Isolation and Initial Characterization of the Single Polypeptide That Synthesizes Uridine 5′-Monophosphate from Orotate in Ehrlich Ascites Carcinoma. Purification by Tandem Affinity Chromatography of Uridine-5′-monophosphate Synthase†

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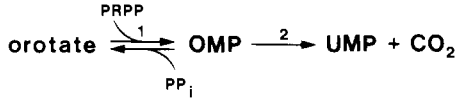
ABSTRACT: UMP synthase, or multienzyme *pyr-5,6* (orotate phosphoribosyltransferase:orotidine monophosphate decarboxylase), has been purified from Ehrlich ascites carcinoma to apparent homogeneity. The purification was achieved by the use of 5-[2-[*N*-(2-aminoethyl)carbamyl]ethyl]-6-azauridine 5′-monophosphate–agarose and phosphocellulose affinity columns linked in tandem by a flow dialysis system. The purified protein has a molecular weight of approximately 51 500 as judged by polyacrylamide gel electrophoresis in sodium dodecyl sulfate. Both enzyme activities cosediment with an $s_{20,w}$ value of 3.7 S, which corresponds to a molecular weight of about 50 000. Two-dimensional electrophoresis of UMP synthase shows that the protein exists as two isomeric forms with isoelectric points of 5.85 (major form) and 5.65 (minor form). Both forms have the same molecular weight of 51 500 and contain both active centers. These results clearly show that the last two enzyme activities of de novo UMP biosynthesis occur on a single polypeptide chain of approximately 51 500 daltons and that this polypeptide exists in at least two isomeric forms.

The last two steps of the de novo synthesis of UMP are catalyzed by orotate PRTase[1] (1) and OMP decarboxylase (2):



These two enzyme activities have been characterized in several mammalian tissues. These include bovine erythrocytes (Hatfield & Wyngaarden, 1964), human erythrocytes (Grobner & Kelley, 1975; Brown et al., 1975; Brown & O'-Sullivan, 1977), calf thymus (Kasbekar et al., 1964), bovine brain (Appel, 1968), mouse leukemia (Reyes & Guganig, 1975), mouse liver and brain (Reyes & Intress, 1978), and Ehrlich ascites carcinoma (Shoaf & Jones, 1973; Kavipurapu & Jones, 1976; Traut & Jones, 1977, 1979; McClard et al., 1979, 1980).

Several properties of the two enzyme activities show striking similarities in various mammalian tissues. The activities cosediment in sucrose gradients (Shoaf & Jones, 1973; Grobner & Kelley, 1975; Reyes & Guganig, 1975; Kavipurapu & Jones, 1976; Reyes & Intress, 1978; Traut & Jones, 1979), coelute from columns of molecular sieves under nondenaturing conditions (Grobner & Kelley, 1975; Brown et al., 1975; Reyes & Guganig, 1975; Traut & Jones, 1979), and coelute during ion-exchange chromatography (Hatfield & Wyngaarden, 1964; Kasbekar et al., 1964; Appel, 1968; Reyes & Guganig, 1975; Kavipurapu & Jones, 1976; Brown & O'Sullivan, 1977). In addition, heat treatment can cause a parallel loss in the two enzyme activities (Kasbekar et al., 1964; Appel, 1968). These observations are consistent with the hypotheses presented by Jones (1971, 1972) that the two activities comprise a bifunctional complex (Jones, 1971) and that both activities could reside on a single polypeptide (Jones, 1972).

However, occasional reports have indicated that the two enzyme activities could be on separate polypeptides. Kasbekar et al. (1964) observed that the two activities separated during electrophoresis in a starch gel, although the orotate PRTase activity was frequently lost; Appel (1968) could not recover the transferase activity after electrophoresis. Brown & O'-Sullivan (1977) inferred from experiments on a partially purified protein from human erythrocytes that the orotate PRTase:OMP decarboxylase complex was composed of two subunits (approximately 13 000 daltons) responsible for the orotate PRTase activity plus two subunits (approximately 20 000 daltons) responsible for the OMP decarboxylase activity. Unfortunately, this conclusion was based, in part, on the finding that these species were observed after prolonged storage of the impure protein in the presence of dilute guanidinium chloride. The possible role of proteases in their system was not discussed.

These studies thus provided a confusing basis for the understanding of the structure of this complex. We, therefore, chose to purify UMP synthase,[2] previously Complex U, from Ehrlich ascites carcinoma and to analyze its structure. Shoaf & Jones (1973) first described UMP synthase from this cancer cell line. The protein has been partially purified by using salt fractionation and chromatography on diethylaminoethylcellulose (Kavipurapu & Jones, 1976). The sedimentation behavior of UMP synthase in crude form has been well characterized (Traut & Jones, 1979). We have reported recently that this protein can be purified by using a combination of phosphocellulose[3] and AECE-azaUMP–agarose (Brody &

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[1] Abbreviations used: AECE-, 5-[2-[*N*-(2-aminoethyl)carbamyl]-ethyl]-; azaUMP, 6-azauridine 5′-monophosphate; IEF, isoelectric focusing; OMP, orotidine 5′-monophosphate; PEG, poly(ethylene glycol); PRPP, 5-phosphorylribose 1-pyrophosphate; PRTase, phosphoribosyltransferase; $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

[2] UMP synthase (orotate PRTase:OMP decarboxylase) is a term suggested as a synonym for multienzyme *pyr-5,6* (Jones, 1980).

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Westheimer, 1979) affinity columns (McClard et al., 1979). In the present communication we present an expedient purification procedure in which the two affinity columns are linked in tandem by an intervening flow dialysis system. We also report that the purified UMP synthase, which contains both orotate PRTase and OMP decarboxylase activities, is a single polypeptide chain, which exists in at least two isomeric forms. A preliminary account of this work has been presented (McClard et al., 1980).

Experimental Section

Materials. The following materials and chemicals were obtained from the designated sources: carboxymethyl-Bio-Gel A, Biolytes 5/7 and 3/10, acrylamide, *N,N'*-methylenebis(acrylamide), *N,N,N',N'*-tetramethylethylenediamine, and riboflavin 5'-phosphate (Bio-Rad Laboratories); phosphocellulose P11 (Whatman); OMP, UMP, PRPP, and yeast OMP decarboxylase (Sigma Chemical Co.); sodium [7-^{14}C]orotate, sodium [7-^{14}C]OMP, and [*methyl*-^{14}C]-hemoglobin (New England Nuclear); YM-10 ultrafiltration membranes (Amicon, Incorporated). Hollow fiber bundles (176 fibers, 2.0-mL nominal volume) were purchased from Spectrum Medical Industries. The bundles were stored in 1.5% formalin and washed thoroughly with water before use. PEG (20 000-dalton size) was obtained from either Sigma or Tridom/Fluka (Hauppauge, NY). Sigma PEG contained a polymer contaminant which was opalescent and displayed a strong fluorescence emission spectrum with λ_{max} at about 310 nm when excited at 265 nm. The Tridom/Fluka product lacked these characteristics and was preferred in later work. Yeast OMP decarboxylase (Sigma) contained amounts of orotate PRTase that interfered with assays. Therefore, for some experiments the commercial enzyme was repurified by adsorbing the protein to a small column of AECE-azaUMP–agarose (described below) and eluting with 5×10^{-5} M azaUMP as described by Brody & Westheimer (1979). Ehrlich ascites cells were harvested from mall Swiss Webster mice approximately 10 days after injection with 2×10^6 cells.

Enzyme Assays. OMP decarboxylase activity was determined by measuring $^{14}CO_2$ formed from [7-^{14}C]OMP at 37 °C using the method previously described (Prabhakararao & Jones, 1975). Each stock solution of [7-^{14}C]OMP was assayed for apparent specific radioactivity by incubating aliquots of the radioactive substrate with vast excesses of yeast OMP decarboxylase. Orotate PRTase was assayed as previously described (Prabhakararao & Jones, 1975). The specific radioactivity of each [7-^{14}C]orotate solution was determined as described for [7-^{14}C]OMP above, except that excess orotate PRTase was also included. Enzyme activity is expressed as international units (micromoles of CO_2 formed per minute) at 37 °C.

Composition of Buffers. Tris–citrate buffers were prepared by titration of 20 mM Tris base with 1 M citric acid to the desired pH. These buffers are abbreviated in the text as follows: TCD buffer (20 mM Tris–citrate plus 2 mM dithiothreitol) and TCDP buffer (TCD buffer plus 1% PEG).

Affinity Resins. AECE-azauridine was synthesized according to the six-step method of Brody & Westheimer (1979). This compound was found to be extremely deliquescent and required handling under dry N_2. AECE-azauridine was then phosphorylated with $POCl_3$ in dry trimethyl phosphate, and the product was isolated by ion-exchange chromatography (Brody & Westheimer, 1979). The product AECE-azaUMP was identical with authentic material (a gift of R. S. Brody and F. H. Westheimer) as shown by TLC, ^{1}H NMR, and ^{31}P NMR (Brody & Westheimer, 1979). AECE-azaUMP was dissolved in H_2O and used to derivatize activated carboxymethylagarose as described (Brody & Westheimer, 1979). The resin, AECE-azaUMP–agarose, contained approximately 12 μmol of AECE-azaUMP per mL of settled resin as measured by phosphate determinations of ashed samples.

Phosphocellulose P11 was washed for 15 min in 10 volumes of 0.1 M KOH and rinsed with water until the pH was below 8. The resin was then washed with 3 volumes of 0.25 M HCl and stirred gently for 30 min in 10 volumes of fresh HCl solution. The phosphocellulose was rinsed with water until the pH was above 4. Fines were removed, and the settled resin was suspended in 0.5 M Tris-HCl, pH 6.6, and stirred intermittently for 1 h. The resin was transferred to a column, equilibrated with 20 mM Tris-HCl (pH 6.6), rinsed with 100 volumes of deionized water, and then stored under N_2 at 4 °C.

$NaDodSO_4$–Polyacrylamide Gel Electrophoresis Tube Gels. $NaDodSO_4$ tube gels were prepared in 5.5 mm (inside diameter) × 12.5 cm glass tubes by using the procedure described for $NaDodSO_4$ slab gel electrophoresis (see below). Tube gel electrophoresis was conducted in a Buchler Polyanalyst apparatus. Electrophoresis was initiated at 1.5 mA/tube (constant current). After 30 min the current was raised to 2.5 mA/tube (constant current). Electrophoresis was terminated after the tracking dye migrated to about 1 cm from the end of the gel (about 4.5 h). The gels were stained in 0.1% Coomassie blue G-250 in 50% methanol–5% acetic acid and were destained in 5% methanol–7.5% acetic acid. Scanning of the gels was performed at 700 nm by using a Beckman Model 250 spectrophotometer with a scanning attachment.

Isoelectric Focusing and Localization of Enzyme Activity. IEF was performed with a Bio-Rad Laboratories Model 1405 horizontal slab gel apparatus using an 11 cm (direction of focusing) × 9.5 cm × 0.8 mm polyacrylamide slab gel. The composition of the gel was 4.85% acrylamide, 0.15% *N,N'*-methylenebis(acrylamide), 5% glycerol, 1.2% Biolyte 5/7, 0.8% Biolyte 3/10, and 0.0005% riboflavin 5'-phosphate. Anode and cathode electrolytes were 1 N in H_3PO_4 and NaOH, respectively. The carrier ampholytes were prefocused for 1.5 h at 400–1000 V (voltage was adjusted manually to maintain power at ≤8 W) at 4 °C before application of samples containing 0.5–4 μg of protein. The samples were applied in wells cast into the gel 3.5 cm from the alkaline end of the gel. Constant voltage (1000 V) was then applied for 6.5 h at 4 °C. Nitrogen saturated with water was passed into the electrophoresis chamber during focusing of the enzyme.

Upon termination of focusing a strip containing two samples (1 and 5 μg of protein) was cut from the slab gel. The portion of the gel containing 5 μg of protein was cut at 0.25–1.00-cm intervals along the length of the gel such that the cuts extended into the portion of the strip containing the second sample. The parts of the slices containing the larger sample were cut off and incubated in 0.3 mL (per 0.25 cm of gel) of 0.1 M phosphate buffer, pH 7.4, containing 2 mM dithiothreitol and 0.1 mM PRPP, for 1 h at room temperature in order to elute the enzyme from the gel. Enzymatic activities were measured by incubating 50 μL of the eluate for 15 min (OMP decarboxylase) or 200 μL for 80 min (orotate PRTase) using the assay procedures described above.

The remaining portion of the gel strip (with slits along one edge at the positions of the gel slices) was stained for protein according to the procedure of Jäckle (1979). The enzymatic

[3] The enzyme binds P_i at both the OMP decarboxylase and orotate PRTase sites. This led to the finding that the complex binds to phosphocellulose and elutes with OMP (T. W. Traut and M. E. Jones, unpublished results; McClard et al., 1979).

Table I: Outline of Purification of UMP Synthase from Ehrlich Ascites Carcinoma (220 g)

fraction	vol (mL)	mg of protein[a]	OMPDase[b] units	OMPDase[b] sp act.[c]	OMPDase[b] % recovery	OPRTase[b] units	OPRTase[b] sp act.[c]	OPRTase[b] % recovery	ratio OMPDase/OPRTase
(1) streptomycin supernatant	1040	11700	40.4	0.0034		20.5	0.0018		2.0
(2) dialyzed $(NH_4)_2SO_4$ fraction	144	311	24.3	0.0078	60	8.7	0.0028	42	2.8
(3) affinity eluate (concentrated)	0.475	0.51	4.0	7.8[d]	10	0.35	0.69	3.3	11.4

[a] Measured by the method of Lowry et al. (1951). [b] OMPDase = OMP decarboxylase; OPRTase = orotate PRTase. [c] Specific activity. [d] This value represents a 2300-fold purification from fraction 1.

activity in the gel slices and the bands on the stained strip were accurately aligned by the slits on the stained gel. A strip containing a third sample (up to 10 μg of protein) was cut from the slab gel upon completion of focusing and stained immediately.

An alternate procedure was used for the determination of enzymatic activities in some experiments. Two strips containing 0.5 and 3 μg of protein were cut into 0.25–1.00-cm slices, and the slices were assayed for OMP decarboxylase and orotate PRTase, respectively. The slices were assayed directly, without elution of the protein from the gel. The use of this procedure avoided some loss of enzyme activities due to incomplete elution from the gel. However, proper alignment of the two enzyme activities was made difficult by inaccuracies in the slicing of the two gel strips. Values of pH along the gels were determined directly by measuring at points along the length of each gel using a surface pH electrode (Bio-Rad Laboratories).

Two-Dimensional Electrophoresis. A strip of the IEF gel containing the focused enzyme sample was subjected to Na-DodSO$_4$–polyacrylamide gel electrophoresis in the second dimension (Jäckle, 1979) by using a BRL (Model V16) vertical slab gel apparatus. Separating gel dimensions were 14 cm (direction of electrophoresis) × 17 cm × 1.5 mm. The stacking gel was 1 cm long. Gel preparation and electrophoresis were carried out as described by Laemmli (1970) with the following modifications. The final concentrations of *N,N,N′,N′*-tetramethylethylenediamine and ammonium persulfate were each 0.04% in both gels. Dithiothreitol (1%) rather than β-mercaptoethanol was used to denature samples, which were immersed in boiling water for 3 min. A strip from the IEF gel that had been stained for protein was prepared for electrophoresis in the second dimension by the procedure of Jäckle (1979). The equilibration buffer contained dithiothreitol (50 mM) instead of β-mercaptoethanol (5%). Protein samples and the equilibrated gel strip were positioned on wells of the stacking gel, and electrophoresis was initiated at 15 mA (constant current). After 1 h the current was raised to 30 mA (constant current) and electrophoresis continued for 4–5 h at room temperature until the tracking dye (bromophenol blue) approached the end of the gel. The gel was stained and destained as described by Laemmli (1970).

Sucrose Density Gradient Centrifugation. Sedimentation of purified UMP synthase was performed in a 10–40% linear gradient of sucrose essentially as described by Traut & Jones (1979). Centrifugation of 12-mL gradients proceeded for 66 h with an SW41 rotor and a Beckman L5-75 ultracentrifuge. The following protein markers were included in each gradient: lysozyme from chicken egg white, $s_{20,w}$ = 1.91 S (Sophianopoulos et al., 1962); horse hemoglobin, $s_{20,w}$ = 4.09 S (Chiancone et al., 1966); alcohol dehydrogenase from horse liver, $s_{20,w}$ = 5.11 S (Ehrenberg & Dalziel, 1958); lactate dehydrogenase from bovine heart, $s_{20,w}$ = 7.45 S (Pesce et al., 1964). The gradient was fractionated by forcing it up from the tube with 60% sucrose plus 0.2% blue dextran to mark the end of the gradient. Fractions were assayed for orotate PRTase, OMP decarboxylase, lactate dehydrogenase, alcohol dehydrogenase, hemoglobin (Traut & Jones, 1979), and lysozyme (Shugar, 1952).

Assay of Proteolytic Activity. Proteolytic activity of extracts was measured by a modification of the general method of Anson (1938). Reaction mixtures contained 50 μL of buffered extract plus [*methyl*-^{14}C]hemoglobin in a total volume of 100 μL. The mixtures contained either 0.05 M potassium formate (final pH 4) or 0.02 M Tris-HCl (final pH 7.5). Reactions were performed at 37 °C and were stopped by the addition of 100 μL of trichloroacetic acid (10%). Precipitated protein was removed by centrifugation, and soluble radioactivity was determined in a toluene–Triton X-100 (2:1) scintillation fluid. Rates of proteolysis were calculated as micrograms of total protein hydrolyzed per hour per milligram of extract protein added.

Results

Purification of UMP Synthase. The following procedures were carried out at 0–5 °C. Two hundred and twenty grams (wet weight) of Ehrlich ascites cells was suspended in 4 volumes of deionized water. The cell suspension was stirred for 45 min and then homogenized gently for a total of 8 min by using a Tekmar Tissumizer set at 35 V. To the extract was added 0.02 volume of 1 M Tris-HCl, pH 8.4, 10^{-3} volume of 0.1 M disodium ethylenediaminetetraacetate, and solid dithiothreitol (30 mg/100 mL of extract). Solid streptomycin sulfate was added (1.5 g/100 mL of homogenate) over a period of 20 min. The mixture was stirred for 20 min and centrifuged for 7×10^5 *g* min. The supernatant, fraction 1 (Table I), was 1040 mL.

To the above supernatant was added 239 g of solid $(NH_4)_2SO_4$ over a period of 20 min. After being stirred for 20 min, the precipitated protein was removed by centrifugation for 2×10^5 *g* min. To the supernatant was added an additional 120 g of $(NH_4)_2SO_4$ per L of the original solution over a period of 20 min. The resulting suspension was then stirred for 40 min and centrifuged for 1.2×10^6 *g* min. The supernatant was decanted and discarded. The precipitate was dissolved in TCDP buffer, pH 8.0. The volume of protein solution was generally chosen to maintain the protein concentration at approximately 15–20 mg/mL. The dissolved protein was then dialyzed against 40 volumes of TCDP buffer, pH 8.0. The dialyzing buffer was degassed and saturated with N_2 prior to dialysis; nitrogen was dispersed continuously into the dialysate during the entire dialysis period. Some precipitation occurred during dialysis, and this material was removed by centrifugation (9×10^5 *g* min). The resulting supernatant is fraction 2 (Table I).

The dialyzed supernatant was pumped onto a column (80 mL, 4.9-cm^2 cross section) of AECE-azaUMP–agarose, previously equilibrated with TCDP buffer, pH 8.0, at about 50–60 mL/h. The protein was washed onto the column with about 100 mL of buffer, and the column was washed (approximately

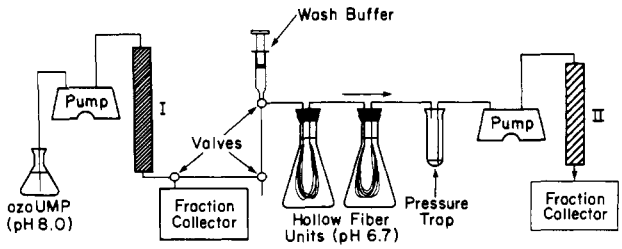

FIGURE 1: Description of the tandem affinity column system. Two column bed volumes of 5×10^{-5} M azaUMP in TCDP buffer, pH 8.0, were used to elute the protein from the column of AECE-azaUMP–agarose (I). The effluent was routed through successive hollow fiber units (see text) to bring the pH of the effluent protein to 6.7. The protein, in TCDP buffer, pH 6.7, was pumped from the pressure trap (reservoir open to 1 atm of N_2) onto the column of phosphocellulose (II) equilibrated with TCDP buffer, pH 6.7. Homogeneous UMP synthase was eluted from column II with OMP–azaUMP as described in the text.

100–120 mL/h) with about 10 bed volumes of TCDP buffer, pH 8.0, plus 5×10^{-5} M UMP and 0.02 M KCl. The column was then rinsed with 50 mL of TCDP buffer, pH 8.0. The complex was eluted from the AECE-azaUMP–agarose column by pumping a 5×10^{-5} M solution of azaUMP dissolved in TCDP buffer, pH 8.0, onto the column at about 50 mL/h. At this point the effluent of the column was routed through a linked pair of hollow fiber units (Figure 1) and pumped directly onto the phosphocellulose column (described below). The first hollow fiber unit was equilibrated with 2 L of TCD buffer, pH 6.5, and the second with 1 L of TCD buffer, pH 6.7. After the AECE-azaUMP–agarose column had been washed with 2 bed volumes of the 5×10^{-5} M azaUMP solution, the lines were rinsed toward the phosphocellulose column with a syringe loaded with TCD buffer, pH 6.7 (Figure 1). A pressure trap and second pump were included in the system to avoid the accumulation of excess back-pressure and subsequent explosions of plumbing connections (Figure 1). After all of the effluent and washes from the first column had been washed onto the column of phosphocellulose (equilibrated with TCDP buffer, pH 6.7), the column (45 mL, 8.0-cm^2 cross section) was then washed with 18 column volumes of TCDP buffer, pH 6.7, plus 5×10^{-5} M UMP. The column was then washed with about 5 column volumes of TCD buffer, pH 6.7, to remove the PEG. UMP synthase was eluted with a solution of 10^{-5} M azaUMP plus 2×10^{-5} M OMP in the above buffer. The pooled enzyme was concentrated by ultrafiltration using a YM-10 membrane. The concentrated protein (fraction 3, Table I) has been stored at −20 °C for 4 months with little loss of OMP decarboxylase activity (<30%) and no detectable loss of orotate PRTase activity. The chromatogram of the entire tandem-column procedure is shown in Figure 2. The overall preparation of purified UMP synthase is outlined in Table I. The specific activity of OMP decarboxylase was about 7.8 units/mg. This value represented approximately a 2300-fold purification over fraction 1 (Table I).

During column chromatography a significant loss of orotate PRTase activity was observed (Table I). This loss of activity is manifested as an increase in the ratio of OMP decarboxylase to orotate PRTase. The ratio increases from 2.0 (fraction 1) to 11.4 after chromatography on the tandem affinity columns. The loss of orotate PRTase activity was observed whether the enzyme (fraction 2) was subjected to chromatography on AECE-azaUMP–agarose alone, phosphocellulose alone, or the tandem-column system (data not shown). In these control experiments, no other fractions which contained orotate PRTase activity could be detected when excess repurified yeast OMP decarboxylase was added to the assay mixtures (data not shown). We attempted to stabilize the orotate PRTase activity during chromatography by the addition of 5×10^{-5}

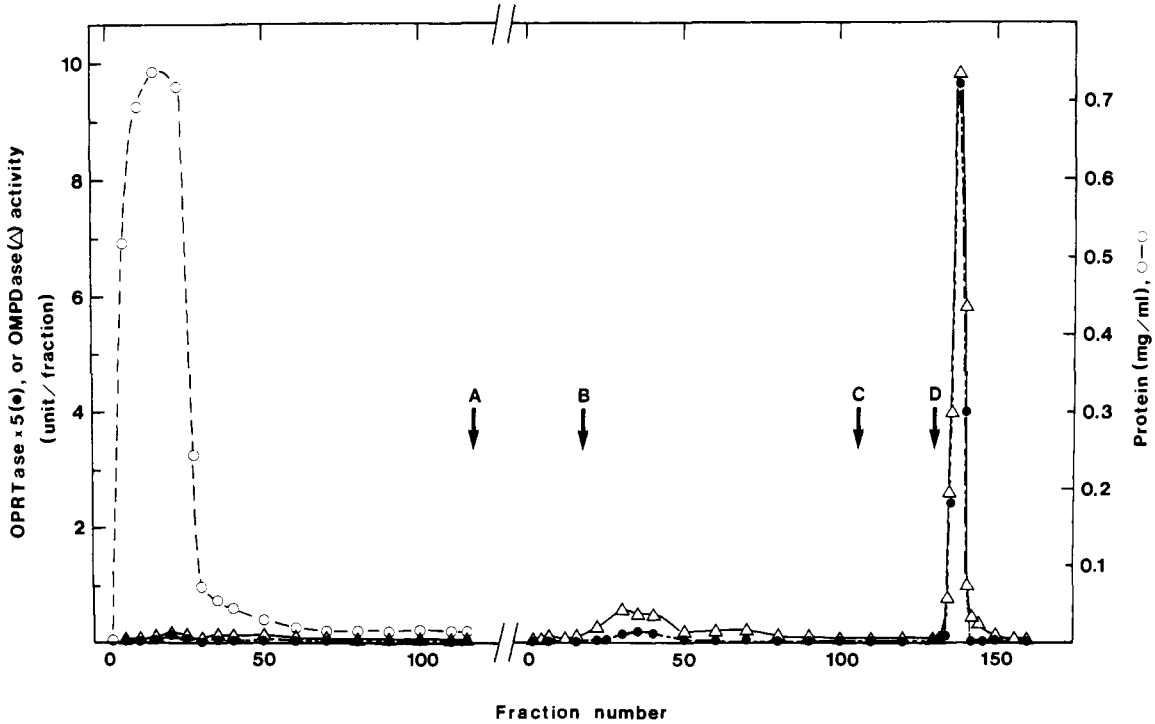

FIGURE 2: Elution profile of tandem affinity columns (described in Figure 1). The first 120 fractions contain the excluded peak of protein and washes of the AECE-azaUMP–agarose column. Event A denotes the point where UMP synthase was eluted with 5×10^{-5} M azaUMP; the discontinuity represents the period where effluent from the first column was routed through the hollow fiber units (see Figure 1). Event B marks the start of the phosphocellulose column rinse with TCDP buffer, pH 6.7, plus 5×10^{-5} M UMP. Event C marks the point where PEG was removed from the wash buffer, and D marks the start of elution of UMP synthase from the column of phosphocellulose. Fractions were each approximately 9 mL until D, where the size was reduced to 4.5 mL. Protein was assayed by the method of Bradford (1976) (OMPDase, OMP decarboxylase; OPRTase, orotate PRTase).

Table II: Effect of Various Incubation Conditions[a] on the Relative Activity of Orotate PRTase

	% of control[b]
buffer	17
0.1 mM PRPP	22
1% PEG	30
0.1 mM PRPP + 1% PEG	42
0.1 mM UMP + 1% PEG	78[c]
0.5 mg/mL bovine serum albumin + 1% PEG	47
300 mM potassium phosphate buffer, pH 7.4	33

[a] Material from the ammonium sulfate fraction (see Table I) was diluted to 20 μg of protein/mL in buffer [20 mM Tris-HCl buffer, pH 8.2 (4 °C), 2 mM dithiothreitol, and 5 mM $MgCl_2$] and the stabilizers as indicated. These mixtures were preincubated in covered scintillation vials for 20 h at 4 °C. The orotate PRTase assays were then performed at 37 °C in the same vials by initiation of the reaction with substrates. [b] Samples which had been preincubated for 20 h were compared to similar samples which were assayed immediately (controls). [c] Preferred conditions during enzyme isolation.

UMP and 1% PEG to the solvents. These agents were found to stabilize the orotate PRTase against loss of activity when fraction 2 was diluted 1000-fold (Table II). These results, taken together, indicated that this loss, which occurred during the final stage of purification, results from a perturbation of the PRTase site and not from the loss of a separate orotate PRTase protein. This interpretation is consistent with the findings of several workers that the orotate PRTase activity of UMP synthase is unusually unstable (Kasbekar et al., 1946; Appel, 1968; Brown et al., 1975; Grobner & Kelley, 1975; Reyes & Guganig, 1975; Kavipurapu & Jones, 1976; Brown & O'Sullivan, 1977).

Homogeneity. The protein obtained by the tandem affinity column method was analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis in a tube gel. A single protein band was observed after the gel was stained with Coomassie blue (Figure 3). A scan of the gel (Figure 3) showed that only a single major peak of stained material could be detected. Upon integration of the major and all minor peaks, this protein comprised ≥98% of the total. Comparison of the relative migration distance (R_f) of UMP synthase with R_f values of known standard proteins (ovalbumin plus those listed in the legend to Figure 6) showed that UMP synthase has an apparent molecular weight of about 51 500. This molecular weight compares well with the value obtained by sucrose density gradient centrifugation for the protein which contains both OMP decarboxylase and orotate PRTase activities (see below).

Sedimentation of UMP Synthase in a Sucrose Density Gradient. When purified UMP synthase was sedimented in a density gradient of sucrose, both the orotate PRTase and OMP decarboxylase cosedimented (Figure 4). A plot of $s_{20,w}$ values (for marker proteins) vs. fraction number yields a value of 3.7 S (by interpolation) for UMP synthase. This value agrees well with the value (3.6 S) that Traut & Jones (1979) reported for the monomeric form of UMP synthase. The value of 3.7 S corresponds to a molecular weight of about 50 000, assuming values of 0.743 for $\bar{v}$[4] and 1.30 for f/f_0 (Traut & Jones, 1979). This value of molecular weight agrees quite well with the value of 51 500 obtained by $NaDodSO_4$–polyacrylamide gel electrophoresis.

Two-Dimensional Electrophoresis. IEF of the enzyme produced two major bands with average isoelectric points of

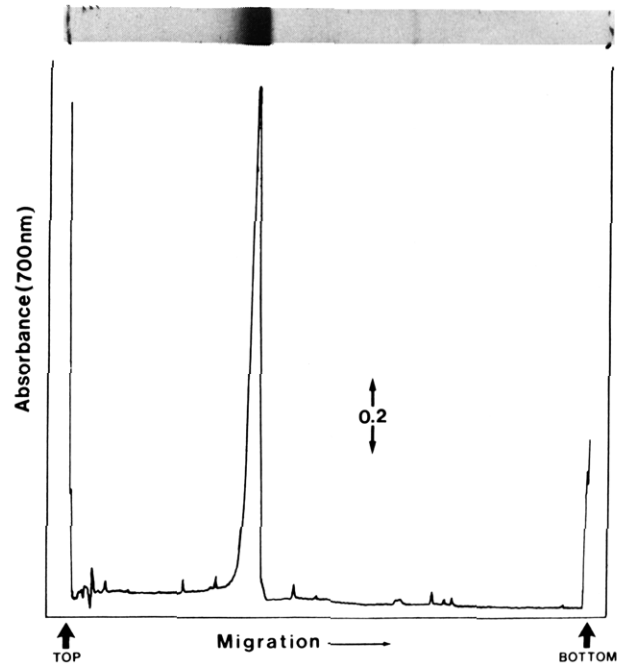

FIGURE 3: $NaDodSO_4$–polyacrylamide gel electrophoresis of purified UMP synthase. Electrophoresis of 5 μg of UMP synthase as depicted was from left to right. The actual length of the gel was 8.2 cm. Scanning of the gel is described in the text.

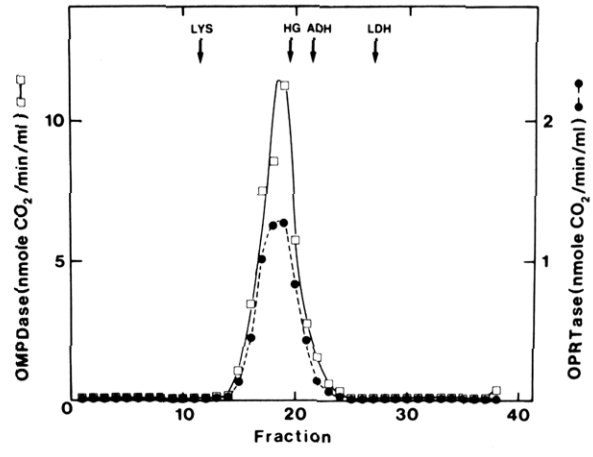

FIGURE 4: Sedimentation profile of the orotate PRTase (OPRTase) and OMP decarboxylase (OMPDase) activities of purified UMP synthase (2 μg). Centrifugation was performed in 10–40% gradients of sucrose as described in the text. Marker proteins were lysozyme (LYS), hemoglobin (HG), alcohol dehydrogenase (ADH), and lactate dehydrogenase (LDH).

5.85 and 5.65 (Figure 5). Densitometric scanning of stained gels showed that the alkaline form (p*I* 5.85) is the major species (70% of the stained protein) and the acidic form (p*I* 5.65) is the minor species (30% of the stained protein). OMP decarboxylase and orotate PRTase activities were detected in both protein bands. One enzyme band (p*I* 5.85) contained 59% (OMP decarboxylase) and 75% (orotate PRTase) of the total activities measured in the gel. The other band (p*I* 5.65) contained 20% (OMP decarboxylase) and 8% (orotate PRTase) of the total activities measured in the gel. The ratios of decarboxylase to PRTase activities were 2.7 (p*I* 5.85) and 9.0 (p*I* 5.65) for the experiment shown in Figure 5.[5] A small

[4] This value was calculated from approximate molar ratios of the component amino acids of purified UMP synthase (R. W. McClard and M. E. Jones, unpublished results).

[5] In similar experiments where gel slices were assayed directly (see Experimental Section), these ratios appeared to be similar (5–6) in each of the two major bands. We have no evidence to indicate the existence of a species which contains only the decarboxylase activity.

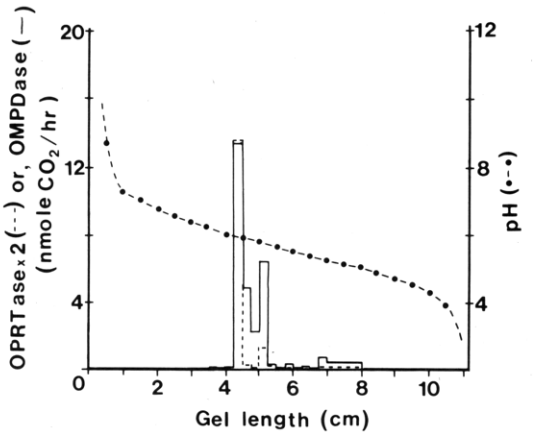

FIGURE 5: Focusing of purified UMP synthase in a pH gradient. The purified protein was subjected to IEF as described in the text. The photograph shows a strip of the gel stained for protein (3 μg of protein). The profiles of the orotate PRTase (dashed line) and OMP decarboxylase (solid line) are presented as histograms. On the average, 8% of the OMP decarboxylase and 21% of the orotate PRTase activities were recovered in the elution buffer. At least 14% of the protein remained in the gel after elution was terminated. Enzyme activity is expressed as nanomoles of CO_2 released per hour per 0.25 cm of gel length per microgram of protein.

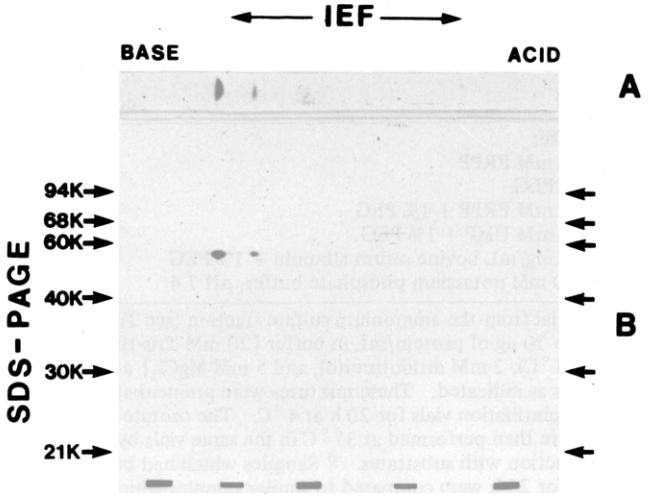

FIGURE 6: Photographs of the two-dimensional electrophoresis analysis of UMP synthase. The portion of the IEF gel which contained stained material (see Figure 5) is shown (A). The IEF gel was then subjected to electrophoresis in the second dimension at a 90° angle on Na-DodSO$_4$–polyacrylamide gel electrophoresis (SDS–PAGE) slab as described in the text (B). Horizontal arrows indicate the positions of marker proteins with their respective molecular weights (K = 10^3). Marker proteins (listed in order of descending molecular weight) were phosphorylase *b*, bovine serum albumin, catalase, aldolase, carbonic anhydrase, and soybean trypsin inhibitor. The bold dashed line at the bottom of the photograph marks the position of the tracking dye (bromophenol blue) front. Staining artifacts due to carrier ampholytes appear in the second-dimension gel as faint horizontal streaks.

amount of decarboxylase activity was found in the gel slices between pH 5.0 and 5.5. This activity possibly indicates the presence of other minor forms of the enzyme.

NaDodSO$_4$–polyacrylamide gel electrophoresis in the second dimension showed that both enzyme forms observed on the IEF gel have the same molecular weight of about 51 500 (Figure 6). This molecular weight of the enzyme was determined by running a solubilized sample of enzyme in the second dimension alongside the IEF gel (not shown). This value of molecular weight is in agreement with the value obtained from NaDodSO$_4$–polyacrylamide gel electrophoresis in a tube gel (Figure 3).

Discussion

UMP synthase has been purified from Ehrlich ascites carcinoma to apparent homogeneity by using a rapid tandem affinity column method (Figures 1 and 2). The first column is AECE-azaUMP–agarose, first described by Brody & Westheimer (1979) for the purification of OMP decarboxylase from yeast. For the Ehrlich ascites protein, this column provides only an initial, partial purification (McClard et al., 1979). Proteins which bind loosely to the column, and thus leach slowly, could not be removed completely without extensive washing of the column or use of a high concentration of salt. We found that 0.02 M KCl was the maximum concentration that could be used without removing UMP synthase from the column. This limitation was also observed by Brody & Westheimer (1979) with OMP decarboxylase from yeast.

Complete purification of UMP synthase was achieved by chromatography on phosphocellulose. The complex adsorbs to the resin at pH 6.7 in a specific manner (McClard et al., 1979) rather than as a response to the net charge of the protein; i.e., the isoelectric point(s) of UMP synthase forms are below 6 (Figure 5), giving the protein a net negative charge during chromatography. The use of phosphocellulose as an affinity adsorbent is not new. The method has been applied to the purification of a wide variety of proteins (Takagahara et al., 1978). The introduction of the in-line dialysis system, which links the two affinity columns, allows rapid equilibration of the effluent from the AECE-azaUMP–agarose column to the conditions of chromatography with phosphocellulose. The two main advantages of such rapid purification of UMP synthase are (1) assays of fractions from the AECE-azaUMP–agarose column, pooling of fractions, and dialysis are eliminated and (2) the protein is exposed much less to the potential hazards of proteases.

It is not clear whether proteolysis is a particular hazard in this instance, but numerous cases of partial proteolysis during purifications of enzymes and enzyme complexes have been described. Recent notable examples of multienzymes include fatty acid synthetase complex from chicken liver (Stoops et al., 1978), the "*arom* aggregate" in *Neurospora* (Gaertner & Cole, 1977), and the *his β* gene product of *Salmonella typhimurium* (Staples & Huston, 1979). We have found that the buffered homogenate of Ehrlich ascites carcinoma displays about 20% of the proteolytic activity found in an extract of mouse liver.[6]

Using the rapid purification procedure, we have increased the specific activity of the protein approximately 2300-fold to a final value of approximately 7.8 units/mg for the OMP decarboxylase activity (Table I). Upon purification by tandem affinity chromatography a loss of orotate PRTase activity has been observed. This loss of orotate PRTase is not surprising, despite efforts to provide maximal stablizing conditions (Table II). Several workers have demonstrated that the orotate PRTase activity of UMP synthase is far more labile than the OMP decarboxylase activity (Kasbekar et al., 1964; Appel, 1968; Brown et al., 1975; Grobner & Kelley, 1975; Reyes & Guganig, 1975; Kavipurapu & Jones, 1976; Brown & O'-Sullivan, 1977). We feel that the partial loss of orotate PRTase activity results from an abrupt change, induced by

[6] The rates of proteolysis (values are reported in units of micrograms of protein hydrolyzed per hour per milligram of extract protein added) were 64 (pH 4) and 0.9 (pH 7.5) for Ehrlich ascites carcinoma and 310 (pH 4) and 2.9 (pH 7.5) for mouse liver. Benzamidine (0.2 mM) or phenylmethanesulfonyl fluoride (0.2 mM) did not significantly reduce the rates of proteolysis in these extracts.

the extensive purification of the protein, in the structure of the PRTase domain of UMP synthase. It is very unlikely, for two reasons, that a separate catalytic unit of orotate PRTase is lost during purification. First, the activity appears in no other fractions other than with OMP decarboxylase. Second, the orotate PRTase activity is notably unstable.

$NaDodSO_4$–polyacrylamide gel electrophoresis of purified UMP synthase (Figure 3) has demonstrated that the protein is apparently homogeneous (>98% pure) and has a molecular weight of approximately 51 500. The purified protein also sediments with an $s_{20,w}$ value of 3.7 S, which corresponds to the monomer form (Traut & Jones, 1979) of the protein, which has a molecular weight of approximately 50 000. Both enzyme activities cosediment with the same apparent molecular weight. We feel that the protein species observed on $NaDodSO_4$–polyacrylamide gel electrophoresis (51 500 daltons) and sucrose density gradient centrifugation (50 000 daltons) are indeed identical. The fact that both enzyme activities occur on a protein with a molecular weight identical with that observed on $NaDodSO_4$–polyacrylamide gel electrophoresis provides compelling evidence that UMP synthase is a single polypeptide. The possibility that separate OMP decarboxylase and orotate PRTase proteins could share identical $NaDodSO_4$–polyacrylamide gel electrophoresis mobility (Figure 3), sedimentation coefficient (Figure 4), isoelectric points of isomeric forms (Figure 5), ion-exchange chromatographic behavior (Kavipurapu & Jones, 1976; R. W. McClard, L. R. Livingstone, and M. E. Jones, unpublished results), and affinity chromatographic behavior[7] seems remote. There appear to be at least two forms of the UMP synthase multienzymic polypeptide as demonstrated by isoelectric focusing (Figure 5); these forms have the same apparent molecular weight (Figure 6). Studies are now under way to determine how closely related the structures of these apparent isozymes are.

It is now easier to reconcile the proposed or implied structures of the complex between orotate PRTase and OMP decarboxylase from various sources (Kasbekar et al., 1964; Appel, 1968; Brown & O'Sullivan, 1977). Brown & O'Sullivan (1977) proposed that UMP synthase from human erythrocytes was composed of two 13 000-dalton subunits of orotate PRTase and two 20 000-dalton subunits of OMP decarboxylase; the molecular weight of the intact protein was about 62 000. Two major problems in their study confound the conclusion. First, the protein which they were studying was not pure. Second, the 13000-daltons species was detected only upon prolonged storage of the protein in 0.2 M guanidinium chloride. We feel that the most likely explanation for the results of Brown & O'Sullivan (1977) is that the authors were possibly characterizing fragments produced by proteolytic digestion of the native protein. In fact, Reyes & Guganig (1975) have pointed out that the same protein, obtained from mouse leukemia P1534J cells, can undergo limited cleavage by elastase to yield active fragments containing either OMP decarboxylase (about 45 000 daltons) or orotate PRTase (about 32 000 daltons) activities. The observation by Brown & O'Sullivan (1977) of a 26000-dalton fragment containing orotate PRTase activity (twice the 13000-dalton form) may reflect a type of homodimer formation between like fragments. Such a dimer would be likely considering that intact UMP synthase readily forms higher aggregation states (Traut & Jones, 1979; Reyes & Guganig, 1975; Grobner & Kelley, 1975). Studies are now under way in our laboratory to purify the complex from human erythrocytes and conclusively determine the structure of the protein(s).

The existence of a single polypeptide, which encompasses both the OMP decarboxylase and orotate PRTase activities, provides a consistent explanation for the several observations that the two activities co-vary in fibroblasts with the orotic aciduric trait (Pinsky & Krooth, 1967a,b) and in mammalian cells that produce altered levels of the enzymes (Suttle & Stark, 1979; Levinson et al., 1979). In the same pathway, multienzyme *pyr-1,3*, also named CAD (Kempe et al., 1976; Coleman et al., 1977), is apparently also a single polypeptide which contains the first three enzymes of the synthesis of pyrimidines de novo (Coleman et al., 1977). In transformed Syrian hamster cells resistant to phosphonoacetyl-L-aspartate, a single mRNA which codes for CAD overaccumulates (Padgett et al., 1979) due to gene amplification (Wahl et al., 1979). Thus, it appears that, at least in some mammalian cells, the first three and last two steps of the synthesis of UMP de novo are carried out by two multienzymic polypeptides.

Acknowledgments

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[7] The orotate PRTase activity is retained by AECE-azaUMP–agarose (Figure 2) and is eluted by 5×10^{-5} M azaUMP. This concentration of nucleotide is sufficient to saturate the OMP decarboxylase site but has no apparent effect on the orotate PRTase site (Traut & Jones, 1977).

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β-Carotene as a Probe of Lipid Domains of Reconstituted Human Plasma Low-Density Lipoprotein: Induced Circular Dichroism†

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ABSTRACT: Mixtures of neutral lipids containing cholesteryl esters and β-carotene were used to reconstitute the lipid core of heptane-extracted low-density lipoproteins (LDL). The resulting preparations of reconstituted LDL, referred to as r-[cholesteryl ester + β-carotene]LDL, exhibited temperature-dependent circular dichroism (CD) in the visible region similar to that of native LDL. Since β-carotene lacks intrinsic optical asymmetry, the observed CD must be induced by environmental constraint. LDL reconstituted with cholesteryl oleate, cholesteryl linoleate, or cholesteryl linolenate in the presence of β-carotene exhibited thermotropic transitions at approximately 40, 35, and 30 °C, respectively. These temperatures are similar to the liquid-crystalline to isotropic liquid phase transition temperatures observed for pure cholesteryl ester model systems. The addition of triolein to r-[cholesteryl linoleate + β-carotene]LDL lowered the transition temperature and decreased the specific ellipticity of the visible CD bands. When triolein accounted for 70% or more of the total neutral lipid content of r-[cholesteryl linoleate + triolein + β-carotene]LDL, specific ellipticity was zero. The addition of low levels of unesterified cholesterol to r-[cholesteryl linoleate + β-carotene]LDL did not alter the transition temperature but did reduce the specific ellipticity by 40%. The current data indicate that the organization of the core of neutral lipids in reconstituted LDL resembles that of native LDL with respect to environmental constraint on the β-carotene molecule and that the helicity of the protein moiety resembles that of native LDL. In addition, the data are consistent with the possibility that the core cholesteryl esters of reconstituted LDL undergo phase transitions similar to their transitions in the free state.

The molecular organization of the lipid core of human plasma low-density lipoproteins (LDL)[1] has recently been investigated (Deckelbaum et al., 1975, 1977a,b; Sears et al., 1976; Tardieu et al., 1976; Atkinson et al., 1977; Laggner et al., 1977; Mateu et al., 1978; Hamilton et al., 1979). Data from differential scanning calorimetry and small-angle X-ray scattering studies suggest that LDL contain an apolar core of neutral lipids surrounded by a polar surface shell. The neutral lipid core, which comprises ~50% of the total mass of the particle, consists predominantly of cholesteryl esters, chiefly cholesteryl linoleate (Skipski, 1972). This cholesteryl ester rich core undergoes a reversible phase transition around body temperature associated with an ordered liquid-crystalline–liquid phase change of cholesteryl esters within the intact LDL. Cholesteryl esters isolated from LDL (Deckelbaum et al., 1975, 1977a,b) undergo similar phase changes.

Chen & Kane (1974) have shown that the carotenoids normally present in trace amounts in LDL exhibit induced optical activity between 37 and 2 °C. β-Carotene comprises ~90% of the native carotenoids of LDL. As the temperature of LDL is lowered below 37 °C, the β-carotene, which is a symmetrical molecule lacking intrinsic optical activity, begins

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[1] Abbreviations used: CD, circular dichroism; LDL, low-density lipoproteins; r-[lipid + β-carotene]LDL, heptane-extracted LDL reconstituted with the indicated lipid and β-carotene; r-LDL, reconstituted LDL; EDTA, ethylenediaminetetraacetic acid; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

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