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HORMONE-INDUCED REARRANGEMENT OF LOCUST HAEMOLYMPH LIPOPROTEINS

THE INVOLVEMENT OF GLYCOPROTEIN C_2

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Abstract—Formation of lipoprotein A^+ and elevation of lipoprotein fraction O in locust (*Locusta migratoria migratorioides*) haemolymph as induced by adipokinetic hormone (AKH) includes the participation of non-lipid carrying proteins (fraction C), which was examined in more detail. By using gel filtration chromatography, the rather heterogenous C-proteins were resolved into three protein fractions, only one of which (C_2) appeared to be actually involved in the lipoprotein reassociation. The changes in amino acid composition of the elevated lipoprotein fractions as compared with those from the lipoproteins in the resting situation are accounted for by the contribution of the rather specific amino acid composition of this C_2-fraction. Polyacrylamide gel electrophoresis (PAGE) indicates that the C_2-protein is migrating as only one band; SDS–PAGE revealed that the C_2-protein consists of one single polypeptide chain with an approximate molecular weight of 20,000. This chain is also recovered in the subunit structure of the lipoprotein fractions induced by AKH-injection (A^+, O_{AKH}) in contrast with that of the lipoprotein fractions in resting haemolymph. Unlike the other C-proteins, protein C_2 displayed immunoreactivity with antiserum raised against lipoprotein A^+. From carbohydrate analyses, C_2 appeared to be a glycoprotein containing approx. 12.5% carbohydrate. *In vivo* pilot studies on the dynamics of C_2-proteins using ^{3}H-labelled glycoprotein C_2 gave evidence for the incorporation of radiolabel into both A^+ and O_{AKH}. Possible functions of the involvement of the glycoprotein to A^+ formation are discussed.

Key Word Index: *Locusta migratoria migratorioides*, lipoproteins, lipid transport, energy supply, adipokinetic hormone, glycoprotein

INTRODUCTION

One of the major functions of haemolymph lipoproteins in adult locusts is to supply lipids to the working flight muscles. Characteristically, flight-induced stimulation of lipid mobilization from the fat body is predominantly controlled by adipokinetic hormone (AKH) and manifested by a considerable increase in the level of haemolymph diacylglycerol, which is transported associated with carrier lipoproteins to the flight muscles (for reviews, see Beenakkers *et al.*, 1981a, b, 1984a, b). In resting locusts, haemolymph diacylglycerol is transported by lipoprotein fraction A_{yellow} which is eluted by gel filtration chromatography in addition to lipoprotein fraction O which is recovered in the void volume. However, promotion of lipid mobilization as induced by flight or injection of AKH is attended by the formation of a "new" lipoprotein of higher molecular weight (A^+) along with an increase in both lipid and protein contents of fraction O (Mwangi and Goldsworthy, 1977; Van der Horst *et al.*, 1979). These changes in lipoproteins originate from combinations of two existing haemolymph protein components, e.g. lipoprotein A_{yellow} and non-lipid containing proteins (fraction C), as was concluded from both immunological evidence and experiments using radioiodinated lipoprotein A_{yellow} or protein fraction C (Van der Horst *et al.*, 1981a, b). The resulting lipoprotein fractions display a high capacity for diacylglycerol binding which accounts for the progressive turnover of diacylglycerol during flight (Van der Horst *et al.*, 1978, 1979). Hence this lipoprotein rearrangement is of vital importance. Since virtually nothing is known about the mechanism of this arrangement, which is particularly true for the participation of the heterogenous protein fraction C, the present paper explores some biochemical features of the changes in the lipoproteins and especially the C-proteins, during the action of AKH.

MATERIALS AND METHODS

Insect handling

Locusta migratoria migratorioides were taken from the colony reared *ab ovo* in the laboratory under crowded conditions as described previously (Van der Horst *et al.*, 1978). Adult male insects (12 days after the imaginal ecdysis) were used in all experiments. Synthetic adipokinetic hormone (AKH) (Peninsula Laboratories, San Carlos, California), prepared as a stock solution in absolute methanol, was diluted with water and injected at a dosage of 10 pmol in a volume of 10 μl/locust to induce elevation of haemolymph diacylglycerol levels. Haemolymph was collected from resting and AKH-injected (90 min after injection) insects from a puncture in the ventral neck membrane made with a Hamilton microsyringe. Samples of 25 μl/locust were immediately pooled in ice-cooled centrifugation tubes containing 1–2 ml of buffer (pH 7.5) consisting of 130 mM NaCl, 5 mM KCl, 1.9 mM NaH_2PO_4 and 1.7 mM KH_2PO_4, to which 2.5 mM glutathione and 0.02% NaN_3 were added. Pooled haemolymph samples (final dilution, 50%; final volumes 2–4 ml) were centrifuged at 12,000 *g* for 2 min at

4°C to remove haemocytes, yielding clear, unclotted sera which were used directly.

Isolation of haemolymph proteins

Gel permeation chromatography of freshly prepared haemolymph samples (2–4 ml) was performed on glass columns (200 × 1.6 cm i.d.) packed with Ultrogel AcA 22 (LKB Ltd) as described earlier (Van der Horst *et al.*, 1979). Except for the absence of glutathione, the buffer used for elution of proteins was as described above. The eluates containing lipoprotein fractions O and A_{yellow} from resting haemolymph and those containing O_{AKH} and A^+ from elevated haemolymph were concentrated by ultrafiltration using an Amicon Cell (50 ml) with a PM 10 membrane as described before (Van der Horst *et al.*, 1981b). For isolation of the various C-proteins before and after hormone injection, the eluates containing the protein fractions B and C (which due to the fractionation range of the column cannot be resolved completely) were pooled and concentrated as indicated above except for the use of YM 2 membranes and subjected to gel filtration chromatography on Sephadex G-75 (Pharmacia) packed into glass columns (200 × 1.6 cm i.d.). Alternatively, C-proteins were isolated directly from fresh haemolymph samples by chromatography on Sephacryl S-200 Superfine (Pharmacia) columns (200 × 1.6 cm i.d.). Elution buffer was as above. Separated B- and C-protein fractions obtained by either method were concentrated by ultrafiltration using YM 2 membranes. In view of the observed susceptibility to degradation of one of the isolated C-protein fractions on storage for more than a few days at 4°C in the dark, 10 μM of the serine protease inhibitor phenylmethylsulphonyl fluoride (Sigma) was included where indicated.

Amino acid compositions

Amino acid compositions of lipoprotein and protein fractions were analyzed after hydrolysis of about 5 mg samples in 6 M HCl at 110°C *in vacuo* for 24, 48 and 72 hr with a Biocal analyzer (LKB), using chromax-11 resins and lithium citrate buffers according to Benson *et al.* (1967). Lipoprotein fractions were delipidated with chloroform–methanol (Bligh and Dyer, 1959) prior to hydrolysis. Lipoprotein A_{yellow} was purified from contaminating protein A_{blue} by chromatography on a glass column (25 × 1.6 cm i.d.) packed with Cellex D (Bio-Rad), using 0.05 M NaH_2PO_4 (pH 6.3) for elution of A_{blue} followed by 0.10 M NaH_2PO_4 + 0.5 M KCl (pH 6.3) for isolation of A_{yellow}, which was concentrated by ultrafiltration (PM 10 membrane).

Electrophoresis

Polyacrylamide gel electrophoresis (PAGE) of locust protein fractions was carried out on 7.5% gels in Tris (0.045 M)–glycine (0.100 M) buffer at pH 8.9, using the LKB 2117 Multiphore horizontal system. Since particularly C-protein components were not detected with the generally used Coomassie Brilliant Blue R 250 dye, gels were stained for protein with Amido black 10 B.

To analyze the subunit structure, following denaturation in 1% of sodium dodecyl sulphate (SDS), samples of the protein and lipoprotein fractions were separated by SDS–PAGE on 12.5% gels in a modified discontinuous buffer system containing 0.1% SDS according to Laemmli and Favre (1973), using the equipment described above. Prior to denaturation, lipoproteins were delipidated with alcohol–ether according to the method of Shore and Shore (1962). Protein bands were stained with Amido black 10 B. For calculation of molecular weights, protein markers of known molecular weight (Electrophoresis Calibration Kit; Pharmacia) were used.

Protein determination

Initially protein contents of all chromatography samples were routinely measured after staining with Coomassie Brilliant Blue G250 according to Bradford (1976). As, however, in contrast to other proteins, intensity of staining of the C-proteins appeared to be too low to be used for quantification, the Lowry method as modified by Schacterle and Pollack (1973) was adopted.

Determination of carbohydrates

Carbohydrate content of glycoproteins was assayed colorimetrically with phenol–sulphuric acid after the method of Dubois *et al.* (1956).

Immunological procedures

The preparation of specific antiserum against lipoprotein A^+ and the conditions for the Ouchterlony double diffusion test were described in a previous paper (Van der Horst *et al.*, 1981a).

Protein labelling

Individual C-protein fractions (approx. 1 mg) isolated by gel filtration chromatography on Sephacryl S-200 Superfine as described above were taken up in 100 μl 0.1 M borate buffer (pH 8.5) and reacted for 15 min at 0°C with 0.5 mCi *N*-succinimidyl [2,3-^{3}H]propionate (sp. act. 249 mCi/mg, Amersham International) essentially after the method of Bolton and Hunter (1973). Excess reagent was reacted for 5 min with 500 μl of 0.2 M glycine in 0.1 M borate buffer (pH 8.5). Reaction products were separated by gel filtration chromatography on a Bio-Gel P-6 (Bio-Rad) column (50 × 1.0 cm i.d.), using the buffer for Ultrogel AcA 22 gel filtration described above. The voided protein fraction was concentrated by ultrafiltration (Amicon YM 2 membrane) and its purity checked by SDS–PAGE and re-chromatography on Sephacryl S-200 as described above.

In metabolic experiments, incorporation of radiolabel from [^{3}H]C-proteins into lipoprotein A^+ was assayed 60 min after simultaneous injection of locusts with a dose of the labelled protein in a volume of 20 μl and 10 pmol AKH in a 10 μl volume. Haemolymph samples were fractionated by gel filtration on Ultrogel AcA 22 (column dimensions 100 × 1.6 cm i.d.) as described above.

Radioassay

Aliquots of protein fractions containing tritium were counted by liquid scintillation spectrometry in Emulsifier 299 (Packard) using a Packard Tri-Carb Model 4550 spectrometer and corrected for quenching.

RESULTS

The amino acid compositions of the lipoprotein fractions and total protein fractions C isolated by gel filtration chromatography of Ultrogel AcA 22 of haemolymph from resting locusts (O, A_{yellow}, C_{rest}) or AKH-injected insects (O_{AKH}, A^+, C_{AKH}) are shown in Table 1. Obviously, hormone injection leads to a marked change in amino acid composition of the induced lipoprotein fractions as compared with those in the resting situation, e.g. a significant increase in for instance glutamate and alanine and a singificant decrease in glycine, which is accounted for by the contribution of part of the C-protein fraction. From the data of the remaining fraction C_{AKH} it is apparent that fraction C_{rest} is heterogenous, since its amino acid composition differs appreciably from that of C_{AKH}.

Chromatography on Sephadex G-75 of the combined B- and C-protein fractions recovered from Ultrogel AcA 22, or direct fractionation of fresh haemolymph samples on Sephacryl S-200 yielded essentially identical results, revealing that protein fraction C is resolved into three fractions (C_1–C_3),

Table 1. Amino acid composition (in mol %) of haemolymph lipoprotein fractions* and protein fraction C* isolated from resting and AKH-injected locusts

Amino acid	O	A_{yellow}	O_{AKH}	A^+	C_{rest}	C_{AKH}
Asp	12.3	12.2	12.1	12.2	12.0	13.5
Thr	6.0	5.8	6.1	6.2	6.4	6.0
Ser	7.2	6.9	6.5	6.1	7.5	9.4
Glu	12.0	11.9	15.8	15.4	17.5	11.3
Pro	5.1	4.9	3.9	3.9	4.0	5.6
Gly	6.7	6.3	4.9	4.5	5.4	8.6
Ala	7.0	7.4	10.8	12.1	15.2	10.9
Val	8.1	8.0	6.6	6.5	5.0	6.3
½Cys	ND†	ND	ND	ND	0.3	0.4
Met	ND	ND	ND	ND	ND	ND
Ile	5.2	5.6	5.0	4.9	3.8	4.3
Leu	10.4	10.9	11.1	11.0	8.9	7.5
Tyr	1.1	1.3	1.3	1.4	0.2	0.6
Phe	5.0	5.0	3.2	3.2	2.1	3.3
Lys	8.1	8.1	7.3	7.4	3.9	4.0
His	2.4	2.2	3.0	3.0	5.0	4.8
Arg	3.4	3.4	2.4	2.2	1.8	3.4

*Hydrolyzed for 24, 48, 72 hr. Labile amino acids extrapolated to zero time. Values are means from three independent analyses; maximum deviation from mean values is <5%.
†ND—not detectable.

Table 2. Concentrations of B- and C-protein fractions recovered by gel filtration on Sephadex G-75 of haemolymph from resting and AKH-injected locusts

Protein fraction	Rest (mg)	AKH-injected (mg)
B	4.2 ± 0.3	4.1 ± 0.3
C_1	5.0 ± 0.2	5.0 ± 0.3
C_2	14.4 ± 0.9	4.0 ± 0.3
C_3	1.0 ± 0.1	1.0 ± 0.1

Values represent the means ± SD for 1.0 ml haemolymph samples obtained from three different groups of locusts.

only one of which (C_2) actually decreases after AKH-injection (Fig. 1). Quantitative data on the concentrations of B- and C-proteins in both resting and elevated haemolymph recovered after Sephadex G-75 chromatography are given in Table 2. Recoveries after Sephacryl S-200 fractionation were usually higher; for protein fraction C_2 in resting haemolymph values up to 19 mg/ml were found (see Fig. 1). In contrast to the other fractions, the C_2-fraction, particularly when kept isolated from the other C-proteins, appeared to be rather susceptible to degradation processes. Upon standing at 4°C in the dark, an increasing number of protein bands was observed by PAGE within a few days. Addition of 2.5 mM glutathione to the samples was ineffective in preventing degradation; however, inclusion of 10 μM of the serine protease inhibitor phenylmethylsulphonyl fluoride (PMSF) completely prevented degradation and was therefore routinely added to C_2-protein-containing chromatography fractions prior to ultrafiltration.

The amino acid composition of fraction C_2 is rather different from those of the C_1- and C_3-fractions; besides, the decrease in C_2-protein concentration induced by injection of AKH does not result in an apparent change in composition (Table 3). It should be remarked that in contrast to the other experimental procedures described, for amino analysis of the C_3-fraction the total C_3-protein peak was used in view of the low concentration of this fraction in the haemolymph. For fraction $C_{3_{rest}}$ this results in some contamination with the dominant fraction $C_{2_{rest}}$ (see Fig. 1).

In Ouchterlony immunodiffusion with antiserum prepared against pure lipoprotein A^+, only the C_2-fraction gave a single precipitation line (which was

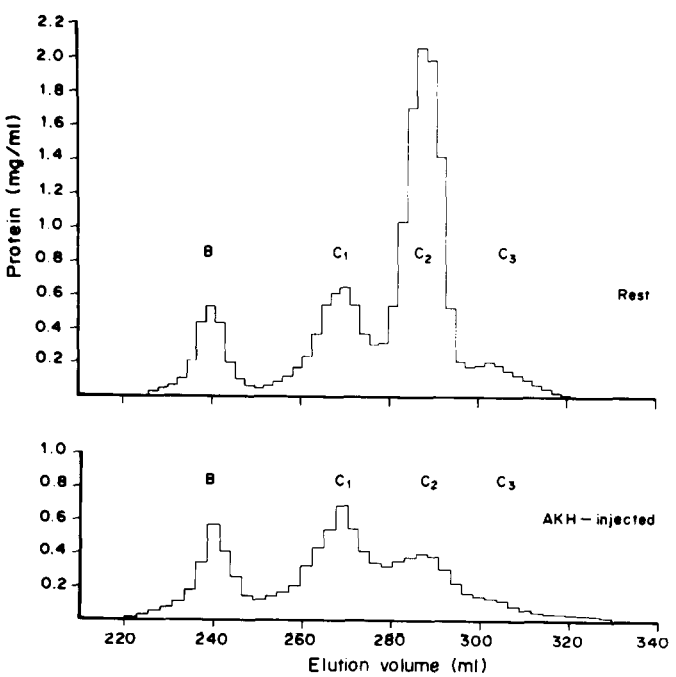

Fig. 1. Elution profiles of locust haemolymph B- and C-protein fractions on Sephacryl S-200. Haemolymph samples (1.0 ml) were taken from resting and AKH-injected locusts.

Table 3. Amino acid composition (in mol %) of haemolymph B- and C-protein fractions* from resting and AKH-injected locusts

Amino acid	B_{rest}	B_{AKH}	C_{1rest}	C_{1AKH}	C_{2rest}	C_{2AKH}	C_{3rest}	C_{3AKH}
Asp	13.5	13.3	10.1	9.8	12.5	12.9	12.2	11.5
Thr	4.6	4.6	4.2	4.5	6.7	6.3	7.9	9.0
Ser	6.2	6.3	12.8	12.9	6.3	6.8	9.3	9.9
Glu	10.1	10.0	12.7	12.2	20.1	19.4	10.8	9.6
Pro	5.3	5.4	6.0	6.0	3.6	3.8	9.0	8.0
Gly	8.0	8.4	10.0	10.2	3.4	3.8	8.1	9.1
Ala	10.5	10.6	10.0	10.0	17.9	17.4	11.5	9.6
Val	7.6	7.2	6.5	6.3	4.7	4.8	6.7	8.0
½ Cys	1.1	1.3	2.5	2.6	0.2	0.2	ND†	ND
Met	0.4	0.4	ND	ND	ND	ND	ND	ND
Ile	3.0	3.0	4.8	5.0	3.7	3.8	4.1	5.2
Leu	10.9	11.0	7.2	7.3	11.0	10.9	6.4	5.8
Tyr	3.4	3.5	2.7	2.7	0.3	0.4	0.9	0.9
Phe	3.1	3.1	3.8	3.9	1.3	1.6	3.3	4.0
Lys	5.7	5.5	2.0	2.0	3.2	3.0	2.7	2.6
His	2.1	2.0	1.4	1.4	4.1	3.9	5.0	4.3
Arg	4.5	4.3	3.3	3.3	1.0	1.1	2.1	2.5

*Hydrolyzed for 24, 48 and 72 hr. Labile amino acids extrapolated to zero time. Values are means from three independent analyses; maximum deviation from mean values is $< 5\%$.
†ND—not detectable.

also revealed when the total protein fraction C was used, see Van der Horst *et al.*, 1981a) whereas the antibody cross-reacted neither with the C_1-proteins nor with the C_3-fraction (results not shown).

Molecular weights of the B- and C-protein fractions were estimated after calibration of both the Sephadex G-75 and Sephacryl S-200 columns with protein standards of known molecular weights (Boehringer Combithek calibration proteins for chromatography), yielding identical results. The molecular weight of fraction B was about 73,000, whereas for the C-proteins values of 45,000 (C_1), 33,000 (C_2) and 25,000 (C_3) were calculated (Fig. 2).

PAGE of the B- and C-protein fractions obtained by Sephacryl S-200 gel filtration of 1.0 ml haemolymph samples from resting and AKH-injected locusts revealed that in contrast to the other C-protein fractions, the C_2-protein consists of only one protein band. Apart from a quantitative change in protein C_2, no obvious changes in the C-proteins are observed (Fig. 3).

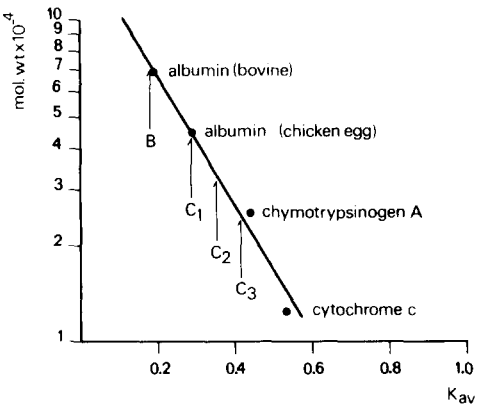

Fig. 2. Molecular weight determination of locust haemolymph B- and C-protein fractions by gel filtration on Sephacryl S-200. Calibration curve was obtained using protein standards. Molecular weights are: bovine albumin 68,000; chicken egg albumin 45,000; chymotrypsinogen *A* 25,000; cytochrome *c* 12,500.

SDS–PAGE demonstrates that protein fraction C_2 consists of only one subunit (Fig. 4), which is also present in the elevated haemolymph lipoprotein fractions (O_{AKH}, A^+) in contrast to the lipoproteins in the resting situation (O, A_{yellow}) in which such a subunit is absent. Molecular weight of the C_2-polypeptide chain is about 20,000 (Fig. 5). All lipoprotein fractions contain a subunit of about mol. wt 77,000 apart from a very heavy one (mol. wt over 200,000). The subunit from protein B has a mol. wt of about 74,000, the major polypeptide chains of C_1 are mol. wt 54,000 and mol. wt 39,000; C_3 contains only one major subunit with an identical mol. wt as C_2 (Figs 4 and 5).

The obvious discrepancy in molecular weight of the C_2-protein estimated by gel filtration chromatography (33,000) and the value obtained by SDS–PAGE (20,000) prompted us to assay this fraction for carbohydrate. Indeed, C_2 appeared to be a glycoprotein; carbohydrate content amounted to $12.5 \pm 1.2\%$ (mean $\pm$ SD of four analyses).

Preliminary studies on the dynamics of ^{3}H-labelled C_2-protein in which a fixed incubation time (60 min) was used are illustrated in Fig. 6. Groups of 20 locusts were injected each with 20 μl aliquots of [^{3}H]C_2-protein (containing 248,000 dpm) along with 10 pmol AKH in a 10 μl vol. After 60 min, haemolymph was collected and pooled samples fractionated by gel filtration on Ultrogel AcA 22. Both the A^+ lipoprotein fraction formed and, to a lesser extent, O_{AKH} appeared to contain radiolabel (Fig. 6). When, by way of control, similar experiments were repeated with [^{3}H]C_1-protein prepared in the same way and containing 238,000 dpm per 20 μl, no incorporation of radiolabel into lipoprotein fractions was detected (data not shown).

DISCUSSION

Though a number of insect species, particularly belonging to Lepidoptera and Orthoptera, are known to rely on lipid oxidation to sustain prolonged flight (for reviews see Gilbert and Chino, 1974; Beenakkers *et al.*, 1981b, 1984a, b), the functioning of the lipoprotein system transporting lipids in the haemolymph

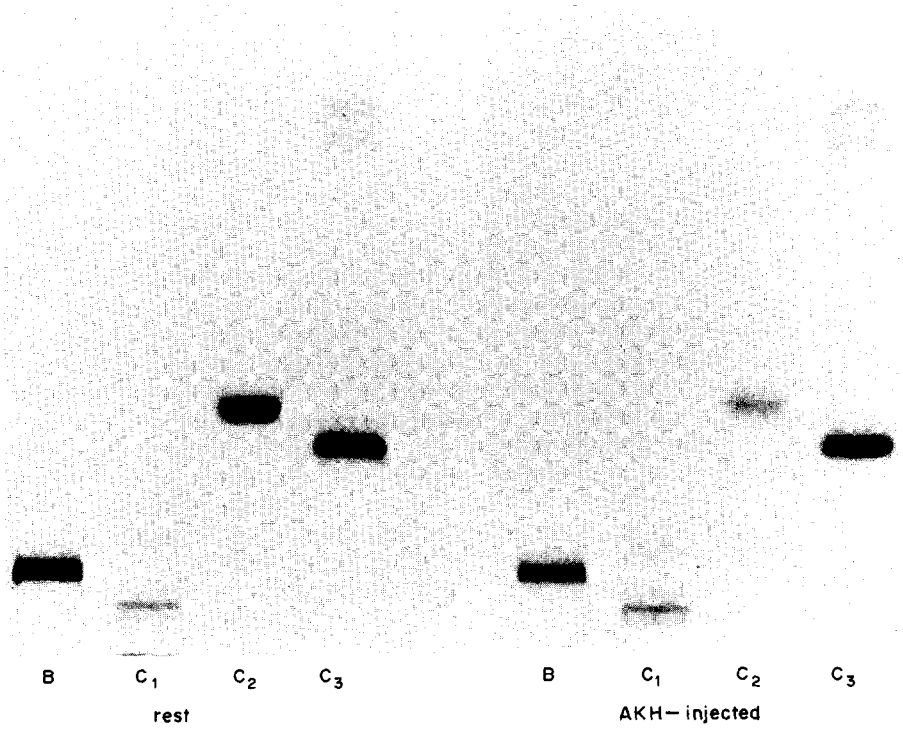

Fig. 3. Polyacrylamide gel electrophoresis (PAGE) in 7.5% gels of haemolymph B- and C-protein fractions obtained by Sephacryl S-200 gel filtration of 1.0 ml haemolymph from resting and AKH-injected locusts. Proteins were stained with Amido black 10 B.

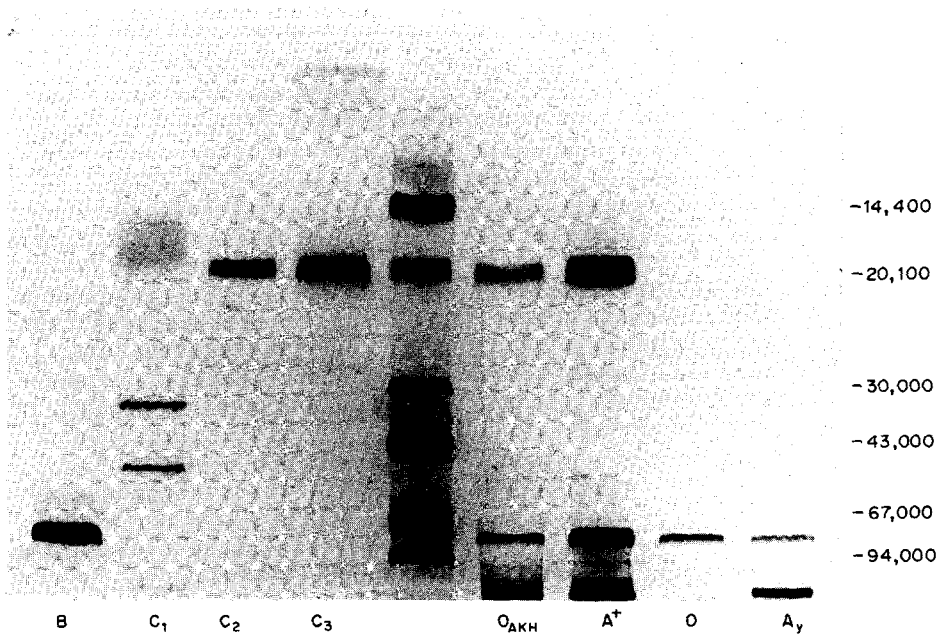

Fig. 4. SDS–polyacrylamide gel electrophoresis (PAGE) in 12.5% gels of haemolymph protein and lipoprotein fractions obtained from resting and AKH-injected locusts. In the middle lane, a reference sample of protein standards of known molecular weights (see Fig. 5) was run. Gels were stained for protein with Amido black 10 B.

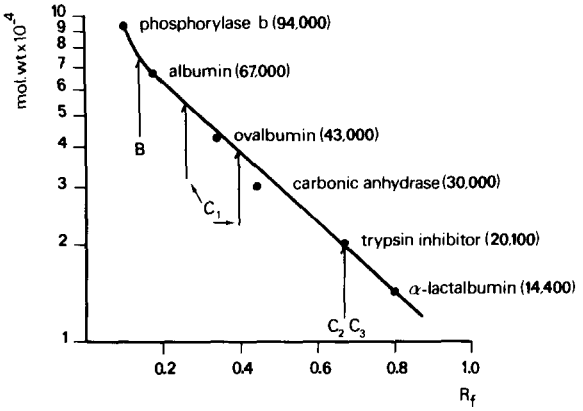

Fig. 5. Molecular weight determination of locust haemolymph B- and C-protein subunits by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) in 12.5% gels. Calibration curve was obtained using protein standards of known molecular weight (Electrophoresis Calibration Kit; Pharmacia).

has been studied extensively only in the locust. In *Locusta migratoria*, several lines of evidence indicate that formation of lipoprotein A^+ results from a rearrangement of protein originating from both lipoprotein A_{yellow} and the non-lipid carrying protein fraction C; the same mechanism holds for the elevation of the voided lipoprotein fraction O (Van der Horst *et al.*, 1981a, b). In contrast, most of the lipid originates from the increased release of diacylglycerol from the fat body, though some diacylglycerol is contributed by A_{yellow} (Van der Horst *et al.*, 1981b). An association of C-fraction proteins with lipoprotein A^+ has been confirmed recently using ^{3}H-labelled preparations of fraction C (Wheeler and Goldsworthy, 1983a). Lipoprotein A^+ apparently is the specific transport protein for the supply of diacylglycerol to the working flight muscles as evidenced in locust half-thorax preparations (Robinson and Goldsworthy, 1977) as well as in flying insects after [^{125}I]lipoprotein injection (Van der Horst, Storm, Van Doorn and Beenakkers, to be published). The function of the chylomicron-like fraction O and the elevation of this fraction after AKH-injection (including the participation of protein fraction C) remains the subject of further study.

From the present results it is concluded that only one specific protein component (C_2) of the C-fraction participates in the lipoprotein reorganizations. This C_2-protein is the sole protein fraction decreasing in concentration during A^+-formation as induced by injection of AKH (Figs 1 and 3, Table 2). In addition, the protein subunit patterns of the two elevated lipoprotein fractions (A^+ and O_{AKH}) reveal the presence of a subunit with an identical molecular weight as that of the C_2-protein (Fig. 4). The changes in amino acid composition of the lipoprotein fractions A^+ and O_{AKH} by comparison with the compositions of the resting lipoprotein fractions A_{yellow} and O, indicative of the contribution of the specific amino acid composition of the C_2-protein (Tables 1 and 3), give further support, as does the immunoreactivity of the C_2-component with antiserum prepared against pure lipoprotein A^+ (data not shown). Furthermore, *in vivo* pilot studies on the dynamics of C_2-protein using [^{3}H]protein C_2 showed the incorporation of radiolabel into both elevated lipoprotein fractions (Fig. 6), whereas in contrast [^{3}H]protein C_1 was not incorporated. Though the principal subunit of the C_3-fraction has the same molecular weight as C_2-protein (Figs 4 and 5), both the very low level of

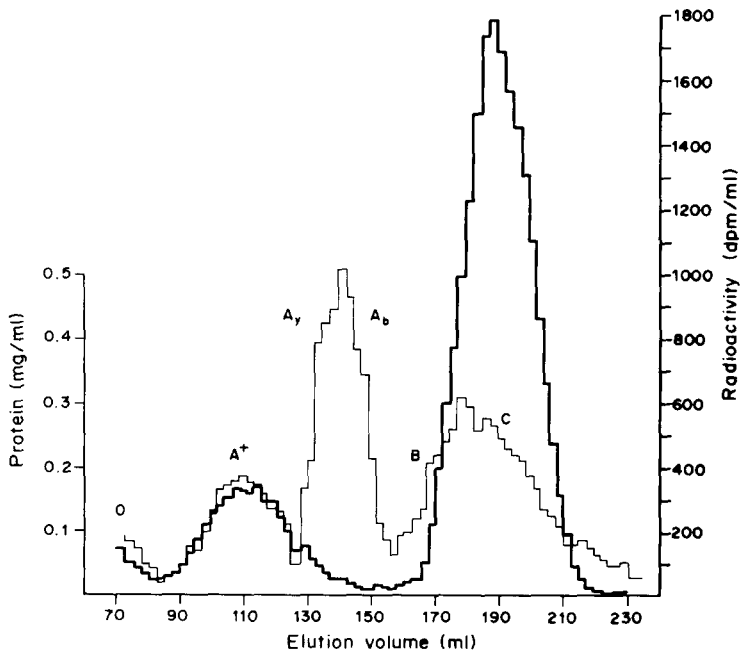

Fig. 6. Distribution of radiolabel over (lipo)protein fractions after gel filtration of 0.7 ml haemolymph on Ultrogel AcA 22. Haemolymph was collected from locusts 60 min after simultaneous injection of [^{3}H]C_2-protein and 10 pmol AKH. Light lines represent protein concentration; heavy lines represent radioactivity.

C_3-protein in the haemolymph which is unaffected by hormone injection (Figs 1 and 3, Table 2) and its amino acid composition which is markedly different from that of C_2-protein render any contribution of C_3-protein to the lipoprotein rearrangements unlikely. The absence of immunoreactivity of the C_3-proteins with specific antiserum raised against lipoprotein A^+ (data not shown) confirms this conclusion.

Considering the subunit structure of the locust haemolymph protein fractions studied (B, C_1–C_3), protein B apparently contains one subunit, the molecular weight of which determined by SDS–PAGE is estimated as 74,000 while by gel filtration chromatography a nearly identical value (73,000) was obtained for the native protein (Figs 2 and 4). Protein fraction C_1 apparently is composed of two proteins which are not resolved by gel filtration, since molecular weights of the two most prominent subunits in SDS–PAGE are 54,000 and 39,000 (Fig. 4), whereas by gel filtration a molecular weight of 45,000 was obtained (Fig. 2). Very recently, gel filtration of the C_1-fraction on Bio-Gel P-200 (Bio-Rad) resulted in the separation of both proteins, revealing the presence of a predominating component with a mol. wt of 39,000 and only a minor component with a mol. wt of 54,000 (unpublished results). Protein fraction C_3 consists of only one subunit (mol. wt about 20,000); by gel filtration an approximate value of 25,000 was estimated (Figs 2 and 4). Molecular weight of protein C_2 as determined by gel filtration (33,00) deviates appreciably from the single protein subunit molecular weight obtained by SDS–PAGE (20,000) (Figs 2 and 4). However, the C_2-component appeared to contain about 12.5% carbohydrate and the molecular weight of glycoproteins may be overestimated by gel filtration chromatography since, due to a greater hydration in solution induced by the carbohydrate units, they have a more expanded structure than proteins not containing carbohydrate (Spiro, 1973; Aldaheff, 1978). Though migration of glycoproteins in SDS–PAGE may be also anomalous at low gel concentrations (5%), at high acrylamide concentrations (10–12.5%) as used in the present experiments migrations close to those expected from the molecular mass are observed (Segrest *et al.*, 1971). Therefore, we conclude that the C_2-glycoprotein is composed of one single polypeptide chain with an approximate mol. wt of 20,000. The complex sugar composition of glycoprotein C_2 will be published together with the sugar compositions of the lipoprotein fractions (Abbink, Van der Horst, Van Doorn and Beenakkers, in preparation).

As to the protein subunit structure of the lipoprotein fractions, apart from the C_2-subunit in the elevated lipoprotein fractions A^+ and O_{AKH}, all lipoprotein fractions contain a subunit with a mol. wt of about 77,000 (Fig. 4). Besides, a heavy chain subunit of over 200,000 is revealed to which the molecular weight due to its minimal migration is difficult to assess. For locust lipophorin, which we have called lipoprotein A_{yellow}, a subunit of about 85,000 has been reported apart from a component of about 240,000 which was considered to be an artificially aggregated product of the 85,000 subunit (Gellissen and Emmerich, 1980; Gellissen and Wyatt, 1981). However, Chino and Kitazawa (1981) also identified both a light-chain (85,000) subunit and a heavy-chain (250,000) subunit in locust lipophorin, but demonstrated that in contrast to the light chain, the heavy-chain subunit is associated with carbohydrates, rendering an unphysiological aggregation of light-chain subunits to the heavy chain less likely.

Though the present metabolic pilot studies with radiolabelled glycoprotein C_2, in which a fixed time of 60 min after injection of locusts with both [^{3}H]protein C_2 and AKH was used, indicate the incorporation of C_2 into protein A^+ (as well as O_{AKH}) (Fig. 6), the mechanism of A^+-formation is not yet clear. It has been shown recently that injection of a ^{3}H-labelled preparation of the total protein fraction C into locusts in which lipoprotein fraction A^+ was already present in the haemolymph, resulted in a very rapid association of radiolabel with A^+, thus suggesting a competition for binding sites at the surface of lipoprotein A^+, which was supported by *in vitro* studies (Wheeler and Goldsworthy, 1983a). Besides, injection of a preparation of ^{3}H-labelled lipoprotein A^+ into locusts in which the endogenous titre of A^+ was decreasing resulted in the recovery of considerably more radiolabel in protein fraction C than in lipoprotein A_{yellow}. Therefore, latter authors suggested lipoprotein A_{yellow} to be vital for the structural integrity of lipoprotein A^+ while C-proteins may play only a relatively minor structural role. However, in other work studying the time course in A^+-formation, it was argued that lipoprotein A_{yellow} does not contribute to A^+ until some 15–20 min after hormone injection whilst A^+ is formed without a delay, as is the association of radiolabel from injected [^{3}H]protein fraction C with A^+ (Wheeler and Goldsworthy, 1983b). Very recently, *in vitro* incubation studies using locust fat body preparations and isolated haemolymph protein fractions have demonstrated that both lipoprotein fraction A_{yellow} and protein fraction C are required for the formation of lipoprotein A^+ whereas individual fractions were ineffective (Van Heusden *et al.*, 1984). Interestingly, when only lipoprotein A_{yellow} was present in the medium, AKH failed to stimulate any lipid release from the fat body.

The net result of the mutual interactions between lipoprotein A_{yellow}, protein C_2, and diacylglycerol released from the fat body, being that lipids are efficiently delivered to the contracting flight muscles, will be allowed by the structural features of insect lipoproteins which apparently differ from those of mammalian serum lipoproteins (see Pattnaik *et al.*, 1979; Van der Horst, 1983). The rationale for the involvement of protein C_2 is not yet clear though a few explanations have been advanced, including some specific interaction with the flight muscles (Van der Horst, 1983; Wheeler and Goldsworthy, 1983a) possibly resulting in activation of flight muscle lipoprotein lipase (Wheeler *et al.*, 1984), which would resemble the activation of mammalian lipoprotein lipase by apoproteins (for reviews, see Cryer, 1981; Sparrow and Gotto, 1982). In this respect it is of interest that protein C_2 is a glycoprotein; it is, therefore, tempting to speculate that one of the functions of the sugars included in mammalian proteins, namely determinants of protein recognition by its

target cells (for a review, see Olden *et al.*, 1982) is applicable to the locust as well. This possibility is presently under investigation. In contrast to the other C-protein fractions, the isolated C_2-protein appeared to be rather susceptible to degradation. As the inclusion of the serine protease inhibitor PMSF completely prevented this degradation, this effect apparently must be due to protease activity. Interestingly, plasma serine proteases co-isolate with human very low density lipoproteins (VLDL) which specifically degrade apolipoprotein E and therefore cause a loss of ability to interact with the receptor-mediated uptake of VLDL by human skin fibroblasts (Bradley *et al.*, 1982). This protease activity which affects VLDL structure and biological activity was also inhibited by PMSF. It remains, however, to be established whether this breakdown serves a functional role in regulation of receptor interaction; a similar suggestion may be proposed for the locust lipoprotein-bound C_2-protein.

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