (Fig. 3).

Lipid release in incubation medium containing isolated lipoprotein fractions. Figure 4 shows the time course of the effect of adipokinetic hormone on lipid release from fat body *in vitro*, in an incubation medium containing isolated protein fractions to a concentration of approx. 50% of untreated haemolymph. In a medium containing only lipoprotein A_y, no lipid release from fat body occurred. When the hormone was added, the lipid content of the medium seemed to decrease. This decrease, however, was not significant ($P = 0.1$).

When incubated in a medium containing only fraction C, the fat body released a certain amount of lipid, this amount being significantly higher than in the case of incubation in saline ($P < 0.01$). Addition of adipokinetic hormone had no influence on this lipid release.

Incubation in a medium containing both lipoprotein A_y and fraction C resulted in lipid mobilization from fat body which was significantly higher than that in control incubations in 50% haemolymph ($P \ll 0.01$). Adipokinetic hormone had a stimulating effect on lipid mobilization in this medium ($P < 0.01$). When incubated in saline, fat body did not release lipid, neither in control conditions nor with hormone added.

Lipoprotein interconversions in incubation medium containing isolated protein fractions. During fat body incubation in a medium containing either lipoprotein

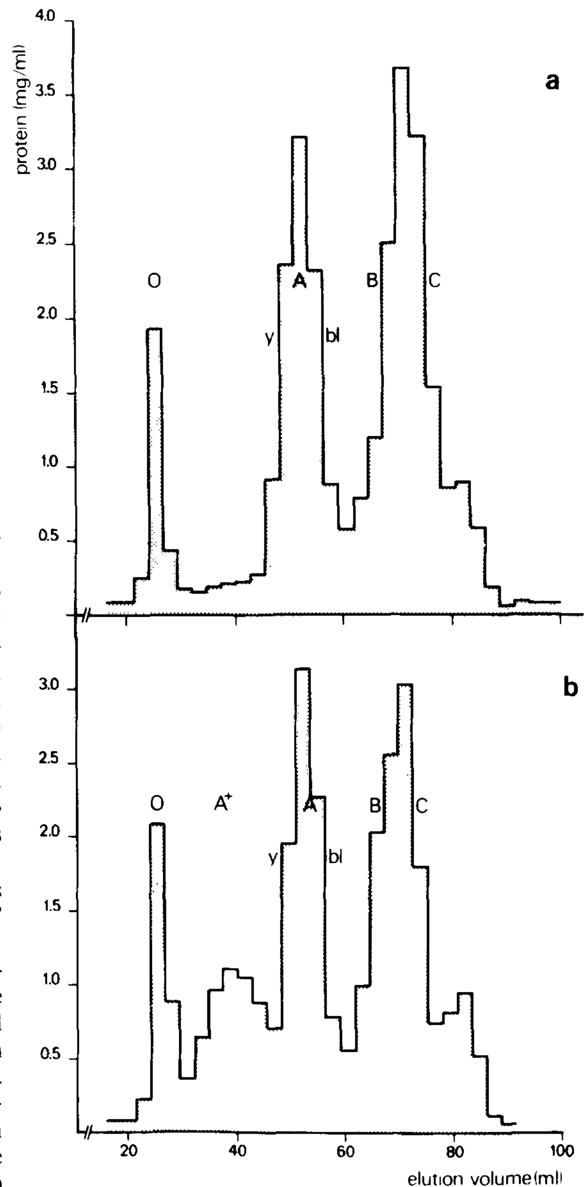

Fig. 2. Elution profile on Ultrogel AcA 22 of incubation media after 3 h of incubation in 50% haemolymph: (a) control incubation, (b) incubation with 10 pmol adipokinetic hormone added. Protein values are expressed as mg protein/ml incubation medium.

Table 1. Interconversions of haemolymph lipoproteins during *in vitro* incubation of fat body in 50% haemolymph

	%Of total protein			
	0	A^+	A_y	B + C
Control	12.75 ± 2.63	—	37.33 ± 2.08	49.93 ± 2.50
10 pmol adipokinetic hormone	12.61 ± 1.63	16.53 ± 0.42	32.40 ± 0.50	38.46 ± 1.86

Incubation media of 2–3 incubation tubes were combined after a 3-h incubation, and submitted to gel filtration as described in the Methods section. Values are expressed in % of total protein/ml incubation medium. Results are mean values ±SD of three experiments.

A_y or protein fraction C, no alterations in the protein pattern occurred. During incubation in a medium containing both lipoprotein A_y and protein fraction C, lipoprotein A^+ was formed in control incubations as well as in experiments with adipokinetic hormone added (Fig. 5). However, the amount of lipoprotein A^+ formed in the presence of the hormone was significantly higher ($P \ll 0.01$) than the amount formed in control incubations. A decrease in concentration of both fraction C and lipoprotein A_y is apparent from the elution profile of incubations with hormone.

Incubation medium containing lipoprotein A_y, fraction C and 10 pmol adipokinetic hormone, but lacking fat body, showed no alterations in protein pattern after a 3 h incubation.

Effects of octopamine on in vitro *incubation of fat body*

Lipid release in 50% haemolymph. The time course of the effect of octopamine on lipid release from fat body *in vitro* is shown in Fig. 6. The addition of octopamine at a final concentration in the incubation medium of 5 μM, had a significant effect ($P \ll 0.01$) on lipid release from fat body. Octopamine stimu-

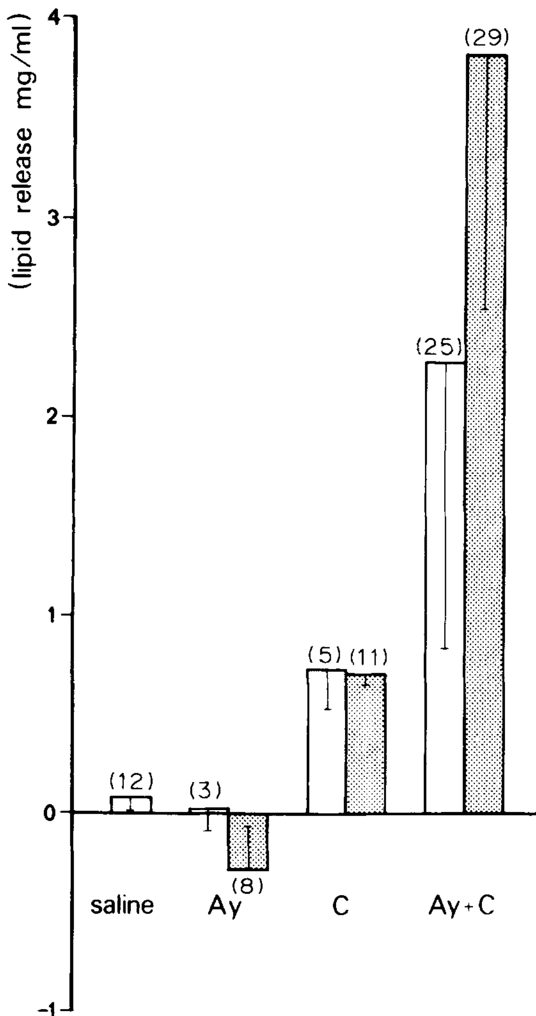

Fig. 4. The effect of adipokinetic hormone (10 pmol) on the lipid release from fat body *in vitro* after 3 h of incubation in a medium containing isolated protein fractions: open bars without hormone and shaded bars with hormone. Values of lipid release are expressed as mg lipid released/ml incubation medium/fat body. Values ±SD are given for the number of incubations in parenthesis.

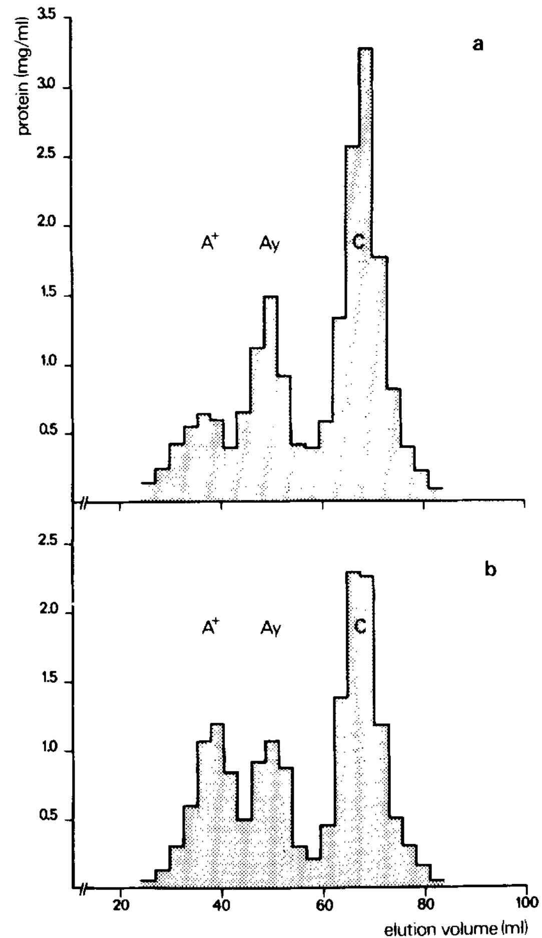

Fig. 5. Elution profile on Ultrogel AcA 22 of incubation media after 3 h of incubation in medium containing both lipoprotein A_y and protein fraction C: (a) control incubation, (b) incubation with 10 pmol adipokinetic hormone added. Protein values are expressed as mg protein/ml incubation medium.

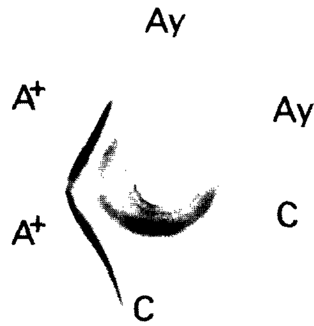

Fig. 3. Ouchterlony immunodiffusion test of antiserum anti A^+ (in central hole) against lipoprotein A, protein fraction C and lipoprotein A^+ formed *in vitro* in 50% haemolymph.

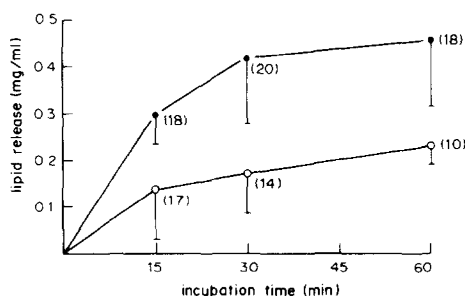

Fig. 6. Time course of the effect of octopamine (final concentration 5 μM) on lipid release from fat body *in vitro*. Incubations were performed in 50% haemolymph with (●——●) and without (○——○) octopamine. Values of lipid release are expressed as mg lipid released/ml incubation medium/fat body. Each point is the mean $\pm$SD for the number of incubations in parenthesis.

lated the release up to between 15 and 30 min of incubation; after that period lipid release was identical to that of control incubations.

Interconversions of lipoproteins in 50% haemolymph. Octopamine had no effect on the lipoprotein pattern of the incubation medium: after 60 min of incubation, the elution profile appeared to be similar to that of resting haemolymph (results not shown).

DISCUSSION

The involvement of adipokinetic hormone in the elevation of haemolymph lipid levels during locust flight has long been established (Beenakkers, 1969; Mayer and Candy, 1969). The present results clearly show that the hormone is indeed capable of stimulating lipid release from fat body *in vitro*, when the incubation medium contains haemolymph or haemolymph protein fractions. Chino and Gilbert (1965) and Tietz (1967) already mentioned the importance of haemolymph proteins in the loading and transporting of diacylglycerols in the haemolymph. The present results using 30 and 50% haemolymph as incubation medium suggest that lipid release increases almost linear with protein concentration. The increase in the amount of lipid released is almost linear for up to 3 h of incubation and hence the lipoproteins in the incubation medium appear to be not fully saturated with diacylglycerols during this period of incubation.

In resting locusts, the diacylglycerols are carried in the haemolymph by a yellow lipoprotein A_y. After injection of corpora cardiaca extracts a new, higher molecular weight lipoprotein A^+ is formed, carrying the increased amount of diacylglycerols (Mwangi and Goldsworthy, 1977). In addition to lipoproteins A_y and A^+, an even higher molecular weight, chylomicron-like lipoprotein fraction O was reported, carrying diacylglycerols at rest; after injection of corpora cardiaca extracts, load of lipid and protein content of O is augmented (Van Der Horst *et al.*, 1979).

The present *in vitro* studies show that during incubation of fat body in 50% haemolymph, the amount of newly formed lipoprotein A^+ accounts for 16.5% of the total amount of protein present in the medium (Table 1), this value being comparable to data found *in vivo* (Van Der Horst *et al.*, 1979).

In vivo, following elevation of the adipokinetic hormone titre in the haemolymph, transfer of protein from both lipoprotein A_y and protein fraction C to lipoprotein A^+ has been demonstrated (Van Der Horst *et al.*, 1981a). In accordance with these *in vivo* results, A^+-formation *in vitro* is accompanied by a decrease in concentration of both lipoprotein A_y and fraction C, indicating that to mimic the flight-related process of lipid mobilization and A^+-formation *in vitro*, the following factors are sufficient: (diluted) haemolymph, fat body and adipokinetic hormone.

Based upon the above *in vivo* results, *in vitro* experiments were performed with isolated protein fractions A_y and C. Values of lipid release and of interconversions of haemolymph lipoproteins varied quantitatively between series of experiments, which may be due to the preparation of protein fractions.

Adipokinetic hormone has no significant effect on lipid release when fat body is incubated in a medium containing only lipoprotein A_y or protein fraction C (Fig. 4). Furthermore, the presence of only one of these isolated proteins in the incubation medium is not sufficient for A^+-formation. In the case of incubation in isolated protein C, however, some lipid is released, independent of the addition of hormone. The amount is significantly higher than lipid increase in the medium following incubation of fat body in saline, or in 50% haemolymph without addition of hormone. C-Proteins have been proposed to be involved in the early increase in protein content of the A^+-fraction (before lipoprotein A_y does contribute), by binding to a lipoprotein fraction eluted by gel filtration at the leading edge of A_y (Wheeler and Goldsworthy, 1983a). The results reported here seem to indicate that isolated C-proteins might induce some lipid release, apparently without the participation of other haemolymph proteins, as no higher molecular weight lipoprotein could be detected by protein determination in the medium after incubation. Since, *in vivo*, protein fraction C does not contain any lipid, this result cannot be explained as yet and further investigation on the role of C-proteins, in which particularly glycoprotein C_2 appears to be involved (Van Der Horst *et al.*, 1984), with more reliable methods, is necessary.

When both lipoprotein A_y and protein fraction C were present in the incubation medium, adipokinetic hormone always significantly stimulated lipid release from fat body (Fig. 4). Control incubations also showed some increase in the amount of lipid released into the medium, significantly higher than in control experiments with 50% haemolymph. The average lipid release ratio with/without hormone was about 1.73. Lipid release in control incubations was accompanied by A^+-formation; however, when adipokinetic hormone was added to the incubation medium, the amount of lipoprotein A^+ formed was significantly higher (Fig. 5). The average A^+-formation ratio with/without hormone was about 1.78. These results indicate the existence of a relation between A^+-formation and lipid release from fat body.

When fat body was incubated with lipoprotein A_y and protein fraction C, both lipid release and

A^+-formation are already accomplished to some extent without the intervention of adipokinetic hormone. The *in vivo* resting situation apparently is not mimicked by the fat body *in vitro* in the presence of latter protein fractions. During preparation of the protein fractions some modifications might have been introduced, which could result in this hormone-independent A^+-formation. It, however, is more likely that a factor (or possibly more) in the haemolymph, other than lipoprotein A_y or fraction C, can be held responsible for the prevention of lipoprotein A^+-formation and lipid release in control incubations in 50% haemolymph. In resting locusts this blocking factor would prevent A^+-formation; release of adipokinetic hormone would abolish it. In the case of incubation in isolated protein fractions, the blocking factor would be absent from the medium, thus allowing A^+-formation without intervention of the hormone. Lipid release from the fat body would be possible to some extent; adipokinetic hormone would enhance the process by stimulating the conversion of triacylglycerols into diacylglycerols. This would mean that the hormone has an influence on both lipoprotein metabolism in haemolymph and lipid mobilization from fat body, as has been suggested earlier (Van Der Horst *et al.*, 1979).

The *in vitro* results of lipoprotein interconversions reported here differ from the changes occurring *in vivo*, as far as lipoprotein O is concerned. During incubation in 50% haemolymph, the protein content of fraction O was not elevated after addition of hormone. During incubation using isolated protein fractions, in contrast to lipoprotein A^+ lipoprotein O was never formed in the medium. Probably, in the process of lipid mobilization from fat body and transport in the haemolymph, lipoprotein A^+ has a more fundamental role than lipoprotein O.

Octopamine has been reported to be widely distributed in invertebrates. In locusts, octopamine, besides playing a role as neuromodulator, also functions as a neurohormone (Orchard, 1982). The effect of octopamine on lipid mobilization from fat body has been investigated *in vivo*. The stress-induced short-term lipid-mobilizing effect of saline injection into resting locusts (Van Der Horst *et al.*, 1980) can be enhanced and prolonged by octopamine injection (Van Marrewijk *et al.*, 1983). *In vitro* studies have demonstrated that octopamine has a direct effect on the fat body by stimulating lipid release (Orchard *et al.*, 1982). In the present study the stimulating effect of octopamine on lipid release *in vitro* also appeared to be a short-term effect: up to between 15 and 30 min of incubation the amount of lipid released in octopamine incubations is significantly higher than in control incubations. During the second 30-min of incubation, there is no longer any difference between time course of lipid release in both experimental conditions. During 1 h of incubation, about 40 μg lipid is released. The difference between this value and that of 160 μg lipid released during 1 h of incubation reported by Orchard *et al.* (1982) could possibly be due to the experimental conditions; as the latter incubations were performed in pooled haemolymph of both male and female adult locusts, the female-specific lipoprotein vitellogenin could be involved. In *Phylosamia cynthia*, vitellogenin carries diacylglycerols, but accepts the diacylglycerols from the sex-unspecific lipoprotein, rather than directly from fat body (Chino *et al.*, 1976). If vitellogenin can accept diacylglycerols from A_y in locusts too, this would increase the capacity of the incubation medium to accept diacylglycerol from the fat body.

An elevation in two phases of the haemolymph lipid level has been suggested, in which octopamine has been proposed to be responsible for the initial lipid increase (Orchard and Lange, 1983). The limited amount of lipid released by the action of octopamine could be due to the disappearance of the neurohormone from the haemolymph, mainly via the Malpighian tubes (Goosey and Candy, 1982).

Our *in vitro* findings suggest that octopamine could indeed stimulate a limited lipid release from the fat body during the first period of flight. In the *in vitro* situation, it seems possible that this limitation originates from the lipid-transporting system in the haemolymph: the lack of A^+-formation in the presence of octopamine could limit the capacity of the medium to accept diacylglycerols from the fat body. This hypothesis could be applied to the *in vivo* situation as well.

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