IV. FAT METABOLISM IN VITAMIN A DEFICIENCY: THE BLOOD-SERUM ESTERASE.

By HARRY NORMAN GREEN.

From the Department of Pharmacology, University, Sheffield.

(Received November 16th, 1933.)

THE blood-serum of all animals investigated contains no true fat-splitting enzyme but contains enzymes capable of hydrolysing in greater or less degree the esters of the lower fatty acids, of which the mono- and tri-butyryns have been the esters chiefly used in experimental work.

Much knowledge has accumulated concerning the source, distribution and function of these esterases, but it is impossible to summarise it briefly for the conclusions reached are manifold and often contradictory. It has been suggested that they play a specific rôle in hydrolysing the lower fatty esters in the blood prior to their oxidation, but the esterases are widely distributed in all tissues, and therefore the most plausible hypothesis is that the serum esterases are derived from the blood and tissue cells, and as such have no specific function distinct from that of the tissue esterases generally. On this hypothesis variations in the serum esterase activity are an indication of variations in the esterase content of the tissues as a whole, or of the liver, which by its size and richness in esterase probably constitutes the main source of supply to the blood stream. The work of Kameo [1932], in which it was shown that X-ray irradiation of the liver caused a marked fall of the serum esterase content within 24 hours whereas direct irradiation of the blood had no effect, supports this theory. Observations on the tissue and serum esterases in the sarcoma-bearing rat [Green, 1934] also indicate a parallelism between the blood-serum esterase content and that of the liver, lung and kidney.

The esterase content of the serum can be affected by many factors. An increase has been observed after feeding large amounts of fat [Abderhalden and Rona, 1911; Greene and Summers, 1916], on a raw soy bean diet [Howarth and Chang, 1926], after starvation [Aguma, 1924] and after the injection of pilocarpine [Prewitt, 1925]. A decrease has been noted in conditions of severe intoxication with damage to cell metabolism [Fanconi, 1924; Caro, 1920], in lipaemia [Sakai, 1914; Fanconi, 1924], in starvation where Greene and Summers [1916] found a later rise above normal, and in the later stages of scurvy [Palladin and Normark, 1924]. Jobling and Bull [1912] found an increase in the serum esterase in immune haemolytic sera, whilst Bach [1922] found that it was independent of the type of food even when there was much fat in the diet.

The following work was undertaken primarily to determine the effect of vitamin A deficiency on the serum esterase content and the relation between this latter factor and the spontaneous infections arising in this deficiency disease.

EXPERIMENTAL.

The following method of estimating esterase activity was finally used after a trial of more complicated methods, since it proved efficient enough for the purpose in hand, which was a purely comparative one. Duplicate readings were reasonably close ($\pm 2\%$).

To 20 ml. of $M/80$ phosphate buffer of p_H 8.0 measured into a 100 ml. Erlenmeyer flask, 0.25 ml. phenolphthalein solution and 0.25 ml. of ester are added and the flask is brought to 37° by gentle heat; 0.5 ml. of blood-serum is then added and the flask incubated at 37° for 2 hours. The flask is then rapidly cooled and the contents are immediately titrated against $N/100$ NaOH until a pink tint appears matching that obtained with the blank control. The control flask contains no serum but is otherwise similar. In earlier experiments serum heated at 80° for 30 mins. to destroy the esterase was added to the control flask, but as this made an insignificant difference it was discontinued.

Continual shaking of the flask during the experimental period is recommended by most workers. Since it was observed, however, that this procedure produced a slight but definite diminution, varying with the rate of shaking, in the rate of hydrolysis, in the present work the flasks were not shaken. No antiseptic was added, as it was found that significant bacterial contamination did not occur over the experimental period of 2 hours.

The substrates used in the majority of experiments were ethyl butyrate and tributyrin. Similar results were obtained using ethyl acetate, phenyl acetate and triacetin as substrates, but as only a few determinations were made they are not reproduced.

Most of the experiments were carried out using young white rats from which blood was obtained by severing the vessels of the neck after previous stunning with a head blow. The blood was immediately centrifuged and the serum used within a maximum period of 6 hours. Blood was obtained from dogs by bleeding from an ear vein.

A standard basal diet [Green and Mellanby, 1928], deficient in vitamin A, was given to the experimental rats. The control rats received a supplement of some source of vitamin A, cod-liver oil (5 mg. daily), dried cabbage (0.5 g. daily) or carotene (0.02 mg. daily) being used. In some cases an ordinary stock diet was given but no significant differences in the serum esterase contents of the control animals were found whatever the source of vitamin A given. All rats received a supply of vitamin D in the form of "radiostol" (5 mg. daily).

In all cases the degree of hydrolysis, under the defined conditions, is expressed in terms of ml. $N/100$ NaOH required to neutralise the fatty acid liberated. The amount of esterase in normal rat-serum appears to vary within fairly wide limits. In 61 young rats, reared on complete diets, figures for ethyl-butyrase varying from 41 to 18 were obtained. The upper range was exceptional and the lower range was more often reached, but in the majority of cases values between 25 and 30 were obtained. The average figure for 30 control rats in the present series was 27.4 (Table II). Rats from the same family and from groups reared on the same diet and under similar conditions tended to give similar values. The serum content of tributyrinase appeared to be less variable; 10 control rats gave an average figure of 10.7 (Table II). It will be noted that rat-serum hydrolyses ethyl butyrate about three times as rapidly as tributyrin, and ethyl butyrate was therefore the substrate of choice where the amount of serum available made the use of only one substrate possible.

Criteria of vitamin A deficiency.

For the purpose of analysing the results it was necessary to formulate rough standards for the estimation of the degree of deficiency and infection in the vitamin A-deficient rat. The large individual variations which occur in rats on the same diet are well known; some die shortly after the decline period has set in whilst many survive for 2-3 weeks, the survival time depending to a great extent on the maximum weight reached before the decline commences. Neither the degree of xerophthalmia nor the severity of infection nor the loss in weight from the maximum reached is an absolute indication of the degree of deficiency. A sudden rapid fall of weight with an early death may be associated with only mild infection though the animal presumably died from rapid exhaustion of its vitamin A reserves, whilst a gradual fall in weight may be associated with a more severe infection which has had time to develop to the stage of more obvious lesions. In the former case the rat may be killed shortly before impending death and therefore at the last stage of deficiency, and in the latter case the rat killed at the same time may be in a definitely earlier stage of deficiency. All three criteria of deficiency have therefore been used, but it is obvious that no such clinical method of assessment can be exact. The results have however been averaged over many determinations, and the groupings should be reasonably accurate.

The criteria used for assessing the degrees of infection and deficiency are given below.

Degree of infection	Criteria.
+	Small tongue abscess and/or mild infection elsewhere (<i>e.g.</i> nasal sinuses)
++	Large tongue abscess or other definitely infected focus (<i>e.g.</i> lung, genito-urinary tract)
+++	Multiple infected foci
Degree of vitamin A deficiency	
+	Very slight xerophthalmia and/or constant weight for about 7 days or slight fall in weight for a few days
++	Infection (+) and/or mild xerophthalmia and/or constant weight for about 14 days or gradual fall for about 7 days
+++	Infection (++) and/or advanced xerophthalmia and/or weight falling gradually for about 14 days
++++	Infection (+++) and/or very rapid loss in weight and/or very severe xerophthalmia (panophthalmitis)

Results.

In earlier experiments the amount of hydrolysis in normal saline, instead of buffer solution, over a period of 18 hours was determined. The results obtained with 23 deficient and 17 control rats and 7 rats on a complete but quantitatively restricted diet were proportionately similar to those obtained in the later work and are not therefore given in detail.

Typical experiment. The results of one typical experiment are given in Table I in order to show the general plan of the experiments from which the average results were derived.

Table I. *Illustrative experiment.*

Days on diet	Degree of deficiency	Degree of infection	Serum esterase		Weight curve
			Ethyl butyrate (ml. N/100 NaOH)	Tributyrin	
I. Basal diet alone:					
27	None	None	30.0	9.7	Rising
48	+	None	18.3	8.0	Rising very slowly
55	+	None	19.8	7.5	Rising very slowly
70	++	+	14.1	5.4	Plateau—20 days
59	++	+	9.5	4.8	Falling—10 days
59	+++	++	7.2	3.9	Falling—10 days
59	++++	+++	2.5	2.9	Falling rapidly—6 days
II. Basal and cod-liver oil (5 mg.). Food intake restricted.					
27	—	—	30.2	10.3	Rising
48	—	—	30.8	10.1	Rising
55	—	—	22.1	8.4	Plateau—7 days
55	—	+	19.9	7.2	Falling slowly—12 days
70	—	—	24.5	8.3	Falling slowly—30 days
70	—	—	23.8	8.5	Falling rapidly—10 days
III. Basal and excess cod-liver oil (3 ml.).					
27	—	—	47.9	11.3	Falling slowly—14 days
48	—	—	36.1	10.8	Rising slowly
59	—	—	42.8	10.6	Rising slowly
70	—	—	42.9	11.2	Rising slowly
IV. Basal and excess olive oil (3 ml.) and cod-liver oil (5 mg.).					
27	—	—	31.2	.	Plateau—6 days
27	—	—	28.4	.	Slight fall—4 days
48	—	—	27.0	.	Rising slowly
70	—	—	26.8	12.6	Rising slowly
V. Basal and ethyl butyrate (0.25–1.0 ml.) and cod-liver oil (5 mg.).					
27	—	—	45.0	.	Rising
27	—	+	18.3	.	Rising
48	—	—	19.4	8.0	Falling slowly
70	—	—	26.7	10.2	Falling slowly

Rats from the same family, of the same sex and of roughly similar weight were distributed as equally as possible in the different groups. In the experiment tabulated in Table I, 5 groups of rats received the following diets:

I. The basal diet, deficient in vitamin A; intake unrestricted.

II. The basal diet and cod-liver oil (5 mg. daily). Average amount of diet per rat restricted to average amount per rat consumed by rats of group I.

III. The basal diet and a relatively large amount of cod-liver oil (3 ml. daily). Intake unrestricted.

IV. The basal diet and an amount of olive oil equivalent to the cod-liver oil given to group III (3 ml.). Cod-liver oil (5 mg. daily). Intake unrestricted.

V. The basal diet and ethyl butyrate (0.25 ml. daily for 27 days, 0.5 ml. 27–48th day and 1 ml. 48–70th day). Cod-liver oil (5 mg. daily). Intake unrestricted.

The results of several experiments planned on similar lines have been averaged and the results in this one experiment are not therefore discussed further. They serve to indicate the type of individual variation in the serum esterase content met with in all experiments.

Average results. The results obtained in 47 rats at various stages of vitamin A deficiency, in 30 which had received a complete diet and in 20 which had re-

ceived a complete diet but restricted in amount to that consumed by the rats on the deficient diet, are summarised in Table II.

Table II. *Serum esterase content of rat-serum at progressive stages of vitamin A deficiency. Average results.*

Degree of deficiency	No. of rats	Substrate		Diet	
		Ethyl butyrate (ml. <i>N</i> /100 NaOH)	No. of rats		Tributyryn (ml. <i>N</i> /100 NaOH)
0	30	27.4	10	10.7	Complete. No restriction of intake
0	20	25.0	7	8.4	Complete. Intake restricted
+	10	20.4	5	7.5	Basal
++	10	11.6	5	4.3	Basal
+++	20	7.3	6	4.1	Basal
++++	7	2.5	3	2.9	Basal

It will be seen that there was a progressive fall in the serum esterase content of the rat on a diet deficient in vitamin A, which commenced at the time when the rat ceased to grow and reached a very low figure near the time of spontaneous death. The rats in the last stage of deficiency (++++) were almost moribund, and, as it was considered probable that animals on the verge of death from any cause would give similar figures, only a few determinations were made. Excluding this group, there was a fall in the serum esterase content from 27.4 to 7.3 when the substrate was ethyl butyrate and from 10.7 to 4.1 with tributyrin. When loss of weight was produced by a quantitative restriction of a complete diet the average serum esterase content was only slightly below the normal. In this group individual results varied more widely from the normal than is usual. In some instances very low values were obtained but in such cases evidence of infection was always found, and these values were rejected in averaging the results.

Evidence was obtained that the serum esterase content returns to normal if the vitamin A-deficient rat is given vitamin A. Small doses of cod-liver oil were given to 6 rats at the 2nd (++) or 3rd (+++) stages of deficiency, and they were killed individually at intervals of one week. Except in one case the serum esterase content showed a progressive rise, the rat killed at 6 weeks giving a value within the normal range. The exception gave a low figure (10.5) at the 5th week and was found still to have infected foci.

Vitamin A deficiency in dogs.

In a much smaller series of dogs¹ it was found that vitamin A deficiency affected the serum esterase content in a similar manner to that observed in rats.

Table III. *Serum esterase content in vitamin A-deficient dogs. Average figures.*

Diet	No. of dogs	Substrate	
		Ethyl butyrate (ml. N/100 NaOH)	Tributyrin
Basal and vitamin A	8	6.5	23.5
Basal alone	7	3.5	10.7

¹ Prof. E. Mellanby kindly allowed me to obtain blood samples from his dogs.

As only a relatively few animals were investigated no attempt has been made to classify the degree of deficiency. When blood samples were taken all the deficient dogs showed nervous symptoms such as incoordination of gait; some had conjunctivitis but all were fairly active. The results are summarised in Table III and it will be noted that in the dog the tributyrinase content of the serum is much greater than that of ethyl butyrase. Vitamin A deficiency has obviously produced a marked fall in the serum esterase content.

Effect of vitamin B deficiency.

Though loss of weight alone did not appear to affect the serum esterase it was interesting to determine whether another vitamin deficiency disease would bring about similar changes to that observed in vitamin A deficiency. To this end rats were given a diet deficient in the vitamin B complex; a diet which consisted of the basal vitamin A-deficient diet was used from which the chief sources of the B vitamins (marmite and lemon juice) were omitted. Each rat was given cod-liver oil (10 mg. daily) as a source of vitamin A. On this diet growth ceased very rapidly, and decline in weight set in within a few days in rats weighing 70–100 g. The average serum esterase contents of two groups of rats in which loss of weight had occurred as a result of feeding this diet are given in Table IV.

Table IV. *Serum esterase content of vitamin B-deficient rats.*
Average results.

Diet	No. of rats	Substrate		Weight curve
		Ethyl butyrate (ml. N/100 NaOH)	Tributyrin	
Basal + vitamin B	7	32.6	11.3	Rising
Basal alone	12	27.8	11.1	Falling—4–10 days
Basal + vitamin B	6	24.5	—	Rising
Basal alone	7	21.1	—	Falling—10–21 days

The basal diet was the vitamin B-deficient diet described in text. Vitamin B was given in the form of dried brewer's yeast (1.0 g. daily).

Under these conditions there was only a slight fall in the serum esterase content though the loss of weight was roughly similar to that observed in vitamin A-deficient rats at the 2nd (++) and 3rd (+++) stages of deficiency at which there was a decided fall in the serum esterase content.

Effect of infection.

In view of the frequency of infection in vitamin A deficiency as compared with other deficiency diseases the question arose as to how far the effects described could be due to infection itself apart from the fundamental metabolic disorder. The serum esterase content was therefore determined in a number of rats suffering from spontaneous infections or in others in which infection had been induced by inoculation with material derived from infected lesions in vitamin A-deficient rats. All these rats received ample vitamin A. The type of spontaneous infection usually observed was an acute enteritis which may occur in epidemic form in the rat, though their diet is complete. The results have been classified together, as the type or source of the infection did not appear to affect the response in the serum esterase content. It will be observed from the summarised results given in Table V that infection itself produces a distinct

fall in the serum esterase content, though the fall is not so pronounced as in the later stages of vitamin A deficiency.

Table V. *Effect of infection on serum esterase in rats on a complete diet (ethyl butyrate as substrate).*

Degree of infection	No. of rats	Serum esterase (ml. N/100 NaOH)
0	8	26.2
++	8	15.0
+++	5	9.9

It is obvious therefore that infection is an important factor in producing the serum changes observed in vitamin A deficiency. An analysis of the lesions found in 47 rats at various stages of vitamin A deficiency (see Table II) showed, however, that 13 of these showed no macroscopic signs of infection, and yet the average figure for the serum esterase content was 13.9. It is possible that infection sufficient to affect the serum esterase level is present before definite localisation of infection occurs, but experience with experimental infections does not suggest that a mild infection would produce the degree of change actually observed. Moreover in some rats in which rapid loss of weight had occurred and in which only slight or no signs of infection were present, a fall in the serum esterase content to very low levels was observed. It is therefore probable that the metabolic disturbance associated with vitamin A deficiency is responsible for the serum change, and that infection plays a secondary part in a greater or less degree.

Effect of excess vitamin A.

Since a deficiency of vitamin A produced a diminution of the serum esterase activity it was an obvious step to determine the effect of feeding relatively large amounts of vitamin A. Rats were therefore given the basal diet and amounts of cod-liver oil of 2 or 3 ml. daily. Control rats were given similar amounts of olive oil and a third group was given ethyl butyrate in an amount of 0.25 ml. gradually increased to 1 ml. daily. The results are summarised in Table VI.

Table VI. *The effect of supplementing a complete diet with relatively large amounts of cod-liver oil, olive oil or ethyl butyrate on the esterase content of rat-serum. Average results.*

Supplement to complete diet	No. of rats	Substrate		
		Ethyl butyrate (ml. N/100 NaOH)	No. of rats	Tributylin (ml. N/100 NaOH)
None	10	27.6	5	8.5
Cod-liver oil (2-3 ml. daily)	8	46.1	3	11.0
Olive oil (3 ml. daily)	6	28.3	—	—
Ethyl butyrate (0.25-1.0 ml. daily)	6	27.4	3	9.1

* Complete diet: basal vitamin A-deficient diet + cod-liver oil (5 mg. daily).

In all of 10 rats given the large amount of cod-liver oil the capacity of the serum to hydrolyse ethyl butyrate has significantly increased. One such experiment is given in more detail in Table I and here it will be observed that there is little or no associated rise in the serum content of tributyrinase. The exact stage at which the increase occurs has not been determined; it was evidently at almost its maximum extent at the 27th day of feeding and even at the 125th day no significant change from this level was observed. The highest figure

obtained under these feeding conditions was 59.6 and the lowest 36.1; the majority gave figures between 40 and 50. No rat in the olive oil group showed this effect; the serum esterase content remained at the normal level. Only one of six rats in the ethyl butyrate group showed a rise to above normal and experiments now in progress show a tendency for a fall to occur under these conditions rather than a rise.

The rise in the serum esterase content was also observed in a few adult rats which had received excessive amounts of cod-liver oil (progressively increased up to 50 % of the diet), but where a chronic lung infection had supervened there was actually a definite fall to below normal.

No significant alteration in the serum esterase content was observed in 3 rats which received excessive doses of irradiated ergosterol (10,000 units daily) as a supplement to a normal diet for 3-5 weeks.

The rise in the serum esterase content due to feeding relatively large doses of cod-liver oil was obviously not due to an increased fat intake since a similar dose of olive oil had no effect. It is not however certain as yet whether the effect is due to excessive amounts of vitamin A. Work is in progress to determine the effects of feeding very potent vitamin A concentrates and large doses of carotene as a possible method of deciding this point.

The effect of adding vitamin A to serum *in vitro* has been determined, though a positive effect was not to be expected. Vitamin A and carotene emulsions, stable over the experimental period, were formed by adding one drop of an alcoholic solution of a very potent vitamin A concentrate or of a saturated alcoholic solution of carotene to the buffer mixture. A similar amount of alcohol was added to the control flasks. In three determinations using both normal serum and serum from vitamin A-deficient animals no increase but a slight decrease was observed in the degree of hydrolysis of ethyl butyrate. Carotene was without effect in a similar number of experiments.

DISCUSSION.

It has been shown that vitamin A deficiency brings about a pronounced fall in the esterase content of the blood-serum. It is obvious from previous work on the subject and from my own findings that such a fall is not a change peculiar to vitamin A deficiency; amongst other causes infection and, in the rat, inoculation with sarcoma tissue [Green, 1934], have been observed. There is some reason therefore for considering it a secondary change and one unlikely to throw direct light on the physiology of vitamin A. The fact however that a similar change does not occur, except in a very much less degree, in other conditions producing loss of weight (underfeeding, vitamin B deficiency), suggests that the change may have some significance in the pathological processes due to vitamin A deficiency. Infection probably plays a part as it certainly produces a fall in the serum esterase content both in animals and in man [Green, 1934], but it has already been indicated that this is not the primary cause. Several workers have attempted to identify serum esterase with serum complement. There appears to be a parallelism between the contents of these two factors in the serum of different animals, according to Jobling and Bull [1913] but Gordon and Wormald [1929] have shown that whilst the complement and esterase contents of the serum tend to run parallel there are occasional discrepancies, and they were able to show that there was a significant amount of esterase in serum which had been heated to the extent necessary to destroy all the complement. In view however of the quantitative association of these two factors it is

interesting to compare the present results with those obtained by Osborne [1931] on the serum complement of vitamin A-deficient rats. He found a tendency towards a lowered serum complement content in vitamin A deficiency in the rat, though it is not stated at what stage of the deficiency the observations were made. It is tentatively suggested that this fall may be responsible for the lowered resistance of the vitamin A-deficient rat to infection. If there is a quantitative relationship between the serum esterase and complement it seems possible that the decrease in complement may be the result rather than the cause of infection, for the present findings show that inoculated infection produces a very definite reduction in the serum esterase content.

Future work may show that a fall in the serum esterase content occurs in many conditions in which there is a profound metabolic disturbance in the body cells. Until we know more in this connection and in regard to the function of the esterases in general, and the serum esterase in particular, it may be impossible to assess the real significance of the present findings.

SUMMARY.

1. Vitamin A deficiency in the rat produces a large and progressive decrease in the esterase content of the blood-serum.
2. The infected rat on a complete diet also shows a considerable fall in the serum esterase content, but in rats which have lost weight on account of under-feeding or deficiency in the vitamin B complex, there is, on the average, only a very slight fall.
3. Relatively large amounts of cod-liver oil in the diet produce a rise in the serum esterase content well above normal.
4. The relation of these findings to infection in vitamin A deficiency is discussed.

My best thanks are due to Prof. E. Mellanby for his interest in this work. The author is in receipt of a personal grant from the Medical Research Council.

REFERENCES.

- Abderhalden and Rona (1911). *Z. physiol. Chem.* **75**, 30.
 Aguma (1924). *J. Biochem.* **4**, 239.
 Bach (1922). *Z. klin. Med.* **95**, 103.
 Caro (1920). *Z. klin. Med.* **89**, 49.
 Fanconi (1924). *Ferm. Forsch.* **7**, 307.
 Gordon and Wormald (1929). *Biochem. J.* **23**, 730.
 Green (1934). *Brit. J. Exp. Path.* (in press).
 — and Mellanby (1928). *Brit. Med. J.* **ii**, 691.
 Greene and Summers (1916). *Amer. J. Physiol.* **40**, 146.
 Howarth and Chang (1926). *Amer. J. Physiol.* **78**, 224.
 Jobling and Bull (1912). *J. Exp. Med.* **16**, 483.
 — (1913). *J. Exp. Med.* **17**, 61.
 Kameo (1932). *J. Biochem.* **15**, 229.
 Osborne (1931). *Biochem. J.* **25**, 2137.
 Palladin and Normark (1924). *Biochem. Z.* **152**, 420.
 Prewitt (1925). *Amer. J. Physiol.* **73**, 1.
 Sakai (1914). *Biochem. Z.* **62**, 387.