Serum and intracellular retinol transport in the equine

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1. Serum and intracellular distribution of retinol was determined in equines maintained on four levels of vitamin A intake.

2. The form of retinol transported in serum was determined by gel filtration and chromatography to be a complex of retinol bound to a protein of molecular weight (MW) of approximately 20000, which was in turn complexed probably with prealbumin to yield a complex with a MW of 75000 to 80000.

3. Increasing dietary vitamin A levels enhanced the concentration of lipoprotein-bound retinyl esters in the plasma.

4. Vitamin A in the liver cytosol was found predominantly as retinyl esters in a lipid–protein aggregate of MW approximately 2×10^6 and hydrated density of 1·063–1·111. In the kidney and adrenal gland, two lipid–protein entitites were found with MW of approximately $1 \cdot 8 \times 10^6$ and $1 \cdot 7 \times 10^5$ respectively. These fractions contained approximately 40 and 20% lipid respectively and had densities of 1·063–1·111 and approximately 1·21.

5. All lipid–protein aggregates were associated with retinyl palmitate hydrolase activity and guanidine treatment released a 15000 MW material, presumably intracellular retinol-binding protein.

6. Increasing dietary vitamin A enhanced the proportion of retinol in the $1 \cdot 7 \times 10^5$ fraction.

7. Findings in equine plasma and liver resemble previous observations in other species. The characterization of two new lipid–protein aggregates in equine kidney and adrenal glands, which have hydrolase activity, may be important in intracellular retinol transport and metabolism, especially in animals subjected to high intakes of vitamin A.

Liver contains the major stores of vitamin A in mammals, and this is usually in the form of retinyl esters. Hepatic retinyl esters are hydrolysed to retinol which becomes complexed to a specific carrier, retinol binding protein (RBP), of molecular weight (MW) approximately 20000 before release to the blood. A further complexation to prealbumin then occurs and in this form retinol is transported to tissues (Smith & Goodman, 1979). RBP does not appear to cross tissue membranes and an intracellular binding protein, which differs in MW (14600) and immunologically from RBP has been isolated from cytosol (Ong & Chytil, 1978, Ross *et al.* 1978). A lipid–protein aggregate contains the majority of the retinyl esters in rat eye (Heller, 1976) and liver cytosol (Heller, 1979). It is associated with retinyl palmitate hydrolase activity (Chen & Heller, 1979).

Little is known concerning retinol transport systems in the equine. In a recent study (Donoghue *et al.* 1981), the equine appeared to be particularly susceptible to abnormal intakes of vitamin A with significant changes in growth, haematology and serum biochemistries before development of clinical signs. These findings indicate the possibility that retinol transport systems may be affected by abnormal vitamin A intakes, and elucidation of these transport systems in the equine was the object of the present study.

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Table 1. *Composition (g/kg) of the low-carotene diet*

Ingredient	
Beet pulp	330·0
Crimped oats	400·0
Soya-bean meal	160·0
Molasses	90·0
Monosodium phosphate	6·7
Limestone	6·7
Trace mineral mix*	6·7

* Contained (g): 6·3 sodium chloride, 0·0335 zinc, 0·0268 manganese, 0·0168 iron, 0·0036 copper, 0·0007 iodine and 0·007 cobalt; cholecalciferol was added at 21·1 μg.

EXPERIMENTAL

Animals and diets. Fifteen horse and pony fillies less than 18 months old were housed and managed essentially as described previously (Donoghue *et al.* 1981). Fillies were given a vitamin A-free low-carotene diet (Table 1) in amounts equal to 30 g/kg body-weight per d. Following accommodation and standardization, fillies were grouped randomly according to age and size into four groups receiving retinyl proprionate daily equivalent to 0 (four fillies; mildly deficient), 12 (three fillies; control group), 1200 (four fillies; mildly intoxicated group) and 12000 (four fillies; severely intoxicated group) μg retinol/kg body-weight. This supplement was diluted in maize oil and administered orally.

Fillies were weighed and blood sampled monthly. After 40 weeks, fillies were killed by an intravenous injection of sodium pentobarbitol. Livers, kidneys and adrenal glands were removed, weighed and frozen at −20° until analysed.

Results of the monthly blood analyses and post-mortem examination will be reported elsewhere; this report characterizes the form of retinol transported in plasma and tissues in selected fillies.

Treatment of tissues. Tissues were homogenized in a high-speed homogenizer (Ultraturrax; Janke-Kunkel KG, Stauffen i. Br., W. Germany) with 3 ml 0·25 M-sucrose and 0·025 M-potassium chloride/g tissue. Cytosol was prepared by spinning the homogenate at 105000 *g* for 50 min in the Ti-50 rotor of a Beckman L3-50 centrifuge at 4°. Floating fat was sliced off and the clear cytosol collected.

Column chromatography. All stages were carried out in dim light and at 4°. Gel filtration was carried out on columns of Sepharose 6B and 2B (Pharmacia Fine Chemicals, Piscataway, NJ) in 50 mM-Tris-hydrochloride, pH 8·0. Some samples were brought to 3·0 M-guanidine hydrochloride and chromatographed in the presence of 1·5 M-guanidine in the eluting buffer.

Ultracentrifugation. The density distribution of retinol and RPH was determined in cytosols, plasma and in various column fractions from Sepharose columns by ultracentrifugation at different sucrose densities, at 145000 *g* for 40 h. Plasma samples were centrifuged at densities prepared with potassium bromide. Tubes were sliced in order to obtain top fractions.

RPH activity. RPH activity was determined by incubation with retinyl [^{14}C]palmitate prepared as described by Harrison *et al.* (1979) and free fatty acids formed were extracted as described by Belfrage & Vaughan (1969).

Chemical methods. Retinol was separated from retinyl esters by alumina column chromatography, located in fractions from columns with a spectroflorimeter (Perkin Elmer Model No. 203) and levels in plasma and tissues determined as previously described

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Table 2. *Concentrations (μg/g) of retinol and retinyl esters in the plasma and tissues of fillies given four levels of vitamin A*

(Results are means of duplicate determination of tissues from the fillies for intracellular studies)

	Mildly-deficient		Control		Mildly-intoxicated		Severely-intoxicated	
Group*...	Retinyl esters	Retinol	Retinyl esters	Retinol	Retinyl esters	Retinol	Retinyl esters	Retinol
Plasma (μg/l)	38	50	4	307	2012	353	797	253
Liver	2·2	2·8	76·2	13·1	1840·0	96·4	1330·0	57·5
Kidney	0·1	0·1	1·2	0·8	6·2	1·1	52·9	92·0
Adrenal gland	0·6	0·3	1·5	0·3	23·8	31·2	33·8	17·8

* For details, see p. 274.

(Donoghue *et al.* 1981). The linearity of the fluorescence was calibrated against chemical determinations and, where necessary, dilutions made. In addition, selected column fractions were checked by the chemical method to allow for differences in the quantum efficiencies of retinol bound to different proteins. Triglycerides, phospholipids (Sklan *et al.* 1975) and cholesterol were determined as previously described (Sklan & Budowski, 1979).

RESULTS

Plasma and tissue concentrations of retinol and retinyl esters increased with vitamin A intake (Table 2).

Gel filtration of plasma from control animals on Sepharose 6B and determination of fluorescence and retinol, following extraction and alumina chromatography, in the fractions collected, showed the majority of retinol to be concentrated in the MW range 75000–80000 (Fig. 1). Additional vitamin A fluorescence was observed at a MW of approximately 5×10^5, and this appeared to be mainly retinyl esters, whereas the lower MW peak consisted only of the retinol as determined by alumina chromatography. Ultracentrifugation of plasma from control animals showed that approximately 5% of total retinol was of hydrated density less than 1·21, whereas in intoxicated animals this proportion increased. Alumina chromatography showed the fraction of density less than 1·21 to be retinyl esters. Chromatography of plasma or of the concentrated 75000–80000 MW peak in the presence of guanidine resulted in almost complete disappearance of the original peak (MW 75000–80000) and appearance of a new retinol containing peak with MW 20000 (Fig. 1).

In animals given high levels of vitamin A, the percentage of retinyl esters in the high MW peak increased to well over 50% of total retinol in severely-intoxicated animals.

Incubation of plasma or fractions of plasma from the Sepharose 6B columns with retinyl [^{14}C]palmitate resulted in hydrolysis of the order of 250 pmol free fatty acid released/h per ml plasma. Higher values were obtained with plasma column fractions from the Sepharose 6B columns and the major hydrolytic activity was located in the region of MW 70000.

Gel filtration of liver cytosol from a control animal determined that over 90% of total retinol (by fluorescent and chemical methods) was located close to the void volume of the Sepharose 6B column (Fig. 2), and on Sepharose 2B appeared to have a MW of approximately 2×10^6. This peak contained approximately 90% retinyl esters of total vitamin A and 48% lipids, which were predominantly triglycerides (83%) and small amounts of cholesterol and phospholipids; it was associated with approximately 70% of total cytosol

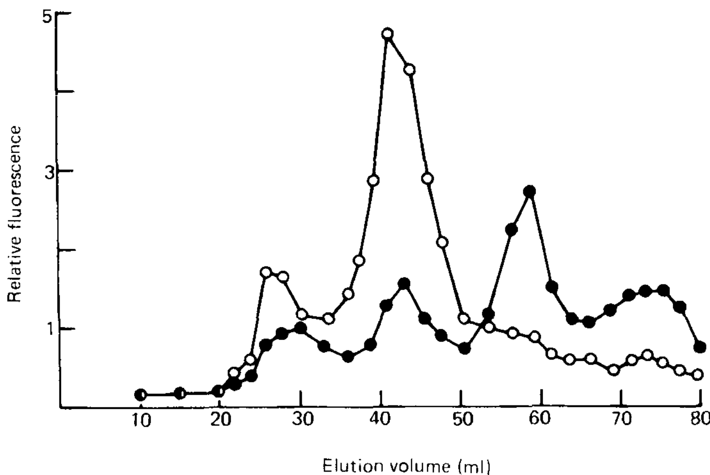

Fig. 1. Gel filtration of plasma from control (○——○) or toxic (●——●) animals treated with 3·0 M-guanidine and chromatographed with 1·5 M-guanidine on Sepharose 6B (450 × 17 mm) in 50 mM-tris hydrochloride pH 8·0 plasma (1·0 ml) was loaded on the columns and fractions of approximately 2·0 ml were collected. For fluorescence determination excitation was set at 350 nm and emission determined at 470 nm, and a spectrum run to ascertain presence of retinol. Columns were calibrated with blue dextran, thyroglobulin, apoferritin, phosphorylase B, albumin, ovalbumin, myoglobin, lysozyme and cytochrome C. The void volume of the column was 26·0 ml and albumin eluted at 52·5 ml, myoglobin at 65·0 ml and the salt volume was 77·0 ml.

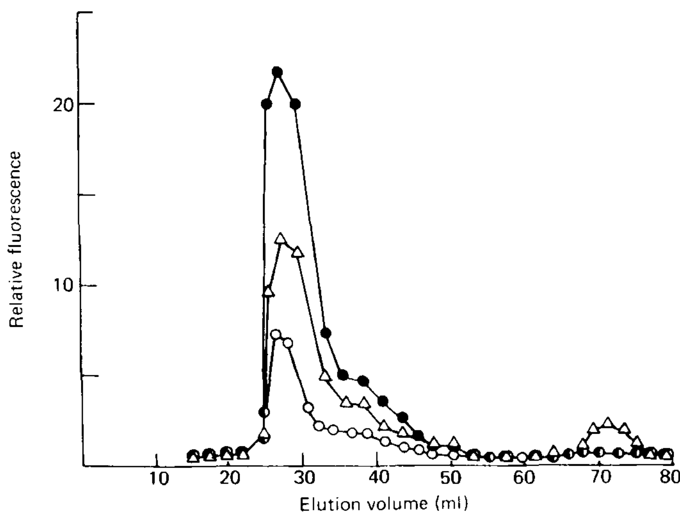

Fig. 2. Gel filtration of liver cytosol from control (○——○) or toxic (●——●) animals on Sepharose 6B (450 × 17 mm) in 50 mM-tris hydrochloride pH 8·0. Cytosol (2·0 ml) was loaded onto the column and fractions of approximately 2·0 ml collected. The sample from the toxic liver was treated with 3·0 M-guanidine and chromatographed in the presence of 1·5 M-guanidine (△——△). Fluorescence was determined and column calibrated as described in Fig. 1.

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Table 3. *Distribution of retinol fluorescence and retinyl palmitate hydrolase (RPH) activity on ultracentrifugation of liver, kidney and adrenal cytosol of horses*

(Results are means of two duplicate samples and cytosol levels set at 100%)

Tissue	Density	Fluorescence in top layer (%)	RPH in top layer (%)
Liver			
	1·063	4·2	2·8
	1·111	85·8	83·6
	1·210	107·5	95·3
	1·250	103·2	94·4
Adrenal			
	1·063	1·2	3·8
	1·111	66·8	56·5
	1·210	96·0	78·4
	1·250	98·5	84·6
Kidney			
	1·063	1·4	2·7
	1·111	68·3	58·6
	1·210	88·7	83·2
	1·250	96·6	91·8

Table 4. *Distribution of retinol fluorescence and retinyl palmitate hydrolase (RPH) activity in the retinol containing peaks isolated on Sapharose 2B from kidney cytosol of horses*

(Results are means of duplicate determinations; 1·25 top was set at 100%)

Approximate MW	Density	Fluorescence in top layer (%)	RPH in top layer (%)
2×10^6			
	1·063	2·8	4·3
	1·090	35·8	43·2
	1·111	86·2	84·6
	1·210	113·2	103·8
2×10^5			
	1·063	0·8	0·8
	1·090	1·3	0·6
	1·111	2·3	1·9
	1·210	88·7	53·4

RPH activity. The fluorescence spectrum exhibited excitation maximum at 350 nm and emission maximum at 470 nm. Ultracentrifugation of this peak or of total liver cytosol (Tables 3, 4) indicated that the density was in the range 1·063–1·111, and that distribution of RPH activity paralleled that of retinol. In horses given the high level of vitamin A, concentrations of retinyl esters in this peak were enhanced, and a small shoulder with MW of approximately 2×10^5 appeared. Treatment of kidney cytosol in a similar manner (Fig. 3) showed that some 75% of total cytosol retinol was located in a similar peak which was close to the void volume of Sepharose 6B but in the included volume on Sepharose 2B and had a MW cf approximately $1{\cdot}8 \times 10^6$. This peak contained 50% retinyl esters and some 42% lipids which were predominantly triglycerides. In addition, a second peak of retinol

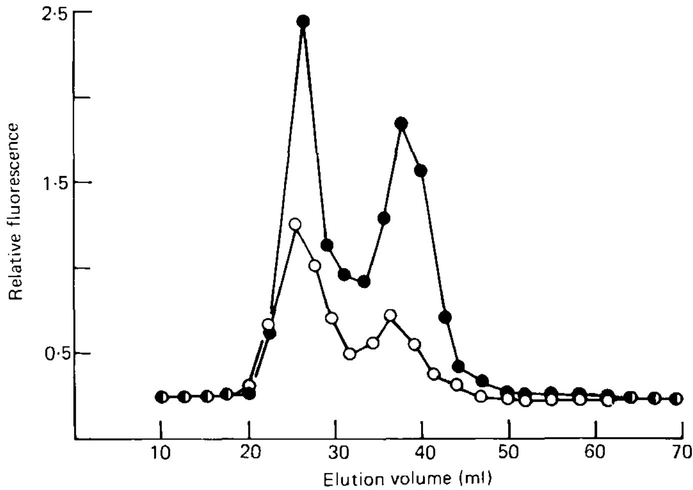

Fig. 3. Gel filtration of kidney cytosol from control (○——○) or toxic (●——●) horses on Sepharose 6B (430 × 15 mm). Cytosol (1·0 ml) was loaded onto the column and fractions of about 2·3 ml were collected. Fluorescence was determined as described in the legend to Fig. 1. The column was calibrated with standards as described in Fig. 1, and void volume was 20·0 ml, albumin eluted at 49·5 ml, myoglobin at 63·0 ml and salt volume was 73·0 ml.

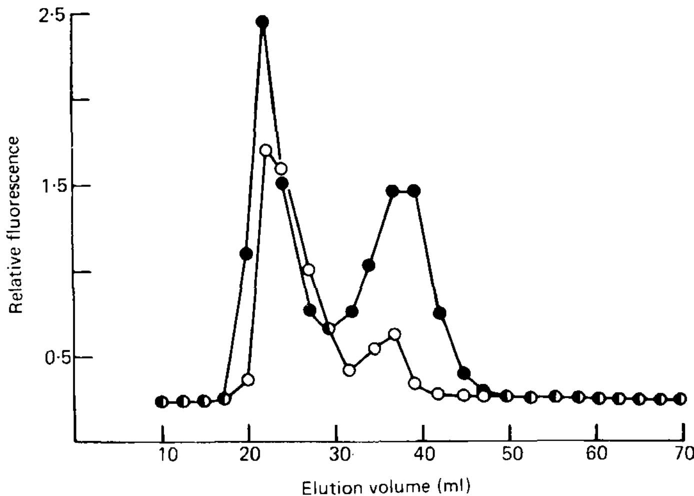

Fig. 4. Gel filtration of adrenal cytosol from control (○——○) or toxic (●——●) horses on Sepharose 6B (430 × 15 mm). Cytosol (1·0 ml) was loaded and fractions of approximately 2·3 ml were collected. Fluorescence was determined as described in the legend to Fig. 1, and column calibrated as described in Fig. 3.

was clearly observed with a MW in the range of $1{\cdot}5\text{–}2 \times 10^5$. This fraction contained 30% retinyl esters, 70% retinol and approximately 20% lipid which was mainly triglycerides.

Both retinol-containing fractions were associated with RPH activity which was of the order of 2·0 μmol free fatty acid released/h per mg protein in both peaks. Ultracentrifugation of cytosol (Table 3) and of the two fractions (Table 4) from control animals indicated that the density of the higher MW fraction was in the range 1·063–1·111, and of the lower MW fraction, approximately 1·21. The RPH activity was distributed similarly to the retinol. Increasing dietary intakes of vitamin A enhanced the lower MW fraction (Fig. 3) which contained over 40% of total vitamin A in severely intoxicated animals.

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Similar studies with adrenal cytosol revealed a similar pattern (Fig. 4) with two fractions of retinol containing proteins of MW $1\cdot8 \times 10^6$ and $1\cdot5$–2×10^5; the percentage of retinyl esters of vitamin A in the two fractions being 26 and 23 respectively, with lipid comprising 41 and 19% respectively. RPH activity was of similar magnitude per mg protein in both fractions and densities (Table 4) similar to those found in the kidney.

Chromatography of both retinol containing fractions isolated from cytosols on Sepharose 6B in the presence of guanidine resulted in partial disappearance of the original peaks and appearance of retinol containing fractions at MW approximately 65000, 42000 and 15000 (Fig. 2).

Gel filtration of cytosol from mildly-vitamin A-deficient fillies which had been preincubated with ^{3}H retinol showed distribution of the label similar to the retinol distribution in normal animals with two peaks apparent in kidney and adrenal cytosol.

DISCUSSION

Our results indicate that the transport of retinol in the plasma of the equine is similar to that described for other species (Muto *et al.* 1973; Glover *et al.* 1974). Retinol is complexed to a protein with MW of approximately 20000, presumably RBP, which is, in turn, complexed with prealbumin yielding a complex with MW in the range 75000–80000. In the rat, the RBP-prealbumin complex has a MW of 75000 and can be dissociated by reducing the ionic strength (Petersen, 1971; Muto & Goodman, 1972) or by guanidine treatment (McGuire & Chytil, 1980). Most of the plasma retinol in horses with a normal vitamin A intake was RBP-prealbumin bound with the remainder in the form of lipoprotein-bound retinyl esters. In animals with a high vitamin A intake, levels of retinyl esters increased in lipoproteins of hydrated density less than 1·21 as has been previously described in the hypervitaminotic A rat (Mallia *et al.* 1975). The in vitro hydrolysis of retinyl esters in the plasma may be due to the action of lipoprotein lipase which would fit the MW region where hydrolysis was found (Augustin *et al.* 1978). The significance of the release of non-RBP-bound retinol in the plasma is unknown and could become significant in intoxicated animals.

We also found a retinyl ester-containing lipoprotein in equine tissues. The involvement of such an aggregate in retinol transport in rats has been previously suggested by Heller (1976, 1979). Equine liver contains an aggregate with MW approximately 2×10^6, with a hydrated density of 1·063–1·09 and containing approximately 80% triglycerides. This aggregate is associated with RPH activity. Guanidine treatment releases a protein-binding retinol, but not retinyl esters, with a MW of approximately 15000. In rat liver, it has been found by immunological means that this is intracellular retinol binding protein (Sklan, Blaner, Adachi, Smith & Goodman, unpublished results).

The presence of two retinol-containing aggregates in kidney and adrenal cytosol may be of metabolic significance. The major, higher MW aggregate was similar to that observed in the liver although there was marginally-less lipid present and the MW was slightly smaller. The second lower MW lipoprotein contained only 20% lipids and had a MW of $1\cdot7 \times 10^5$. This aggregate also was associated with RPH activity, and guanidine treatment released a 15000 MW retinol-binding fraction. The nature of this $1\cdot7 \times 10^5$ MW fraction is intriguing; the presence of a higher proportion of free retinol may indicate that this is a stage in the release of retinol from intracellular retinol-binding protein. Furthermore, almost all the vitamin A is in the form of retinyl esters in the liver, whereas less than half the retinol is esterified in the kidney and adrenal gland. Thus, the functions of the lipid–protein aggregates may represent successive stages in the transport of retinol and the hydrolysis of retinyl esters to retinol for binding to the intracellular binding protein.

Intracellular retinol transport in the equine liver appears to be similar to that reported

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in the rat, with a high MW lipoprotein playing a role. The two lipid–protein aggregates which we found in equine kidney and adrenal glands may be of particular significance in animals with high vitamin A intakes.

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