Carotene-cleavage activity in chick intestinal mucosa cytosol: association with a high-molecular-weight lipid–protein aggregate fraction and partial characterization of the activity

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1. A fluorescent high-molecular weight lipid–protein aggregate was isolated from the cytosol of chick intestinal mucosa or liver by gel filtration on columns of Sepharose 4B or 6B.

2. This aggregate exhibited carotene-cleavage activity.

3. On incubation of this aggregate, dissociation occurred and low-molecular weight fractions containing Cu and Zn and exhibiting carotene-cleavage activity were found. This fraction appeared on sodium dodecyl sulphate polyacrylamide electrophoresis to have a molecular weight of 7000–11000 and resembled the previously described Cu chelatins in amino acid composition.

4. Carotene cleavage may be effected by a copper–zinc metalloprotein of low-molecular weight, associated in intestinal cytosol with a lipid–protein aggregate.

β-Carotene is the major dietary precursor of vitamin A in man and many animals. β-Carotene is largely converted to vitamin A in the intestinal mucosa during absorption (Mattson *et al.* 1947; Mattson, 1948; Thompson *et al.* 1949; Olson, 1961; Goodman & Huang, 1965).

The conversion of carotene to retinol involves first the central cleavage of carotene to two molecules of retinal (Olson, 1961; Harashima, 1964; Huang & Goodman, 1965; Olson & Hayaishi, 1965; Goodman *et al.* 1967; Fidge *et al.* 1969). The newly-formed retinal is reduced to retinol (Fidge & Goodman, 1969) which is esterified with fatty acids and released to the lymph as chylomicrons (Huang & Goodman, 1965; Fidge *et al.* 1968). The enzymes involved in these reactions are soluble cytosolic enzymes and the initial cleavage reaction was shown to be a dioxygenase reaction (Olson & Hayaishi, 1965; Goodman *et al.* 1967; Fidge *et al.* 1969). Carotene cleavage is inhibited by thiol inhibitors and chelating agents and the presence of thiols, bile salts and lipids stimulated the reaction (Olson & Hayaishi, 1965; Goodman *et al.* 1967; Fidge *et al.* 1969). The optimal pH range for this reaction was narrow, between 7·8 and 8·2 (Goodman *et al.* 1967) and the protein was purified twenty-seven-fold by ammonium sulphate precipitation, ion-exchange chromatography and gel filtration (Fidge *et al.* 1969). The enzyme appeared to have a molecular weight (MW) between 100000 and 200000 (Fidge *et al.* 1969). Reduction of retinal to retinol was NADH- or NADPH-dependent and had a narrow pH optimum of approximately 6·3. The two activities overlapped only in the pH range 7·0–7·4 (Fidge & Goodman, 1969).

The present report describes the association of the carotene-cleavage enzymes with a high-MW lipid–protein aggregate (LPA) previously described in the liver (Heller, 1979; Sklan *et al.* 1982; Sklan & Donoghue, 1982*a*) and kidney (Sklan *et al.* 1982; Sklan & Donoghue, 1982*a*) and describes the presence of copper and zinc in the cleavage enzyme and reports a partial purification.

EXPERIMENTAL PROCEDURE

Animal experiments. Male chicks (150–200 g) were injected with 10 μCi ^{65}Zn (Amersham International, Amersham, Bucks) intravenously and not permitted access to food for

13 h. The chicks were then administered 10 μCi [^{3}H]β-carotene (Hoffmann-La Roche, Basel) in 1 ml soya-bean oil. Animals were allowed free access to food for 3 h and then killed with an intracardiac overdose of sodium pentothal.

Cytosol preparation and fractionation. Chick liver or mucosal cytosols were prepared by centrifugation of a homogenate of chick liver or mucosal scrapings from the proximal 300 mm of the small intestine. The tissue was homogenized with 1 vol. (w/v) buffer (0·25 M-sucrose, 0·1 M-potassium chloride containing 10 mg soya-bean trypsin inhibitor/ml, pH 8·0) using a Janke-Kunkle homogenizer (Stauffen i Br., Germany) (Sklan *et al.*1982) at setting no. 6 for 30 s. The homogenate was then centrifuged at 104000 *g* for 60 min in the Ti 50 rotor of a Beckman L3-50 centrifuge. The upper fatty layer was removed by piercing the tube with an 18 gauge needle. The clear supernatant fraction beneath the floating fat was used in further experiments. All procedures were carried out in dim light at 4°. Cytosol was fractionated by gel filtration on columns of Sepharose 4B, 6B or Sephadex G-50 (Pharmacia Fine Chemicals, Piscataway, New Jersey). In addition, ion-exchange chromatography was carried out on DEAE cellulose (Whatman Inc., Clifton, New Jersey) with increasing concentrations of Tris-hydrochloride as previously described (Sklan & Donoghue, 1982*b*).

The hydrated density of the LPA was estimated by ultracentrifugation in solutions of increasing density (between 1·006 and 1·21 with sucrose) for 40 h at 105000 *g* in the Ti 50 rotor of a Beckman L3-70 centrifuge.

Carotene-cleavage assays. [15-15′-^{3}H$_2$]β-carotene was used as a labelled substrate. Vials were opened under nitrogen and a small portion of labelled β-carotene was chromatographed on to a small column of alumina (Goodman *et al.* 1967; Fidge *et al.* 1969) and previously chromatographed unlabelled β-carotene added to give the required specific activity before each experiment. The incubation mixture (2 ml) contained (μmol): potassium phosphate buffer (pH 7·8) 50, NADH 20, reduced glutathione 10, sodium taurocholate 12, phosphatidyl choline 10, α-tocopherol 0·6; 0·5 μg [^{3}H]β-carotene was added in 20 μl propan-2-ol. Following incubation at 37° for 2 h, the samples were extracted with chloroform–methanol (2:1 v/v) and a mixture of non-radioactive carriers (carotene, retinal, retinol and retinyl esters, 5 μg each) was added to the samples and the chloroform phase separated and subjected to alumina chromatography with hexane, hexane–benzene and benzene as described by Huang & Goodman (1965). Activity was calculated from the ^{3}H found in retinal.

Other methods. Hydrolase activities towards retinyl palmitate and triolein were determined as described by Prystowsky *et al.* (1981) using retinyl [1-^{14}C]palmitate (25 μCi/mmol). Polyacrylamide gel electrophoresis was carried out with 200 g/kg gels with and without the presence of sodium dodecyl sulphate (SDS) (Weber & Osborn, 1975).

^{3}H was determined by liquid scintillation counting and ^{65}Zn by γ-counter. Amino acid composition was determined after hydrolysis in 6 M-hydrochloric acid with and without the presence of dimethylsulphoxide in an LKB Biocal 3201 Amino Acid Analyser (Spencer & Wold, 1969). Retinal fluorescence was determined with a Perkin Elmer model 202, and metal concentrations by atomic absorption spectroscopy with a Perkin Elmer 303.

Protein (Bradford, 1976), triglycerides, phospholipid and free fatty acid concentrations (Sklan *et al.* 1975) were determined by the indicated procedures.

<div align="center">RESULTS</div>

When mucosal cytosol was pre-incubated with [^{3}H]carotene and subjected to gel filtration on a Sepharose 6B column, over 90% of the label was found in a fraction which eluted close to the void volume (Fig. 1). The ^{3}H was extracted and, following alumina chromatography, was found to be β-carotene; the same fraction contained the retinyl esters.
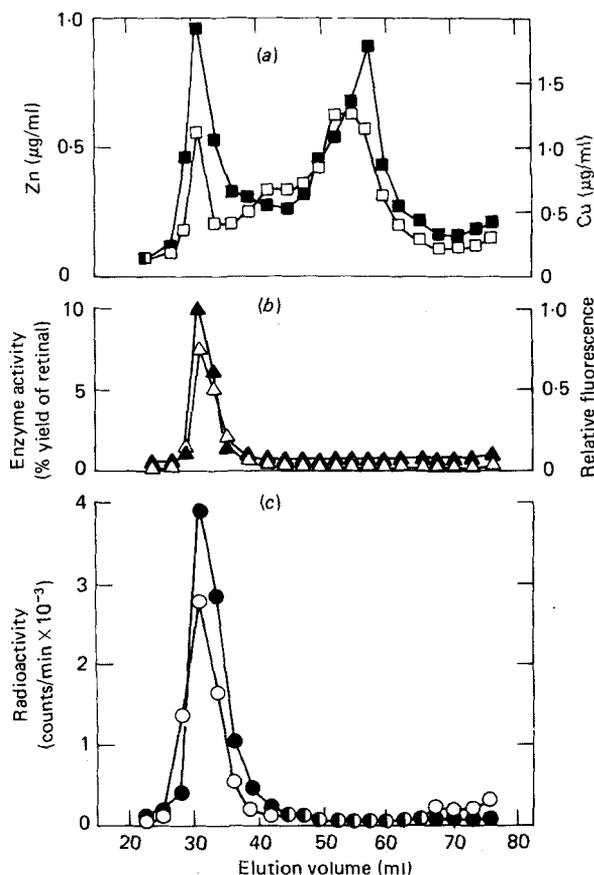
Fig. 1. Gel filtration of mucosal cytosol on a Sepharose 6B column. Cytosol (1 ml) was prepared from 0·5 g mucosal scrapings, pre-incubated for 60 min at 4° with [³H]β-carotene or retinyl esters, applied to the column (480 × 17 mm) and elution carried out with 50 mM-Tris hydrochloride buffer, pH 7·8, containing 12 mM-monothioglycerol; fractions of approximately 3·0 ml were collected. (*a*), Zinc (■) and copper (□) concentrations (μg/ml); (*b*), carotene-cleavage activity (▲, calculated from the ³H in retinal) and relative fluorescence (△, excitation 350 mm, emission 470 nm); (*c*), [³H]β-carotene (○) and ³H-labelled retinyl esters (●) radioactivities (counts/min × 10⁻³).

Pre-incubation of the cytosol with ³H-labelled retinyl esters yielded a similar elution pattern (Fig. 1).

Determination of Cu and Zn concentrations in the elution fractions revealed that both metals co-eluted with the carotene and retinyl esters, although a major amount of Cu and Zn eluted at higher volumes. Determination of the carotene-cleavage activity in the column fractions showed that almost all cytosolic activity was located in this high-MW fraction (Fig. 1). Furthermore, retinyl palmitate and triolein hydrolase activities were also found in the same fraction. Chromatography on Sepharose 4B (not shown) resulted in elution of a fraction within an elution volume very close to that of dextran blue (MW 2 × 10⁶). This fraction contained 458 mmol fatty acids/mol, which were distributed 71% in triglycerides, 18% in phospholipids and 3% in free acids.

Similar treatment of cytosol which was not freshly prepared or had been previously subjected to high ionic strengths resulted in the appearance of an additional lipid–protein
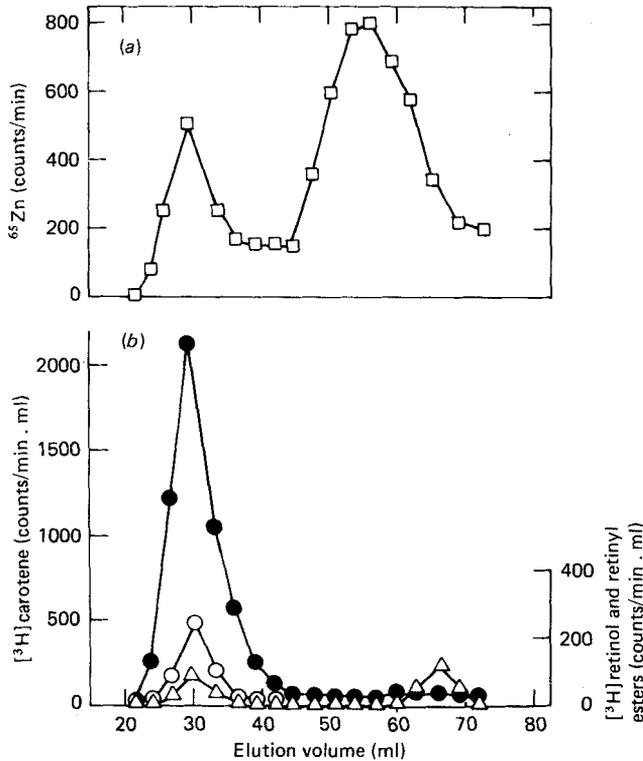
Fig. 2. Gel filtration on a Sepharose 6B column of intestinal mucosa from chicks injected with ^{65}Zn and intubated with [^{3}H]β-carotene. Cytosol (1 ml) was prepared from 0·5 g mucosal scrapings and was applied to the column (480 × 17 mm) and elution carried out with 50 mM-Tris hydrochloride buffer, pH 7·8, containing 12 mM-monothioglycerol; fractions of approximately 3·0 ml were collected. (a), ^{65}Zn (□); (b), [^{3}H]β-carotene (●), ^{3}H-labelled retinyl esters (○), [^{3}H]retinol (△).

fraction with an apparent MW between 100000 and 200000 which exhibited carotene-cleavage activity.

Mucosal cytosol of chicks injected with ^{65}Zn and incubated with [^{3}H]β-carotene and subjected to similar gel filtration on Sepharose 6B (Fig. 2) showed approximately 15–20% of Zn radioactivity in the high-MW LPA and major amounts of radioactivity at MW of less than 75000. Extraction of the eluent fractions and alumina chromatography showed that the high-MW LPA contained more than 95% of the labelled carotene together with labelled retinyl esters and retinol. A further peak of ^{3}H was found following alumina chromatography at an MW of approximately 15000.

The hydrated density of the LPA isolated from a Sepharose 4B column was estimated by ultracentrifugation (Table 1). [^{3}H)β-carotene was found in the top floating fraction in the density range 1·063–1·13 and ^{65}Zn was distributed in the same manner in the LPA. In cytosol, [^{3}H]β-carotene was distributed similarly and approximately 20% of cytosolic ^{65}Zn was in the top fraction at 1·13. These 'top' fractions also exhibited carotene-cleavage activity.

LPA isolated from a Sepharose 4B column, as described previously, was incubated with [^{3}H]β-carotene at pH 7·4 for 90 min at 37° and subjected to gel filtration on Sepharose 6B or Sephadex G-50. In addition to the LPA, which eluted close to the void volume on Sepharose 6B, a 100000–200000 MW fraction containing [^{3}H]β-carotene, Zn, Cu and [^{3}H]retinol was observed. This fraction appeared to be similar to that obtained from cytosol

Table 1. *Density distribution of* [³H]*carotene and* ⁶⁵*Zn in cytosol and high-molecular-weight (MW) lipid–protein aggregate*

(Values are the means of two separate samples. Samples were adjusted to the indicated densities and centrifuged at 105 000 *g* for 40 h in the Ti 50 rotor of a Beckman L3-50 ultracentrifuge. The tubes were sliced in the clear zone below the upper floating layer)

| | Density distribution (% in top floating layer) | | | |
| | Cytosol | | Lipid–protein aggregate* | |
Density	[³H]carotene	⁶⁵Zn	[³H]carotene	⁶⁵Zn
1·006	5·6	0·5	0·7	0·9
1·063	16·5	4·0	5·9	3·0
1·090	26·9	8·9	14·0	20·1
1·130	87·1	21·8	83·6	88·4
1·210	86·2	23·6	82·6	86·6

* High-MW lipid–protein aggregate prepared by gel filtration of cytosol on Sepharose 4B.

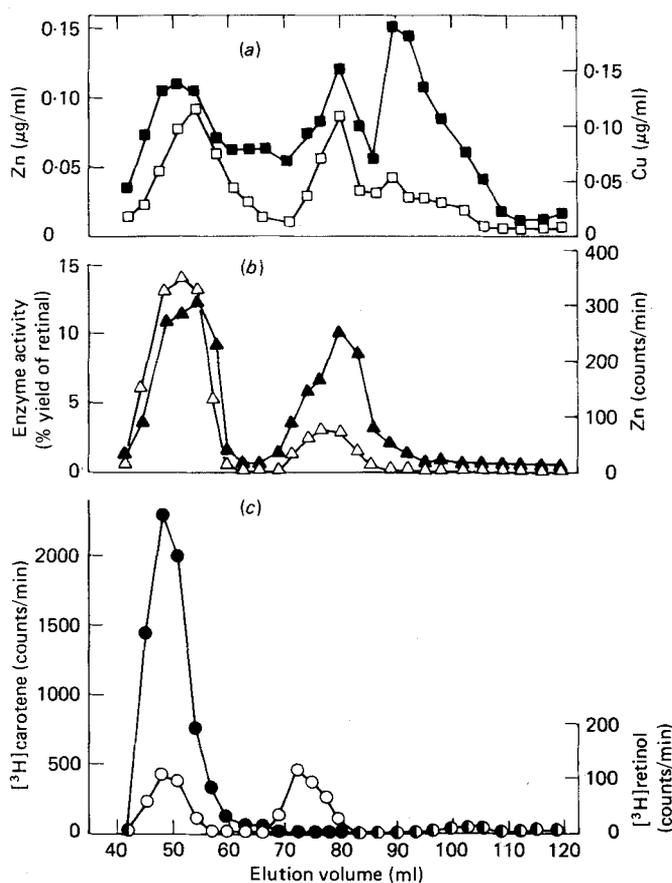Fig. 3. Gel filtration on a Sephadex G-50 column (600 × 15 mm) of the lipid–protein aggregate isolated from a Sepharose 4B column and incubated at pH 7·4 for 90 min at 37°, in the dark in air. The eluting buffer was 50 mM-potassium phosphate containing 12 mM-monothioglycerol and fractions of approximately 2·3 ml were collected. (*a*), Copper (□) and zinc (■) concentrations; (*b*), carotene-cleavage activity (△, calculated from the ³H in retinal) and ⁶⁵Zn (△); (*c*), [³H]β-carotene (●) and retinol (○) radioactivities.
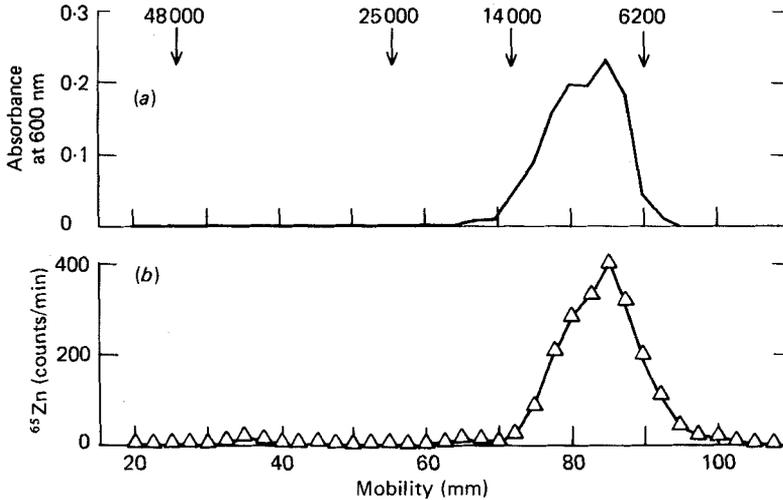
Fig. 4. Sodium dodecyl sulphate electrophoresis of the copper–zinc material (fraction 4, Table 2) separated after incubation of lipid–protein aggregate and Sephadex G-50 and DEAE-cellulose chromatography from an animal injected with ^{65}Zn. (a), The gel scan after staining; (b), ^{65}Zn following slicing. ↓, Molecular weights indicated.

Table 2. *Partial purification of carotene-cleavage enzyme from chicken mucosa*

(Values are means of triplicate determinations)

Purification stage	Specific enzyme activity (g retinol/h per ng protein)	Apparent purification
Cytosol	0·024	—
Sepharose 4B (LPA)	0·48	× 20
Sephadex G-50	1·48	× 62
DEAE-cellulose	2·17	× 90

which had been subjected to high ionic strength. Sephadex G-50 chromatography of the [^{3}H]β-carotene-incubated LPA, yielded (Fig. 3) a [^{3}H]retinol-containing fraction with MW of approximately 14500, a Cu–Zn fraction with MW of 10000–13000 and a Zn-containing fraction at MW of about 8000–10000. The Cu–Zn fraction co-eluted with carotene-cleavage activity (Fig. 3). This Cu–Zn fraction was subjected to ion-exchange chromatography on DEAE cellulose with increasing concentrations of Tris-hydrochloride buffer and the resulting material showed MW between 7500 and 11000 on SDS electrophoresis (Fig. 4). Similar studies with material prepared from an animal injected with ^{65}Zn showed radioactivity localized at approximately 8000 MW. This fraction contained (μg/mg protein) 18·6 Cu and 17·7 Zn and its specific carotene-cleavage activity is shown in Table 2. The purification achieved from mucosal cytosol by these procedures was ninety-fold.

The ^{65}Zn-labelled fraction on incubation with LPA (isolated from Sepharose 4B as described previously) for 60 min at 37° was found to be 79% incorporated into the LPA on rechromatography on Sepharose 6B.

Determination of the amino acid composition of this Cu–Zn fraction after DEAE

Table 3. *Effect of various compounds on carotene-cleavage enzyme activity*

(Incubation was carried out for 60 min at pH 7·8 and the values are the means of triplicate determinations using the fraction obtained from a Sepharose 4B column and a Sephadex G-50 column (see Table2))

	Relative yield of retinol (%)
Without glutathione	16
Without sodium taurocholate	22
Glutathione (10 μmol)	100
5,5'-Dithiobis-(2-nitrobenzoic acid) (10 μmol)	65·4
EDTA:	
(15 μmol)	72·7
(60 μmol)	19·3
α,α-Dipyridyl (5 μmol)	31·2
1,10-Phenanthroline:	
(0·3 μmol)	67·6
(0·6 μmol)	34·1

cellulose chromatography (fraction 4, Table 2) showed a low cysteine content (1·4%) and relatively low concentrations of basic amino acids, other than lysine.

Carotene-cleavage activity was determined in the Sephadex G-50 fraction in the presence of various additives (Table 3). Omission of glutathione or bile salts considerably reduced activity, as did the presence of α,α-dipyridyl, 1,10-phenanthroline and 5,5'-dithiobis-(2-nitriobenzoic acid).

Similar but less-extensive studies with liver cytosol revealed the presence of carotene-cleavage activity in a similar LPA. On incubation of this LPA at 37°, low-MW Cu–Zn fractions with carotene-cleavage activity were also observed.

DISCUSSION

A high-MW LPA containing the cytosolic retinyl esters has been previously described in the livers of rats (Heller, 1979; Sklan *et al.* 1982), horses (Sklan & Donoghue, 1982*a*) and chicks (Sklan & Donoghue, 1982*b*). This LPA was associated with retinyl ester and acyl glyceride hydrolase activities (Chen & Heller, 1979; Sklan *et al.* 1982; Sklan & Donoghue, 1982*a, b*) and, in addition, the presence of the intracellular retinol-binding protein (cRBP) was demonstrated (Sklan *et al.* 1982). Zn and Cu have also been shown to be associated with this aggregate in chick liver (Sklan & Donoghue, 1982*b*). The presence of high-MW aggregate containing retinyl esters in intestinal mucosa was indicated by Hollander *et al.* (1978) and this aggregate has been further characterized in the present study. The MW, lipid content, presence of Zn and Cu, hydrated density and the presence of hydrolases all indicate that the aggregate fraction in intestinal mucosa is very similar to that of the liver. The present study also adds an additional observation that this aggregate is also associated with carotene-cleavage activity. The cleavage activity reported here had a similar pH range and was inhibited by sulphydryl inhibitors and by chelating agents as previously described for carotene-cleavage enzyme (Olson & Hayaishi, 1965; Goodman *et al.* 1967; Fidge *et al.* 1969). In these previous studies a twenty-seven-fold purification was achieved using ammonium sulphate precipitation and ion-exchange chromatography, and the partially-purified material had an apparent MW of 100000–200000 (Goodman *et al.* 1967; Fidge *et al.* 1969). The LPA showed changes in chromatographic behaviour on exposure to high ionic concentrations, as previously reported (Sklan *et al.* 1982). On incubation at 37°, further dissociation of the LPA occurred and two additional fractions, containing Cu and

Zn, were observed with carotene-cleavage activity. One was the 100000–200000 MW fraction and the other was of approximately 10000 MW. This fraction was not completely purified but appeared, on the basis of the amino acid composition and chromatographic characteristics, to be similar to those previously reported for an intestinal Cu-binding protein (Evans & Leblanc, 1976). This low-MW Cu–Zn protein could be re-incorporated into the LPA on incubation. Previous studies on a similar low-MW 'Cu-chelatin' in liver have also noted a tendency of the Cu-chelatin to polymerize (Winge *et al.* 1975).

Incubation of the mucosal LPA with carotene at pH 7·4 resulted in the formation of retinol found both in the LPA and bound to a protein of approximately 15000 MW. Similar retinol-binding fractions in a mucosal LPA were reported by Hollander *et al.* (1978) and this probably represents the intracellular cRBP. In the liver, cRBP was found in the LPA and incubation of labelled retinyl esters with the LPA resulted in the release of retinol–cRBP (Sklan *et al.* 1982; Sklan & Donoghue, 1982*a, b*).

Previous studies (Olson & Hayaishi, 1965; Goodman *et al.* 1967; Fidge *et al.* 1969) have shown sensitivity of the carotene-cleavage enzymes to chelating agents, and Bliss (1951) and Zachman & Olson (1961) have indicated that the hepatic 'retinene reductase' is alcohol dehydrogenase (*EC* 1.1.1.1), which is a Zn metalloenzyme (Vallee, 1976). Zn deficiency reduced the reduction of retinal to retinol (Huber & Gershoff, 1975; Sundaresan *et al.* 1977) and reduced the efficiency of carotene utilization (Chhabra *et al.* 1980). However, in the present study, the cleavage of carotene to retinal, shown to be a dioxygenase reaction (Olson & Hayaishi, 1965; Goodman *et al.* 1967; Fidge *et al.* 1969) has been shown to be associated with both Cu and Zn. Cu has been found in several dioxygenase systems (Malmstron *et al.* 1975; Harrison & Hoare, 1980) and could act in this fashion in carotene cleavage.

Additional clarification of the function of the LPA reported in the present and previous studies (Heller, 1979; Sklan *et al.* 1982; Sklan & Donoghue, 1982*b*) is required. The possibility of a single specific particle combining carotene-cleavage, retinol ester hydrolysis and retinol transport proteins is attractive, but the possibility of a non-specific hydrophobic aggregate cannot be ruled out.

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