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Biophysical studies on the lipid transfer particle from the hemolymph of the tobacco hornworm, *Manduca sexta*

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Hydrodynamic studies conducted in the analytical ultracentrifuge provided evidence for two populations of lipid transfer particle (LTP) when centrifuged in a buffer solution containing 10 mM Tris, pH 8.0/100 mM KCl. The apparent sedimentation coefficients of the two species was 23.3 S and 15.3 S. Upon changing the buffer pH to 7.0 or 5.7, two species of LTP were still present but the ratio of their relative abundance was altered. When the KCl concentration in the buffer was lowered to 50 mM the sample sedimented as a single species with an apparent $s_{20,w}$ of 22.9 S. In higher ionic strength buffers (10 mM succinate, pH 5.7/500 mM KCl) LTP sedimented with an apparent $s_{20,w}$ of 14.8 S. Further experiments revealed that these two forms are interconvertable as a function of buffer ionic strength. Given previous estimates of the molecular size of LTP we concluded that the slower sedimenting peak observed at high ionic strength represents monomeric LTP while the faster sedimenting material observed at low ionic strength is likely to be an aggregated state of LTP. This interpretation is supported by molecular weight determinations made by sedimentation equilibrium experiments conducted in 10 mM succinate, pH 5.7/500 mM KCl which yielded a particle $M_r = 887000$. Circular dichroism spectra of monomeric LTP sample revealed 6% α-helix, 49% β-sheet, 7% β-turn and 35% random coil while aggregated LTP contained 13% α-helix, 66% β-sheet and 21% random coil. The transfer activity of the two LTP forms was assayed and found to be the same indicating that either the state of LTP aggregation did not affect transfer activity or that upon exposure to a large excess of lipoprotein substrate disaggregation, without loss of activity, occurs.

Sedimentation velocity; Sedimentation equilibrium; Circular dichroism; Lipid transfer; Lipoprotein

1. INTRODUCTION

Facilitated lipid transfer is an important mechanism for redistribution of lipid among plasma lipoproteins as well as between lipoproteins and biomembranes. As such it has been proposed that transfer proteins play an important role in lipoprotein metabolism [1]. In the tobacco hornworm, *Manduca sexta*, a hemolymph lipid transfer particle (LTP) has been isolated and shown to catalyze exchange and net transfer of lipid between a number of lipoprotein particles [2–6]. As a result of LTP-mediated lipid redistribution dramatic changes in the density, size and lipid composition of donor/acceptor lipoproteins has been observed. When lipoprotein particles bearing low molecular weight, soluble apoprotein components are employed in transfer experiments apoprotein migration oftentimes results in further alteration of the properties or lipoprotein substrates [7]. In *M. sexta*, the major hemolymph lipoprotein, lipophorin, is comprised of one molecule each of two nonexchangeable, integral apoliproteins, apolipophorin I and apolipophorin II [8]. These two apoproteins plus approximately 80 phospholipid molecules are hypothesized to constitute a basic lipoprotein matrix structure to which lipids may be added or removed without destruction of the basic matrix [9]. Dramatic interconversions of lipophorin subspecies which occur during development [9] or under the influence of the nonapeptide adipokinetic hormone (AKH) have been shown to result from alteration of lipid and, in the case of AKH-induced transformations, the apoprotein content of preexisting particles [10,11]. Recently it has been shown that LTP functions in the transfer of DAG from fat body tissue to lipophorin during formation of low density lipophorin [12].

The intrinsic capacity of *M. sexta* LTP to catalyze vectorial net mass transfer of lipid is a unique property which distinguishes it from other known plasma transfer proteins, which generally facilitate homo or hetero exchange of lipid among lipoprotein substrates [13]. A second distinguishing feature of *M. sexta* LTP is its apparent large size. LTP exists as a very high density lipoprotein with three glycosylated apoprotein components, apoLTP-I ($M_r \sim 320\,000$), apoLTP-II ($M_r = 85\,000$), apoLTP-III ($M_r = 55\,000$) and 14% noncovalently associated lipid [4]. Importantly, the lipid component of LTP is in dynamic equilibrium with the lipid component of potential donor/acceptor lipoproteins and it is thought that the entire LTP complex,

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Abbreviations: LTP, lipid transfer particle; DAG, diacylglycerol; AKH, adipokinetic hormone; HDLp-L, larval high density lipophorin; LDL, low density lipoprotein; CD, circular dichroism

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rather than an individual apoprotein component, is required for transfer activity. LTP exhibits unusual morphological properties when examined by electron microscopy [14]. The particle has two major structural features, a quasispherical head region coupled to an elongated, tapered cylindrical tail, which has a flexible hinge at its approximate midpoint. The exact apoprotein stoichiometry in the native complex is not known nor is its molecular mass. Estimates ranging from $>500\,000$ to 1.4×10^6 Da have been reported using gel permeation chromatography, pore limiting native PAGE and volume measurements based on electron microscopic data [2,4,14]. The relative abundance of LTP in *M. sexta* plasma, its high lipid exchange and net transfer activity [15,16] together with its capacity to utilize a variety of lipid particle substrates makes this an excellent model for lipid transfer protein/paticle structure-function studies. We report here on hydrodynamic experiments, conducted in the analytical ultracentrifuge, which provide evidence of a reversible, ionic strength-dependent LTP self-aggregation and on sedimentation equilibrium experiments which indicate that monomeric native LTP has a molecular mass of $\sim900\,000$. Parallel circular dichroism measurements indicate that large changes in secondary structure do not accompany the ionic strength induced changes in sedimentation coefficient.

2. MATERIALS AND METHODS

2.1. *Animals and lipoprotein and LTP isolation*

Manduca sexta were obtained from a continuing laboratory colony reared as described by Prasad et al. [9]. LTP was isolated from the hemolymph of 7-day-old fifth instar larvae according to established procedures [14]. Larval high density lipophorin (HDLp-L) was isolated from 4-day-old fifth instar larvae by vertical rotor density gradient ultracentrifugation according to Shapiro et al. [17]. [^{3}H]-Diacylglycerol HDLp-L was prepared by instilling 10 μCi [9,10-^{3}H]oleic acid (5 Ci/mmol) in ethanol, directly into the midgut of each of 16 starved larvae (4 h) through a piece of microbore polyethylene tubing fitted onto the tip of a Hamilton syringe. Thirty minutes later the animals were bled and the HDLp-L isolated by density gradient ultracentrifugation. Human low density lipoprotein (LDL) was isolated from freshly collected plasma between the density limits 1.006 and 1.063 g/ml by sequential density gradient ultracentrifugation.

2.2. *Lipid transfer assays*

LTP was assayed by measuring the facilitated transfer of labeled diacylglycerol (DAG) from [^{3}H]DAG HDLp-L (spec. act. = 150 000–300 000 cpm/mg protein) to human LDL as described by Ryan et al. [14]. Briefly, 0.5 mg of LDL protein and 0.25 mg of [^{3}H]DAG HDLp-L protein were incubated for 30 min at 33°C in a given buffer with or without 1 μg LTP protein (final volume 0.5–1.0 ml). Following incubation the reaction was stopped by placing the samples on ice and transferring to Beckman Quick-Seal centrifuge tubes. The solutions were adjusted to a density of 1.23 g/ml by the addition of KBr, brought to a volume of 2.5 ml, overlayered with saline and centrifuged at 65 000 rpm in a Beckman VTi 65.2 rotor for 75 min at 4°C. Following centrifugation the LDL fraction was collected and the amount of labeled DAG present determined by liquid scintillation spectrometry. In all LTP activity experiments control incubations, lacking LTP, were run in parallel, permitting correction for spontaneous DAG transfer. In all assays the donor, acceptor and LTP concentrations were in the linear range of the transfer activity response.

2.3. *Analytical ultracentrifugation*

Purified LTP samples were dialyzed against an appropriate buffer solution for 48 h at 4°C prior to ultracentrifugation. Buffer solutions of various ionic strengths and pH were used throughout in sedimentation velocity and sedimentation equilibrium experiments with details given in the text. A Beckman model E analytical ultracentrifuge equipped with an electronic speed control system, RITC temperature control system, and titanium rotor was used for all runs. The Schlieren optical system was used for sedimentation velocity experiments and the Rayleigh Interference optical system was employed for sedimentation equilibrium experiments. Measurements of photographic plates from both optical systems were performed on a Nikon model 6 microcomparator.

Sedimentation velocity experiments were run at either 40 000 or 60 000 rpm at 10°C. Samples (350 μl) were loaded into a single-sector 2° Kel-F cell equipped with quartz windows and wide aperture window-holders. When two samples were run simultaneously, a 1° positive wedge window was used in one cell. Observed sedimentation coefficients were corrected to standard conditons according to Chervenka [18]. Determination of molecular weights were made using the meniscus depletion sedimentation equilibrium technique described by Chervenka [18]. Samples (120 μl) were loaded into a 12 mm double-sector charcoal filled Epon cell equipped with sapphire windows. Runs were performed at 20°C at speeds ranging from 8000 to 11 000 rpm for a minimum of 36 h before equilibration photographs were taken. Molecular weight calculations were carried out using a computer program written in the APL language. The in Y versus r^2 data were fitted to a 2nd degree polynomial equation using least squares techniques and the point-average molecular weights were calculated from the slope of this equation.

2.4. *Circular dichroism*

Circular dichroism (CD) was performed on a Jasco J-500C spectropolarimeter with DP500N data processor and thermostatted cell-holder with a waterbath at 25°C. The cells employed were 0.05 cm in the far-UV and 1 cm in the near-UV. Sample size was 100 μl and protein concentration ranged from 0.84 to 0.95 mg/ml. The instrument was calibrated with d(+) 10 camphor sulphonic acid at 290 nm and with pantoyllactone dissolved in water at 219 nm. Four scans were performed for each sample in addition to the appropriate blanks. The ellipticity was calculated according to [19] and the CONTIN (version 1; 1980) program was used for structural analysis of helix, β-sheet, β-turn and random coil.

3. RESULTS AND DISCUSSION

To determine the native molecular weight of LTP as well as to gain further insight into its apoprotein stoichiometry and solution properties, hydrodynamic experiments were conducted in the analytical ultracentrifuge. For initial sedimentation velocity experiments LTP (1.17 mg/ml) was dialyzed against 10 mM Tris, pH 8.0, 100 mM KCl prior to centrifugation. Under these conditions evidence of two distinct species was obtained. The faster sedimenting material had an apparent $s_{20,w}$ of 23.3 S while the slower sedimenting material had an apparent $s_{20,w}$ of 15.3 S. This result was unexpected since previous studies employing electron microscopy [14] and native PAGE [4] provided no evidence for two populations of LTP. In an effort to

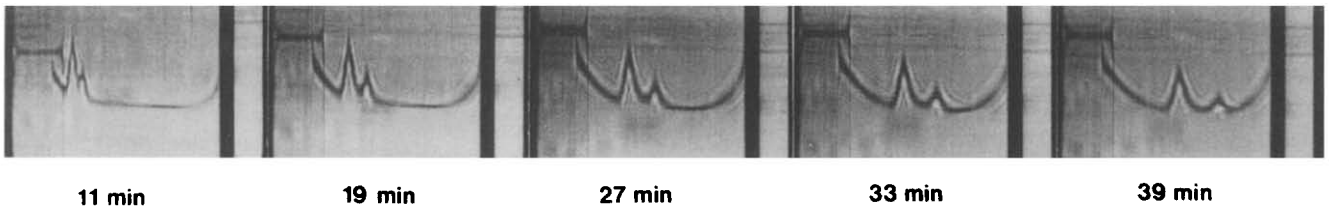

Fig. 1. Schlieren photographs of LTP. LTP (2.27 mg/ml) was dialyzed against 50 mM succinate, pH 5.7/200 mM NaCl. A sedimentation velocity run was performed at 60000 rpm and apparent $s_{20,w}$ values of 20.8 S and 13.6 S were determined for the two peaks.

further examine this apparent discrepancy, alterations were made in buffer composition, pH and ionic strength. When LTP (1.94 mg/ml) was dialyzed against 50 mM sodium phosphate, pH 7.0, 150 mM NaCl sedimentation velocity experiments again revealed the presence of two peaks (apparent $s_{20,w}$ values of 19.0 S and 13.7 S, respectively). Likewise, upon performing sedimentation velocity experiments with LTP (2.27 mg/ml) that was dialyzed against 50 mM sodium succinate, pH 5.7, 200 mM NaCl, a similar result was obtained (apparent $s_{20,w}$ values of 20.9 S and 13.6 S, respectively; Fig. 1). Although all three conditions yielded peaks which had similar sedimentation coefficients the ratio of the peak sizes was variable suggesting the two populations may be interconvertable. Thus over a broad pH range (5.7–8.0) LTP displayed two distinct subpopulations when subjected to analytical ultracentrifugation. Inasmuch as the pH of insect hemolymph is known to be slightly acidic [20] we selected 10 mM sodium succinate, pH 5.7, as buffer to examine the effect of ionic strength on the LTP sedimentation velocity pattern. The results revealed an ionic strength-dependent alteration in the sedimentation rate of LTP. In the presence of 50 mM KCl, LTP (1.05 mg/ml) sedimented as a single species (Fig. 2A) with an apparent $s_{20,w}$ of 22.9 S. This species corresponded to the faster migrating material in Fig. 1. When the ionic strength was increased to 500 mM KCl (protein concentration = 1.78 mg/ml), a single major peak with an apparent $s_{20,w}$ of 14.8 S (Fig. 2B) was observed. Based on previous studies of LTP which revealed a particle M_r in the range of 500 000–1.4×10^6, it was concluded that the faster sedimenting material observed at low ionic strength likely represents an aggregated form of LTP particles while the slower sedimenting species observed at high ionic strength corresponds to monomeric LTP particles.

Using buffer ionic strength as a means to completely convert LTP to one of the two subpopulations, we

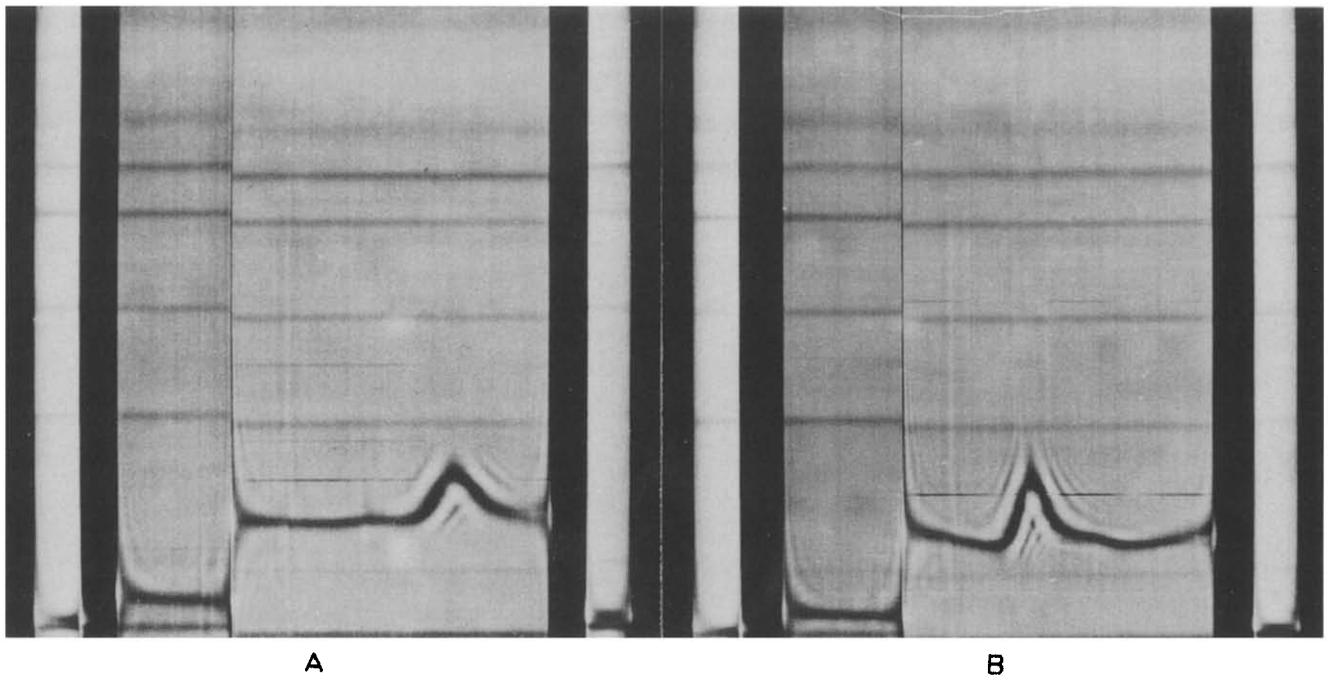

Fig. 2. The effect of ionic strength on the sedimentation rate of LTP. LTP was dialyzed against 10 mM succinate, pH 5.7/50 mM KCl (panel A) or against 10 mM succinate, pH 5.7/500 mM KCl (panel B) for 48 h at 4°C prior to ultracentrifugation. The LTP protein concentrations were 1.05 mg/ml (A) and 1.78 mg/ml (B), respectively. Samples were run at 40000 rpm and both exposures were taken at 61.5 min.

tested the possibility that the faster sedimenting species represents an irreversible aggregation of LTP particles. A sample of LTP (0.85 mg/ml) was dialyzed against succinate buffer containing 500 mM KCl and its sedimentation coefficient determined ($s_{20,w}$ 14.5 S). To induce formation of the faster sedimenting species the remaining sample was dialyzed against succinate buffer containing 50 mM KCl. An aliqout of the sample was then removed and shown to sediment as a single species with an $s_{20,w}$ of 23.0 S. Further dialysis of the remaining sample into 10 mM succinate containing 500 mM KCl resulted in conversion of the material back to the slower sedimenting species ($s_{20,w}$ 14.4 S). These results indicate that the observed conversion of LTP is a reversible phenomenon that is dependent upon the ionic strength of the solution.

Sedimentation equilibrium experiments were then performed to determine the molecular weight of the monomeric species. LTP samples were dialyzed against 50 mM succinate, pH 5.7, 500 mM NaCl buffer. A total of 8 separate runs were performed from which an average molecular mass of 887 000 was determined. Fig. 3 shows a ln$Y$ versus r^2 plot obtained from a typical run. This value is in general agreement with results obtained from pore-limiting native PAGE and electron microscopy. Taken together with previous results on LTP particle apoprotein molecular weights and their presence in a mass ratio of 4.5:1.0:0.34 (based on densitometric scans of stained gels; [4]) as well as the presence of 14% of the particle mass as noncovalently associated lipid the present data are consistent with a native particle structure composed of 2 apoLTP-I, 2 apoLTP-II and 1 apoLTP III plus 14% lipid. The calculated molecular mass based on these components yields a value of 986 000 Da which is within 10% of the M_r determined by sedimentation equilibrium experiments in this study.

CD spectra were obtained for LTP samples which had been dialyzed against succinate buffer which contained 50 mM KCl and 500 mM KCl, respectively (Fig. 4). The results reveal that, although these different ionic strength conditions induce a dramatic alteration in sedimentation coefficient of LTP, alterations in secondary structure are limited. In 50 mM KCl LTP was found to contain 13% α-helix, 66% β-sheet, 7% β-turn and 21% random coil whereas LTP in 500 mM KCl contained 6% α-helix, 49% β-sheet and 35% random coil. This result indicates that large changes in secondary structure do not accompany the ionic strength-induced changes in sedimentation coefficient.

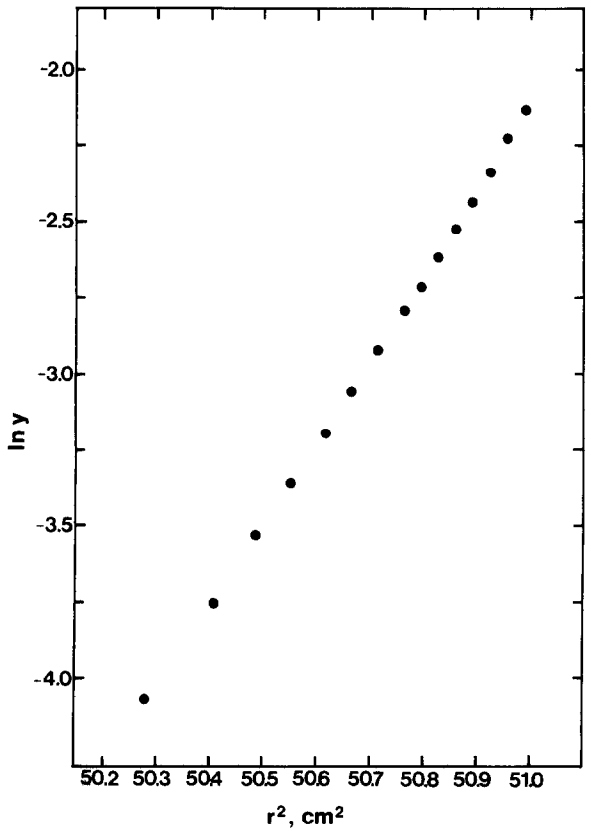

Fig. 3. ln$Y$ vs r^2 plot from a typical sedimentation equilibrium run at 9000 rpm. Point average molecular masses ranged from 817 000 to 968 000. The weight average molecular mass determined from this run was 897 000.

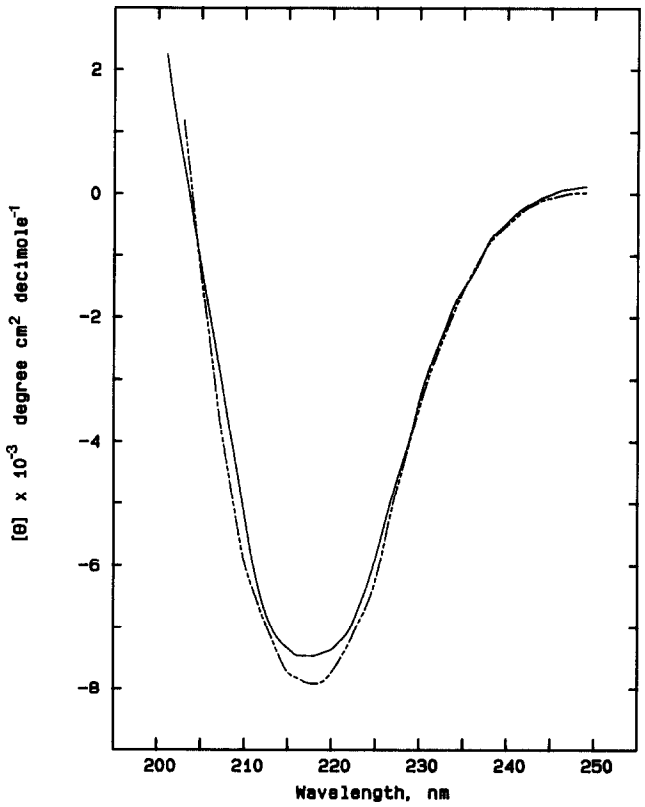

Fig. 4. Circular dichroic spectra of LTP samples. Solid line = LTP in a buffer solution containing 10 mM succinate, pH 5.7/50 mM KCl; broken line = LTP in a buffer solution containing 10 mM succinate, pH 5.7/500 mM KCl. The protein concentration ranged between 0.84 and 0.95 mg/ml and measurements were taken at 25°C.

It is possible that the observed ionic strength-dependent aggregation of LTP may alter its ability to transfer lipids and conceivably could play a role in controlling LTP activity in vivo. To test this hypothesis LTP and substrate lipoproteins were dialyzed against buffers of different ionic strength and facilitated lipid transfer between [^{3}H]DAG HDLp-L and human LDL was measured. The results presented in Table I reveal that there was no significant difference between LTP in the 23 S form and the 14 S form suggesting that the extent of LTP aggregation did not alter its capacity to facilitate lipid transfer. Alternatively, it is possible that the assay conditions, which employ a large excess of potential donor/acceptor lipoprotein substrates (750:1 substrate particle:LTP protein ratio) may have induced a change in the aggregation state of LTP. The observed reversibility of LTP aggregation as well as the relative small changes observed in secondary structure lends credence to the latter explanation of the transfer assay results. Thus, it is likely that although the ionic strength conditions in *M. sexta* larval hemolymph (conductivity = 7.1 mMho) would favor formation of aggregated LTP, the presence of an approximate 150-fold excess of lipoprotein in hemolymph may dictate that LTP interacts, with these potential substrates rather than self associates, thereby facilitating its biological function.

It has been reported [12] that LTP is unstable after its isolation and loss of activity ensues within days upon storage at 4°C. In our experience, however, over 90% of LTP activity is retained after storage of isolated LTP for up to one month at 4°C under N_2 atmosphere in the presence of buffer containing 3 M KBr. It is possible that this apparent discrepancy may be explained by the fact that Van Heusden and Law [12] employed ion-exchange chromatography as the final step in the isolation of LTP and stored the sample in the elution buffer (50 mM phosphate, pH 7.6, containing NaCl at a concentration between 0 and 200 mM). From the experiments reported here these conditions could lead to partial or complete formation of the faster sedimenting, aggregated LTP. Although we have not examined the effect of time of exposure to different buffer salt conditions on the transfer activity of purified LTP, it is possible that storage conditions which promote LTP aggregation destabilize the particle as a function of time. In support of this possibility is the observation that storage of LTP in 10 mM Tris, pH 8.0, 100 mM KCl resulted in sample turbidity and formation of an insoluble precipitate.

Previous studies have demonstrated that LTP can employ a wide variety of potential lipid particle and/or membrane donor/acceptor substrates and is capable of inducing dramatic alterations in the distribution of lipid among these substrates. It is also clear that insect plasma LTP is unique when compared to mammalian plasma or intracellular lipid transfer proteins. The high specific transfer activity [15] displayed as well as its propensity to facilitate net mass transfer of lipid substrates [16] suggest it may provide a useful tool capable of facilitating controlled modification of the lipid content and composition of lipoproteins as well as membranes. Before the potential utility of LTP as a biochemical tool can be fully exploited, however, more information is required about its structural and catalyic properties. The present study provides evidence that the properties of LTP are affected by specific solution parameters. Through further study it should be possible to correlate these observations with its catalytic activity, stability and function.

Table I

The effect of buffer ionic strength on LTP activity[a]

Buffer	LDL specific activity (cpm/μg protein)
10 mm succinate/50 mM KCl, pH 5.7	32.6 ± 1
10 mm succinate/500 mm KCl, pH 5.7	31.4 ± 1
Control (no LTP)	6.2

[a] LTP and substrate lipoproteins were dialyzed against the appropriate buffer for 24 h. Tubes containing 0.5 mg human LDL protein and 0.25 mg [^{3}H]DAG-HDLp-L (150 000 cpm/mg protein) in a final volume of 0.5 ml were incubated in the presence or absence of LTP (1 μg) for 30 min at 33°C. After incubation the substrate lipoprotein particles were reisolated by density gradient ultracentrifugation and the amount of radiolabeled DAG transferred to LDL determined. Values given are the mean ± SD (n=3). The control value represents 8% transfer whereas over 40% of the radiolabeled lipid transferred in LTP containing incubations

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