CXVII. THE WATER-SOLUBLE B-VITAMINS
VII. GROWTH-PROMOTING PROPERTIES OF LACTOFLAVIN

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(*Received 1 May 1937*)

The more heat-stable portion of the vitamin B complex formerly known as vitamin B_2 has recently been proved to be a mixture of at least two constituents. One of these constituents, lactoflavin, first isolated from whey [Ellinger & Koschara, 1933, 1, 2; Kuhn *et al.* 1933, 1, 2], was soon recognized to possess only part of the physiological properties of "vitamin B_2" and to need supplementing in order to demonstrate the full physiological activity [György *et al.* 1933; 1934].

In this laboratory it has been shown that a substance supplementing lactoflavin is present in

(*a*) alcoholic extracts of various cereals;

(*b*) autoclaved aqueous yeast extracts after removal of the lactoflavin present by adsorption on fuller's earth at *p*H 1·4.

A substance with supplementary action for lactoflavin is also contained in yeast extracts which have been autoclaved in alkaline solution (*p*H 9), a procedure which would destroy the lactoflavin present; this may be similar to or identical with the factor Y, described by Chick & Copping [1930], found necessary for supplementing an egg white concentrate, which may be regarded as an impure solution of lactoflavin.

Supplementary materials from many other sources (fish muscle, liver, molasses etc.) have been investigated in other laboratories. György [1934; 1935] provisionally introduced the name "vitamin B_6" for a factor specifically active in curing dermatitis and restoring growth of rats receiving lactoflavin and vitamin B_1 only of the vitamin B complex.

A long series of experiments in this laboratory has shown that young rats can be fairly satisfactorily reared for several weeks from weaning on a synthetic diet in which B-vitamins are provided by the following three materials.

(1) Small daily doses (=0·3–0·6 g. yeast, dry wt.) of Peters's vitamin B_1 concentrate from yeast, or 10–15γ daily of crystalline vitamin B_1 hydrochloride.

(2) Small daily doses, 10–20γ, of crystalline lactoflavin.

(3) A heat-stable supplement contained in the filtrate from yeast extracts after treatment with fuller's earth, given in daily amount equivalent to 0·5 g. yeast, dry wt. This material we have called the yeast fuller's earth filtrate fraction.

Copping [1936] found, however, that an alcoholic cereal extract was more effective than the fuller's earth filtrate from yeast extract for restoration of normal growth and cure of the dermatitis developed in rats which had received

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only lactoflavin in addition to vitamin B_1. This indication that the supplement required with lactoflavin might be composed of more than one factor, and that lactoflavin might need some supplement other than the fuller's earth filtrate from yeast extracts for complete nutrition, is in accord with the results of other investigators working on other lines using preparations derived from other materials [Lepkovsky *et al.* 1936; Halliday & Evans, 1937]. In the following paper further evidence will be brought forward to show that at least two essential heat-stable dietary factors are present in yeast in addition to lactoflavin.

The present paper is, however, concerned with the special growth-promoting properties of lactoflavin for young rats.

Kuhn *et al.* [1935] obtained weight increases in young rats of about 10 g. weekly over a 4-week period by supplementing the Bourquin-Sherman diet [1931] with 10γ daily of lactoflavin and with an aqueous yeast extract which had been treated with fuller's earth. Since the Bourquin-Sherman diet contains an alcoholic extract of wheat as a source of vitamin B_1, this must be regarded as an additional source of supplementary material. Euler *et al.* [1934; 1935] reported a similar weight increase when employing a synthetic diet supplemented only by the fuller's earth filtrate from yeast extracts. An average weight increase of approximately 11 g. weekly for 4 weeks was obtained by György [1935] when 10γ daily of lactoflavin were fed with "vitamin B_6", as contained in large doses of Peters's vitamin B_1 concentrate from yeast, but the growth rate was not enhanced by doubling the lactoflavin dose. Ansbacher *et al.* [1936], however, found that the growth rate of rats was increased with increasing doses of lactoflavin, when supplemented by an extract from rice polishings.

In the absence of other members of the vitamin B_2 complex and supplemented only by vitamin B_1, lactoflavin has been found by us to support a low, subnormal rate of growth. When, however, supplements were provided in the form of preparations from wheat germ or from yeast, the growth rates were substantially increased. When the animals received optimum doses of these supplements the growth response showed a fair proportionality with the amount of lactoflavin given, until the optimum was reached; moreover the growth response appeared to be the same, within the limits of experimental error, for the same dose of lactoflavin whatever the source of the supplementary substance.

Methods

Growth tests were carried out by the methods generally employed in this laboratory [Chick & Roscoe, 1928; Chick *et al.* 1935]. Young rats, weaned at 21 days, weighing between 40 and 50 g. received a basal diet consisting of commercial light white casein[1] 100, rice starch 300, cotton-seed oil 60, lard 15, salt mixture (McCollum's No. 185) 25 and water 500; the diet was steamed for 3 hr. The lard was added to the diet to ensure an adequate supply of the essential unsaturated fatty acid in the hope of eliminating scaly tails and associated conditions [Burr & Burr, 1930; Hume, unpublished experiments]. The diet was supplemented by 0·08–0·1 ml. of cod liver oil daily, to supply vitamins A and D, and by vitamin B_1. In the earlier experiments this was given as 0·05–0·1 ml. of a Peters's concentrate from brewer's yeast, prepared according to the modified method described by Kinnersley *et al.* [1933]; when supplies of crystalline

[1] Although traces of factors of the vitamin B_2 complex may be present in commercial light white casein, the amounts present are so small that this casein has been found satisfactory for experiments with rats on the vitamin B_2 complex [cf. Chick & Roscoe, 1928; Roscoe, 1933].

vitamin B_1 hydrochloride became available the rats received 10–15γ of this daily. It was found that aqueous solutions of vitamin B_1 hydrochloride could be kept free from moulds and without loss of activity if solutions containing 1 mg. per ml. in N/1000 HCl, were stored in the cold. More dilute solutions, also containing N/1000 HCl, suitable for dosing were made from the stock solution at least once weekly.

In preparing litters for this work we did not find it necessary, in order to render the young rats sensitive to deficiency of the B_2-vitamins, to remove the yeast from the stock diet of the mothers during the whole of the lactation period, as was previously the custom in this laboratory. The mothers received the full stock breeding diet, except during the last week of lactation, when yeast was not included.

The rats were weighed 3 times weekly. Their growth had usually ceased by the end of the second week after weaning, and when the weight had remained stationary for several days, the control animals received lactoflavin only, while the others received in addition the selected fraction from yeast extract or wheat germ, to provide the further heat-stable vitamins of the B_2 group. The lactoflavin (hepatoflavin) was prepared from liver and was kindly provided for us by Dr Sidney Smith, to whom we wish to express our great indebtedness.

Preparation of supplementary materials

Filtrate from fuller's earth adsorption of autoclaved yeast extract ("yeast fuller's earth filtrate"). A dilute acetic acid yeast extract, prepared as described by Chick & Roscoe [1930], was autoclaved at *p*H 5 at 120° for 5 hr. to destroy vitamin B_1. 1 l. of this extract (1 ml. = 0·5 g. yeast, dry wt.) was adjusted to *p*H 1·4 by addition of approximately 20 g. of H_2SO_4. 50 g. of fuller's earth (B.D.H. for adsorption purposes) were added and after stirring at intervals for 30 min. the fuller's earth was filtered off. The adsorption was repeated and the final filtrate was treated with $Ba(OH)_2$ to remove sulphate and adjusted to *p*H 3 for storage (1 ml. = 0·5 g. yeast, dry wt.).

Eluate from the norite adsorbate of the fuller's earth filtrate. 1 l. of the above fuller's earth filtrate was extracted 4 times with 12 g. portions of norite charcoal at *p*H 2·5. The norite adsorbate was washed with N/100 HCl, dried in a vacuum desiccator and extracted with glacial acetic acid on a water-bath until the extract was nearly colourless (3 extractions). The acetic acid was removed *in vacuo* and the light brown residue dissolved in 167 ml. of water (1 ml. = 3 g. yeast, dry wt.).

Alcoholic extract of wheat germ. Wheat germ was air-dried to constant weight at 37°, defatted with ether and exhaustively extracted in a Soxhlet extractor with hot absolute alcohol. The alcohol was removed *in vacuo* and the remaining gummy material dissolved in water (1 ml. = 1 g. wheat germ, dry wt.).

Results

Growth-promoting action of lactoflavin without supplement

The administration of 12γ daily of lactoflavin to rats fed on the basal diet unsupplemented by other members of the vitamin B_2 group caused a small but definite increase in weight during the first 2 weeks, after which the growth of the animals practically stopped (Fig. 1). The animals receiving vitamin B_1 as the Peters's concentrate showed a slightly greater weight increase, average of 6·7 g.

Table I. *Growth of rats receiving 12γ daily of lactoflavin with no supplement*

Vitamin B_1 given as	No. of rats	Average weekly increase in wt. over 3 weeks g.	Standard deviation
Peters's concentrate from yeast (= 0·3–0·6 g. yeast, dry wt., daily)	50	6·7	1·7
Vitamin B_1 hydrochloride (10γ daily)	26	5·3	2·3

weekly for 3 weeks, than those having crystalline vitamin B_1, 5·3 g. weekly (Table I). This indicates the presence of a small amount, doubtfully significant, of some growth factor, in addition to vitamin B_1, in the Peters's concentrate.

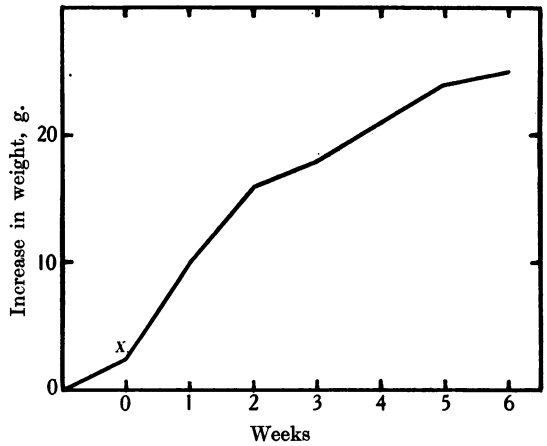

Fig. 1. Growth of young rats on a basal diet free from B-vitamins, receiving 10γ daily of vitamin B_1 hydrochloride, and, from point *X* only, 12γ daily of lactoflavin. Average of 6 rats.

Growth-promoting action of lactoflavin when supplemented

Table II shows the weight increase of rats given varying amounts of lactoflavin supplemented by the different extracts. With the supplementary doses fed without lactoflavin the rats showed only a slow weight increase, approximately 3·5 g. weekly; this indicated either an absence of lactoflavin from these solutions or a very low content.

When the diet containing lactoflavin was supplemented by the above heat-stable fractions the growth of the rats seemed to depend on the amount of lactoflavin given and, for a given dose, was the same, within experimental error, for the supplementary materials tested. Rats receiving 6γ lactoflavin daily, supplemented by the preparation from yeast extract or wheat germ showed average weekly increases in weight of 9·6 and 10·0 g. respectively, and those having 12γ lactoflavin daily, of 12·4 and 12·2 g. respectively. Increasing the doses of the supplements beyond a certain amount did not cause increased growth, as was demonstrated by experiments in which daily doses of 12γ lactoflavin were supplemented by fuller's earth filtrate in doses ranging from the equivalent of 0·25 to 1·0 g. of yeast, dry wt., daily, or by wheat germ extract equivalent to 1·0–2·0 g. wheat germ dry wt. Since the degree of growth appeared to be independent of the source or quantity of the supplements when these were given beyond a certain minimum amount, it seems probable that the different extracts contained the same growth factors although the possibility of this being a coincidence is not excluded.

Care was taken in all cases to give sufficient amounts of the supplementary materials, and the doses were increased with increasing amounts of lactoflavin, in case the resulting augmented growth stimulated by the increased ration of lactoflavin might raise the requirements of the supplement. It can be seen from Table II that the optimum dose of wheat germ extract for the 12γ lactoflavin level lay between 0·5 and 1·0 ml. so that the 1·5 ml. dose given with larger doses of lactoflavin was considered to be adequate.

Table II. *Growth of rats receiving vitamin B_1, various doses of lactoflavin and other heat-stable supplements from yeast or wheat germ*

Daily dose of lacto-flavin γ	Supplement given	Equivalent of yeast or wheat germ, dry wt. g.	No. of rats	Average weekly increase in wt. over 4 weeks g.	Standard deviation	Average for group g.
0	Yeast fuller's earth filtrate	0·5	6	3·9	—	3·5
	Wheat germ extract	1·5–2·0	5	3·2	—	
6	Yeast fuller's earth filtrate	0·75	3	9·6	—	9·8
	Wheat germ extract	1·5	4	10·0	—	
9	Wheat germ extract	1·5	4	10·7	—	10·7
12	Eluate from norite adsorbate of yeast fuller's earth filtrate	1·0	6	12·5		12·3
,,	Yeast fuller's earth filtrate	0·25	2	11·7	1·7	
		0·5	26	12·3		
		0·75	2	12·6		
,,	Wheat germ extract	0·5	2	7·6	1·3	
		1·0	4	12·5		
		1·5	3	11·7		
		2·0	2	12·4		
25	Eluate from norite adsorbate of yeast fuller's earth filtrate	3·0	15	15·6	2·5	16·6
,,	Yeast fuller's earth filtrate	0·75–1·0	6	17·4	2·4	
,,	Wheat germ extract	1·5	2	16·7	—	
37	Yeast fuller's earth filtrate	1·0	3	18·9	—	18·9
50	Yeast fuller's earth filtrate	1·0	8	18·2	3·4	18·2
75	Yeast fuller's earth filtrate	1·5	2	18·1	—	18·1

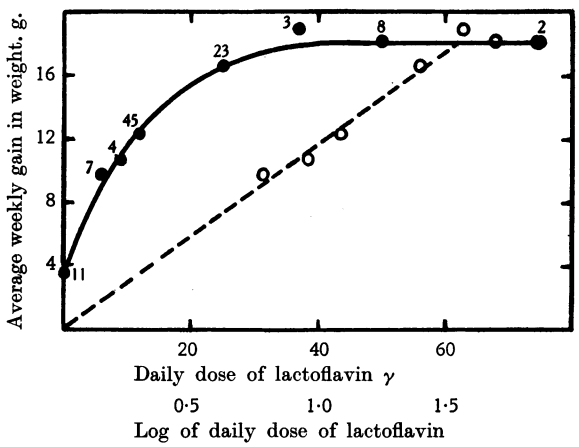

Fig. 2. Growth of young rats on a basal diet free from B-vitamins, receiving vitamin B_1 as either 0·05–0·1 ml. daily of Peters's B_1 concentrate from yeast or 10–20γ daily of crystalline vitamin B_1 hydrochloride, and graded doses of lactoflavin supplemented by yeast fuller's earth filtrate or alcoholic extract of wheat germ.

The figures by each point on the curve indicate the number of rats (♂ and ♀) on each dose of lactoflavin. The continuous line represents the growth plotted against the daily dose of lactoflavin and the dotted line the growth plotted against the log of the daily dose.

Increasing the daily dose of lactoflavin from 6γ to 37γ caused the average gain in body weight to increase from 9·8 to 18·9 g. weekly, over a 4-week period. The increases in weight showed a fair proportionality to the lactoflavin dose,

and the experimental points derived from all observations with the different supplementary materials lay on a smooth logarithmic curve (Fig. 2). The optimum daily dose of lactoflavin under these conditions was approximately 40γ, since growth was not increased beyond an average of 18 g. weekly, over a 4-week period, when much larger doses were given.

Speed of response of rats to lactoflavin

Young rats, growing normally on a synthetic diet which contained vitamin B_1 and all the vitamins of the B_2 group as provided by yeast extract, showed immediate cessation of growth if the extract was withheld (Fig. 3, curve *A*), and their body weight even fell during the first succeeding days. The addition to the diet of the fuller's earth filtrate did not restore growth (curve *B*), suggesting that the sudden cessation of growth was due to withdrawal of the lactoflavin. This was confirmed by an experiment in which the rats received lactoflavin only, when the yeast extract was removed from the diet, and the weights of the animals continued to increase for 1–2 weeks (curve *C*).

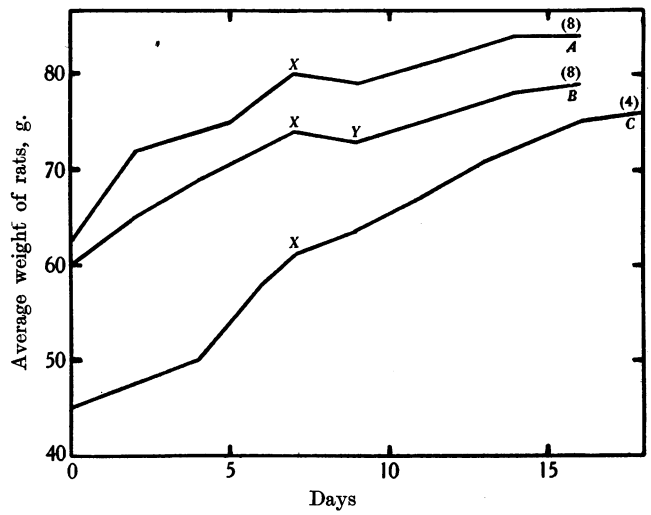

Fig. 3. Growth of rats on a basal diet free from B-vitamins, receiving daily 10γ vitamin B1 hydrochloride and, to *X* only, autoclaved yeast extract = 0·25 g. yeast, dry wt.

Curve *A*. No vitamins of B_2 complex after *X*.
Curve *B*. At *Y* fuller's earth filtrate = 0·5 g. yeast, dry wt. daily.
Curve *C*. At *X* 25γ lactoflavin daily.

The figures in brackets indicate the number of rats from which the growth curves were derived.

SUMMARY

1. The body weight of rats receiving a diet deficient in the heat-stable vitamin B_2 complex increased only slowly when lactoflavin was added to the diet; the growth response was greatly enhanced when lactoflavin was supplemented by extracts from wheat germ or from yeast.

2. The growth rate of rats receiving adequate doses of these supplements was increased regularly with increasing doses of lactoflavin; optimum growth of about 18 g. weekly over a 4-week period was attained when the rats received approximately 40γ daily of lactoflavin.

3. Alcoholic extracts of wheat germ and autoclaved aqueous yeast extracts after treatment with fuller's earth enhanced the growth-promoting action of lactoflavin to an equal degree. When these materials were given without lactoflavin, no increase in weight occurred.

4. Lactoflavin may be considered as the member of the heat-stable vitamin B_2 complex which is especially concerned in the growth of rats.

We wish to thank Dr H. Chick for her continued interest and advice and gratefully acknowledge the gifts of lactoflavin from Dr Sydney Smith of Messrs Burroughs Wellcome & Co., of a specimen of natural vitamin B_1 hydrochloride from Prof. Suzuki and of a specimen of synthetic vitamin B_1 hydrochloride, prepared by I. G. Farbenindustrie, from Sir Henry Dale.

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