Effect of vitamin A deficiency on protein catabolism in chicks

By I. BRUCKENTAL, I. ASCARELLI and A. BONDI

*Department of Animal Nutrition and Agricultural Biochemistry,
Faculty of Agriculture, Hebrew University, Rehovot, Israel*

1. The changes in the concentration of some enzymes and their metabolites were studied in the first stages of vitamin A deficiency in chicks.

2. Kidney arginase (*EC* 3.5.3.1) and liver xanthine dehydrogenase activities had increased even before complete disappearance of vitamin A from the plasma. Similarly, an increase was found in plasma uric acid, and plasma urea also increased but to a lesser extent. Liver proteolytic activity also was slightly increased by vitamin A deficiency.

3. Kidney D-amino acid oxidase (*EC* 1.4.3.3) activity and plasma concentrations of total protein and free amino acids were not affected, at least in the first stages of the deficiency.

4. Oral dosing of deficient chicks with retinyl palmitate to provide 300 μg retinol, 24 h before killing, brought about a decrease in the activities of both enzymes and of plasma uric acid, and an increase in plasma urea.

5. Dietary levels of vitamin A were reflected not only in the liver concentrations of the vitamin but also in the plasma concentrations.

Some authors have drawn attention to the importance of vitamin A in the biosynthesis of proteins and other compounds of metabolic interest. Vitamin A deficiency has been shown to cause a decrease in the concentration of enzymes connected with the activation of inorganic sulphur (Varandani, Wolf & Johnson, 1960), in the biosynthesis of glycopeptides in intestinal goblet cells (DeLuca, Schumacher & Wolf, 1970) and the biosynthesis of corticosteroids (Grangaud, Nicol & Desplanques, 1969). Most workers agree today that vitamin A is not acting as a coenzyme (Rogers, 1969); however, it may be that lack of this vitamin causes derangements in the biosynthesis of enzyme proteins. Indeed, Chiba, Brady & Johnson (1971) showed that there is a decrease in the synthesis of rapidly labelled nuclear RNA.

It is very difficult to establish if the effects found are a primary consequence of the deficiency or a secondary one. Most of the work on vitamin A deficiency has been done with rats, i.e. with animals in which the development of this deficiency is relatively slow. On the contrary, the chick can be rapidly depleted during its period of most rapid growth, quickly and without excessive concern for the many secondary changes that occur with prolonged depletion (Pitt, 1966). However, vitamin A deficiency has been investigated much less in the chick than in the rat, especially with regard to its effects on protein metabolism. Elvehjem & Neu (1932) found a rise in plasma uric acid in vitamin A-deficient chicks; they also mentioned the deposition of urates in the excretory system. Stoewsand & Scott (1961 a), working with poults, also found that plasma uric acid concentration was increased by vitamin A deficiency; however, in subsequent experiments with chicks, no increase was found, in spite of the fact that the test was carried out at an advanced stage of deficiency. A similar lack of

response in chicks had also been mentioned by Lowe, Morton, Cunningham & Vernon (1957).

The reduction in growth caused by lack of vitamin A indicates the occurrence of marked changes in the rate of protein metabolism. Accordingly it has been our purpose to assess the influence of the deficiency on some processes of protein metabolism before the stage was reached at which growth is affected.

EXPERIMENTAL

Animals and diets

All chicks were cross-bred male New Hampshire × White Leghorn. They were kept in thermostatically controlled, electrically heated batteries equipped with raised wire-mesh floors.

Semi-synthetic diets were used of the type described by Nir & Ascarelli (1967). The different protein levels were obtained by altering the amounts of soya-bean meal, fortified with 20 g DL-methionine/kg, included in the diet. The dietary protein level was 240 g/kg, unless otherwise stated.

The diets were fortified with stabilized gelatin-coated beadlets of retinyl palmitate (Chas. Pfizer and Co. Inc., New York); the levels of supplementation will be expressed as the corresponding retinol equivalents.

In all the experiments the control group was fed on a diet containing 900 μg retinol/kg.

Expt 1. Day-old chicks were maintained on a vitamin A-free diet (basal diet) or on the same diet supplemented with 900 or 9000 μg retinol/kg. Every treatment included six replicates. At 1, 3, 5, 8, 11, 15 and 18 d of age, one chick was taken at random from each replicate and its liver and plasma were assayed for vitamin A.

Expt 2. Chicks having been depleted of their vitamin A stores over a 2-week period were maintained on the basal diet but twice a week were dosed by mouth to provide 0, 15, 30, 45, 60, 90 or 150 μg retinol per week. For each treatment, there were three replicates of eight chicks each. The trial was continued for a period of 32 d during which the chicks were weighed every 3rd day.

Expt 3. This experiment was a 3 × 2 factorial design in which the effect of the vitamin A deficiency obtained by giving a dietary level of 150 instead of 900 μg/kg diet was measured in the presence of three different levels of dietary protein, namely 60, 240 and 360 g/kg. Chicks were depleted of their vitamin A stores for 2 weeks and were then divided into groups of eight chicks each, three replicates being assigned to each treatment. When the chicks were 5 weeks old, three samples of two chicks each were taken from every group, the samples having been selected at the start of the experiment. The blood, livers and kidneys from each sample were pooled for analysis.

Expt 4. Day-old chicks were fed on the basal diet or on the same diet fortified with 900 μg retinol/kg (Expt 4A). For every treatment there were six replicates of eighteen chicks each. When 1, 6, 11, 15 and 22 d old, three chicks were taken at random from each group; the blood, livers and kidneys of the chicks from each

replicate were pooled and the following assays were carried out: liver xanthine dehydro-genase (XDH), kidney arginase (*EC* 3.5.3.1) and plasma urea, uric acid, total protein, albumin and globulins. Moreover, liver proteolytic and kidney D-amino-acid oxidase (*EC* 1.4.3.3) activities were measured in five individual chicks from each treatment at the age of 20 or 28 d. At the latter age, the plasma from the five chicks was pooled and assayed for free amino acid concentrations. Liver proteolytic and kidney D-amino-acid oxidase activities were assayed also in five individual chicks in a similar experiment (4B) in which the effect of protein deficiency (60 g protein/kg diet) was also investigated.

Expt 5. Day-old chicks were maintained on the basal diet or on the same diet supple-mented with 150, 900 or 9000 μg retinol/kg. The chicks were divided into groups of eighteen each and five replicates were assigned to every treatment. When 16 d old, half of the chicks were dosed by mouth with retinyl palmitate in aqueous solution, to provide 300 μg retinol. Twenty-four hours later, four dosed and four undosed chicks were taken at random from every group; their blood, livers and kidneys were pooled and the same assays were carried out as in Expt 3.

Preparation of vitamin A solution. An aqueous solution of retinyl palmitate was prepared by the technique described by Nir, Bruckental & Ascarelli (1967). The solution was made to contain 300 mg retinol/l, and 1 ml was given by mouth with a tuberculin syringe.

Analytical methods

Liver vitamin A was measured according to Ames, Risley & Harris (1954), and plasma vitamin A as described by Nir & Ascarelli (1966). Vitamin A concentration was determined by the Carr-Price reaction.

Enzyme assays. Liver XDH activity was determined by Udenfriend's (1962) fluori-metric method for rat liver xanthine oxidase, but with the addition of 300 μg methylene blue/ml reaction mixture as hydrogen acceptor. This addition is necessary since chick liver contains this enzyme, according to Remy, Richert & Westerfeld (1951), as a dehydrogenase, whereas rat liver contains an analogous oxidase (*EC* 1.2.3.2). One unit of activity was defined as the amount of enzyme which causes an increase of 1 % in the fluorescence intensity of a 3 ml reaction mixture after incubation at 30° for 60 min at pH 7·2, the intensity emitted by a quinine solution (1 part/10^6) in 0·05 M-H_2SO_4 being taken as 100 %.

Kidney arginase activity was measured as described by Dror & Gertler (1967). One unit of activity was defined as the amount of enzyme which causes the release of 1 μmol urea/min at 37°.

Liver proteolytic activity was determined by Kunitz's method as described by Nitsan & Alumot (1960). To adapt the method for the determination of the relatively low activity in chick liver, the following modification was used: the reaction was started by the addition of 2 ml of a casein solution (25 g/l) in 0·1 M-phosphate buffer, pH 8·0, to 5 ml of liver homogenate (100 g/l 0·14 M-NaCl solution). At zero time, a 1 ml sample of the reaction mixture was transferred into 5 ml trichloroacetic acid (TCA) solution (50 g/l). After 10 min at 37°, another 1 ml sample was similarly

transferred. The two solutions were filtered and the difference in extinction at 280 nm was determined. The results were calculated on the basis of wet weight of tissue.

Kidney D-amino-acid oxidase (D-AAO) was determined by the method of Burton (1955). Oxygen variations in the enzymic system were measured using an YSI Model 53 Biological Oxygen Monitor (Yellow Springs Instrument Co., Yellow Springs, Ohio) equipped with a Clark electrode. The absolute changes in oxygen quantity in the reaction system were determined by calibration with standard solutions. The results were calculated on the basis of wet weight of tissue.

Plasma uric acid concentration was determined by the uricase method of Praetorius & Poulsen (1953), and plasma urea concentration by that of Coulombe & Favreau (1963), in a TCA filtrate (100 g/l). Total protein in plasma or in tissue homogenates was determined by the biuret method as described by Gornall, Bardawill & David (1949).

Plasma protein fractions were separated by a Beckman Model R-199 Microzone (Beckman Instruments, Inc., Fullerton, California) on cellulose-acetate membranes, as described in the Beckman Technical Bulletin no. 7086. The different fractions were determined quantitatively with a Beckman Analytrol. Plasma free amino acid levels were determined using a BC-200 Amino Acid Analyser (BioCal GmbH, München-Gräfelfing, West Germany) after plasma deproteinization by picric acid as described in the BioCal Instrument Handbook.

The results of each experiment were subjected to analysis of variance. Significance among the different groups was tested by the Studentized range test (Federer, 1955). The terms 'significant' and 'highly significant' indicate statistical significance $P < 0.05$ and $P < 0.01$ respectively.

RESULTS

Expt 1. The results are presented in Fig. 1. The increase in plasma vitamin A which occurred in all groups between the 1st and 3rd day after hatching presumably originated from the transfer of vitamin from the yolk to the liver. From the 3rd day onwards, there was a similar decrease in liver and plasma concentration of vitamin A in the chicks given the vitamin-free diet. No remaining vitamin A was detectable in the liver at 15 d of age or in the plasma 3 d later. The increase in dietary vitamin A from 900 to 9000 μg/kg diet was reflected in the amounts of the vitamin stored in the liver. The plasma concentrations of vitamin A also showed similar trends, that of the chicks given the 9000 μg/kg diet remaining in the last days of the experiment at values about twice as high as those of the chicks given the 900 μg/kg diet.

Expt 2. The results, presented in Fig. 2, clearly show that body-weight gains were directly correlated with the log of the weekly vitamin A dose. Indeed the correlation coefficient between these two variables showed a high statistical significance ($r = 0.868, P < 0.01$).

One of the aims of this experiment was to find out at which age it was best to terminate the subsequent experiments. We intended to examine some enzymic activities and metabolites in the initial stages of deficiency, i.e. before the effect on growth becomes apparent. From the growth curves obtained in this experiment it was learnt
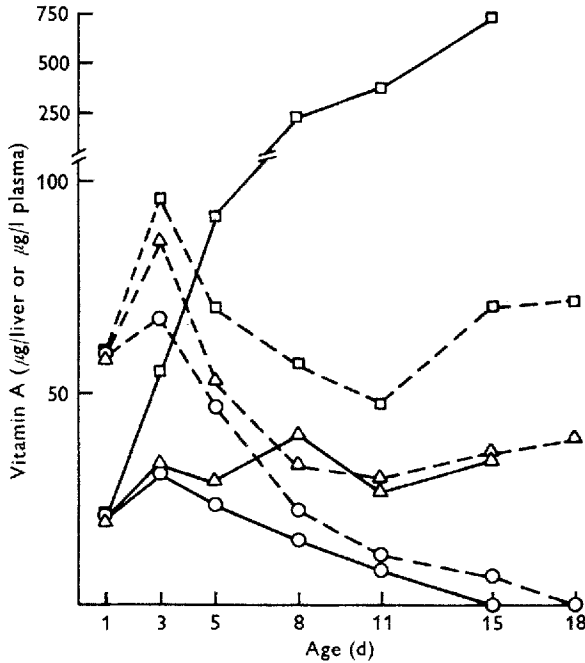
Fig. 1. Expt 1. Changes in total liver and plasma vitamin A concentrations (expressed as retinol) in chicks given diets containing 0, 900 or 9000 μg retinol/kg. ———, liver vitamin A; –––––, plasma vitamin A; O, vitamin A-free diet; △, 900 μg retinol/kg diet; □, 9000 μg retinol/kg diet.
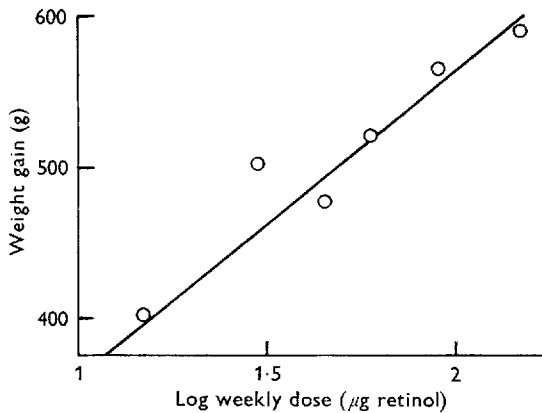
Fig. 2. Expt 2. Dependence of body-weight gains of chicks between ages of 15 and 47 d upon the log weekly dose of retinyl palmitate. Each point represents the mean of three replicates.

that, under our experimental conditions, the growth rate of the chicks given the vitamin A-free diet began to decline during the 3rd week of life. Accordingly, later experiments in which a vitamin A-free diet was given were terminated by the end of the 3rd week. However, it could be deduced from the growth curves also that a marginal dietary level of the vitamin (150 μg/kg diet) would allow the experiments to be prolonged up to the age of 30–35 d: under these conditions the vitamin would be

Table 1. *Expt 3. Comparison of effects of control (C, 900 µg retinol/kg diet) and marginal (M, 150 µg retinol/kg diet) vitamin A intakes, at dietary protein levels of 60, 240 and 360 g/kg, on kidney arginase and liver xanthine dehydrogenase (XDH) activities and plasma urea, plasma uric acid and total vitamin A concentrations in 5-week-old chicks*

(Mean values with their standard errors)

Value at dietary protein level of:

Variable measured	60 g/kg			240 g/kg			360 g/kg			SEM
	C	M	M:C	C	M	M:C	C	M	M:C	
Body-wt gain‡ (g)	88a	88a	—	364c	330bc	—	316bc	305b	—	4·1
Kidney arginase (units*/g wet tissue)	32·0a	27·3a	0·85	94·8b	148·1c	1·56	235·3d	279·0d	1·18	17·3
Plasma urea (mg/l)	9·0a	6·4a	0·71	23·1b	26·8b	1·16	35·1c	40·4c	1·15	1·6
Liver XDH (units*/mg protein)	30·0a	29·0a	0·97	454·0b	570c	1·25	854d	953e	1·11	23·8
Plasma uric acid (mg/l)	11·1a	26·2b	2·36	50·3c	58·8c	1·17	97·9d	103·1d	1·05	4·8
Total liver vitamin A† (µg)	24·3	ND	—	36·6	ND	—	29·7	ND	—	—

ND, not detectable.

Differences between all groups not designated with the same letter are statistically significant ($P < 0.05$).

* See p. 3 for definition.

† Means of only three pooled analyses.

‡ Body-weight gain between the ages of 15 and 35 d.

supplied in such an amount that differences in growth would become apparent only at that age.

Expt 3. The aim of this experiment was to find out at which dietary protein level the effects of marginal lack of vitamin A are most conspicuous. The results presented in Table 1 show that the increase in dietary protein caused significant increases in both the enzymic activities and the plasma concentration of the metabolites assayed. The effects of marginal lack of vitamin A on the different variables were not of the same intensity even at the same dietary protein levels. At the level of 60 g protein/kg diet, lack of vitamin A caused an increase only in plasma uric acid although liver XDH activity was not affected. At the other dietary protein levels, lack of the vitamin caused an increase in all the variables considered, which, however, reached statistical significance only for kidney arginase in the chicks given the 240 g protein/kg diet and for liver XDH at both levels. Since the increases were more marked at the level of 240 g protein/kg diet, this protein level was used in all the subsequent experiments.

Expt 4. Liver XDH activity and plasma uric acid concentration were higher in the deficient chicks at all times during Expt 4A; however, the differences between the treatments became most marked towards the end of the experiment and had reached statistical significance for uric acid at 15 d of age and for XDH at 22 d of age (Fig. 3). Kidney arginase activity also increased as a result of lack of the vitamin, and this increase also reached statistical significance at 15 d of age. Plasma urea, which is very
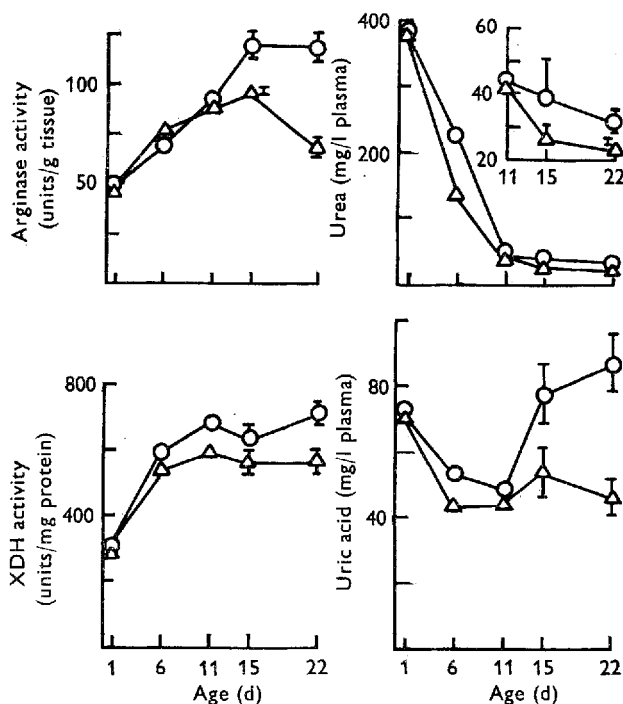
Fig. 3. Expt 4A. Effect of lack of dietary vitamin A upon kidney arginase and liver xanthine dehydrogenase (XDH) activities (for definition of units see p. 3), and plasma urea and uric acid concentrations in chicks. ○, vitamin A-free diet; △, control, 900 μg retinol/kg diet. The vertical lines represent the standard errors of the means.

Table 2. *Expt 4. Levels (expressed as ratios to control values) of liver proteolytic activity and kidney D-amino-acid oxidase (D-AAO) activity in vitamin A-deficient chicks and in protein-deficient chicks*

Expt	Age (d)	Body-wt	Liver proteo-lytic activity	Kidney D-AAO activity
4A: vitamin A-deficient†	20	1·01	1·09	0·88
	28	0·92	1·14	1·20
4B: protein-deficient,*	13	0·73	0·33	0·91
vitamin A-deficient	19	0·90	1·18	0·82

* Protein-deficient diet, 60 g protein/kg; protein control diet, 240 g protein/kg; vitamin A control diet, retinyl palmitate to provide 900 μg retinol/kg.

† Similar to Expt 4A, but both vitamin A and protein deficiencies were investigated.

For both enzymes, the results were calculated on the basis of wet weight of tissue.

high at hatching, decreased greatly during the initial period of the experiment and by day 15 the levels returned to normal. Under the influence of the lack of vitamin A, the concentration of this metabolite increased and by day 22 this increase was statistically significant. Total plasma protein and its fractions, albumin and globulins, showed no significant differences between the two treatments. Vitamin A deficiency caused a consistent, even if not statistically significant, increase in liver proteolytic activity (Table 2), whereas kidney D-amino-acid oxidase activity did not
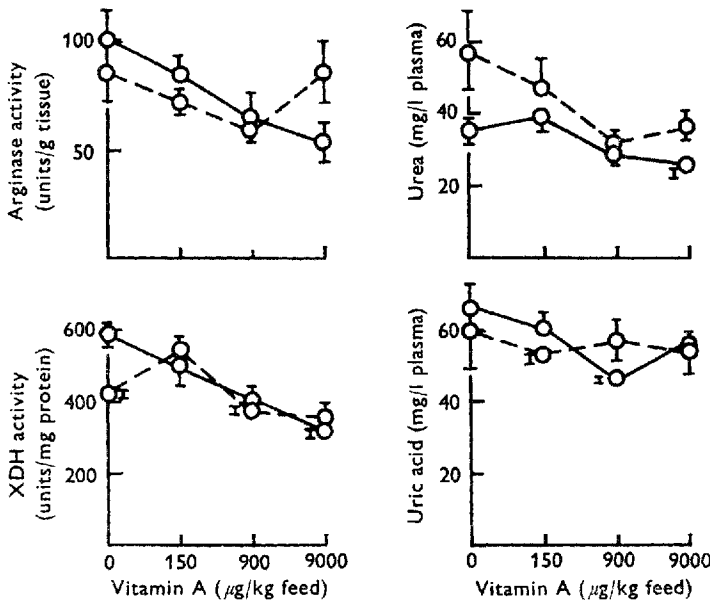
Fig. 4. Expt 5. Effect of dietary level of vitamin A and of dosing with retinyl palmitate to provide 300 μg retinol, 24 h before killing, upon kidney arginase, liver xanthine dehydrogenase (XDH) activities (for definition of units see p. 3), and plasma urea and uric acid concentrations in 17-d-old chicks. ———, undosed; ————, dosed. The vertical lines represent the standard errors of the means.

show a constant trend and no conclusion can be drawn. Included in the table are also results from Expt 4B in which the effect of protein deficiency on these two enzymic activities was measured. It is clear that also in response to this new type of stress the two activities differed, liver proteolytic activity being strongly reduced whereas kidney D-amino-acid oxidase activity was not much affected. Plasma free amino acids were determined at the end of the experiment (28 d of age). No change was caused by vitamin A deficiency in the concentration of total free amino acids or in the concentrations of phenylalanine and tyrosine, which have been reported to be increased in the rat (Malathi, Seshadri Sastry & Ganguly, 1961).

Expt 5. The results presented in Fig. 4 confirmed those of Expt 3. Vitamin A deficiency caused a significant increase in liver XDH and kidney arginase activities and in plasma uric acid concentration, all these changes taking place when growth had not yet been influenced; plasma urea was also increased, though not to a significant extent. The effect of a single dose of retinyl palmitate providing 300 μg retinol was also tested. It can be seen (Fig. 4) that only 24 h after the dosing, there was a decrease in plasma uric acid concentration and in the enzymic activities of the deficient chicks, which reached statistical significance for XDH; in contrast, the plasma urea concentration was consistently increased by the dosing, and this increase was of significant extent in the deficient chicks. At this initial stage of deficiency, the concentrations of plasma protein and of its albumin and globulin fractions were not significantly altered either by lack of the vitamin from the diet or by a single dose of it.

DISCUSSION

During the first few days of the chick's life, there is a transitory increase in plasma and liver concentrations of vitamin A (Fig. 1), which obviously is the result of transfer of the vitamin from the yolk to the liver. The amount of vitamin A in the liver remained stable during the short experimental period only in the control group. Feeding with a diet containing 900 μg retinol/kg caused increases in both liver and plasma vitamin A. According to Moore (1957), there is in rats an efficient mechanism which keeps the plasma vitamin A concentration constant independently of its content in liver or diet. It seems that in chicks such a control mechanism is much less efficient. Whereas in rats' plasma, vitamin A levels begin to decline only after complete depletion of the liver (Dowling & Wald, 1959), a parallel decrease was seen in liver and plasma vitamin A in the chicks fed on the vitamin A-free diet. A similar relationship between chick liver and plasma vitamin A during vitamin A depletion has been previously reported (Ascarelli, 1969). In this work it has also been shown that increasing the dietary vitamin A content tenfold can bring about a doubling of the plasma vitamin A concentration, i.e. a rise which is not obtainable in the rat.

The importance of vitamin A in the growth processes of rats was demonstrated by Bieri, McDaniel & Rogers (1969), who were able to keep non-growing, germ-free rats on a vitamin A-deficient diet for a prolonged period. Similarly, Amine, Corey, Hegsted & Hayes (1970) showed that manifestations of vitamin A deficiency were reduced when growth was hindered by a superimposed iron deficiency, and were precipitated by resumption of growth when the supply of iron was renewed. A direct dependence of growth on the supply of vitamin A was shown by the finding of Ellenberger, Guerrant & Chilcote (1949) that there is a linear relationship between weight gain and the log of the daily vitamin A dose given to rats. Olsen, Harvey, Hill & Branion (1959) did not find a similar dependence in chicks, apparently because (1) they did not deplete their chicks before the beginning of the experiment and (2) the treatments used by them included different dietary levels of the vitamin, not different individual doses. In the present work we used chicks that had been depleted for 15 d and dosed them twice a week. Under these conditions we obtained, also with chicks, a linear correlation between body-weight gain and the log of the weekly vitamin A dose (Fig. 2).

Growth depression may by itself bring about changes in protein metabolism; therefore, the influence of vitamin A deficiency on factors characteristic of protein metabolism should be tested before the onset of growth depression. From a preliminary experiment (Ascarelli, Nir, Dror & Bruckental, unpublished results) we had reason to believe that liver xanthine oxidase and kidney arginase activities are increased by vitamin A deficiency in chicks. In the present work the increase in liver XDH and kidney arginase activities and in the plasma concentrations of uric acid and urea in chicks given the vitamin A-deficient diet appeared before growth depression set in, at an age when vitamin A was presumably still present in their plasma (Fig. 1). To the best of our knowledge there are no previous reports on the effect of vitamin A deficiency on arginase. Plasma urea concentrations have been shown to be increased also in vitamin A-deficient ewes (Webb, Mitchell & Little, 1971). Xanthine oxidase has been assayed

in rats fed on a vitamin A-deficient diet for 25 d (Esh & Bhattacharya, 1961) and an increase was found which did not reach statistical significance. Conflicting results have been reported on the influence of the deficiency on blood uric acid. Stoewsand & Scott (1961b) and Lowe *et al.* (1957) found no change, whereas Elvehjem & Neu (1932) found an increase in the blood concentration of this metabolite. In agreement with the latter workers, we consistently found increases in uric acid in all our experiments. Liver proteolytic activity was also raised somewhat, even if not to a statistically significant extent, by vitamin A deficiency; however, this activity was not assayed in the very first stages of the deficiency. It can also be pointed out that all the above-mentioned factors are involved in protein catabolism. Other enzymes, such as kidney serine dehydratase (Ascarelli & Bruckental, 1968) and D-amino-acid oxidase, or plasma nitrogen fractions, e.g. free amino acids, albumin or globulins, that are not directly involved in protein catabolism, were not influenced at all, even in advanced stages of deficiency.

Many workers have demonstrated the response of liver XDH and kidney arginase in different animals, including chicks, to changes in dietary protein level (Dror & Gertler, 1967; Scholz & Featherston, 1968), to starvation (Della Corte & Stirpe, 1967) or as a result of endogenous hormonal factors, such as adrenalectomy, hypophysectomy or hormone injection (Fraenkel-Conrat, Simpson & Evans, 1942–3; Clark & Pesch, 1956; McLean, 1961). The effect of dietary protein level on these two enzymes was confirmed also in the present work (Table 1). The liver proteolytic activity of the chicks was also shown to be influenced by changes in dietary protein level (Table 2), but not kidney serine dehydratase (Ascarelli & Bruckental, 1968) or D-amino-acid oxidase (Table 2).

It can be concluded that there are changes in protein catabolism that are significant biochemical signs of vitamin A deficiency and appear before other gross signs.

In the literature little work can be found concerning this aspect of vitamin A deficiency, except for the effect of proteolysis which has been investigated for a long time. Dingle, Sharman & Moore (1966) found that the liberation of proteolytic enzymes from the lysosomes of rat liver was greatly increased in hypervitaminosis A and also slightly increased in avitaminosis. However, lysosomes may undoubtedly be influenced by factors such as age, starvation and sexual changes, i.e. processes in which the action of vitamin A does not appear to have a central role (Moore, 1970). It would be premature, therefore, to conclude that vitamin A has a specific effect on lysosomes. Our finding that vitamin A deficiency causes an increase in the enzymic activities connected with protein catabolism, in spite of their being of cytoplasmic origin (XDH and arginase), supports the hypothesis that vitamin A function is to be searched for in a central system that is involved also in protein metabolism.

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