CXVIII. WATER-SOLUBLE B-VITAMINS

VIII. ESSENTIAL DIETARY FACTORS FOR THE RAT PRESENT IN AUTOCLAVED YEAST EXTRACTS IN ADDITION TO LACTOFLAVIN

BY CONSTANCE ELIZABETH EDGAR AND THOMAS FOTHERINGHAM MACRAE

From the Division of Nutrition, Lister Institute, London

(*Received 1 May 1937*)

IN this laboratory it has long been recognized that although rats could be reared satisfactorily for some weeks on a diet containing, as sources of the B-vitamins, vitamin B_1 and lactoflavin supplemented by the filtrate from autoclaved yeast extract after treatment with fuller's earth, this diet was not complete, since better growth and development resulted when the animals received autoclaved aqueous extracts of yeast which had not been subjected to further fractionation. The results of experiments carried out by Copping in this laboratory [1936] showed that the dermatitis, developed in rats receiving lactoflavin only of the vitamin B_2 group, was more effectively cured by alcoholic extracts of cereals than by the yeast fuller's earth filtrate. This indicated that at least one additional factor, not contained in the yeast filtrate, was present in alcoholic cereal extracts.

As reported in the following paper [Edgar & Macrae, 1937], the chemical and physical properties of the dietary factor present in the yeast fuller's earth filtrate show that this factor is not identical with György's "vitamin B_6" [1935, 1], which, in his experiments, together with lactoflavin, appeared to provide the whole vitamin B_2 complex. It was possible that some supplementary factor for lactoflavin, not contained in the yeast fuller's earth filtrate, was adsorbed with the lactoflavin on the fuller's earth; this paper describes experiments in which treatment of this adsorbate has yielded an additional active, lactoflavin-free fraction which, when fed with lactoflavin and the fuller's earth filtrate, appears to satisfy the whole vitamin B_2 requirements of rats.

Whilst these investigations have been in progress, communications bearing on this subject have appeared from other laboratories. Euler & Malmberg [1936] suggested that two supplements for lactoflavin besides vitamin B_1 are present in yeast extracts, though adequate experimental evidence was not provided for this hypothesis. Lepkovsky *et al.* [1936] showed that the vitamin B_2 requirements of rats were satisfied only when (1) lactoflavin, (2) an eluate prepared from the fuller's earth adsorbates of rice bran extracts and (3) a fuller's earth filtrate from aqueous liver extracts [Elvehjem & Koehn, 1935] were supplied. These results have been supported recently by Halliday & Evans [1937], whose work has further indicated the presence of the above supplementary factors, (2) and (3), in alcoholic extracts of wheat. Elvehjem *et al.* [1936] have reported the presence of a further factor for nutrition of rats in an ether-alcohol precipitate from aqueous liver extracts, required presumably in addition to the factors described by other investigators; Halliday & Evans [1937] have, however, adversely criticized this work.

Experimental

The methods employed in the present work were similar to those described in the preceding paper [Edgar *et al.* 1937], the criterion being growth of young rats. Rats were deprived of the vitamins of the B_2 group at weaning and after 10–14 days, when all weight increase had ceased, received the test doses of the different fractions supplemented by 50γ daily of lactoflavin, an amount in excess of the optimum requirements [Edgar *et al.* 1937]. Crystalline vitamin B_1 hydrochloride was supplied from the time of weaning, the doses being 10–20γ daily according to the body weight.

Comparison of the growth-promoting action of the B-vitamins provided as (a) untreated aqueous yeast extract, (b) yeast extract autoclaved at pH 5, supplemented by crystalline vitamin B_1 hydrochloride and (c) the fuller's earth filtrate from autoclaved yeast extract supplemented by vitamin B_1 and lactoflavin

A litter of rats was divided into 3 groups as follows: 2 males received doses of an untreated aqueous yeast extract equivalent to 1 g. yeast, dry wt. daily; 2 males received similar doses of the yeast extract after autoclaving at *p*H 5 at 120° for 5 hr., supplemented by 10–20γ daily of vitamin B_1 hydrochloride; 2 males and 2 females received doses of the yeast fuller's earth filtrate [see Edgar *et al.* 1937] equivalent to 1–1·5 g. yeast, dry wt. daily, supplemented by 10–20γ daily of vitamin B_1 and 50–75γ daily of lactoflavin.

The growth rates of the rats receiving the untreated yeast extract (curve *A*, Fig. 1) were almost identical with those of the rats having autoclaved yeast extract supplemented by crystalline vitamin B_1 hydrochloride (curve *B*); both

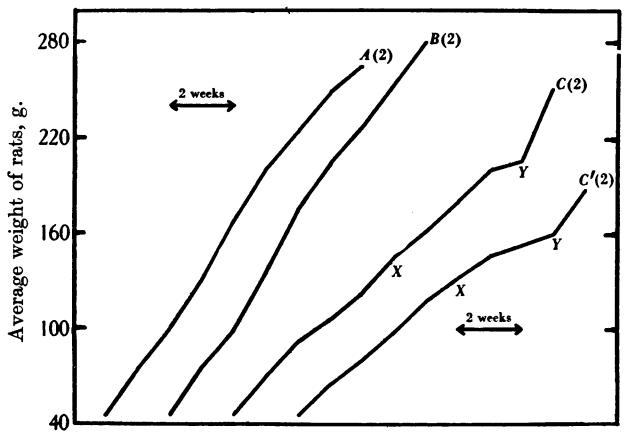

Fig. 1. Growth of young rats on a basal diet free from B-vitamins and receiving daily as sources of B-vitamins: curve *A* (♂ rats): untreated yeast extract = 1 g. yeast, dry wt.; curve *B* (♂ rats): autoclaved yeast extract = 1 g. yeast, dry wt. + 10–20γ vitamin B_1 hydrochloride; curve *C* (♂ rats) and *C'* (♀ rats): yeast fuller's earth filtrate = 1 g. yeast, dry wt. + 50γ lactoflavin + 10–20γ vitamin B_1 hydrochloride. (At *X* the doses were increased to fuller's earth filtrate = 1·5 g. yeast, dry wt. and 75γ lactoflavin. At *Y* the doses were changed to autoclaved yeast extract = 1 g. yeast, dry wt. + 20γ vitamin B_1 hydrochloride.) The figures in brackets indicate the number of rats from which the growth curves were derived.

pairs showed rapid and steady growth during the experimental period of 8 weeks. The rats receiving the fuller's earth filtrate supplemented by lactoflavin and vitamin B_1 showed lower growth rates (curves *C* and *C'*); when these doses were

Table I. *Growth of rats receiving the constituents of the vitamin B_2 complex as different fractions from yeast extract*

Unless otherwise stated, 10–20γ vitamin B_1 hydrochloride were given daily

	No. of rats ♂	No. of rats ♀	Lactoflavin daily γ	Yeast preparation given	Daily dose as equivalent of yeast, dry wt. g.	Average weekly gain in weight over period of 4 weeks g. ♂	Average weekly gain in weight over period of 4 weeks g. ♀
Exp. 1	2	0	0	Untreated yeast extract; no additional vitamin B_1	1·0	31·1	—
	2	2	0	Autoclaved yeast extract	1·0	31·3	22·75
	2	2	50	Fuller's earth filtrate	1·0	19·3	18·3
	1	1	75	Fuller's earth filtrate	1·5	19·25	17·0
Exp. 2	1	3	50	Purified fuller's earth filtrate	1*	22·5	18·5
	2	3	50	Fuller's earth eluate	2*	12·1	11·9
	3	5	50	Fuller's earth filtrate + fuller's earth eluate	1 + 2	32·6	24·0
						Average weekly gain in weight over period of 2 weeks g. ♂	Average weekly gain in weight over period of 2 weeks g. ♀
	—	1	0	Fuller's earth eluate; after 2 weeks, given fuller's earth filtrate in addition	2 1	—	2·5 8·5
	—	1	0	Fuller's earth eluate + fuller's earth filtrate	2 + 1	—	3·5

* Dose doubled after 2 weeks.

replaced by untreated yeast extract a sharp increase in growth rate resulted immediately. Further experiments, summarized in Table I, Exp. 1, confirmed these results.

These experiments demonstrate that autoclaving yeast extracts at *p*H 5 does not destroy any of the B-vitamins concerned with growth other than vitamin B_1, and that treatment with fuller's earth removes from an autoclaved yeast extract at least one growth factor in addition to lactoflavin.

Investigation of the yeast fuller's earth adsorbate

Preliminary experiments in which fuller's earth adsorbate was itself fed directly to young rats in addition to the fuller's earth filtrate and lactoflavin suggested that the adsorbate had some additional growth-promoting action. The growth responses were, however, irregular, and seemed to indicate that elution of the adsorbed material from fuller's earth was not accomplished satisfactorily in the alimentary canal of the rats. Eluates were therefore prepared, the following method being finally adopted.

The fuller's earth adsorbate was made by adding 50 g. of fuller's earth (B.D.H. "for adsorption purposes") to 1 l. of aqueous yeast extract (1 ml. $\equiv$ 0·5 g. yeast, dry wt.), which had been autoclaved at *p*H 5 at 120° for 5 hr. and then adjusted to *p*H 1·4. This adsorbate was washed twice by grinding in a mortar with 250 ml. of 0·1 *N* HCl and then, after filtering, was thoroughly mixed with 600 ml. of 0·1 *N* $Ba(OH)_2$ in a mortar. After standing for 16 hr. in the cold, the fuller's earth was filtered off and the elution repeated. The eluates were

neutralized with H_2SO_4 immediately after being filtered from the fuller's earth. In order to remove the lactoflavin present, the combined eluates were treated at *p*H 8 with a slight excess of a solution of basic lead acetate (containing approximately 7 g. basic lead acetate). After standing 16 hr. in the cold, the precipitate was filtered off, the filtrate treated with H_2S, the lead sulphide filtered off and the final filtrate reduced *in vacuo* to a volume of 250 ml. (1 ml. = 2 g. yeast, dry wt.). This material will be referred to as the yeast fuller's earth eluate.

For the following experiments the yeast fuller's earth filtrate was further purified by three additional adsorptions with fuller's earth at *p*H 3, 50 g. fuller's earth per litre of filtrate being used for each adsorption.

The additional growth-promoting action of the eluate fraction on rats receiving vitamin B_1, lactoflavin and the purified fuller's earth filtrate was striking (Table I, Exp. 2 and Fig. 2). Rats receiving daily the eluate solution

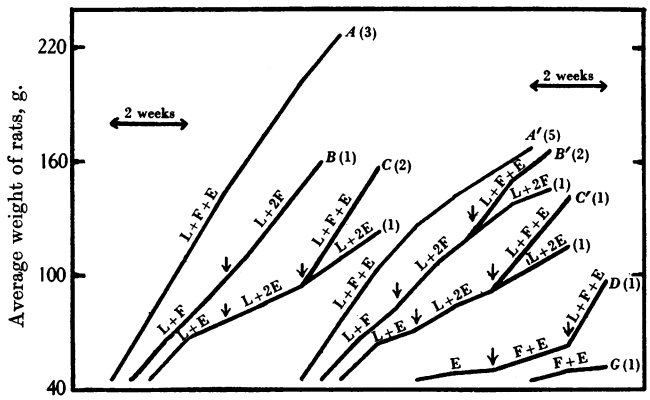

Fig. 2. Growth of young rats on a basal diet free from B-vitamins and receiving daily 10–20γ vitamin B_1 hydrochloride and one or more of the following components of the vitamin B_2 complex: L = 50γ lactoflavin. F = purified fuller's earth filtrate = 1 g. yeast, dry wt. E = fuller's earth eluate = 2 g. yeast, dry wt. The arrows indicate the points at which the doses were changed. Curves *A*, *B*, *C*, ♂ rats. Curves *A′*, *B′*, *C′*, *D*, *G*, ♀ rats. The figures in brackets indicate the number of rats from which the growth curves were derived.

(= 2 g. yeast, dry wt.) supplemented by the filtrate fraction (= 1 g. yeast, dry wt.) and 50γ of lactoflavin showed weight increases of about 24–32 g. weekly, according to their sex, over a 4-week period (curves *A′* and *A*). This is a growth rate of the same order as that attained by rats maintained on experimental diets with untreated yeast extract as source of the B-vitamins, or on a good mixed diet of natural foodstuffs. The growth rate obtained with litter-mate rats receiving lactoflavin supplemented only by the fuller's earth filtrate (1 g. yeast, dry wt. daily) was approximately 15–20 g. weekly for 4 weeks (curves *B′* and *B*), and was little affected by doubling the dose of the filtrate fraction, but it was greatly enhanced by the addition of the eluate fraction. Feeding of the eluate fraction in doses equivalent to 2 g. dried yeast daily supplemented by lactoflavin caused an initial increase in growth (curves *C* and *C′*) similar to that obtained when the filtrate fraction was fed with lactoflavin, but, after about 10 days when the animals had gained approximately 30 g., the growth slackened sharply; doubling the eluate dose at the end of 2 weeks had little or no effect on the growth rate, while the addition of the filtrate fraction to the diet caused a marked increase.

Since only a slight or negligible effect on the growth rate was observed by doubling the doses of either the filtrate fraction or the eluate fraction it may be assumed that a fairly complete separation of these two growth-promoting vitamins had been attained. The eluate fraction, having been treated with basic lead acetate, contained no lactoflavin, and rats receiving this fraction and vitamin B_1 only did not show any increase in weight, nor did they grow when these were supplemented with the filtrate fraction (curves *D* and *G*). This again illustrates the fact [see Edgar *et al.* 1937] that lactoflavin is the most active of the growth-promoting vitamins of the B_2 group, since in its absence all growth was checked, while the absence of one or both of the other factors resulted only in limitations of the growth if the lactoflavin were present in the diet.

The regularity of the growth response of the animals to the various factors was striking, the effects being evident in 1 week or even less. When all three vitamin B_2 components were present, the average increase in weight during the first week was 28–35 g.; in the absence of either the eluate fraction or the filtrate fraction it was 18–23 g. By the end of the second week a difference was apparent, absence of the filtrate fraction resulting in a marked slackening in the growth rate during the second week, while in the absence of the eluate fraction the initial suboptimum growth rate was maintained for 3–4 weeks.

Relation of the filtrate and eluate fractions to rat dermatitis

The curative actions of the yeast fuller's earth filtrate and the yeast fuller's earth eluate for the symmetrical dermatitis, developed by rats maintained on a diet containing only vitamin B_1 and lactoflavin of the vitamin B complex, are being investigated. The generalized skin affection developed in rats deprived of lactoflavin is known to be a specific sign of this deficiency and responds promptly when lactoflavin is given [Copping, 1936].

Discussion

Yeast contains all the vitamins of the B group; indeed, the B-vitamins are often defined as the water-soluble essential food factors contained in yeast. The complex nature of yeast extracts, however, has induced many investigators to use other materials as sources of the B-vitamins. This policy has met with considerable success, the most important achievement of recent years being the isolation of lactoflavin from whey by Ellinger & Koschara [1933, 1, 2] and from whey and egg white by Kuhn *et al.* [1933, 1, 2, 3]. Warburg & Christian [1932], however, had previously obtained preparations of the yellow oxidation enzyme from yeast which have since been shown to contain the lactoflavin molecule and to possess vitamin activity. In this laboratory fractionation of yeast extracts has offered the most hopeful method for complete biological characterization of the vitamin B complex, although isolation of individual factors may be found simpler from other materials.

Although our understanding of all the dietary factors present in the vitamin B complex is still very incomplete, the differentiation of the factors present in yeast described in this paper will, it is hoped, clarify this problem. The "vitamin B_6" from wheat germ or fish muscle, described by György [1935, 1, 2] and Birch & György [1936], does not appear from its chemical and physical and dermatitis-curing properties to be identical with the rat growth factor present in the yeast fuller's earth filtrate fraction [see Edgar & Macrae, 1937] but resembles rather the factor present in the eluate fraction; further investigations must decide this point. The fuller's earth filtrate factor, however, appears to be

similar in its properties to the filtrate obtained from fuller's earth treatment of liver extract, first described by Elvehjem & Koehn [1935], and later investigated and named the "filtrate factor" by Lepkovsky & Jukes [1936]. This factor prevents dermatitis and restores growth in chicks fed on a heated grain diet [Kline *et al.* 1932], but only manifests its activity in rats by its growth-promoting action. Whether this chick antidermatitis factor is identical with the rat growth factor is open to discussion.

The factor obtained by Lepkovsky *et al.* [1936] from the fuller's earth adsorbate from rice bran extracts, which cured rat dermatitis, may be similar to, or identical with, the factor described in this paper, contained in the eluate fraction from fuller's earth adsorbates of yeast extract. Their preparation from rice bran stimulated further the growth rates of rats receiving lactoflavin and the "filtrate factor", just as in our observations the growth rates of animals receiving lactoflavin and the yeast extract filtrate fraction were enhanced by the addition of the yeast eluate preparation.

In this laboratory the growth rates of rats receiving both the filtrate and eluate fractions from yeast supplemented by optimum doses of lactoflavin (50γ daily) were comparable with those of rats receiving good mixed diets, weekly averages of 32 and 24 g. being maintained by the males and females respectively. Lepkovsky *et al.* [1936] obtained similar growth rates by feeding 40γ daily of lactoflavin with the eluate fraction from rice bran and the filtrate fraction from liver. The importance of lactoflavin as a growth-promoting factor is illustrated by the experiments described in our previous paper [Edgar *et al.* 1937]. Halliday & Evans [1937] reported only subnormal growth rates (average gain of 22–32 g. in 4 weeks) when they fed both eluate and filtrate fractions from wheat and other sources, supplemented by only 5γ daily of lactoflavin; in their experiments the effect on growth of omitting either the eluate or the filtrate fraction was much less noticeable than in those carried out in this laboratory, where the rats were receiving much larger doses of lactoflavin. It appears to be necessary, in order to distinguish other factors of the vitamin B_2 complex, to supply the optimum requirement of lactoflavin in the diet. This would have the further advantage of eliminating any effect on growth rate that would arise from the presence of lactoflavin in the substance tested.

An additional rat growth factor present in the alcohol-ether precipitate from liver extracts has been described by Elvehjem *et al.* [1936], but from the method given for preparation of this fraction, it would appear that any or all of the water-soluble essential rat factors present in liver might be contained in this precipitate.

The work of Reader [1929; 1930] postulating the existence in the B group of vitamins of "vitamin B_4", a second heat-labile factor, distinct from vitamin B_1, has not been confirmed [Kinnersley *et al.* 1935; O'Brien, 1934]. Recently, however, the existence of "vitamin B_4" has been supported by Keenan *et al.* [1933] and by Kline *et al.* [1936, 1, 2]. The fact that yeast extracts autoclaved at *p*H 5 at 120° for 5 hr. completely satisfy the vitamin B requirements of rats when supplemented with crystalline vitamin B_1 hydrochloride indicates that no other heat-labile dietary factor is essential for rat nutrition.

Fouts *et al.* [1936] have reported successful treatment of human pellagra with their "filtrate factor" from liver, while Koehn & Elvehjem [1936] used it for the cure of black tongue in dogs. It is pointed out by Jukes [1937] that foods found to be rich in the "filtrate factor", when tested by the chick growth method, are not those rich in pellagra-preventive (P-P) factor, i.e. not found to be of value in the prevention of human pellagra. It seems therefore probable

that this chick growth factor is not identical with the pellagra-preventive (P-P) factor. It would be of interest to repeat these tests using a rat growth method, for it is possible, as already stated, that the chick-antidermatitis and growth factors might not be identical with the rat growth factor.

SUMMARY

1. Two heat-stable factors, necessary for the optimum growth of rats maintained on a vitamin B-free diet and receiving lactoflavin and vitamin B_1, have been separated from yeast. One factor is present in the filtrate, after exhaustive extraction with fuller's earth of an autoclaved acid aqueous yeast extract, while the second is contained in the eluate from the fuller's earth adsorbate after the removal of the lactoflavin.

2. The growth rates of rats receiving both the eluate and filtrate fractions supplemented by vitamin B_1 and optimum doses of lactoflavin were similar to those of rats receiving a good mixed diet of natural foodstuffs.

3. Vitamin B_1 appears to be the only member of the B group of vitamins which is destroyed by autoclaving at *p*H 5 at 120°.

We wish to thank Dr H. Chick for her continued interest and advice and Sir Charles Martin for his helpful suggestions. We gratefully acknowledge 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.

REFERENCES

Birch & György (1936). *Biochem. J.* **30**, 304.
Copping (1936). *Biochem. J.* **30**, 845.
Edgar & Macrae (1937). *Biochem. J.* **31**, 893.
—— —— & Vivanco (1937). *Biochem. J.* **31**, 879.
Ellinger & Koschara (1931, 1). *Ber. dtsch. chem. Ges.* **66**, 315.
—— —— (1933, 2). *Ber. dtsch. chem. Ges.* **66**, 808.
Elvehjem & Koehn (1935). *J. biol. Chem.* **108**, 709.
—— —— & Oleson (1936). *J. biol. Chem.* **115**, 707.
Euler & Malmberg (1936). *Biochem. Z.* **284**, 455.
Fouts, Lepkovsky, Helmer & Jukes (1936). *Proc. Soc. exp. Biol., N.Y.*, **35**, 245.
György (1935, 1). *Biochem. J.* **29**, 741.
—— (1935, 2). *Biochem. J.* **29**, 767.
Halliday & Evans (1937). *J. biol. Chem.* **118**, 255.
Jukes (1937). *J. biol. Chem.* **117**, 11.
Keenan, Kline, Elvehjem, Hart & Halpin (1933). *J. biol. Chem.* **103**, 671.
Kinnersley, O'Brien & Peters (1935). *Biochem. J.* **29**, 701.
Kline, Keenan, Elvehjem & Hart (1932). *J. biol. Chem.* **99**, 295.
—— Elvehjem & Hart (1936, 1). *Biochem. J.* **30**, 780.
—— Bird, Elvehjem & Hart (1936, 2). *J. Nutrit.* **11**, 515.
Koehn & Elvehjem (1936). *J. Nutrit.* **11**, 67.
Kuhn, György & Wagner-Jauregg (1933, 1). *Ber. dtsch. chem. Ges.* **66**, 317.
—— —— —— (1933, 2). *Ber. dtsch. chem. Ges.* **66**, 576.
—— —— —— (1933, 3). *Ber. dtsch. chem. Ges.* **66**, 1034.
Lepkovsky & Jukes (1936). *J. biol. Chem.* **114**, 109.
—— —— & Krause (1936). *J. biol. Chem.* **115**, 557.
O'Brien (1934). *J. Soc. chem. Ind., Lond.*, **53**, 452.
Reader (1929). *Biochem. J.* **23**, 689.
—— (1930). *Biochem. J.* **24**, 77.
Warburg & Christian (1932). *Biochem. Z.* **254**, 438.