Retinol deficiency and *Dipetalonema viteae* infection in the hamster

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ABSTRACT

Following chronic retinol (vitamin A) deprivation leading to exhaustion of liver vitamin A reserves below 50 I.U. per liver hamsters were fed diets either deficient in ("Rd": 250 I.U.A/kg in experiment I, 1000 I.U.A/kg in experiment II) or enriched with retinol ("Rw": 10000 I.U.A/kg in experiment I and II). After 4 weeks some of the animals (36 in experiment I, 30 in II) were infected with 150 3rd-stage larvae of *D. viteae*, while clean animals were kept as controls. The retinol status, the immune response (indirect fluorescent antibody test: IFAT) and parasitological parameters were examined up to 8 (experiment I) and 12 weeks (experiment II) post infection (p.i.). Rd hamsters had levelling off of weight gain or weight loss, severely deficient retinol levels in serum and liver, and high mortality. Weight gain was less in infected than in uninfected hamsters, and the capacity of infected Rw animals to restore liver retinol was significantly lower than that of uninfected Rw animals. IFAT titres were similar in Rd and in Rw animals, but microfilaraemia was significantly enhanced at 8 and 10·5 weeks p.i. in Rd hamsters. While the number of worms recovered from Rd and Rw hamsters was similar, there was a significant increase in the ratio of female to male worms in Rd hamsters. Rd hamsters in experiment I produced 3·3 times the worm mass per 100 g body-weight than Rw hamsters. Also, the average mass per female worm was significantly higher in Rd than in Rw hamsters, and this parameter was negatively correlated with the liver retinol concentration in experiment I ($r = -0.89$). Retinol deficiency has a marked effect on growth and fertility of *D. viteae* in hamsters.

INTRODUCTION

The parasitological and immunological features of *Dipetalonema viteae* infection in the golden hamster (*Mesocricetus auratus*) have been described in detail (WEISS 1970; MUELLER 1980; WEISS & TANNER 1981). After subcutaneous (s.c.) inoculation, third-stage larvae (L3) moult twice, then develop into fertile males and females within about six weeks. Patency (microfilaraemia) begins shortly thereafter and lasts for 9 to 14 weeks, until microfilaraemia is suppressed by the host's immune response, while adult worms survive in the host (WEISS & TANNER 1981). Peak microfilaraemia occurs 10 to 13 weeks post inoculation (p.i.).

Interactions of nutrition with immunity and parasitic infections in mammalian hosts are complex (ISLIKER & SCHUERCH 1981; KEUSCH 1982). In rats, enhancing and depressing effects of protein, pyridoxine, thiamine and retinol deficiencies on infections with filariid worms and immunity have been reported (JAYAPRAGASAM *et al.* 1977; STOREY 1981, 1982; PRASAD *et al.* 1980 a, b). We were interested in the effects of latent retinol deficiency in man on onchocerciasis and schistosomiasis (STÜRCHLER *et al.* 1981, 1983) and were looking for an experimental model for further study. We report on the effects of experimental retinol deficiency in golden hamsters on growth and reproduction of *D. viteae* and on the immune response of the host.

MATERIALS AND METHODS

Male golden hamsters (*M. auratus*) of the LAKZ strain were used. They were from a random bred colony from the Institute for Bio-Medical Research Ltd. CH-4414 Fuellinsdorf. They were maintained three per cage and received feed and water ad libitum.

During breeding they received commercial feed pellets (Naehr- und Futtermittel AG, CH-9202 Gossau, NAFAG product 854) containing analytically determined vitamin A and D_3 concentrations of 3000 and 500 I.U./kg, respectively. With this diet the retinol reserves of the colony were slowly reduced over several generations while reproduction of the animals continued.

Four-week-old hamsters were transferred to the experimental laboratories for enforced retinol depletion by feeding retinol-free diet ("A 103", table I) for 7 to 10 days. Hamsters were then separated into two groups, retinol well-fed (Rw) and retinol deficient (Rd) by adding various amounts of retinyl palmitate (ROVIMIX A-500) to the A 103 diet: in Expt I Rd animals received 250 I.U. of retinyl palmitate per kg of feed; in Expt II this was increased to 1000 I.U.A/kg because of high mortality of Rd animals in Expt I. Rw animals received 10000 I.U.A/kg in both experiments. For convenience pellets with 10000 I.U.A/kg were coloured red with canthaxanthin which lacks provitamin A activity.

Four weeks later animals were s.c. inoculated ("day 0") with 150 L3 of *D. viteae*. In Expt I 18 Rw and 18 Rd animals were infected, while 12 Rw and 12 Rd animals were kept as clean controls. In Expt II 30 animals were infected (15 each Rw and Rd) and 14 were controls (7 each Rw and Rd).

Blood was collected by puncturing the retro-orbital sinus of animals anaesthetized with ether.

Thick drops (20 μl of blood) were stained with Delafield's haematoxylin and the number of microfilariae (mff) counted in one preparation.

At necroscopy male and female adult worms were removed from the connective tissue, blotted to dryness on filter paper and weighed. The recovery ratio is the proportion of the number of adult worms to the number of larvae inoculated.

The embryogenesis of female worms was determined as follows: batches of five randomly selected females recovered from the same hamster were homogenized in a

Table I.   Rodent diet A 103

Composition per kg

A.	Casein: extracted, vitamin-free	180 g
	Rice starch	530 g
	Coconut oil: heated for 8 h and aerated	130 g
	Brewer's yeast (dry)	100 g
B.	Vitamins: D_3	2000 I.U.
	E	10 I.U.
	Biotin	0·1 mg
	B_1	10·0 mg
	B_2	10·0 mg
	B_6	10·0 mg
	B_{12}	40·0 μg
	Ca-D-pantothenate	20·0 mg
	Nicotinic acid amide	70·0 mg
	p-Aminobenzoic acid	70·0 mg
	Folic acid	1·0 mg
	meso-Inositol	30·0 mg
	C	70·0 mg
	Tetrasodium salt of 2-methyl-1,4-naphthohydroquinone	2·0 mg
	sugar, ad	10·0 g
C.	Salts, trace elements	50·0 g

glass tissue grinder containing 200 μl of normal saline. After gently grinding for 30 sec, 800 μl of saline was added, the suspension shaken and 10 μl examined under a light microscope for mff and late embryonic stages.

The indirect fluorescent antibody test (IFAT) was used for the determination of antifilarial antibodies in the sera of infected animals (WEISS 1970). Female, adult *D. viteae* frozen sections were used as antigen. For statistical analysis, the reciprocal titres were log. transformed.

Livers, sections of the dorsal skin and worms were gassed with N_2 and, like the sera, kept at $-20°C$ for retinol determination. Sera (approximately 100 μl) were diluted with water to 0·4 ml and with ethanol to 0·8 ml for de-proteination. 0·8 ml n-hexane was added, the solution shaken and centrifuged. The supernatant which contains the hexane-retinol mixture was pipetted and high performance liquid chromatography performed under the following conditions. Stationary phase: Li Chrosorb Si 60 5 μm 25 cm; mobile phase: n-hexane with 2% isopropanol; detection: UV 325 nm with Uvikon LCD 725. The peak area was evaluated, compared with an external standard and expressed as I.U.A (1 I.U.A corresponds to 0·33 μg retinol).

Retinol was extracted from tissues by saponification at 90 to 95°C in a water bath for 20 min with 30 ml of ethanol and 10 ml of 50% potassium hydroxide. The saponified solution was cooled, poured into a separating funnel and extracted with 100 ml of ether three times. The extract was washed with water to neutral, dried over sodium sulphate and concentrated to dryness in a rotation evaporator. The residue was dissolved in n-hexane and processed in the same way as the sera.

For statistical evaluation the t-test and the U-test of Wilcoxon, Mann and Whitney were used when indicated.

RESULTS

Growth and mortality

Four to eight weeks p.i., Rd animals gained less weight than Rw animals or lost weight (Fig. 1). The growth curve was more strongly influenced by retinol deficiency than by infection. The only significant difference in mean body weights was observed at week 8 in Expt I between Rw and Rd animals (in the uninfected: t-value 3·8, $p \approx 0·001$, in the infected: t-value 5·4, $p < 0·001$). Mortality was elevated in Rd hamsters (in Expt I eight of 30 by 8 weeks p.i., in Expt II six of 22 by 12 weeks p.i.) compared with Rw hamsters (one of 52 at the end of experiments). When Rd animals developed a coarse fur and eyes became covered with a sticky mucous discharge suggestive of clinical retinol deficiency, they usually died within a few days.

Vitamin A status

Hamsters from another series fed the commercial product (NAFAG 850 with vitamin A) had a median retinol liver concentration of 176 I.U./g (range 139–231, $N = 10$).

After feeding a diet in the breeding-station without vitamin A addition followed by a vitamin-A-free experimental diet (A 103) retinol liver reserves of hamsters in this study were exhausted (Table II a). They were somewhat replenished by the 12th week p.i. in animals on 10000 I.U.A/kg with significant differences between infected and uninfected animals in both experiments. However, in all Rd animals (I and II, uninfected and infected) liver reserves declined to 0.

Retinol serum concentrations (table II b) reflect the dietary intake: they are adequate in Rw animals, low in animals given 1000 I.U.A/ and virtually undetectable in those receiving 250 I.U.A/kg.
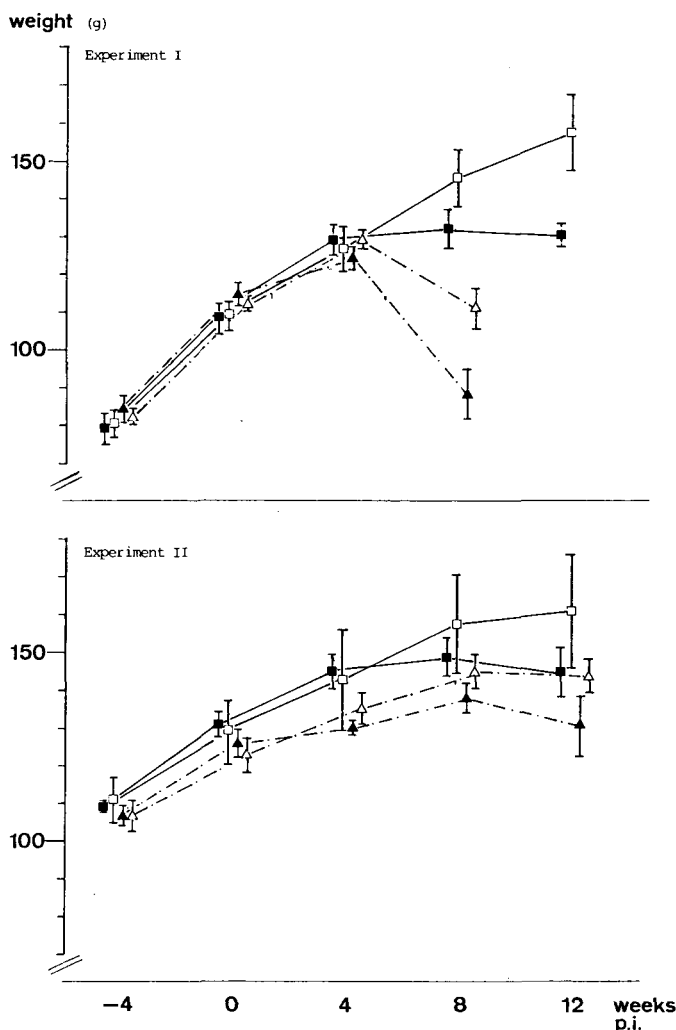
FIG. 1. Weight of hamsters on A 103 diet plus various additions of retinol, before and after infection (p.i.) with 150 L3 *D. viteae* s.c., and in controls. Well-fed animals (10 000 I.U.A/kg diet in Expts I and II): ■ infected; □ uninfected. Deficiently fed animals (250 I.U.A/kg diet in Expt I, 1 000 I.U.A/kg in Expt II): ▲ infected; △ uninfected.

Skin retinol concentrations which were determined in Expt I were all <0·5 I.U.A per g (N = 43) and no difference was found between Rw and Rd animals and infection status.

Immune response
    IFAT titres in Rd animals were of the same order as in Rw animals (Table III).

Parasitology
    The number of circulating mff was significantly higher in Rd than in Rw animals at 8 and 10·5 weeks p.i. (Table IVa). In Expt II microfilaraemia was looked for already at 5·5 weeks p.i., but it was absent in Rw as well as Rd animals, indicating that the release of mff is not accelerated in Rd animals.

Table II a. Retinol concentration in the liver of hamsters after retinol-free diet (~4 weeks p.i.), after addition of retinol to the diet for 4 weeks (week 0) and after infection with *D. viteae*

Weeks p.i.	Experiment I				Experiment II			
~4	8* (4–22) N=4				9 (5–32) N=3			
0	10,000 I.U.A/kg 41 (36–50) N=4		250 I.U.A/kg 2 (1–18) N=4		10,000 I.U.A/kg 69 (61–77) N=2		1,000 I.U./kg 9 (3–15) N=2	
	inf.	uninf.	inf.	uninf.	inf.	uninf.	inf.	uninf.
8	78 (46–127) N=8	99 (48–129) N=6	0 (0–4) N=10	0 (0–1) N=7				
12	59[a] (45–108) N=9	109[a] (81–141) N=6			75[b] (47–132) N=15	111[b] (82–123) N=7	0 (0–0·3) N=9	0 (0–1) N=7

* I.U.A per g liver, median (and range); N sample size.
[a] $U = 3$, $p < 0.005$.
[b] $U = 17$, $p < 0.01$.

Table II b. Retinol concentration in the sera of hamsters before (week 0) and after infection with *D. viteae**

Weeks p.i.	Experiment I				Experiment II			
	10,000 I.U.A/kg		250 I.U.A/kg		10,000 I.U.A/kg		1,000 I.U.A/kg	
0	379 (292–408) N=4		80 (58–147) N=4					
	inf.	uninf.	inf.	uninf.	inf.	uninf.	inf.	uninf.
4	325[a] (246–367) N=7	401[a] (352–463) N=6	13 (0–31) N=8	16 (6–40) N=6				
8	273 (221–381) N=6	305 (187–363) N=6	0 (0–60) N=9	0 (0–47) N=6	362 (301–473) N=11	378 (314–521) N=7	45 (12–521) N=11	56 (10–88) N=6
12		335 (154–351) N=6			367 (140–407) N=15	354 (308–429) N=7	12 (0–101) N=9	26 (17–60) N=7

* ng per 1000 ml, median (and range); N sample size.
[a] $U = 1$, $p < 0.005$.

Table III. Results of the humoral immune response by indirect fluorescent antibody test (IFAT)

	Reciprocal IFAT titre*		
	4 weeks p.i.	8 weeks p.i.	12 weeks p.i.
Experiment I			
Rw (N=9)	85·1 (1·93±0·18)	89·1 (1·95±0·20)	
Rd (N=9)	93·3 (1·97±0·26)	63·1 (1·80±0·15)	
Experiment II			
Rw (N=15)			346·7[a] (2·54±0·18)
Rd (N=9)			631·0[a] (2·80±0·20)

* geometric mean ($10^{g\bar{x}} \pm$ SD).
[a] $t = 3.0$, $p < 0.01$.

Embryogenesis was only studied in Expt I. At 8 weeks p.i. the geometric mean number of mff and of advanced embryonic stages was $170/10 \mu l$ in Rw animals and $324/10 \mu l$ in Rd animals ($U = 37$, $p < 0.025$). At 12 weeks p.i. this number was $141/10 \mu l$ in Rw animals.

Results on adult *D. viteae* are shown on Table IVb, Fig. 2 and Fig. 3.

Table IV a. Microfilariae in peripheral blood

| | Number of microfilariae*/20 μl blood | |
	8 weeks p.i.	10·5 weeks p.i.
Experiment I		
Rw (N = 8)	20·0[a]	
	(1·30 ± 0·26)	
Rd (N = 7)	416·9[a]	
	(2·62 ± 0·32)	
Experiment II		
Rw (N = 15)	3·9[b]	18·8[c]
	(0·59 ± 0·15)	(1·27 ± 0·20)
Rd (N = 14 at wk. 8,	12·8[b]	186·0[c]
N = 11 at wk. 10·5)	(1·11 ± 0·11)	(2·27 ± 0·11)

[a] $U = 5$, $p < 0.005$.
[b] $U = 60$, $p < 0.05$.
[c] $U = 27$, $p < 0.005$.
* geometric mean $(10 \, g\bar{x} \pm SE)$
To include the number of zero, a $\log(x + 1)$ transformation was used which was reversed when calculating antilog $(x + 1)$.

Table IV b. Recovery rate and weight of adult *D. viteae* worms from hamsters after 8 (experiment I) and 12 weeks p.i. (experiment II)*

| Hamsters Number | Experiment I | | | Experiment II | | |
	Rw 8	Rd 10	Significance	Rw 15	Rd 9	Significance
Worms/hamster						
f	19·6	22·5	ns	19·4	26·8	ns
	(1·6)	(2·2)		(2·8)	(3·6)	
m	26·9	20·7	ns	26·9	26·0	ns
	(2·5)	(2·5)		(3·2)	(4·1)	
Recovery rate	0·31	0·29	ns	0·30	0·35	ns
	(0·02)	(0·03)		(0·04)	(0·05)	
f:m ratio	0·8	1·2	t = 2·9	0·7	1·1	t = 2·6
	(0·1)	(0·1)	p < 0·01	(0·1)	(0·1)	p < 0·02
Worm mass (mg)/hamster						
f	26·9	63·7	t = 2·6	34·8	61·9	t = 2·4
	(3·0)	(13·7)	p < 0·02	(6·3)	(9·6)	p < 0·05
m	14·0	17·9	ns	15·4	17·5	ns
	(1·9)	(3·7)		(2·1)	3·2	
f + m/100 g hamster	30·8	101·9	t = 2·9	36·7	62·7	t = 2·1
	(3·2)	(24·1)	p < 0·01	(7·1)	(10·5)	p < 0·05
f:m weight ratio	2·1	3·9	ns	2·2	4·1	t = 1·9
	(0·3)	(1·0)		(0·2)	(0·6)	p < 0·01

* mean (SE)
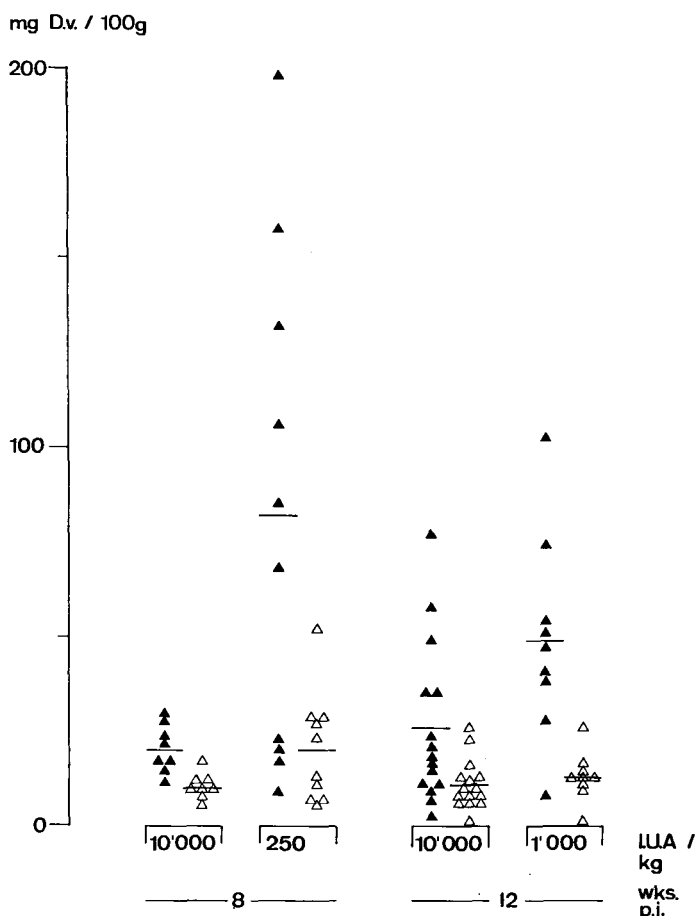f = female, m = male, ns = not significant.

FIG. 2. Mean weight of female and male worms recovered, standardized to a hamster body-weight of 100 g. Data at week 8 are from Expt I, those at week 12 from Expt II. Bars indicate group means. ▲ females; △ males.

The recovery ratio was similar in all groups. However, more females were recovered from Rd than from Rw animals, and the female : male ratio was greater than one in both experiments. Rd animals produced large worm masses. Standardized to a body-weight of 100 g, hamsters on 250 I.U.A/kg produced a 3·3 times higher worm mass than the corresponding Rw hamsters. This increment in worm mass is attributable to both an increase in the number of female worms in Rd hamsters (male worms are lighter than female worms weighing 0·5 to 0·8 mg on an average), and to an increase in mass per female worm; the average weight (S.E.) of females at 8 weeks p.i. in Expt I is 2·5 mg (0·4) in Rd hamsters and 1·4 mg (0·1) in Rw hamsters (t-value 2·6, $p < 0.02$). Corresponding data of Expt II at 12 weeks p.i. are 2·3 (0·2) and 1·7 (0·1) mg (t-value 3·3, $p < 0.01$).

Retinol liver concentrations were negatively correlated with the mean weight per female worm in Expt I only (at 8 weeks p.i. $r = -0.77$, $p < 0.05$, at 12 weeks p.i. $r = -0.89$, $p < 0.01$). Females weighing over 2·5 mg were recovered only from hamsters with retinol concentrations below 1 I.U./g liver and 25 ng/l serum, respectively.

In Expt II the retinol concentration per adult *D. viteae* were determined in four worm batches each from Rd and Rw hamsters respectively; parenchymal retinol concentration was near to zero in all animals.
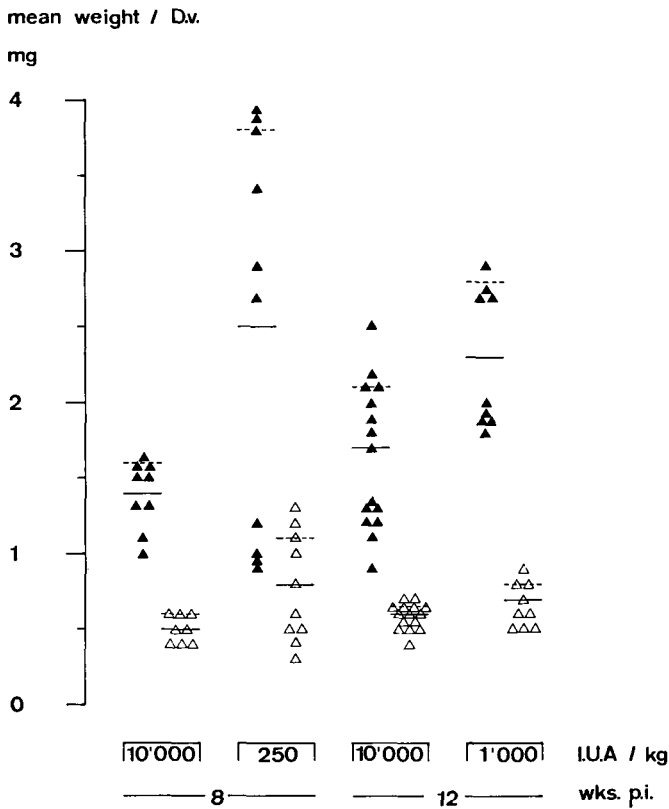
FIG. 3. Mean weight of adult worms recovered per hamster. Data at week 8 are from Expt I, those at week 12 from Expt II. Bars indicate group means, interrupted lines give the standard error (to one side only). ▲ females; △ males.

DISCUSSION

This study demonstrates a strong effect of retinol on the physical condition of hamsters and on growth and reproduction of *D. viteae* worms. It also shows that the host's humoral immune response remains unimpaired during retinol deficiency (even at week 12 p.i. titres were significantly higher in deficient animals), and that the parasite is detrimental to both the growth and the retinol liver reserves of the host but otherwise less harmful than the retinol deficiency.

While this study was in progress STOREY (1982) reported on retinol deficiency in cotton-rats infected with *L. carinii*. Like us, he found better growth of retinol well-fed rats (Rw) than of ill-fed rats (Rd), a more marked influence of retinol than of infection on growth of the rats, longer worms in Rd than in Rw rats, and serum retinol concentrations in proportion to the diet but uninfluenced by infection. However, Storey found an increased recovery rate in Rd rats, and retarded larval development in female worms recovered from them. Possible explanations for this variance are: (i) the type of retinol depletion, maintenance diet and parasitological parameters used are different in the two studies; (ii) it may be more difficult to recover *D. viteae* from the connective tissue of hamsters than *L. carinii* from the pleural cavity of cotton-rats, although in the former model it is easier to quantify the infective dose than in the latter. (iii) the metabolism of retinol in *L. carinii* is different from that in *D. viteae*. We regard this explanation as the most probable. In sharp contrast to *D. viteae* we have found a

median retinol concentration of 12·6 I.U./g in adult *Onchocerca volvulus* from man (STÜRCHLER *et al.*, 1981). In adult female and male *D. viteae* and in microfilariae all obtained from hamsters on commercial diet (NAFAG 850) we have found retinol concentrations of 0·18, 0·20 and 0·01 I.U./g respectively (D. Stürchler, M. Tanner and A. Hanck, unpublished), indicating that the retinol demands of a parasite species may vary with the diet the host is receiving.

It might be argued that inanition and hence protein-calorie malnutrition contributed to the differences between Rw and Rd animals observed in our study. This is not likely, however, since opposite effects are observed in experimentally protein deficient rats which are infected with *L. carinii*, that is a prolonged prepatency, depressed parasitaemia and decreased worm length (JAYAPRAGASAM *et al.* 1977; STOREY 1981).

While some nutritional effects on parasites may be mediated by the altered immune response of the host (ISLIKER & SCHUERCH 1981), others are not, as has been shown by PRASAD *et al.* (1980 b) in pyridoxine deficient rats that were refractory to experimental *L. carinii* infection, although their humoral immune response seemed depressed. In our experiments the retinol effects seem unlikely to be mediated through an altered immune response which was of the expected quality also in Rd animals. How then do adult *D. viteae* females prosper so well in retinol deficient hamsters? A causal relationship is suggested (i) by the negative correlation between retinol liver concentration and average female worm weight in severely depleted hamsters, (ii) by the slower restoration of liver reserves in infected than in uninfected hamsters, (iii) by the fact that bulky female worms grow in retinol-exhausted hosts. Is an adequate host tissue retinol concentration inhibitory of growth and reproduction of female *D. viteae*? Studies on the metabolism of *Brugia pahangi* indicate that this filariid takes up and incorporates retinol, metabolizing it to substances which may be involved in filarial glycoprotein synthesis (COMLEY & JAFFE 1983).

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