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Carotene dioxygenase (*EC* 1.13.11.21) activity in rat intestine: effects of vitamin A deficiency and of pregnancy

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1. Female weanling rats were fed on a purified diet containing either no vitamin A, apart from traces present in casein (deficient groups), or the same diet containing 1·55 mg retinol as retinyl acetate/kg (control groups). In one experiment the deficient groups were given 1 μg retinol/d after 10 weeks, to permit successful reproduction under conditions of marginal vitamin A status. A proportion were mated at 11 weeks after weaning, and fetal development was permitted for 7 d or for 20 d before killing.

2. Carotene dioxygenase (*EC* 1.13.11.21) activity was measured in a supernatant fraction from intestinal mucosal scrapings. For each group, activity was 20–30% greater in the vitamin-A-deficient animals than in the controls, and the difference reached statistical significance for the virgin and 7 d pregnant animals in the first experiment (severe deficiency) and for the 20 d pregnant animals in the second experiment (less-severe deficiency).

3. It is suggested that low tissue vitamin A levels may feedback to increase carotene dioxygenase activity, by mechanisms at present unknown, presumably to ensure a more efficient use of precursor dietary carotenoids.

Little is known about the existence of mechanisms whereby an inadequate supply of trace nutrients may induce metabolic adjustments to improve or conserve supplies, either in humans or in animals. In the case of vitamin A, a possible control point is the enzyme carotene dioxygenase (*EC* 1.13.11.21) in the intestine, which is likely to be rate-limiting for carotenoid utilization and hence the supply of vitamin A under some conditions. Since vitamin A is derived almost entirely from dietary carotenoids in those human populations where deficiencies of this nutrient frequently occur, the existence of a feedback control mechanism affecting this enzyme could be of considerable biological importance. Previous studies of this enzyme have explored its mode of action (Goodman & Huang, 1965; Olson & Hayaishi, 1965; Goodman *et al.* 1966, 1967; Goodman, 1969; Olson, 1969; Olson & Lakshmanan, 1970) and its response to variations in dietary protein levels (Gronovska-Senger & Wolf, 1969), the latter indicating the possibility of diet-induced modulation of activity in an experimental animal model. The purpose of the present study was to examine the effects of dietary vitamin A deficiency and pregnancy on carotene dioxygenase in a rat model. Pregnancy both increases the demand for most nutrients and induces hormonal and other adaptive changes which might have an effect on enzyme activity. It is associated with vitamin A deficiency symptoms in human subjects (Bates, 1983).

A brief description of the present study has been published as an abstract (Villard & Bates, 1984).

MATERIALS AND METHODS

Female Norwegian hooded or Sprague-Dawley albino rats were housed in suspended wire cages at constant temperature and cycles of light and darkness, and received a purified diet from weaning which contained (g/kg): sucrose 706, casein 'low in vitamins' (British Drug Houses, Poole, Dorset) 210, Briggs salt mixture (Greenfield *et al.* 1969) 50, arachis oil 30, choline chloride 2, cystine 1·5, calcium pantothenate 0·02, thiamin hydrochloride 0·004,

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riboflavin 0·015, pyridoxine hydrochloride 0·009, nicotinamide 0·025, biotin 0·001, folic acid 0·001, cyanocobalamin 5×10^{-5}, α-tocopherol 0·16, menadione 0·009, vitamin D_2 $7{\cdot}5 \times 10^{-6}$.

The retinol content of the casein was measured by high pressure liquid chromatography (HPLC) of a hexane extract of saponified material, and was shown to be 0·38 μg/g, which is equivalent to a retinol intake of 1·3 μg/d by rats who were eating approximately 16 g diet/d. No carotene was detectable in the diet. In the first experiment, the deficient group received this diet for 52 d after weaning at 23 d of age, and then continued to receive it after mating and during 7 or 20 d of pregnancy. Since this regimen produced a poor 20 d pregnancy outcome, in a second experiment the 'deficient' animals received the same unsupplemented diet for 68 d after weaning, but were then given an additional 1 μg retinol as retinyl acetate for 11 d before mating, and during their subsequent pregnancy (Moore, 1957). A further difference between the two experiments was the strain of rats, which were Norwegian hooded in Expt 1 and Sprague–Dawley albinos in Expt 2. In both experiments the control group of animals received the same purified diet to which retinyl acetate had been added in an amount sufficient to yield 1·55 mg retinol/kg diet, or approximately 30 μg/d, which was sufficient to maintain normal development and support normal pregnancy in all the animals. The control animals were pair-fed, as a group, with the deficient animals from an early stage up to the time of mating, after which pair-feeding became impracticable.

All except the animals in the virgin groups were individually mated with healthy males fed on a chow diet, and day 1 of pregnancy was counted as the day when a sperm plug was found beneath the cage. In both experiments the largest number of animals was allocated to the 20-d-pregnant groups, in order to increase the chances of observing normal pregnancy development in a significant proportion of the animals despite the stress of deficiency. Successful pregnancy at 20 d was only observed in Expt 2, although the fetuses developed normally until day 7 in both experiments.

The animals were killed on the appropriate day after an overnight fast, by diethyl ether anaesthesia followed by exsanguination, and intestinal mucosal scrapings were prepared, as described later, for the carotene dioxygenase assay. In Expt 2, samples of liver were removed and plasma was separated from heparinized blood samples; these samples were then stored at −20° for vitamin A analyses which are described later.

Carotene dioxygenase assay (based on Goodman et al. *1967)*

The proximal 600 mm of small intestine was removed, saline (9 g sodium chloride/l)-washed free from lumen contents and mucus, and was then slit open longitudinally and spread on an ice-cooled glass plate to expose the mucosa. The mucosa were scraped with the edge of a microscope slide, and the scrapings were suspended at 0° in 6 ml of a buffer, pH 7·7, containing (mol/l): potassium phosphate 10^{-1}, nicotinamide 3×10^{-2}, magnesium chloride 4×10^{-3}. They were gently homogenized in a Potter–Elvehjem homogenizer with loose-fitting pestle, and centrifuged for 15 min at 104000 ***g***. The supernatant fraction was collected and its protein content measured by Biuret assay (Gornall *et al.* 1949).

Sufficient supernatant to provide 5 mg protein was incubated in portions with 2 ml of an incubation mixture, pH 7·7, containing (mol/l): potassium phosphate 2×10^{-4}, nicotinamide 3×10^{-5}, magnesium chloride 4×10^{-6}, sodium dodecyl sulphate 12×10^{-6}, reduced glutathione 1×10^{-5}. It also contained L-α-phosphatidyl choline (Sigma, Poole, Dorset; type III-E) 0·2 g/l, α-tocopherol (dissolved initially in acetone) 0·125 g/l and [15,15′−^{3}H]β-carotene 0·4 mg ($7{\cdot}5 \times 10^7$ disintegrations/min)/l. Incubation was performed in 25 ml Erlenmeyer flasks for 35 min in a shaking water-bath in the dark. The reaction was then stopped by addition of unlabelled β-carotene (60 μg) and retinal (100 μg), and the lipid-soluble components were extracted into 3 ml chloroform–methanol (2:1, v/v).

Sulphuric acid (10^{-2} M; 5 ml) was added and the mixture was shaken vigorously and then centrifuged for 15 min at 500 ***g***. The organic layer was transferred, evaporated under nitrogen, redissolved in toluene (60 μl) followed by addition of *n*-hexane (500 μl), and 50 μl portions were chromatographed on a 20 mm column of 10% water-deactivated neutral alumina (Woelm) in a Pasteur pipette. Four eluent fractions were collected: 5 ml hexane (β-carotene fraction), 4 ml hexane (wash fraction), 7 ml hexane–toluene (1 : 1, vlv) (retinal fraction) and 5 ml toluene (retinol fraction). This elution schedule had been tested and optimized in a preliminary experiment. The fractions were mixed with 5 ml scintillant containing (/l toluene) 5 g 2,5-diphenyloxazole and 0·3 g 1,4-di-(2-(5-phenyl oxazolyl))-benzene, and were counted for 20 min in a Packard model 2245 liquid-scintillation counter, with external standard quench calibration. The weight of β-carotene converted to retinal + retinol was calculated from the ratio, radioactivity in the third and fourth eluent fraction: that in the first eluent fraction, and from the known weight of β-carotene added to the incubation mixture. Blank correction was made for the small, constant amount of radioactivity which appeared in the retinol + retinal fractions from 'zero time' incubations of reagents and extracts. No increase in conversion was observed on incubating the reagents alone in the absence of enzyme preparation.

Vitamin A assay in plasma and liver samples

Plasma was stored at −20° for short periods before analysis; 0·5 ml was mixed with 0·5 ml water and 1·0 ml ethanol containing retinyl acetate as internal standard. After extraction twice with 2 ml *n*-hexane, the hexane extracts were evaporated under N_2, were redissolved in diethyl ether (40 μl) followed by methanol (80 μl), and were chromatographed on a HPLC column, 250 × 3 mm, of μ-Bondapak C_{18} with 50 ml water/l methanol as eluent, flowing at 2 ml/min (International Vitamin A Consultative Group, 1982). Optical density was monitored with a Cecil CE 272 spectrophotometer at 328 nm and peak height ratios were used for quantitative determination.

Frozen liver samples were homogenized in a volume equal to their wet weight of distilled water in a Potter–Elvehjem homogenizer and 1 ml portions were saponified with 5 ml ethanol containing potassium hydroxide, 50 g/l, for 30 min at 60° in a N_2 atmosphere. Retinol was extracted into 5 ml cyclohexane and its concentration measured in a Perkin–Elmer MPF3 spectrofluorimeter with excitation setting 330 nm (4 nm slit) and emission setting 480 nm (40 nm slit), calibrated with retinyl acetate as external standard.

RESULTS

Fig. 1 shows the growth curves of the rats from weaning up to mating in the two experiments. In Expt 1, the growth curve of the deficient group was indistinguishable from that of the pair-fed controls, whereas in Expt 2 the mean weight of the deficient animals was about 10% lower than that of the controls, possibly because pair-feeding was delayed by 19 d at the start of the special-diet feeding schedule. In Expt 2 there was clear evidence of growth-faltering at about 75–91 d of age, which was matched in the control group, and was reversed by an additional 1 μg retinol/d to the diet. In both experiments, the fur of the deficient animals was showing signs of roughness, irritability was increased and, in the second experiment, there was a slight ataxia in the latter stages. In Expt 1, thickening of the bladder wall through infiltration by keratinized cells was a very obvious pathological feature in the deficient animals at autopsy (cf. Moore. 1957).

In Expt 1, all seventeen control animals which were mated supported apparently normal pregnancies either for 7 d (*n* 6) or for 20 d (*n* 11) whereas the deficient animals supported pregnancy only for 7 d (*n* 6) but not for 20 d (*n* 13) since all the latter group resorbed their

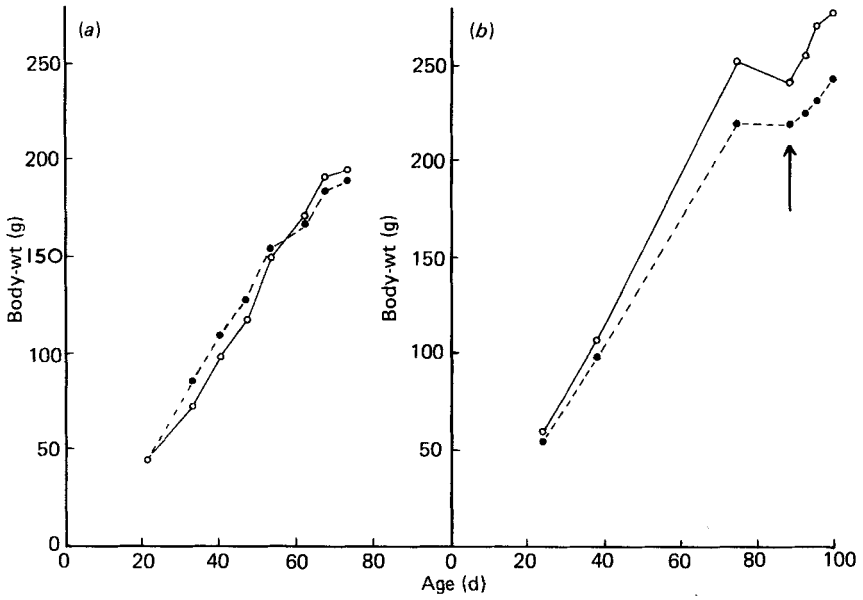

Fig. 1. Growth of control and vitamin-A-deficient rats before mating in (*a*) Expt 1 and (*b*) Expt 2. (○—○), Control; (●—●) vitamin-A-deficient. Mean coefficient of variation at each time-point: Expt 1, 6·7%, Expt 2, 7·5%. ↑, Time-point at which an additional 1 μg vitamin A/d supplement was introduced.

Table 1. *Body-weights (g) of rats at the time of killing*
(Mean values with their standard errors)

	Control group			Deficient group		
	Mean	SE	*n*	Mean	SE	*n*
Expt 1:						
Virgins	219	3	7	212	6	3
7 d pregnant	224	8	6	219	5	6
20 d pregnant	279	6	11	196	5	13
Expt 2:						
Virgins	308	6	7	267	14	5
7 d pregnant	294	8	4	274	4	8
20 d pregnant	378	10	10	333	10	12

fetuses in mid-pregnancy. The failure of pregnancy by 20 d in the deficient animals in Expt 1 is further illustrated by the final body-weights at the time of killing (Table 1). In Expt 2, all except one (which was discarded) of the animals which were mated supported apparently normal pregnancies until the time of killing, including the twelve animals in the deficient group which were killed on day 20 of pregnancy (Table 1). Weights of reproductive organs, including fetuses, were identical between the control and deficient groups at each stage of pregnancy in Expt 2.

Table 2 shows the vitamin A concentrations in plasma and liver of the animals in Expt 2. It is clear that whereas the hepatic retinol content of most of the control animals varied between 80 and 100 μg/g, that of the deficient group was much lower, with mean values

Table 2. *Expt 2. Hepatic and plasma vitamin A concentrations in control and vitamin-A-deficient rats*

(Mean values with their standard errors)

	Retinol concentration											
	Hepatic† (μg/g)						Plasma (μg/l)					
	Controls			Deficient			Controls			Deficient		
	Mean	SE	*n*	Mean	SE	*n*	Mean	SE	*n*	Mean	SE	*n*
Virgins	96	4	7	3***	2	5	640	100	7	260**	80	5
7 d pregnant	82	4	4	4***	2	8	600	70	4	250**	30	8
20 d pregnant	78	6	10	4***	2	12	420	40	10	100**	20	12

Mean values for deficient and control groups were significantly different (Student's *t* test): ** $P < 0{\cdot}002$, *** $P < 0{\cdot}001$.

† Retinol released by saponification.

of 3 or 4 μg/g. The difference in plasma retinol concentrations between the two groups was also highly significant, although less marked than the difference in hepatic levels. Both groups showed a fall in plasma retinol levels as pregnancy progressed, which was not paralleled by a change in hepatic levels. Although vitamin A analyses are not available from Expt 1 (because the samples were destroyed in an accident), a parallel experiment with the same strain of rats maintained for similar periods of time yielded the following mean (with SE) hepatic retinol levels (μg/g): controls (receiving a chow diet) 67·0 (SE 12) (*n* 5), deficient animals (receiving the purified vitamin-A-deficient diet), 4·4 (SE 1·8) (*n* 5); deficient animals which had received an additional 1 μg retinol/d for 3 weeks following 9 weeks of severe deficiency, 5·1 (SE 1·3) μg/g (*n* 5). It is thus clear that the two strains of rats did not differ greatly in their hepatic retinol reponse to the deficient diet, and that an additional 1 μg/d did not raise the hepatic store substantially.

Table 3 shows the carotene dioxygenase activities observed in the two experiments; the specific activity of the enzyme was consistently higher in the deficient than in the control groups, and significantly so in three of the six groups examined. The differences in carotene dioxygenase activity between deficient and control groups were similar if the activities were expressed per g or per mm intestine instead of being expressed per unit supernatant protein. (The 20-d-pregnant animals in Expt 1 are not strictly comparable between the two groups because the fetuses had resorbed in the deficient animals.)

DISCUSSION

The growth curves in the two experiments, together with the onset of mild pathological signs of vitamin A deficiency about 7 weeks after weaning at the lowest level of vitamin A intake suggested that a purified diet providing the equivalent of about 1·3 μg retinol/d was barely sufficient to maintain minimally-adequate amounts of the vitamin for growth and normal health in weanling rats of both strains, and the observations in Expt 1 showed that it was insufficient to support the maintenance of pregnancy up to parturition. Addition of a further 1 μg retinol as retinyl acetate/d was sufficient to restore growth and to support apparently normal pregnancy at least to day 20, without substantially increasing the hepatic stores of the vitamin. Intakes of about 30 μg retinol/d provided sufficient for substantial hepatic stores. It is clear from these observations, as has been reported previously (Moore, 1957;

Table 3. *Intestinal carotene dioxygenase* (EC *1.13.11.21*) *activities in control and vitamin-A-deficient rats*

(Mean values with their standard errors)

	Carotene dioxygenase activity (μmol retinal and retinol formed/mg protein per min $\times 10^{-7}$)					
	Control group			Deficient group		
	Mean	SE	*n*	Mean	SE	*n*
Expt 1:						
Virgins	5·4	0·3	7	6·2**	0·3	3
7 d pregnant	4·3	0·2	4	6·7***	0·6	6
20 d pregnant†	4·9	0·3	11	5·6	0·4	13
Expt 2:						
Virgins	2·4	0·1	7	2·9	0·4	5
7 d pregnant	2·4	0·3	4	3·4	0·3	8
20 d pregnant	2·8	0·2	10	3·7*	0·3	12

† Pregnancy was not supported for as long as 20 d in the deficient group in this experiment (see p. 116).

Mean values were significantly different from those of the control group (Student's *t* test): * $P < 0·02$, ** $P < 0·01$, *** $P < 0·001$.

Takahashi *et al* 1975) that the vitamin A requirement to support normal outcome of pregnancy is substantially greater than that needed for maintenance of the non-pregnant animal. It is also clear that early resorption, not failure to conceive, is the usual result of inadequate vitamin supply.

Preliminary experience with the carotene dioxygenase assay generally supported the observations of Goodman (1969) and of Olson (1969) and showed that a simplified product separation procedure could be achieved by the use of disposable Pasteur pipette microcolumns of alumina. Dependence of the assay on enzyme concentration and incubation time was studied, and a suitable compromise was chosen between optimum linearity of response and optimum sensitivity of product detection.

Using this optimized procedure it was shown that animals in the vitamin-A-deficient groups consistently had higher dioxygenase specific activities of the intestinal carotene dioxygenase than those in the control groups. The difference reached statistical significance for the virgin and 7-d-pregnant animals in Expt 1, but not for the 20-d-pregnant animals, in whom the resorption of fetuses may have temporarily alleviated the shortfall of circulating vitamin. In Expt 2, where the deficient animals had a somewhat higher intake of the vitamin, and where pregnancy was supported to day 20, the most marked and significant difference between the deficient and control groups occurred in the 20-d-pregnant animals (although this is partly a result of higher numbers in this group). No consistent or significant effect of pregnancy *per se* on the enzyme was observed, in either the deficient or the sufficient groups; however this effect of vitamin A deficiency may be greater in pregnant than in non-pregnant animals receiving similar diets.

No significant differences were observed between the groups in the yield of supernatant protein from the mucosal homogenates, thus eliminating the possibility that the difference in specific activity could have been due to differences in overall soluble-protein content of the mucosa. Since neither group of rats had any detectable amount of carotene in their diets, and since both groups had the same basic diet, it is very improbable that traces of carotene

in the mucosal preparation could have affected the result. The pair-feeding protocol eliminated the possibility that differences related to body size or growth rate could have been responsible for the observed differences.

Absolute differences in specific activity between the two experiments were most probably due to differences between the two strains of rats used. However, it is significant that a similar pattern of response to vitamin A deficiency was observed in both strains of rat, suggesting that some general significance may be attached to this phenomenon.

The only previous report of dietary modulation of carotene dioxygenase activity is that of Gronovska-Senger & Wolf (1969) who showed that the activity is deceased in the intestine of protein-deprived animals. Mittal (1983) reported that rats fed on carotene as the only source of vitamin A and later made deficient, responded better (in terms of plasma retinol) to carotene-based repletion than those fed on retinol, but the biochemical basis for this adaptation was not investigated. There have been reports that the level of dietary protein may affect the hepatic deposition of retinol formed from carotene (Jagannathan & Patwardhan, 1960; Deshmukh & Ganguly, 1964; Kamath *et al.* 1972; Kamath & Arnrich, 1973), that the quality of protein may be important (Berger *et al.* 1962; Geervani & Devi, 1981), and that dietary fat can also affect hepatic deposition (Korycka *et al.* 1969; Jayarajan *et al.* 1980: Geervani & Devi, 1981). The nature of the carotene source (Rajalakshmi *et al.* 1975) and the magnitude of the carotene load (Bondi & Sklan, 1984) affect the efficiency of intestinal uptake, and the presence of bile salts (El-Gorab *et al.* 1975) or of zinc deficiency (Honory, 1978; Takruri & Thurnham, 1981) may affect efficiency of conversion. Some of these effects may be mediated by variations in carotene dioxygenase activity, but no evidence is currently available.

The response of carotene dioxygenase to variations in vitamin A status, reported in the present study, is of particular interest in representing possibly a specific response to the deprivation of a particular micronutrient, acting on a metabolic pathway which is involved uniquely in its own economy. The mechanism of the increase in activity seen in the deficient animals is at present unknown. It may arise through synthesis of new enzyme protein by transcriptional or translational induction, by a reduction in enzyme turnover, by changes in the maturation and turnover of the brush-border epithelium, or by changes in modulatory components which may affect enzyme activity without altering the total amount of enzyme present. Further studies are needed to distinguish between these (and other) possible mechanisms.

Questions clearly also remain about the relevance of changes in dioxygenase activity for carotene availability and the vitamin A economy of the animal. A pilot experiment carried out in the authors' laboratory has demonstrated some intriguing differences in the handling of dietary carotene between control and vitamin-A-deficient rats, but while the patterns observed evidently reflected a complex balance between the absorption, conversion, distribution and retention of carotene, they could not be interpreted simply on the basis of the observed changes in dioxygenase activity. Further studies are obviously needed to clarify the relation between carotene dioxygenase activity and other metabolic controls, and overall carotene and vitamin A economy.

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REFERENCES

Bates, C. J. (1983). *Proceedings of the Nutrition Society* **42**, 65–79.
Berger, S., Rechiegl, M., Loosli, J. K. & Williams, H. H. (1962). *Journal of Nutrition* **77**, 174–178.
Bondi, A. & Sklan, D. (1984). *Progress in Food and Nutrition Science* **8**, 165–191.
Deshmukh, D. S. & Ganguly, J. (1964). *Indian Journal of Biochemistry* **1**, 204–207.
El-Gorab, M. I., Underwood, B. A. & Loerch, J. D. (1975). *Biochimica et Biophysica Acta* **401**, 265–277.
Geervani, P. & Devi, A. (1981). *Indian Journal of Medical Research* **74**, 548–553.
Goodman, D. S. (1969). *American Journal of Clinical Nutrition* **22**, 963–965.
Goodman, D. S. & Huang, H. (1965). *Science* **149**, 879–880.
Goodman, D. S., Huang, H., Kanai, M. & Shiratori, T. (1967). *Journal of Biological Chemistry* **242**, 3543–3554.
Goodman, D. S., Huang, H. & Shiratori, T. (1966). *Journal of Biological Chemistry* **241**, 1929–1932.
Gornall, A. G., Bardawill, C. J. & David, M. M. (1949). *Journal of Biological Chemistry* **177**, 751–766.
Greenfield, H., Briggs, G. M., Watson, B. H. J. & Yudkin, J. (1969). *Proceedings of the Nutrition Society* **28**, 43A.
Gronovska-Senger, A. & Wolf, G. (1969). *Journal of Nutrition* **100**, 300–308.
Honory, K. (1978). *Bulletin of the Veterinary Institute of Pulawy* **22**, 77–80.
International Vitamin A Consultative Group (1982). *Biochemical Methodology for the Assessment of Vitamin A Status*. Washington, DC: Nutrition Foundation.
Jagannathan, S. N. & Patwardhan, V. N. (1960). *Indian Journal of Medical Research* **48**, 775–784.
Jayarajan, P., Reddy, V. & Mohanram, M. (1980). *Indian Journal of Medical Research* **71**, 53–56.
Kamath, S. K. & Arnrich, L. (1973). *Journal of Nutrition* **103**, 202–206.
Kamath, S. K., MacMillan, J. B. & Arnrich, L. (1972). *Journal of Nutrition* **102**, 1579–1584.
Korycka, M., Bialek, T., Miler, M., Chabrowski, K. & Berger, S. (1969). *Acta Physiologica Polonica* **20**, 662–667.
Mittal, P. C. (1983). *Nutrition Reports International* **28**, 181–188.
Moore, T. (1957). *Vitamin A*. Amsterdam, London, New York, Princeton: Elsevier Publishing Co.
Olson, J. A. (1969). *American Journal of Clinical Nutrition* **22**, 953–962.
Olson, J. A. & Hayaishi, O. (1965). *Proceedings of the National Academy of Sciences, USA* **54**, 1364–1369.
Olson, J. A. & Lakshmanan, M. R. (1970). In *The Fat Soluble Vitamins*, pp. 213–226 [H. F. DeLuca and J. W. Suttie, editors]. Madison: University of Wisconsin Press.
Rajalakshmi, R., Chari, K. V., Advani, M., Chary, T. M., Patel, M. A., Mutalik, A. M., Bhat, T. H. & Vyas, A. D. (1975). *Baroda Journal of Nutrition* **2**, 103–119.
Takahashi, Y. I., Smith, J. E., Winick, M. & Goodman, D. S. (1975). *Journal of Nutrition* **105**, 1299–1310.
Takruri, H. R. H. & Thurnham, D. I. (1981). *Proceedings of the Nutrition Society* **41**, 53A.
Villard, L. & Bates, C. J. (1984). *Proceedings of the Nutrition Society* **44**, 15A.

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