The ready detection of the rapidly iodlatable tyrosine residue in chymotrypsinogen and the elucidation of the sequence adjoining this residue demonstrate the applicability of radiochemical methods to peptides and proteins labelled with ^{131}I or ^{125}I .

In control experiments it was found that di-iodotyrosine is only slowly deiodinated in concentrated hydrochloric acid at 37° , whereas monoiodotyrosine is largely deiodinated under these conditions. Thus, in 16 hr. at 37° at an iodotyrosine concentration of $0.5 \mu\text{mole/ml.}$ of concentrated hydrochloric acid, di-iodotyrosine was deiodinated to the extent of less than 5%, and monoiodotyrosine to the extent of 75%. This presumably accounts for the absence of monoiodotyrosine derivatives in the chymotrypsinogen hydrolysis.

The occurrence of exchange reactions somewhat limits the scope of the method for the study of active-centre sequences.

SUMMARY

1. The iodination of tyrosine, histidine and their simple peptides with iodine in ammonia and with iodine monochloride has been studied by using radioactive iodine.

2. With iodine in ammonia mono- and di-iodo derivatives are formed with both residues, whereas with iodine monochloride histidine is converted only into the monoiodo derivative.

3. Treatment of the non-radioactive iodo derivatives with radioactive iodine leads to the incorporation of the label by exchange reactions.

4. Iodination of native chymotrypsinogen with

iodine monochloride occurs preferentially at one tyrosine residue which is present in the sequence Thr-Arg-Tyr.

5. Similar results are obtained with native chymotrypsin. The substitution on the readily iodlatable residue does not appear to involve loss in enzymic activity.

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Studies on Metabolism of Vitamin A

5. DIETARY PROTEIN CONTENT AND METABOLISM OF VITAMIN A*

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Dzialeszynski, Mystkowski & Stewart (1945) showed that vitamin A was present in human blood as a protein complex and not in the colloidal state. This idea was further extended by Ganguly, Krinsky, Mehl & Deuel (1952) and by Ganguly & Krinsky (1953), who suggested that a specific carrier protein probably maintained a steady

* Part 4: Mahadevan, Seshadri Sastry & Ganguly (1963).

vitamin A alcohol concentration in the blood. Also, Krinsky, Cornwell & Oncley (1958) have shown that vitamin A alcohol and vitamin A ester are transported by two separate lipoproteins in human blood.

Some recent observations have, however, focused attention on the coincidence of widespread protein malnutrition and vitamin A deficiency in human beings in many parts of the world, and have led to

the general recognition that absorption of vitamin A from the intestine and its mobilization from the liver are dependent on the protein nutritional status of the individual. Thus Arroyave, Viteri, Behar & Scrimshaw (1959) showed that children with kwashiorkor failed to absorb vitamin A even after a massive dose of the vitamin, and that if given skim-milk powder for a few days before the dose normal absorption could take place. Moreover, a direct relationship between the plasma albumin and vitamin A concentrations in humans (Arroyave, Wilson, Castellanos & Scrimshaw, 1961*a*; Arroyave, Wilson, Mendez, Behar & Scrimshaw, 1961*b*) and pigs (Friend, Heard, Platt, Stewart & Turner, 1961) was pointed out. Although the changes in blood concentrations indicate the overall efficiency of absorption, more detailed information can be obtained from an examination of the various phases of absorption, as reflected by the events taking place at the time of absorption in the lumen and mucosal cells of the small intestine, in lymph and in blood. Though previous investigators measured the quantities of the vitamin in the blood and liver, our present work, which was based on detailed examination of the phases of absorption, reveals that several steps involved in the absorption of vitamin A, and the enzyme that catalyses the vital step of oxidation of vitamin A aldehyde to the corresponding acid, are adversely affected by decreased protein intake in rats.

MATERIALS AND METHODS

Animals and diets. Weanling rats of both sexes and of this Institute strain were used throughout this study. Rats weighing 25–30 g. were maintained on a diet consisting of 60 parts of corn starch, 20 parts of casein exhaustively extracted with ethanol and light petroleum (Malathi, Subba Rao, Seshadri Sastry & Ganguly, 1963), 10 parts of cane sugar, and 5 parts each of groundnut oil and salt mixture (Steenbock & Nelson, 1923). The diet was supplemented per rat per day with 0.5 ml. of a dispersion of vitamin B complex which was made up as follows: 1 g. of *p*-aminobenzoic acid, 25 mg. of thiamine hydrochloride, 25 mg. of pyridoxine hydrochloride, 50 mg. of riboflavin, 10 mg. of folic acid, 100 mg. of calcium pantothenate, 2 mg. of biotin, 200 mg. of nicotinic acid, 500 mg. of inositol, and 5 g. of choline chloride, in 250 ml. of water. In addition, every rat received orally a weekly dose of 50 μ g. of vitamin K, 2 mg. of vitamin E and 1.5 μ g. of vitamin D, dissolved in 0.1 ml. of groundnut oil. After the rats had reached an average weight of 50 g., they were divided into three groups, with the litter mates of both sexes distributed equally. The animals of the three groups were then given isocaloric diets whose compositions were similar to the original basal diet, except that the casein was replaced by equivalent amounts of starch so as to give protein contents of 5, 10 and 20%. At this stage, in addition to receiving the vitamin supplements as above, the rats received every week an oral dose of 5 μ g. of vitamin A acetate dissolved in

0.1 ml. of groundnut oil. This procedure of maintaining weanling rats for some time on the vitamin A-free diet was followed to deplete their initial vitamin A reserves. The weekly supplement of 5 μ g. of vitamin A acetate was adequate for optimum growth without leaving any appreciable margin for storage; in fact, the rats usually contained only 20–30 μ g. of vitamin A per liver when killed during the experiment. Diet and water were given *ad lib*.

Rats receiving the diet containing 20% of casein grew best, whereas that containing 5% of casein led to the poorest growth together with a marked loss of hair. However, the tissue protein concentrations in the rats of the three groups did not show any significant difference.

The sources of all the materials used were as described by Mahadevan, Murthy & Ganguly (1962).

Experimental procedure. Rats were maintained on the three different diets for about 30 days. After a period of 24 hr. of starvation, one from each group received in one single dose 10 mg. of vitamin A acetate dissolved in 0.2 ml. of groundnut oil containing 1% of vitamin E. The animals were killed by heart puncture under light ether anaesthesia at the given time-intervals. Blood was collected into oxalated tubes and the liver and intestine were removed into containers maintained at 0°. The intestine was washed out with ice-cold 0.9% sodium chloride and the washings were pooled. The intestinal mucosae were removed from the muscles by scraping, as described by Ganguly, Krishnamurthy & Mahadevan (1959). All the tissues were weighed and maintained at 0°. Subsequent extraction of the tissues and analyses were carried out as described by Ganguly *et al.* (1959).

Preparation of enzymes. Separate rats, without the massive dose of vitamin A, were similarly killed and used for the preparation of the enzymes. The liver, intestinal wall and pancreas were homogenized in a Potter–Elvehjem homogenizer in ice-cold water. The liver homogenate was centrifuged for 1 hr. at 100 000 g in a Spinco model L ultracentrifuge and the supernatant fraction was used; the whole homogenates of all the other tissues were used as such as the sources of the enzymes. All enzymes were assayed as described previously (Mahadevan *et al.* 1962; Mahadevan, Murthy, Krishnamurthy & Ganguly, 1961).

Determination of protein. Tissue proteins were determined by the biuret method (Robinson & Hogden, 1940). Total proteins and albumin of plasma were determined as described by Hiller (1953).

RESULTS

Relative rates of absorption and storage of vitamin A in rats maintained on the diets of the three protein contents. Table 1 shows that, within 1 hr. of dosing, the concentration of vitamin A alcohol in all the samples analysed was highest in the group given the 20% casein diet and lowest in those given the 5% casein diet, and that the first group had the highest concentration of vitamin A alcohol up to 3 hr. after the dose. Fig. 1 shows that the alcohol:ester ratio of the intestinal contents was always highest in the group given the diet with the highest protein content and least when the protein intake was lowest. This strongly suggests that the hydrolytic enzyme

of the lumen of the intestine is more active in the group receiving the 20 % casein diet. The picture was much clearer in the intestinal mucosa and muscles, where at any given time-interval up to 3 hr. the concentrations of both ester and alcohol increased progressively with increasing protein intake. At all the time-intervals studied up to 24 hr., the vitamin A ester and alcohol concentrations of blood and liver were highest in the group given the diet with the highest protein content and least in the group given the diet with the lowest protein content. In all the three groups blood and liver showed the typical tolerance curves, as usually noticed during the active absorption of vitamin A in rats (Ganguly & Krinsky, 1953).

In these studies we did not find any significant sex difference in the vitamin A concentrations of

the tissues at the initial stages after the dose. However, at 24 hr. the females in all the groups had slightly more vitamin A in their livers.

Effect of decreasing the dietary protein, followed by a diet with a higher protein content, on the blood and liver vitamin A concentrations of adult male rats with initial liver stores of the vitamin. The above experiments have shown that the efficiency of absorption of vitamin A is directly dependent on the protein intake of the animal. That the mobilization of the stored vitamin A is related to the protein nutritional status, and more particularly to the blood albumin concentrations of animals, has been shown by many workers (Arroyave *et al.* 1961*a, b*; Friend *et al.* 1961). A systematic investigation was undertaken to study the effect of decreasing the dietary protein content on the blood vitamin A concentrations of normal rats. Adult stock male rats weighing 150–200 g. were given a single dose of 10 mg. of vitamin A acetate dissolved in 0.1 ml. of groundnut oil to build up their liver reserves; subsequently they were kept on the diet containing 20 % of casein for 7 days. On the eighth day, the rats were divided into three batches of six each, which then received the three diets containing 5, 10 and 20 % of casein respectively. Blood samples (about 2 ml.) were withdrawn by direct heart puncture from all the rats just before they were put on the respective diets, and from then onwards similar samples were withdrawn periodically during the experiment, as described in Table 2. Vitamin A alcohol, total proteins and albumin were determined in these samples. At the termination of the experiment the rats were killed by bleeding under light ether anaesthesia and their blood and liver were analysed for vitamin A.

Table 2 shows that vitamin A, total protein and albumin concentrations of blood remained con-

Fig. 1. The ratio of vitamin A alcohol to vitamin A ester in the contents of the small intestine of the rats of the experiments described in Table 1. The diets contained: ●, 5 % of protein; △, 10 % of protein; ○, 20 % of protein.

Table 2. *Progressive effect of lowered protein intake on the vitamin A alcohol, total proteins, albumin and on the albumin:globulin ratio of the plasma of normal adult rats*

Rats with initial liver reserves were put on the diets containing 5, 10 and 20 % of casein, and 2 ml. blood samples were withdrawn from them at the given time-intervals. Each point represents average values obtained from six rats. At the end of the fifth week the 20 % casein diet was restored. Values are expressed as $\mu\text{g.}$ of vitamin A/100 ml. of blood, and g. of protein or albumin/100 ml. of plasma.

	Dietary protein (%)	Weeks							
		0	1	2	3	4	5	6	7
Vitamin A alcohol	5	49	42	30	25	25	23	30	35
	10	44	40	34	31	30	31	38	41
	20	46	50	51	58	56	54	54	51
Albumin	5	3.2	2.7	2.3	1.9	1.7	1.7	2.2	2.5
	10	3.2	2.8	2.6	2.3	2.2	2.3	2.7	2.8
	20	3.2	3.3	3.2	3.2	3.2	3.2	3.1	3.2
Total protein	5	6.2	5.7	5.3	4.5	4.0	4.0	4.7	5.2
	10	6.0	5.7	5.7	5.3	5.0	5.0	5.4	5.7
	20	6.0	6.1	6.0	6.2	6.1	6.1	6.2	6.1
Albumin:globulin ratio	5	1.1	0.89	0.79	0.74	0.71	0.71	0.76	0.93
	10	1.0	0.94	0.85	0.82	0.78	0.83	0.90	0.97
	20	1.2	1.2	1.2	1.1	1.1	1.1	0.99	1.1

stant in the rats receiving the 20 % casein diet throughout. In the rats on the 10 % casein diet, the blood vitamin A, total protein and albumin concentrations progressively decreased up to the fifth week, after which they steadily increased as a consequence of the higher protein diet. The decline in all these substances was most marked in the rats receiving the 5 % casein diet, where also the introduction of the diet with the higher protein content brought about a steady increase in all three.

Table 3 shows that, at the end of the experiment, the rats receiving the diet with the highest protein content throughout had the lowest liver content of vitamin A, whereas those receiving the diet with the lowest protein content for part of the experiment had the highest.

Changes in the activities of the vitamin A esterases of the pancreas and small intestine and of the retinene oxidase of the liver in the rats on the three protein diets. Lower activities of several enzymes of the liver, pancreas and small intestine have been reported in animals on decreased protein intake (Muramatsu & Ashida, 1962; Ramakrishnan, Srinivasan & Nedungadi, 1961; Lyman & Wilcox, 1960). Moreover, significant lowerings of the activities of many enzymes of the pancreas and intestinal mucosal cells have been observed in kwashiorkor cases

(Waterlow, 1959; Thompson & Trowell, 1952). In the present work enzymes of the pancreas and small intestine hydrolysing and synthesizing vitamin A esters and of the liver oxidizing retinene to vitamin A acid were compared in the rats on the diets with the three protein contents; these enzymes were chosen because of their importance in the regulation of absorption and metabolism of vitamin A. Table 4 shows that all the three enzyme activities were markedly decreased in the rats with the lowest protein intake, and that with increasing dietary protein their activities increased progressively.

DISCUSSION

The present results demonstrate that the activities of both hydrolytic and esterifying enzymes for vitamin A esters of the pancreas and mucosa of rats are markedly decreased on a low-protein diet. The loss of the hydrolytic enzyme in rats on the low-protein diet is emphasized by the fact that, at the earlier time-intervals after the dose of the vitamin A ester, the vitamin A alcohol concentrations in the contents of the small intestine are highest in the group given the 20 % casein diet and lowest in those on the 5 % casein diet.

The morphological damage to the structures of the pancreatic and mucosal cells that has been reported in kwashiorkor cases and in rats given low-protein diets (McPhedran & Lucas, 1960; Davies, 1948; Tejada, 1956; Price, 1962) might affect absorption of vitamin A. Moreover, decreased protein intake leads to lower lipoprotein concentrations in human blood (Cravioto, De La Pena & Burgos, 1959; Olson, Jablonski & Taylor, 1958), which makes it possible that in these studies also the blood lipoprotein concentrations might have been significantly decreased in the rats receiving the low-protein diet.

Table 3. *Liver vitamin A contents of the adult rats receiving the 5, 10 and 20 % protein diets*

The values were obtained at the end of the experiment from the rats described in Fig. 2, and are expressed as μg . per whole tissue.

	Casein in diets		
	5%	10%	20%
Vitamin A ester	2900	2200	1400
Vitamin A alcohol	11	7.5	6.9

Table 4. *Effect of the three dietary protein contents on the specific activities of the tissue enzymes of rats*

Incubation mixture for the hydrolytic system was 3.5 ml. of 0.1 M-veronal buffer, pH 8.6, 0.5 ml. of an ethanolic solution of 250 μg . of vitamin A palmitate, 0.5 ml. of a 10 % (w/v) solution of sodium taurocholate and 0.5 ml. of the enzyme preparation containing 18.7–20.2 mg. of protein of the mucosal homogenate, or 14.7–17.5 mg. of protein of the pancreatic homogenate. The incubation mixture for the esterifying system consisted of: 4 ml. of 0.1 M-sodium phosphate buffer, pH 6.6, 0.5 ml. of an ethanolic solution of 250 μg . of vitamin A alcohol plus 500 μg . of lauric acid, and 0.5 ml. of the enzyme preparation used for the hydrolytic system. Both mixtures were incubated at 37° for 30 min. The reaction mixture for the retinene oxidase consisted of: 4 ml. of 0.1 M-sodium phosphate buffer, pH 6.6, 0.5 ml. of a Tween 20 dispersion of 200 μg . of retinene and 9–13 mg. of enzyme protein in 0.5 ml. of the rat-liver soluble fraction. Incubation was at 37° for 1 hr. Values are expressed as μg . of vitamin A alcohol or ester formed or retinene disappeared/mg. of protein/given time of incubation.

Tissue	Enzyme	Casein in diets		
		5%	10%	20%
Pancreas	Hydrolysis of vitamin A esters	1.4	4.0	5.4
	Synthesis of vitamin A esters	2.4	3.4	5.2
Small intestine	Hydrolysis of vitamin A esters	0.37	1.1	1.5
	Synthesis of vitamin A esters	0.42	1.7	1.9
Liver	Retinene oxidase	2.0	5.0	9.0

Vitamin A in blood is usually found in two forms, namely the free alcohol and the ester. The former is present in combination with lipoprotein and represents the normal state in which it is mobilized for tissue requirements, whereas the latter is transported by yet another lipoprotein and represents the newly absorbed vitamin A. No rise in plasma vitamin A concentration occurs when kwashiorkor patients are given massive doses of the vitamin, but skim-milk therapy for only a few days before the dose brings about a normal absorption (Arroyave *et al.* 1959). The blood albumin and vitamin A concentrations of children with kwashiorkor are low, but they rise to normal when skim milk is given (Arroyave *et al.* 1961*a, b*; Bagchi, Halder & Chowdhury, 1959). The blood proteins transporting vitamin A ester and alcohol may behave in the same way as the albumin. It has been shown above that, when normal adult rats with considerable liver reserves of the vitamin are maintained on the diets of lower protein contents, a progressive fall in their blood vitamin A concentrations together with concomitant lowering of the plasma protein concentrations occur, the rats on the diet with the lowest protein content showing the most marked fall of both, and that this can be correlated to the decrease of the albumin fraction of the blood. After the protein depletion, when these rats were put back on the diet with the highest protein content, the concentrations of both vitamin A and protein of plasma increased simultaneously. Our results thus confirm the view that a lowering of the concentration of blood proteins, especially that of albumin, leads to decreased blood vitamin A concentrations.

Malathi *et al.* (1963) have shown that vitamin A acid is markedly more effective than the acetate in causing growth response in vitamin A-deficient rats. The stored vitamin A palmitate is hydrolysed by vitamin A esterase to give the alcohol, which in turn is successively oxidized to retinene by the alcohol dehydrogenase and to the acid by the retinene oxidase (Mahadevan *et al.* 1962; Malathi *et al.* 1963). The low blood vitamin A concentration in protein malnutrition has generally been believed to be the causative factor for the consequent vitamin A deficiency. However, it has been shown above that the liver retinene-oxidase activity is much lower when there is a low protein intake. Therefore, in addition to the low plasma concentrations of vitamin A, the significantly decreased enzymic activity for the formation of the acid would have an added effect. It would then appear that the vitamin A deficiency caused by protein malnutrition is an additive effect of both low plasma vitamin A concentration and poor retinene-oxidase activity.

Several workers have shown that rats with initial stores of vitamin A, when put on a diet with a

lower protein content, contain higher amounts of vitamin A in their livers, as compared with similar rats receiving a diet with a higher protein content (Esh & Bhattacharya, 1960; Jagannathan & Patwardhan, 1960; Rechcigl, Berger, Loosli & Williams, 1962). Our results are in agreement with these observations. The animals receiving diets with lower protein contents grew less, and their blood vitamin A concentrations, as well as the activities of the enzymes metabolizing the vitamin, were decreased. It is therefore possible that less of the vitamin is utilized by such animals, thereby leading to higher retention in their livers. However, in a situation where rats have already been on low-protein diets and then receive vitamin A, 'the consequent reduced absorption appears to outweigh the reduced utilization' (U. K. Vakil & O. A. Roels, personal communication), and thereby they show less storage, as compared with those on diets with higher protein contents (Esh, Bhattacharya & Basu, 1961; Esh & Bhattacharya, 1960).

SUMMARY

1. Growing rats separately maintained on diets containing 5, 10 and 20 % of casein, when given a single massive dose of vitamin A acetate, showed that the efficiency of absorption of the vitamin increases progressively with the increase in the dietary protein content.

2. The enzyme activities of the pancreas and intestinal mucosa hydrolysing and synthesizing vitamin A esters, and of the liver oxidizing retinene, were progressively decreased with the lowering of the dietary protein content.

3. When adult male rats with considerable liver reserves of vitamin A were maintained on the diets of the three protein contents, the blood albumin and vitamin A concentrations of those receiving the diets containing 5 and 10 % of protein fell with the progress of time, those rats receiving the least protein showing the maximum decrease. The decreased blood concentrations were restored on allowing the rats to receive the 20 % protein diet.

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6. THE EFFECT OF VITAMIN A DEFICIENCY ON THE ACTIVATION OF SULPHATE AND ITS TRANSFER TO *p*-NITROPHENOL IN RAT LIVER

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It is generally believed that sulphate metabolism is dependent on the vitamin A nutritional status of the animal (Fell, Mellanby & Pele, 1956; Dziewiatkowski, 1954; Balakhovskii & Kuznetsova, 1958). Wolf & Varandani (1960) have reported that vitamin A deficiency leads to a decreased incorporation of [³⁵S]sulphate into the mucopolysaccharides of colon homogenates of rats. This was claimed to be due to a lowered synthesis of 'active sulphate' (adenosine 3'-phosphate 5'-sulphatophosphate) and not to any loss of the activity of the enzyme transferring the sulphate from adenosine 3'-phosphate 5'-sulphatophosphate to mucopolysaccharides (Varandani, Wolf & Johnson, 1960). It has been shown that the sulphurylation of *p*-nitrophenol is markedly decreased in the tissues of vitamin A-deficient rats (Subba Rao, Seshadri Sastry & Ganguly, 1963). Sulphurylation of phenols is a two-step reaction, the first being the activation of sulphate

by way of the synthesis of adenosine 3'-phosphate 5'-sulphatophosphate, and the second the transfer of sulphate from the adenosine 3'-phosphate 5'-sulphatophosphate to phenols catalysed by phenol sulphokinase (De Meio, Wizerkaniuk & Schreiberman 1955; Spencer, 1960; Gregory & Robbins, 1960). In the present paper it is demonstrated that the rates of both the steps involved in the sulphurylation of phenols are decreased in the liver of the vitamin A-deficient rats, and it is further shown that the lost activity can be restored by the addition of vitamin A to the reaction mixture *in vitro*, the restoration being dependent on the form of vitamin A used.

MATERIALS AND METHODS

Animals. Male rats of this Institute strain, made vitamin A-deficient according to the procedure described by Malathi, Subba Rao, Seshadri Sastry & Ganguly (1963),