Nutrition and Lysosomal Activity

THE INFLUENCE OF THE VITAMIN A STATUS ON THE PROTEOLYTIC ACTIVITY OF EXTRACTS FROM THE LIVERS AND KIDNEYS OF RATS

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1. Young rats were kept for several weeks on a diet deficient in vitamin A. Some were undosed, others were given marginal (25 i.u. weekly), adequate (1000 i.u. weekly) or excessive (50 000 i.u. daily) doses of vitamin A acetate. The undosed rats developed signs of vitamin A deficiency, and the overdosed animals had skeletal fractures indicative of hypervitaminosis A. 2. The rats were decapitated. Their livers, and sometimes their kidneys, were homogenized and processed by centrifugal methods to sediment most of the lysosome fractions. Proteolytic activity was measured, after treatment with a detergent, in the whole homogenate ('total' activity), in the pellet obtained after 20 min. at 15 000 g ('bound' activity) and, without treatment with detergent, in the supernatant ('free' activity). 3. In rats suffering from hypervitaminosis A the free activity and to a smaller extent the total activity were increased. Free activity was also raised in most rats suffering from avitaminosis A, but less than in those suffering from hypervitaminosis. 4. The vitamin A status appeared to have little effect on the proteolytic activity of the kidneys. Results for total and free activities, but not for bound activities, were higher than for corresponding liver preparations. 5. Control experiments were done on starved rats and on rats which were pair-fed with hypervitaminotic animals. Short periods of starvation caused an increase in free activity in young rats, but not in adults. The increases caused by starvation were much less than those caused by hypervitaminosis A. 6. For studies of the distribution of vitamin A more complete separation of the subcellular fractions was carried out on the combined livers from several hypervitaminotic rats. The concentration of vitamin A in the lysosome fraction was less than in the liver as a whole. 7. Our finding that the free proteolytic activity of the liver is increased by toxic oral dosing with vitamin A can be considered an extension of the previous observation that proteolytic enzymes are liberated when lysosomes are treated *in vitro* with vitamin A.

According to de Duve, Pressman, Gianetto, Wattiaux & Appelmans (1955) the acid proteases of cells together with a number of other acid hydrolases are concentrated in small organelles contained in the cytoplasm, to which particles the name 'lysosomes' is given. The treatment of isolated lysosomes *in vitro* with vitamin A causes the liberation of their proteolytic enzymes (Dingle, 1961) and other hydrolases (J. T. Dingle, unpublished work). This release of enzymes probably explains the loss of mucopolysaccharides from cartilage cultured in the presence of excess of vitamin A (3.0 μ g./ml.) (Dingle, Lucy & Fell, 1961) and its consequent loss of metachromasia (Fell & Mellanby, 1952). The distribution of vitamin A

throughout the body, however, is well known to be highly localized, with the liver as the central storage depot and distribution centre. It is therefore always questionable how closely experiments *in vitro* can reproduce the exact conditions pertaining *in vivo*. In particular it might be asked whether ingested vitamin A can reach the lysosomes *in vivo* in concentrations equal to those used in experiments *in vitro*.

It seemed therefore desirable to ascertain whether the proteolytic activity of lysosomes is influenced by vitamin A reaching the cells *in vivo* by the normal routes of absorption and distribution. For this purpose homogenates and subcellular fractions have been made from the livers and kidneys of

groups of rats given graded intakes of vitamin A ranging from none to toxic quantities. A preliminary report of some of our findings (Dingle, Sharman & Moore, 1963) has already indicated that the proteolytic activity of tissue extracts can indeed be influenced by the dietary intake of vitamin A. In this paper our evidence is presented more fully. We have also studied the extent to which lysosomal activity can be directly related to the vitamin A status, rather than indirectly through a reduced food intake and consequent emaciation (Beaufay, van Campenhaut & de Duve, 1959).

EXPERIMENTAL

Preparation of rats with graded vitamin A status. Young piebald rats were obtained from mothers that had received a full stock diet during pregnancy, but from which green vegetables, liver and cod-liver oil were withdrawn when the offspring became 10 days old. The young continued on the same diet until the experiments started, usually when their body weights were about 70 g. At this point measurements of the vitamin A reserves in the livers of three such animals indicated low total reserves of 90, 110 and 140 i.u. respectively.

All the rats were given our standard vitamin A-deficient

Table 1. *Average total, free and bound protease activities in livers of female rats given various intakes of vitamin A (series 1 experiments)*

The free and bound acid-protease activities of rat liver were estimated in animals maintained on diets containing known amounts of vitamin A. Vitamin A content of the liver was estimated and expressed as i.u./g. wet wt. Lysosome stability was measured as the free activity expressed as a percentage of the free plus bound activities, as described in the text, after the tissue had been disintegrated in an all-glass homogenizer.

Group	No. of rats	Mean time on diet (days)	Mean time on excessive vitamin A* (days)	Body wt. (g.)			Vitamin A (i.u./g.)	Activity (μ g. of tyrosine/hr./mg. wet wt.)			Free activity (% of free + bound activities)
				Initial	Maximum	Final		Total	Free	Bound	
1. Deficient (no vitamin A)	5	51	—	69	169	151	2	2.94	0.12	1.56	6.7
2. Marginal (25 i.u. of vitamin A weekly)	5	41	—	71	182	182	13	3.15	0.21	1.47	12.9
3. Adequate (150 i.u. of vitamin A weekly)	5	42	—	72	193	193	170	2.37	0.18	1.98	8.2
4. Excessive (50 000 i.u. of vitamin A daily)	6	48	26	156	162	150	18 000	4.20	0.60	1.71	25.7

* Included in previous column.

Table 2. *Average total, free and bound protease activities in kidneys of female rats given various intakes of vitamin A (series 1 experiments)*

Experimental conditions were as described in Table 1.

Group	No. of rats	Mean time on diet (days)	Mean time on excessive vitamin A* (days)	Activity (μ g. of tyrosine/hr./mg. wet wt.)			Free activity (% of free + bound activities)
				Total	Free	Bound	
1. Deficient (no vitamin A)	5	51	—	5.19	0.78	1.89	28.5
2. Marginal (25 i.u. of vitamin A weekly)	5	41	—	5.52	1.44	1.89	43.3
3. Adequate (150 i.u. of vitamin A weekly)	5	42	—	4.47	0.99	1.32	42.1
4. Excessive (50 000 i.u. of vitamin A daily)	6	48	26	6.30	1.44	1.92	40.9

* Included in previous column.

Table 3. *Total, free and bound protease activities in the livers of male rats given various intakes of vitamin A (series 2 experiments)*
 The experimental conditions were as described in Table 1, except that tissue was disintegrated in a Perspex-and-glass homogenizer (Aldridge *et al.* 1960).

Group	Rat no.	Time on diet (days)	Body wt. (g.)				Liver wt. (g.)	Vitamin A (i.u./g.)	Activity (μg. of tyrosine/hr./mg. wet wt.)		Free activity (% of free + bound activities)	
			Initial	Maximum	Final	Loss			Total	Free		Bound
5. Deficient (no vitamin A)	22	46	55	218	195	23	6.7	1	2.64	0.39	1.50	21
	23	48	67	243	186	57	4.8	2	4.17	1.44	1.83	44
	24	49	65	242	210	32	7.2	1	2.27	0.18	1.68	10
	25	50	64	214	188	26	6.8	1	1.98	0.03	1.32	2
	26	50	64	223	212	11	9.3	1	2.82	0.27	1.89	13
	27	52	63	262	213	49	6.7	1	2.46	0.15	1.53	9
	28	53	56	214	160	54	5.8	1	2.82	1.20	1.35	47
	29	55	51	226	194	32	6.9	1	3.66	0.69	2.10	25
	30	60	61	249	165	84	5.3	1	3.30	0.78	1.59	33
	31	60	57	261	192	69	7.0	1	2.91	0.78	1.32	37
	32	60	57	238	176	62	5.6	1	4.08	1.20	1.41	46
	33	62	72	256	237	19	9.2	1	4.59	0.57	2.19	21
	Average.....	54	61	237	194	43	6.8	1	3.14	0.64	1.64	26
6. Marginal (25 i.u. of vitamin A weekly)	34	48	67	255	255	0	12.9	1	2.43	0.18	1.26	13
	35	49	62	265	265	0	14.4	1	1.02	0.00	0.87	0
	36	51	65	297	297	0	13.6	1	1.77	0.03	1.32	2
	37	51	61	299	299	0	13.6	1	1.98	0.15	1.32	10
	38	52	63	248	243	5	6.6	1	2.79	0.24	1.80	12
	39	77	67	327	327	0	12.7	145	2.46	0.33	1.59	17
	40	83	56	274	262	12	8.0	1	2.58	0.57	1.44	28
	41	86	82	334	334	0	14.3	282	1.86	0.00	1.17	0
	42	88	65	312	312	0	11.1	1	1.53	0.00	1.29	0
	43	88	63	338	338	0	13.8	1	1.59	0.00	1.47	0
	44	88	60	318	318	0	12.5	1	1.80	0.00	1.68	0
	45	90	58	303	303	0	11.9	1	3.54	1.02	1.65	38
	46	90	55	294	294	0	11.7	1	3.15	0.06	1.98	3
	47	90	51	261	261	0	9.4	1	2.94	0.00	1.65	0
	Average.....	74	63	295	293	1.2	11.9	31	2.25	0.18	1.46	9

	Days on excessive vitamin A												
	48	43	172	186	150	36	7.7	6980	6.54	1.41	0.96	59	
7. Excessive (50000 i.u. of vitamin A daily)	49	48	148	167	159	8	7.6	6200	4.05	1.59	1.38	54	
	50	48	154	241	241	0	12.0	13300	4.62	2.28	1.50	60	
	51	50	173	242	238	4	10.8	5500	3.30	1.11	1.29	46	
	52	50	143	184	169	12	8.0	20000	3.06	1.11	1.32	46	
	53	55	194	223	199	24	8.6	8240	4.65	0.75	0.63	54	
	54	55	132	168	156	12	8.1	7630	4.26	1.23	1.20	51	
	55	57	166	235	235	0	11.5	12300	4.56	1.50	1.32	53	
	56	77	153	198	173	25	8.0	4740	4.02	1.11	1.20	48	
	57	86	165	199	194	5	7.0	1980	4.92	0.87	0.81	52	
Average											57	1.16	52

diet, containing: casein (vitamin-free), 20 parts; sucrose, 60; arachis oil, 15; dried yeast, 10; minerals, 5. The mineral mixture was made up of the following: $\text{Ca}_3(\text{PO}_4)_2$, 400 parts; KCl , 250; NaH_2PO_4 , 105; MgSO_4 , 80; NaCl , 50; iron citrate, 35; KI , 1.0; MnSO_4 , 0.2; NaF , 0.04. Adequate weekly doses of vitamins D, E and K were also allowed: these vitamins were dissolved in a little arachis oil and administered orally, separately from the rest of the diet.

Arrangements for the dosing of groups with vitamin A varied in detail between successive experiments, but the intended vitamin A status and the level of dosing were always chosen from the following four gradings of vitamin A status: 'deficient', no vitamin A given; 'marginal', 25 i.u. of vitamin A weekly; 'adequate', 1000 i.u. of vitamin A weekly; 'excessive', 50000 i.u. daily.

Vitamin A was always given in the form of its synthetic acetate, dissolved in arachis oil. At the lower levels the dose was administered into the rat's mouth by a dropping-pipette. At the excessive level the vitamin A was mixed into the diet. To avoid deterioration of the vitamin fresh supplies of diet were made up daily; a solution of vitamin A in the bulk of the arachis oil was mixed with the premixed solid components of the diet. In most of the rats given toxic excess of vitamin A the incidence of the skeletal fractures, typical of hypervitaminosis A, was studied by radiography (Moore & Wang, 1945).

Series 1 experiments. Four groups, each of at least ten female rats, were restricted to the diet deficient in vitamin A at body weights of about 70 g. Groups 1, 2 and 3 received doses of vitamin A equivalent to 0, 4 or 150 i.u. daily. The rats were kept on their diets for different periods, as shown in Table 1. Most of the animals in group 1 stopped growing and declined in body weight, usually with the development of xerophthalmia. In this group only those rats that showed clear signs of deficiency were used for the preparation of subcellular fractions.

The action of toxic excess of vitamin A in arresting growth in rats is much more rapid than that of dietary deficiency. Therefore, to prevent a great disparity in size and age between the avitaminotic and hypervitaminotic animals, the commencement of toxic overdosing in group 4 was delayed until day 20 of the experiment. Until this point the rats received 4 i.u. of vitamin A daily.

The timing of the experiment was made difficult by the laborious procedure necessary for the preparation of the tissue fractions, which did not permit more than two rats to be used on the same day. Some rats in groups 1 and 4 were therefore wasted because they died before they could be used in the way intended. In this series both the liver and kidneys were fractionated (Tables 1 and 2).

Series 2 experiments. In further experiments male rats were used. The group given 'adequate' doses of vitamin A (150 i.u. daily) was omitted. To avoid too many rats becoming available for use at once, the animals were started on their diets in three batches. As far as possible the killing of rats of the same group in succession was avoided. The periods during which the animals received their diets, and their changes in body weight during the experiment, are summarized in Table 3. In this series only the liver was fractionated.

Control experiments on starved or pair-fed rats. In Expt. 3 (Table 4) six young male rats, weights approx. 75 g., were divided into equal groups. One group was starved for 24 hr. before being killed, the other group was not starved.

In Expt. 4 (Table 5) 12 adult male rats, weights 250–300 g., were divided into four groups of three rats each. Two of the groups were starved for 24 or 48 hr. respectively before being killed, the remaining groups serving as unstarved controls. In Expt. 5 (Table 6) four rats given toxic excess of vitamin A were matched with four others, which served as pair-fed controls.

Homogenization of tissues. Immediately after each rat had been killed by stunning and decapitation, the liver and sometimes also the kidney were removed and cooled for 5 min. in 0.25 M-sucrose at 4°. A 2.0 g. sample of tissues was then taken for homogenization and was placed in 20 ml. of ice-cold 0.25 M-sucrose. In the experiments of series 1 an all-glass homogenizer was used. Trials on a number of samples of the same liver gave free proteolytic enzyme activities, as percentages of the total activity (see below), with a standard deviation of 15.3 on a sample mean of 14.4. Estimations of total enzyme activity gave a much lower standard deviation. The wide variations in free activity

therefore were presumably not due to unevenness in the composition of the tissue samples, or to gross inaccuracies in the assay method, but to low efficiency in homogenization. For all our subsequent experiments a homogenizer of the type described by Aldridge, Emery & Street (1960) was used. On 11 samples of the same tissue estimations the percentage of free enzyme activity had a standard deviation of only 4.9 on a sample mean of 37.9. The clearance of the Perspex piston in the glass homogenizer did not vary, and little or no wear was seen after months of use.

Preparation of tissue fractions. For the estimation of 'total' protease activity 1 ml. of the homogenate was taken, and its bound enzyme released by the addition of 0.5 ml. of an aqueous 1% solution of the detergent BRIJ 35, together with 1 ml. of water. The remainder of the homogenate was then spun in 50 ml. tubes in an MSE refrigerated centrifuge (rotor no. 69181) at 600g for 5 min. to remove nuclei and debris. The pellet was discarded and the supernatant was respun at 15000g for 20 min. 'Free' acid-protease activity

Table 4. *Average total, free and bound protease activities in livers of young male rats starved for 24 hr. compared with unstarved controls (series 3 experiments)*

Estimations of total, free, and bound protease activities were carried out as described in the text after starvation for 24 hr. Animals of the same age and sex were used as controls.

Group	No. of rats	Body wt. (g.)		Liver wt. (g.)	Activity (μ g. of tyrosine/hr./mg. wet wt.)			Free activity (% of free + bound activities)
		On day before being killed	On day killed		Total	Free	Bound	
8. Starved	3	81	73	3.15	5.90	0.75	3.53	18.1
9. Controls, not starved	3	80	86	4.27	3.43	0.22	2.30	8.6

Table 5. *Average total, free and bound protease activities in the livers of adult male rats starved for 24 and 48 hr. compared with unstarved controls (series 4 experiments)*

Experimental conditions were as described in Table 4, except that some animals were starved for 48 hr.

Group	No. of rats	Body wt. (g.)		Liver wt. (g.)	Activity (μ g. of tyrosine/hr./mg. wet wt.)			Free activity (% of free + bound activities)
		On day before being killed	On day killed		Total	Free	Bound	
10. Starved for 24 hr.	3	272	256	8.1	6.03	0.66	3.21	16.6
11. Starved for 48 hr.	3	287	275	7.3	6.87	0.69	3.63	16.3
12. Controls, not starved	6	280	286	10.2	3.69	0.51	2.25	16.8

Table 6. *Average total, free and bound protease activities in the livers of hypervitaminotic rats compared with pair-fed controls (series 5 experiments)*

Protease activity was measured in the livers of hypervitaminotic animals and pair-fed controls; the results are the averages for four animals in each group. Rats received the experimental diet for periods ranging from 9 to 31 days.

Group	Body wt. (g.)		Activity (μ g. of tyrosine/hr./mg. wet wt.)			Activity (% of free + bound activities)
	Maximum	Final	Total	Free	Bound	
13. Hypervitaminotic A	100	90	4.55	0.59	2.44	19.8
14. Controls	110	106	4.93	0.32	2.99	9.9

Table 7. *Stability of lysosomal enzymes in liver slices from rats of series 2 experiments*

Rat liver from control and hypervitaminotic animals was sliced on a tissue chopper and incubated in Krebs-Ringer buffer, pH 7.4, for 30 min. at 37°. The tissue was then homogenized and free and total activities of acid protease were estimated in the usual manner. Significance of differences: unincubated, $0.001 < P < 0.01$, i.e. highly significant; incubated, $P < 0.001$, i.e. very highly significant.

Free activity (% of total activity)	Controls		Hypervitaminotic A	
	Unincubated	Incubated	Unincubated	Incubated
	18.4	41.2	39.3	70.8
Based on no. of animals	11		7	

was estimated in the supernatant, without further treatment. In this paper 'free' activity is defined as that enzyme which does not sediment with particulate material at 15000g. For the estimation of 'bound' activity the pellet was resuspended by homogenization with 18.4 ml. of 0.1% BRIJ 35. The 0.1% BRIJ was found to give maximum release of bound activity. No effect of the detergent on the activity of solubilized enzyme was observed up to 0.5%.

Estimation of enzyme activity. The activity of the fractions (1 ml.), prepared as described above, was measured after the addition of 1 ml. of a 4% solution of haemoglobin in 0.1 M-acetate buffer, pH 4, as substrate. The solutions were incubated for 20 min. at 37°. Then 5 ml. of 4.7% (w/v) trichloroacetic acid was added and the precipitate was removed by filtration. Free 'tyrosine' was estimated in the filtrate by means of the phenol reagent of Folin & Ciocalteu (1927). Unincubated controls were prepared for each estimation. The enzyme activity was expressed as $\mu\text{g.}$ of tyrosine liberated/hr./mg. wet wt. of the original tissue. A tyrosine standard was used for each batch of estimations.

Effect of slicing liver, and of the incubation of lysosome preparations, on enzyme activity. To explore further the effect of hypervitaminosis A on the stability of the lysosomes post-mortem experiments of two types were made on the livers of some of the rats.

(1) The liver was sliced on a tissue chopper (McIlwain & Buddle, 1953) and incubated in Krebs-Ringer buffer, pH 7.4, for 30 min. at 37°. This treatment has been shown by Dingle (1963) to increase the ratio of free to bound enzyme in the tissue. For comparing the extent of this effect in hypervitaminotic and control rats measurements were made of the free enzyme activity, expressed as a percentage of the total activity, both before and after incubation. The animals used were from series 2 (Table 7).

(2) A granule fraction, rich in lysosomes, was prepared as described in Table 8 (group 4) from hypervitaminotic and control rats (series 2). The particles were suspended in 0.25 M-sucrose and incubated at 37°. At intervals portions were removed and sedimented, and free protease was measured in the supernatant. The free enzyme was again expressed as a percentage of the total activity (Fig. 1).

Estimation of vitamin A. To check that the graded dietary intakes of vitamin A had the desired effects on the vitamin A contents of the tissue, 1–2 g. samples of liver and sometimes also of kidney were taken from each rat. The tissues were finely chopped with scissors; 2 ml. of ethanol was then added, followed by 0.5 ml. of aqueous saturated KOH. The

Fig. 1. Release of protease from isolated rat-liver lysosomes. $\circ$, Control; $\times$, hypervitaminotic A. A granule fraction rich in lysosomes was prepared from livers of hypervitaminotic and control rats. Particles were suspended in sucrose, incubated at 37°, and at intervals the non-sedimentable acid protease was measured. The results at each time are expressed as a percentage of the total activity of the suspension.

mixture was heated in a boiling tube until the tissues were dissolved (5–10 min.), and was cooled and extracted twice with 20 ml. portions of ether. The ether extracts were combined, washed with water, dried with anhydrous Na_2SO_4 and evaporated on a hot-water bath under reduced pressure. The residue was dissolved in chloroform, and portions of the chloroform solution were taken for treatment with the SbCl_3 reagent, the blue colour being measured in a photoelectric absorptiometer, fitted with a Wratten filter no. 26. The absorptiometer had previously been calibrated with vitamin A acetate. The extraction procedure followed that of Davies (1933).

Distribution of vitamin A in tissue fractions. Several experiments were made on the distribution of vitamin A in the subcellular fractions. Since information was desired on the relative vitamin contents of the various fractions, the method of fractionation was different from that used for the sole purpose of estimating the lysosomal enzymes. Combined livers from four rats from Expt. 1 (group 4) were minced and a sample was taken. The remainder was homogenized in 0.4 M-sucrose and centrifuged in successive portions of suitable size. Nuclei and cell debris were removed by spinning for 5 min. at 600g (fraction 1).

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Table 8. *Distribution of vitamin A in various subcellular fractions of the pooled livers of hypervitaminotic rats (group 4, series 1 experiments)*

Experimental details are given in the text.

No.	Fraction Nature	Vitamin A		
		(i.u./g. of original liver)	(% of total vitamin A in fraction)	(i.u./mg. of protein N in fraction)
	Whole liver	12800		471
	Homogenized and spun			
1.	Spun at 600g: pellet of nuclei and debris	4900	38.3	1100
2.	Respun at 15000g: supernatant (soluble material and ribosomes)	5700	44.5	820
	Pellet from 15000g homogenized			
3.	Spun at 4500g: pellet of mitochondria	840	6.6	620
4.	Respun at 8000g: pellet of lysosomes etc.	30	0.2	130
5.	Soluble material and ribosomes	6	0.05	5

Spinning at 15000g for 20 min. followed and the supernatant was removed (fraction 2). The combined 15000g pellets were rehomogenized with sucrose solution, layered over 0.6M-sucrose and centrifuged first at 4500g, giving a pellet mainly of mitochondria (fraction 3). Further layering and centrifugation at 8000g produced a pellet that on electron-microscope observations was found to contain lysosomes, small mitochondria and a few ribosomes (fraction 4), and a supernatant containing soluble and emulsified material and some ribosomes (fraction 5).

The estimations of vitamin A were difficult owing to the large volumes of the supernatant fractions that had to be extracted and to the presence of sucrose. Various minor modifications of the method of extraction of the vitamin had therefore to be made. Even with these modifications, however, attempts to study the distribution of the vitamin in the livers of the rats given marginal or adequate doses of vitamin A failed. Serious discrepancies were found between the total amount of the vitamin in the various fractions and in the original liver. A reasonably good recovery of vitamin A was obtained in the various fractions from hypervitaminotic liver (Table 8).

RESULTS

The numbering of the Tables in which our results are summarized has already been given. To save space results for individual rats are only given for our series 2 experiments (Table 3), which were the most successful as a demonstration of the effect of the vitamin A status on the lysosomal enzymes. In the other series of experiments average results are given.

DISCUSSION

Technical limitations. In Tables 1-6, our findings for proteolytic activities are given under the headings (1) total, (2) free and (3) bound. If we accept the view that almost all the acid-proteolytic activity of the organs under investigation originates in their

lysosomes, which may not be far from the truth, then the total activity ought to be equal to the sum of the free and bound activities. The consistent tendency, in our results, for the total activity to exceed considerably the sum of the free and bound activities suggests that usually our homogenizer was only 50-70% efficient in breaking the liver cells. In view of the technical difficulties involved this low efficiency is not surprising. Presumably unbroken cells must have been included in our first pellet, obtained by low-speed centrifugation, which was discarded.

Changes in our homogenizer made between series 1 and series 2 appear to have improved its efficiency in disrupting the liver cells. The narrowing of the gap between the piston and cylinder of the homogenizer, however, had to be undertaken cautiously, so as to avoid unwanted breakages in the organelles, including the lysosomes, as this would cause an increase in the free proteolytic activity at the expense of the bound activity. In view of the incomplete breakage of the liver cells it seemed preferable to express the free proteolytic activity as a percentage of the sum of free and bound activities, as found experimentally, rather than as a percentage of the total activity. This percentage, i.e. $(\text{free} \times 100)/(\text{free} + \text{bound})$, is considered to be a measure of the stability of the lysosomes to homogenization. The term 'free', as used in this connexion, does not imply that the enzyme is free in the cell cytoplasm but is an indication of the lability of the lysosomes to mechanical stress.

The high concentration of proteolytic activity in the liver lysosome-rich fraction is shown by the fact that, whereas this fraction contributed only about 1.4% of the nitrogen content of the whole liver, it contained at least 80% of the total proteolytic activity. The concentration of activity

in the lysosomes therefore must be about 60 times as great as that in the liver as a whole.

Our method for separating subcellular fractions was open to the criticism that the products of the homogenization of more than one type of cell were included. Thus the fractions from liver were doubtless derived mainly from parenchymal cells, but contributions were presumably also made by the Kupffer cells and those of the blood vessels and bile ducts. The possibility remains that variations in the vitamin A status may affect some types of liver cells more than others.

Increase of free proteolytic activity in the liver in hypervitaminosis A. In both series 1 and 2 the free proteolytic activity in the liver of those rats that were given toxic doses of vitamin A was much higher than in any of the other groups. In series 1, for example, this difference was statistically significant ($0.001 < P < 0.01$) between the hypervitaminotic group 4 and the deficient group 1, with means of 0.60 and 0.12 $\mu\text{g. of tyrosine/hr./mg. wet wt.}$ respectively. In series 2 the free activity in the hypervitaminotic group 7 was also much greater than in the marginal group 6, with means of 1.30 and 0.18 $\mu\text{g. of tyrosine/hr./mg. wet wt.}$ respectively. In both series the ratio of free activity to the sum of free and bound activities was increased in hypervitaminosis. Total activity was also raised in hypervitaminosis, but not to the same extent as the free activity.

In some of our hypervitaminotic rats the toxic effects of the vitamin on the lysosomes may have been augmented by those of emaciation. In at least some of the rats (e.g. nos. 50, 51, 55 and 57), however, large increases in free proteolytic activity occurred without appreciable loss of body weight, and in the presence of substantial amounts of intraperitoneal fat.

The experiments *in vitro* with sliced liver and with incubated lysosomes indicated that the fragility of the lysosomes towards both these treatments, as indicated by the liberation of their enzymes, was increased in hypervitaminosis A.

Proteolytic activity in the liver in avitaminosis A. Our results for avitaminosis A differed in the experiments of series 1 and 2. In series 1 no evidence of a rise in the free proteolytic activity was found in the avitaminotic rats, as compared with the activity in rats given marginal or adequate doses of vitamin A. In series 2, with much larger groups of animals, free activity was higher in the deficient than in the marginally dosed rats, with means of 0.64 and 0.18 $\mu\text{g. of tyrosine/hr./mg. wet wt.}$ respectively. These findings seem in good agreement with those of Roels, Trout & Guha (1964), who reported that the free β -glycerophosphatase activity of rat-liver preparations is increased in 'frank' vitamin A deficiency but not in 'mild' deficiency. As judged

by the percentage loss of body weight our rats were more severely deficient in series 2 than in series 1. Even in our series 2, however, the free activity was much lower in the deficient than in the hypervitaminotic animals.

Proteolytic activity in the liver in starvation. The results of our experiments on young rats starved for 24 hr. (Table 4) confirmed the finding of Beaufay *et al.* (1959) that starvation may cause the release of lysosomal enzymes. In contrast, the starving of adult rats for 24 or 48 hr. (Table 5) did not increase the release of their enzymes. Possibly this difference was related to the presence of much larger fat deposits in the older animals.

The higher values for free proteolytic activity in liver preparations from the control rats in Expt. 4 as compared with those from the controls, in Expts. 1, 2 and 3, may possibly have been due to their greater age. In rat liver and kidney Franklin (1962) has demonstrated an increase in the percentage of free lysosomal enzyme activity in the first 2 months of life, and in kidney has reported a similar increase as the animals become senile. These results suggest that further studies on the effect of age on lysosome lability are desirable.

The results of Expt. 5 (Table 6) indicate that hypervitaminosis A has a greater effect on the release of lysosomal enzymes than can be explained by the associated voluntary reduction in food intake. It was noticed, however, that the stomachs of the hypervitaminotic rats invariably contained substantial amounts of undigested food, and that the intestinal contents and faecal pellets were putty-coloured. In contrast, the stomachs of the control pair-fed rats were empty, and their intestinal contents and faeces were normal in colour. In this experiment therefore pair-feeding produced equality in the amount of food ingested rather than in the amount of food digested. No simple method of avoiding this complication seems available.

Proteolytic activity of kidneys. In series 1 there was no clear evidence that the vitamin A status affected the proteolytic activity of the kidneys. Values for total and free activities, but not for bound activity, were consistently higher in the kidneys than in the liver of rats of the same series.

Distribution of vitamin A in relation to proteolytic activity. The results in Table 3 indicate that, on the basis of either the total vitamin A content or its concentration per mg. of nitrogen, there was no evidence of a concentration of vitamin A in the lysosome fraction. Indeed, in contrast with the great concentration of proteolytic activity in the lysosomes, it appears that the vitamin is less concentrated in these organelles than in the liver as a whole. This conclusion, of course, refers to the whole lysosomes. It remains possible that the

vitamin may be highly concentrated in the lysosomal membrane.

General. Our results show clearly that the lysosomes of rat liver are more unstable in animals that have been made hypervitaminotic than in control animals. This finding agrees with the action *in vitro* of vitamin A on isolated lysosomes (Dingle, 1961; Fell, Dingle & Webb, 1962); it is also consistent with the effect of the vitamin on skeletal tissues in organ cultures (Fell & Dingle, 1963), and with the hypothesis that the changes seen in hypervitaminosis A in the living animal are associated with the release of bound hydrolytic enzymes (Lucy, Dingle & Fell, 1961). In studies on hypervitaminosis A induced by dosing with vitamin A acid, which differs widely from vitamin A alcohol and esters with regard to its transport and storage in the body, Weissmann & Thomas (1963) have reported increased lysosomal activity in the livers of rabbits, and Weissmann, Uhr & Thomas (1963) in the livers of guinea pigs.

Our findings on rats deficient in vitamin A also support the view that this condition may affect the stability of the lysosomes, although the injury to the lysosomes seems less severe than in hypervitaminosis.

It must be emphasized, however, that the activity of lysosomes can be affected by many other factors besides the vitamin A status. Thus we have confirmed that starvation may raise enzyme activity, as reported by Beaufay *et al.* (1959), although this effect seems smaller and less consistent than in hypervitaminosis A. The increases in free enzyme activity sometimes found in avitaminosis A may probably be partially explainable on this basis. Cortisone and hydrocortisone have been repeatedly implicated as antagonists to vitamin A in its effect on lysosomal activity (Weissmann & Thomas, 1963) or on organ cultures (Weissmann & Fell, 1962). Increased lysosomal enzymes have been found by Tappel, Sawant & Shibko (1963) in various forms of muscular dystrophy, including those induced by avitaminosis E. Dingle *et al.* (1963) have mentioned that the lysosomal cathepsin of rat kidneys is increased in deficiency of vitamin E.

No explanation can yet be given why, if both

starvation and hypervitaminosis A can cause the release of enzymes from lysosomes, skeletal fractures are caused only by hypervitaminosis A. Presumably this question will only be answered when more is known about the relative vulnerability to vitamin A of lysosomes in different tissues, and about other relevant factors.

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REFERENCES

- Aldridge, W. M., Emery, R. C. & Street, B. W. (1960). *Biochem. J.* **77**, 326.
 Beaufay, H., van Campenhaut, E. & de Duve, C. (1959). *Biochem. J.* **73**, 617.
 Davies, A. W. (1933). *Biochem. J.* **27**, 1770.
 de Duve, C., Pressman, B. C., Gianetto, R., Wattiaux, R. & Appelmans, F. (1955). *Biochem. J.* **60**, 604.
 Dingle, J. T. (1961). *Biochem. J.* **79**, 509.
 Dingle, J. T. (1963). *Ciba Found. Symp.: Lysosomes*, p. 384. Ed. by de Reuck, A. V. S. & Cameron, M. P. London: J. and A. Churchill Ltd.
 Dingle, J. T., Lucy, J. A. & Fell, H. B. (1961). *Biochem. J.* **79**, 497.
 Dingle, J. T., Sharman, I. M. & Moore, T. (1963). *Proc. Nutr. Soc.* **22**, x.
 Fell, H. B. & Dingle, J. T. (1963). *Biochem. J.* **87**, 403.
 Fell, H. B., Dingle, J. T. & Webb, M. (1962). *Biochem. J.* **83**, 63.
 Fell, H. B. & Mellanby, E. (1952). *J. Physiol.* **116**, 320.
 Folin, O. & Ciocalteu, V. (1927). *J. biol. Chem.* **73**, 627.
 Franklin, T. J. (1962). *Biochem. J.* **82**, 118.
 Lucy, J. A., Dingle, J. T. & Fell, H. B. (1961). *Biochem. J.* **79**, 500.
 McIlwain, H. & Buddle, H. L. (1953). *Biochem. J.* **53**, 412.
 Moore, T. & Wang, Y. L. (1945). *Biochem. J.* **39**, 222.
 Roels, O. A., Trout, M. & Guha, A. (1964). *Biochem. J.* **93**, 23c.
 Tappel, A. L., Sawant, P. L. & Shibko, S. (1963). *Ciba Found. Symp.: Lysosomes*, p. 78. Ed. by de Reuck, A. V. S. & Cameron, M. P. London: J. and A. Churchill Ltd.
 Weissmann, G. & Fell, H. B. (1962). *J. exp. Med.* **116**, 365.
 Weissmann, G. & Thomas, L. (1963). *J. clin. Invest.* **42**, 661.
 Weissmann, G., Uhr, J. W. & Thomas, L. (1963). *Proc. Soc. exp. Biol., N.Y.*, **112**, 284.