XV. HEAT-STABILITY OF THE (ANTI-DERMATITIS, "ANTI-PELLAGRA") WATER-SOLUBLE VITAMIN B_2.

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When Goldberger and his colleagues [1926] discovered in yeast the water-soluble vitamin B_2, the nutritional factor which they named "P-P" and considered was involved in the prevention and cure of human pellagra, its separation from the antineuritic vitamin B_1 was achieved by heating the yeast in an autoclave for 2½ hours at 15 lbs. pressure. By this degree of heat the antineuritic vitamin was destroyed but the yeast thus heated contained a dietary factor necessary to prevent dermatitis and maintain health and growth in young rats.

In the efforts of subsequent workers [Chick and Roscoe, 1927, 1928, 1929; Williams and Waterman, 1927, 1928; Hogan and Hunter, 1928; Reader, 1929] to unravel the complex formerly known as "vitamin B," autoclaved yeast and autoclaved yeast extracts have frequently been employed as sources of vitamin B_2 apart from vitamin B_1, and the former is usually referred to as the "heat-stable" or "more heat-stable" constituent of the complex.

There is, however, little precise knowledge as to the degree to which vitamin B_2 is stable to heat, or of its sensitiveness to the action of acids and alkalis at high temperature. At the present time such information is especially important in view of the recently reported discovery of other thermolabile and thermostable constituents of the vitamin B complex, in addition to the well-established factors B_1 and B_2 [Hunt, 1928; Williams and Waterman, 1928; Reader, 1928, 1929; Peters, 1929]. These researches are discussed later in this paper.

While the following experiments were in progress, Williams, Waterman and Gurin [1929] published their investigation of the effect of reaction upon the destruction of vitamins B_1 and B_2 in brewer's yeast, when heated for 6 hours at 120° in an autoclave. They observed the growth of rats on diets in which the heated material was the sole source of vitamin B_1 or B_2 respectively. They concluded that much of the original vitamin B_2 survived the above degree of heating if the reaction were that natural to yeast (p_H about 4·5) and more if it were more acid (p_H 1·0–2·0), and further that some at least of the vitamin B_1 could survive this treatment if the reaction were acid. If the material heated were alkaline (p_H 8–14) both vitamins were destroyed.

As far as we are aware the only approximately quantitative data in the literature on the heat-stability of vitamin B_2 are contained in a previous paper of our own [1927], where a comparison was made between the content of vitamin B_1 and vitamin B_2 respectively, in dried brewer's yeast before and after heating for 5 hours in an autoclave at 120°. Of the unheated yeast 0·05 g. sufficed to cure the collapse due to lack of vitamin B_1 observed in young rats on an otherwise complete diet, while no alleviation of symptoms was observed with 0·2 g. of the heated material. When the diet contained vitamin B_1 as Peters's [1924] antineuritic yeast concentrate, 0·2 g. daily of the unheated, or 0·3–0·4 g. daily of the autoclaved, yeast sufficed to maintain growth and prevent dermatitis. That is to say, yeast after exposure to moist heat at 120° for 5 hours, contained about 50 % of the vitamin B_2, but no significant amount of the vitamin B_1, present in the original material.

The present paper contains the results of a more precise determination of the stability to heat of vitamin B_2 as contained in two different yeast products. Washed, pressed brewer's yeast was used in one series of experiments (A, Table I); "Yeast Fraction 5," an extract made from the former with dilute acetic acid, in a second series (B, Table II).

Series A (Table I). Fresh brewer's yeast was washed with cold tap-water and filtered four times; it was then pressed. A weighed sample (No. 1) was removed and dried to serve as control material and for determination of dry weight. Five weighed portions of the remainder were taken, each puddled to a thick cream with an equal weight of water, the p_H being about 5·0 (bromocresol green). One portion (No. 2) was heated in a steamer for 2 hours at 98–100° and another (No. 4) autoclaved at 123° for 5 hours. Of the remainder, portions 3 and 5 were made alkaline by addition of strong sodium hydroxide to a p_H about 9–10 (thymol blue) and heated respectively as portions 2 and 4 above. Portion 6 was acidified with concentrated hydrochloric acid to p_H about 3·0 (thymol blue and bromophenol blue) and autoclaved for 5 hours at 123°, during which process the acidity fell to a p_H of about 3·5. The alkaline material in portions 3 and 5 similarly became less alkaline during heating. After heating, all five portions were dried to a convenient consistency in a hot room under a fan, 3, 5 and 6 being previously acidified to a p_H of about 4–5. Determinations of the dry weight were made in each final product, and, in the subsequent tests, the doses were calculated in terms of the dry weight of yeast contained. The values for p_H given above were obtained colorimetrically with indicators and are approximate only.

The method of vitamin B_2 assay was that previously described [Chick and Roscoe, 1928; Aykroyd and Roscoe, 1929]. Young rats, 35–45 g. weight, immediately after weaning, were placed upon the basal diet free from B vitamins for 1–2 weeks, during which period growth was arrested. Afterwards, they received a suitable daily dose of the material to be assayed for vitamin B_2 and 0·1 cc. (the equivalent of 0·6 g. yeast) of Peters's [1924] antineuritic yeast concentrate, as source of vitamin B_1. The growth of the rat was then observed

Table I. *Heat-stability of vitamin B_2 in brewer's yeast, determined by observations on the growth of young rats receiving doses of the material before and after heating, respectively, as sole source of vitamin B_2 in a diet* complete in other respects and including vitamin B_1 as Peters's antineuritic yeast concentrate (daily dose equivalent to 0·6 g. original yeast).*

Exp.	Material (Yeast XVII)	Reaction during heating p_H approximate	Daily dose in terms of dry yeast g.	Litter	Rat	Body-weight at beginning of the test g.	Weekly increments of body-weight during the period of the test g.	Average (1st week excluded in expts. 1, 2, 4, 6)	Mean
1 Control	Washed, pressed, dried	—	0·25	1366	504 ♂	47	20, 19, 14, 13, 17	16	16
			0·2	1367	516 ♀	33	11, 14, 17, 11, 11	13	
			0·2	1378	525 ♀	32	16, 17, 15, 6, 14	13	14
			0·2	1385	536 ♂	40	14, 12, 18, 16	15·5	
			0·1	1367	515 ♂	35	7, 10, 12, 6, 12	10	
			0·1	1378	524 ♀	35	9, 12, 9, 9, 11	10	10
2	Steamed 2 hrs. 98–100°	5·0	0·2	1385	534 ♀	38	12, 17, 9, 12	13	
			0·2	1385	535 ♂	41	16, 16, 18, 16	17	15
3	„	9–10	0·4	1374	509 ♀	47	3, 1, 5, 4, 12	5	—
			0·4	1367	512 ♂	41	1, −1	0	—
			0·2	1385	533 ♀	39	1, 0, −2, 2	0	—
4	Autoclaved 5 hrs. at 123°	5·0	0·8	1438	545 ♂	47	29, 23, 15, 13	17	17
			0·4	1367	519 ♂	36	16, 14, 9, 11, 11	11	
			0·4	1374	510 ♀	46	26, 14, 13, 11, 11	12	12
			0·2	1378	526 ♀	33	15, 18, 11, 7, 11	12	
			0·2	1367	520 ♀	35	10, 11, 10, 8, 9	9·5	11
5	„	9–10	0·4	1374	508 ♀	56	1, −2, 3, 6, 0	2	
			0·4	1374	505 ♂	47	5, 0	2·5	—
			0·4 (+0·1 g. dry yeast)	„	„	(52)	(3, 6, 7)	(5)	—
			0·4	1374	507 ♀	36	1, −1	0	—
			0·4 (+0·1 g. dry yeast)	„	„	(36)	(8, 9, 8)	(8)	—
			0·4	1367	517 ♀	35	−1, −1	−1	—
			0·4 (+0·1 g. dry yeast)	„	„	(33)	(7, 4, 6)	(6)	—
6	„	3·0–3·5	0·8	1438	540 ♂	44	26. 29, 28, 23	27	27
			0·4	1367	514 ♀	42	12, 18, 12, 8, 9	12	
			0·4	1374	506 ♂	44	19, 17, 7, 7, 10	10	11
			0·2	1378	527 ♀	31	11, 12, 6, 4, 13	9	
			0·2	1367	518 ♀	34	9, 13, 12, 5, 13	11	10

* Specially purified caseinogen 20, rice starch 60, cotton seed oil 15, salt mixture (McCollum and co-workers [1917] No. 185) 5, water 100; cooked 3 hours in a steamer to prevent occurrence of refection [Roscoe, 1927]; cod-liver oil (0·05–0·1 g. according to size) administered daily to each rat to provide vitamins A and D.

for a period of 4–5 weeks. The preliminary period with complete deprivation of B vitamins is inserted in order to make the rats eager to consume the doses when offered subsequently.

The results of Exp. A are set out in Table I, the aim of the separate trials being to compare the various products with the original material, as regards the size of dose needed to induce resumption of growth to an equal degree.

A rat which is limited in growth by absence from its diet for 1–2 weeks of vitamin B_2 and subsequently receives it, appears to be relatively insensitive to large variations in the amount administered, especially if the daily dose is near to that required for normal growth. As large a difference as 100 % in the dose given may be followed by a disproportionately small difference in the amount of growth. The quantitative conclusions drawn from the data in Tables I and II have, therefore, a wide margin of error.

The results in Table I may be summarised as follows. Yeast at its ordinary reaction (p_H about 5·0) suffers no determinable loss in vitamin B_2 content

after being steamed for 2 hours at 98–100°. The average weekly increase in weight of 2 rats receiving doses of the heated material equivalent to 0·2 g. dried yeast was 15 g., while that of 3 rats receiving 0·2 g. of the original dried yeast was 14 g. After being autoclaved for 5 hours at 123° about half the original potency is destroyed; the average weekly increase in weight of rats receiving doses equivalent to 0·2 g. dried yeast of the autoclaved material and 0·1 g. of the original, being similar, viz. 11 g. and 10 g. respectively.

With alkalised yeast (p_H 9–10), however, whether steamed (Exp. 3) or autoclaved (Exp. 5), the destruction of vitamin B_2 appeared to be complete. With the doses given no significant growth was obtained. It was noticed, however, that the animals suffered severely from diarrhoea and it is probable that the total lack of growth was due, not only to deficiency of vitamin B_2, but also to the effect of toxic substances formed in the alkaline yeast during heating. In order to test this point 3 rats (505, 507, 517, Exp. 5), which for 2 weeks had shown no growth with doses of the autoclaved product equivalent to 0·4 g. dry yeast, received in addition 0·1 g. unheated dry yeast and were observed for a further period of 3 weeks. During this period the average weekly increase in weight was 6·4 g. as compared with 10 g. increase shown by animals which received the 0·1 g. dose of dry yeast alone (rats 515 and 524, Exp. 1). This discrepancy points to the presence of some actively deleterious substance in the alkaline-heated yeast. Our next effort, therefore, was to find some substance containing less material of a protein-like nature from which toxic products could be formed when autoclaved. An extract made from yeast with boiling dilute acetic acid was found suitable for the purpose.

Series B (Table II). The material used was the first extract obtained in the preparation of Peters's antineuritic yeast concentrate. Washed, pressed yeast was added gradually to boiling water containing 0·01 % acetic acid in the proportion of 1 kg. (200 g. dry weight) to 2 litres solution. The mixture was brought again to the boil, boiled for 5 minutes and filtered. The filtrate was concentrated on a water-bath to a volume of 400 cc., so that 2 cc. contained the equivalent of 1 g. dry yeast. The material was acidified to p_H about 3·0 for storage. Although still rich in vitamin B_2 the extract contained only one-fifth of the total solids of the original yeast. From the concentrated solution measured volumes were removed, the control sample remaining at p_H 3·3 and others being adjusted by addition of strong NaOH solution to p_H 8·3 and 9·9 respectively.

Treatment was as follows. Steaming for 2 hours at 98–100° at p_H 8·3 (Exp. 9) and autoclaving at 122–125° for 4 hours at p_H 8·3, 9·9 and 3·3 respectively (Exps. 10, 11 and 12, Table II). In each case the p_H before and after heating was determined by means of a hydrogen electrode and it was found that during heating the alkaline material became less alkaline (Table II, Column 3). After being heated the samples were adjusted to the original volume and those which were alkaline were acidified to p_H about 3·0 for

Table II. *Heat-stability of vitamin B_2 in dilute acetic acid (0·01 %) extract made from brewer's yeast XII. Method of assay as in Table I.*

Exp.	Material (Yeast extract XII)	Reaction p_H	Daily doses expressed as equivalent of original dry yeast g.	Litter	Rat	Body-weight at beginning of the test g.	Weekly increments of body-weight during the period of the test g.	Average	Mean
7	Fraction 5	3·0	0·4	1438	542 ♂	47	18, 25, 18	20	18
Control			0·4	1443	560 ♀	42	20, 14, 14	16	
			0·2	1443	558 ♀	41	11, 11, 11	11	11
			0·2	1468	566 ♂	43	12, 10, 12, 12	11·5	
8	Kept at room temp. 10 days	9·9–9·5	0·4	1468	572 ♂	39	17, 10, 14, 20	15	15
			0·2	1468	573 ♀	43	8, 5, 11, 8	8	8
			0·2	1506	597 ♀	44	12, 4, 7, 7	7·5	
9	Steamed 2 hrs. at 98–100°	8·3–8·0	0·8	1468	570 ♀	41	19, 18, 14, 12	16	16
			0·4	1468	571 ♂	44	15, 13, 14	14	14
10	Autoclaved 4 hrs. 122°	8·3–7·1	0·8	1438	543	50	6, 3	4·5	5·5
			0·8	1442	548	40	8, 5	6·5	
11	Autoclaved 4 hrs. 124°–125°	9·9–8·7	0·8	1468	568 ♂	45	3, 9, 4, 10	6·5	—
			0·8 (+0·1 g. dry yeast XVII)	„	„	(71)	(22, 19)	(20·5)	—
			0·8	1468	569 ♀	41	3, −1, 1	1	—
			0·8 (+0·1 g. dry yeast XVII)	„	„	(44)	(18, 23)	(20·5)	—
12	Autoclaved 4 hrs. 123°–124°	3·3–3·0	0·8	1438	544 ♂	45	23, 26, 15	21	21
			0·4	1443	557 ♂	46	16, 11, 13	13	12
			0·4	1468	567 ♀	38	12, 7, 11, 14	11	

storage. Owing to the limited supply of the material fewer rats were used than in Series A and the growth was observed for a shorter period, viz. 2–4 weeks.

The loss in vitamin B_2 content on autoclaving the acidified extract for 4 hours was about 50 %; similar to that observed with the acidified yeast. Rats receiving doses of the original extract equivalent to 0·2 g. and of the autoclaved material equivalent to 0·4 g. yeast showed a similar average weekly increase in weight, viz. 11 g. and 12 g. respectively (Exps. 7 and 12).

The loss of vitamin B_2 on heating in alkaline solution was much greater. About one-half the original potency was destroyed by steaming at 98–100° at p_H 8·3–8·0 and more than three-fourths by autoclaving for 4 hours at 122° (p_H 8·3–7·1). Rats receiving daily doses of the autoclaved extract equivalent to 0·8 g. yeast showed an average increase in weight of only 5·5 g. weekly (Exp. 10), whereas 11 g. weekly was the average for those receiving the original material equivalent to 0·2 g. yeast.

After being autoclaved for 4 hours at 124° in more alkaline solution (p_H 9·9–8·7) one test indicated complete destruction of vitamin B_2, another over 75 % destruction (rats 568, 569, Exp. 11). When, later, these two rats received 0·1 g. dried yeast in addition to their dose of autoclaved extract, the response in growth was instant and complete, showing that the previous failure was not attributable to the effect of any toxic material produced in the alkaline yeast extract during heating (compare Exp. 11, Table II, with Exps. 5 and 1, Table I).

A specimen of the alkalised extract (p_H 9·9) was protected from access of CO_2 and left at room temperature for 10 days during warm weather

(September 6–16, 1929). At the end of the period the material was acidified and the vitamin B_2 content assayed. The solution appeared to have lost about one-third of its original potency, doses equivalent to 0·4 g. yeast caused a growth response intermediate between those evoked by doses of the original material equivalent to 0·4 and 0·2 g. yeast (Exp. 8, Table II).

The results of Exps. A and B may be summarised as follows. Vitamin B_2 is comparatively stable to prolonged heating at high temperatures if the reaction is acid, p_H 5·0–3·0, about 50 % of the original content surviving 4–5 hours' heating at 122–124°. If alkaline, p_H 8·0–10·0, however, although less labile than vitamin B_1, vitamin B_2 loses 30 % potency in 10 days at room temperature, 50 % on steaming for 2 hours and 75–100 % on heating for 4–5 hours at 122–125°.

Discussion.

Since the discovery that "water-soluble B" contains at least two well-defined components, vitamins B_1 and B_2, evidence for the existence of an additional constituent has been brought forward from three different laboratories [Williams and Waterman, 1927, 1928; Hunt, 1928; Reader, 1929]. These researches have been summarised by Peters [1929].

One of these factors is stated to be thermostable and two thermolabile. All appear to be different from one another. None has been identified with prevention or cure of any specific disease or pathological syndrome. Their existence has been postulated to explain the failure of rats to grow to maturity (or of adult pigeons to maintain their weight) on diets which are believed to contain adequate supplies of the known necessary food factors, including vitamins B_1 and B_2. In some of these investigations vitamin B_2 has been supplied as strongly heated yeast or yeast products, the assumption being that the latter vitamin is entirely heat-stable.

Hunt [1928] has produced evidence showing that autoclaved yeast contains a heat-stable B vitamin necessary for rat nutrition in addition to vitamin B_2 and that this factor is contained in whole wheat in addition to vitamins B_1 and B_2.

Williams and Waterman [1927] found that the B vitamins required for normal growth of the rat could be supplied by an antineuritic concentrate prepared with fuller's earth from an aqueous yeast extract supplemented with autoclaved yeast. This combination could not, however, maintain weight in the pigeon. From the fact that this was accomplished by whole unheated yeast, the authors concluded that a third unknown B vitamin, unnecessary for growth of the rat, was required for nutrition of the pigeon and was heat-labile. Peters [1929] has confirmed this observation, using unheated marmite.

A recent paper by Reader [1929] announces the discovery of a "second thermolabile water-soluble vitamin necessary for the nutrition of the rat." The basal diet contained all the necessary food constituents except B vitamins and when these were provided, as 6 % marmite, normal development of

young rats took place. Growth, however, was subnormal and after a few weeks failed altogether, when vitamin B_1 was provided by Peters's antineuritic concentrate and vitamin B_2 as 6 % alkaline (p_H 9) marmite heated for 1 hour at 120°. It is assumed that the latter material contained an adequate supply of vitamin B_2 and the growth failure is attributed to destruction of a third B vitamin, "vitamin B_3," which is present in the raw marmite and is heat-labile. In view of the demonstration in the present paper of the serious degree of destruction suffered by vitamin B_2 in yeast and yeast extracts when these are heated at an alkaline reaction, it cannot be accepted, in the absence of quantitative experiments, that adequate amounts of vitamin B_2 survived the heating to which the marmite was subjected in Reader's experiments. And it seems doubtful whether the effect interpreted as due to qualitative deficiency of a new vitamin, "B_3," might not have been caused by an insufficiency of vitamin B_2.

Reader suggests (p. 693) that traces of "vitamin B_3" are probably present in Peters's antineuritic concentrate as at first obtained after extraction of the norite with dilute acid alcohol, and that these are removed in the further purification by fractionation with alcohol [Kinnersley and Peters, 1927]. In her experiments, vitamin B_1 was supplied as the more purified concentrate; in ours the less pure was used. We have obtained normal growth of rats to maturity (6–7 months) when vitamin B_2 was provided as autoclaved (slightly acid) yeast and vitamin B_1 as the less purified Peters's concentrate (unpublished experiments). It might, therefore, be suggested that in these experiments the "heat-labile vitamin B_3" was contained in our preparation of vitamin B_1. The fact, however, that the same vitamin B_1 concentrate was unable to supplement our vitamin B_2 preparation, when the latter had been strongly heated in slightly alkaline solution, indicates that, notwithstanding Reader's statement to the contrary [1929, p. 690], vitamin B_2 is a "heat-labile factor," and it is unnecessary to postulate the existence of another.

It would be more satisfactory if in the assay of vitamin B_2 the criterion could be the prevention or cure of the dermatitis (? rat-pellagra) developed in rats deprived of this vitamin, rather than the maintenance of growth. The irregularity in the time of onset of symptoms and in the degree of their severity would, however, make such observations untrustworthy and quantitative results impossible. Nevertheless, tests of the curative value of the control and heated materials would be useful to confirm our conclusion that it is vitamin B_2 rather than a third heat-labile vitamin B_3 which is destroyed when yeast or its extracts are heated at high temperature at alkaline reaction.

Summary.

1. The anti-dermatitis (? anti-pellagra) vitamin B_2 contained in brewer's yeast and in an extract made therefrom, was found to be much more stable at high temperatures in acid (p_H 5·0–3·0) solutions than when the reaction was alkaline.

2. A yeast extract made with boiling dilute acetic acid (0·01 %) was found to be more suitable material for the study than yeast itself, owing to the formation of toxic substances in the latter during heating, especially when the reaction was alkaline.

3. At p_H 5·0 no loss in vitamin B_2 potency could be detected on heating yeast for 2 hours at 90–100°; about 50 % was lost on heating the yeast for 4–5 hours at 123°; at p_H 3·0 the loss was the same both with yeast and yeast extract.

4. When the reaction was alkaline (p_H 10–9·5) about 30 % of the vitamin B_2 originally contained in the yeast extract was lost in 10 days at room temperature (summer); on heating for 2 hours at 98–100° (p_H 8·3) the loss was about 50 % and on autoclaving for 4–5 hours at 122–125° (p_H 8·3–10) between 75 % and 100 %.

5. These facts concerning the sensitiveness of vitamin B_2 to high temperatures in alkaline solution are of importance in connection with the recently reported discovery in yeast of a new thermolabile B vitamin, in addition to the antineuritic vitamin B_1, the assumption in one at least of these researches being that vitamin B_2 is heat-stable even in alkaline solution.

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