[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, COLUMBIA UNIVERSITY, No. 424]

A CRITICAL INVESTIGATION AND AN APPLICATION OF THE RAT-GROWTH METHOD FOR THE STUDY OF VITAMIN B

BY H. C. SHERMAN AND ADELAIDE SPOHN

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For the present investigation white rats were selected as the experimental animal and dried skimmed milk as the vitamin-containing food. Our own experience and what we have been able to learn of the experience of others, lead to the belief that more regular results, and therefore a higher degree of precision in quantitative studies, may be expected from experiments with rats than with pigeons. While the gravimetric form of the Williams yeast-growth method is also capable of yielding satisfactorily quantitative data, the use of rats permits the interpretation of results in terms of vitamin B with much greater certainty and is therefore the preferable method.[1]

Selection and Care of Experimental Animals.—The white rats used were placed upon the experimental diets when 28 or 29 days old, this being the standard age adopted in our Laboratory for the separation of young rats from their mothers. These young (four weeks old) rats weighed in all cases between 30 and 65 g., in most cases between 40 and 50 g. The animals all came from mothers on similar diets (a mixture of dried whole milk and ground whole wheat, with or without the addition of meat), with the further precaution that, in each experiment, equal numbers of comparison and control rats came from mothers on exactly the same diet.

The young rats were assigned to the diets to be compared in the following manner. If, for example, in one experiment there were five variations of diet to be compared and ten rats were to be fed on each variation, it would very likely be necessary to make use of six to eight litters to obtain the 50 rats required. Each litter was distributed over the five variations of diet as far as it would go so that each rat on each diet had litter-controls on other diets, and the entire 50 rats were so distributed in the assignment that the total weight of the ten rats and the number of males and females on each modification was practically the same. (At this age the difference in size between the sexes is much smaller than the individual variation in either sex.)

The experiments were continued for eight weeks, from the beginning of the fifth to the end of the twelfth week of the rat's life, this being the period during which the rat grows most rapidly if normally fed. It was demonstrated by continuing several of the experiments for a period of 50%

[1] Sherman and Edgeworth, THIS JOURNAL, **45**, 2712 (1923).

longer time that this extension of the experimental period beyond that here described did not yield any additional significant information.

In the (relatively few) experiments in which the milk was fed mixed with the rest of the diet, the animals were caged in lots of six to eight, each lot in a cage having a floor area of 28 by 35 cm. In all other experiments each rat was kept separately in a round galvanized iron wire cage 22.5 cm. in diameter and 20 cm. high with a raised screen floor which prevented access to the feces. No bedding was used nor was wood in any form accessible to the rats at any time. Fresh water and the basal ration were given freely. The cages were cleaned frequently, usually the group cages every day and the individual cages three times a week. The animals were weighed weekly (or oftener) and the amount of food consumed was determined and recorded for the same weekly intervals.

The Basal Diets.—The basal diets used were designed to be devoid of vitamin B and to contain all the other essential food factors in as nearly as possible the optimum proportions. No attempt was here made to supply vitamin C to the rats as they do not seem to need it and we do not know any source of vitamin C which could be depended upon to be entirely free from vitamin B. The two basal diets used in this work had the following composition.

TABLE I

PERCENTAGE COMPOSITION OF BASAL DIETS

	Diet 94 %	Diet 107 %
Casein (purified by 60% alcohol)[a]	18	18
Salt mixture (Osborne and Mendel[2])	4	4
Butter fat	10	8
Cod liver oil	0	2
Starch	68	68
	100	100

[a] The casein used in the basal rations was freed from vitamin B as follows. One liter of 60% (by weight) alcohol was poured upon 200 g. of casein and the mixture stirred for 1/2 hour and then allowed to stand for 5 1/2 hours, filtered on a Büchner funnel and washed with 500 cc. of 60% alcohol; then again stirred with one liter of 60% alcohol as before, and this time the mixture was allowed to stand for 18 hours, then filtered and washed as before with 500 cc. of 60% alcohol and finally with 500 cc. of 90% alcohol to facilitate subsequent air-drying.

Tests of the Basal Diets.—In order to make sure that the basal diet contained the optimum amounts of its various constituents, it was fed in comparison with variations in which each ingredient in turn was increased in percentage, the increase replacing an equal weight of starch. Thus Diet 94 was compared with Diet 102 in which butter fat was increased to 15%; Diet 103, casein increased to 23%; Diet 105, salt

[2] Osborne and Mendel, *J. Biol. Chem.*, **37**, 572 (1919).

mixture increased to 5%; and Diet 104, casein replaced by meat protein. The results on these various diets were practically identical.

As a further test, similar variations in the constituents of the basal diet were introduced in experiments in which fixed amounts of milk were fed as sources of vitamin B. Again, no better results were obtained when the butter fat, or the casein, or the salt mixture was increased. Furthermore, it was shown by another series of experiments that the introduction of skimmed milk powder from which vitamin B had been extracted into the basal diet did not change the results. Each of the various modifications of both basal diets was tested on about 10 separate animals. Except for the condensed summary of a part of the data in Table III, the results of this extended and critical study of the basal diets are here omitted for the sake of economy of space. We believe it is amply established that the two basal diets used (Diets 94 and 107) furnish practically optimum amounts and proportions of all nutrients required by the rat except vitamin B, and that all increases in growth resulting from the addition of the milk to either of these diets may confidently be attributed to the vitamin B thus furnished.

The individual records of 10 rats receiving Diet 107 plus 8 cc. of milk per day are shown in Table II.

Table II

Weights of Ten Rats Receiving Diet 107 and 8 Cc. of Milk

Rat	4	5	6	7	8	9	10	11	12	Total gain in 8 weeks
	Age in weeks / Weight in grams									
5960 (F)	49	63	79	86	87	80	78	81	91	42
5962 (F)	31	47	63	73	78	77	74	79	83	52
5965 (M)	55	71	81	80	82	84	85	89	96	41
5968 (M)	52	67	76	80	77	73	76	82	87	35
5994 (M)	58	73	87	93	91	84	84	87	93	35
5998 (F)	50	56	68	79	80	83	86	85	87	37
6002 (M)	56	67	82	89	94	96	93	92	89	33
6040 (M)	56	70	77	82	78	72	77	79	85	29
6049 (M)	56	77	88	92	91	97	98	103	104	48
6055 (M)	44	63	77	85	85	80	83	84	92	48
Av.	51	65	78	84	84	83	83	86	91	40

A series of comparative tests in which the same amount of milk was fed with different modifications of the basal diet yielded the average results shown in Table III.

Diet 108 differed from Diet 107 in containing 13% of butter fat; Diet 109 in containing 23% of casein; Diet 110 in containing 5% of the Osborne and Mendel salt mixture. It will be seen that the variability of the animals on the different diets was of the same order and that the gains on the different modifications of the basal diet were essentially the same, the differences being even smaller than their probable errors.

Table III

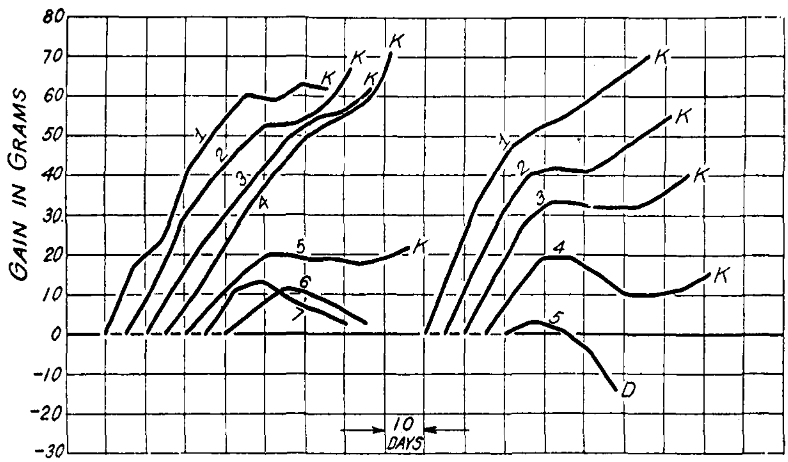
Comparison of Basal Diets 107, 108, 109, 110 (Fed with 8 Cc. of Milk)

Diet	No. of rats	Average gain G.	Probable error[a]	Difference from diet 107 G.	Probable error[b]	Average food intake G.	Coefficient of variation of gains
107	10	35	±3.1	.	...	281	42
108	12	32	±3.6	3	±4.8	260	35
109	11	35	±2.4	0	±3.9	275	34
110	11	31	±2.4	4	±3.9	268	38

[a] Computed according to the classical method as described, for example, by Jevons, "Principles of Science," The Macmillan Co., London and New York, **1905**, p. 387.

[b] Computed according to the usual rule that the probable error of the difference of two means is the square root of the sum of the squares of their probable errors.

The Question of the Best Amount of Milk to Feed in the Study of Heat Destruction of Vitamin B.—Two long series of experiments with the two



Part 1 Part 2

Fig. 1.—Part 1. Average-gain curves of rats on Diet 94 plus various amounts of skimmed milk.

Curve 1, + 15 cc. of milk; Curve 2, + 12 cc. of milk; Curve 3, + 10 cc. of milk; Curve 4, + 8 cc. of milk; Curve 5, + 6 cc. of milk; Curve 6, + 4 cc. of milk; Curve 7, + 2 cc. of milk.

Fig. 1.—Part 2. Average-gain curves of rats on Diet 107 plus various amounts of skimmed milk.

Curve 1, + 12 cc. of milk; Curve 2, + 10 cc. of milk; Curve 3, + 8 cc. of milk; Curve 4, + 6 cc. of milk; Curve 5, no milk.

K = killed D = died

The last point on the curve is the average age and weight at death.

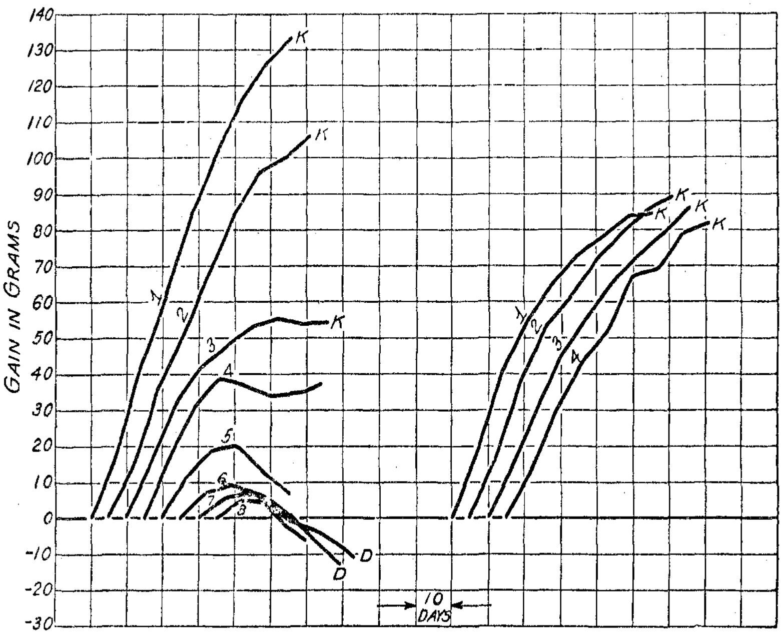
Curves 6 and 7 in Part I could not be completed, as some of the rats died before the end of the experimental period of eight weeks.

basal diets shown in Table I (above) were carried out for the purpose of finding the most advantageous amount of milk to feed in order to detect most readily any decrease in the vitamin B content which may result from destruction by heat or from any other cause. The final average results of these two series of experiments are shown graphically in Parts 1 and 2, respectively, of Fig. 1. It will be seen from these curves that in the first series 8 cc. of milk per rat per day appeared to furnish an optimum amount of vitamin B, no further increase of growth resulting from larger feedings of milk. In the second series the rate of growth continued to increase with the amount of milk fed up to 12 cc. per rat per day, the largest amount here tested. The reason for this difference is not apparent; but in both cases it was found that the largest difference in weight gained from the same difference in milk fed is that between 6 cc. and 8 cc. per rat per day. Hence, for the purpose of the present investigation 8 cc. per rat per day was chosen as the best level at which to feed in order to detect any diminution in vitamin B content of the milk, since it is at this level that we found the largest difference in the total gain in weight for 8 weeks as compared with the next lower amount fed. (This presumably furnishes somewhat less than the optimum amount of vitamin B, since further addition of milk gave increased growth in many cases and in the general average; and it furnishes considerably more than the amount required for maintenance of constant weight which is the level of feeding found advantageous in some of our other experiments, as described in a subsequent paper.)

In another series of experiments the amount of milk given was varied in accordance with the weight of the rat, each animal receiving daily 5, 7.5, 10 or 12.5 mg. of the skimmed milk powder per gram of body weight. It was expected that this method might yield more regular results, but such did not prove to be the case. A comparison of the weight curves of the rats fed the same allowance of milk daily throughout the experiment, with those of rats whose daily allowance of milk was increased weekly in proportion to their increasing body weight, shows that the former are as uniform as the latter. Hence, in experiments in which the milk was fed separately from the basal ration we continued the method of giving each rat his predetermined daily dose regardless of changes in his body weight.

Since our plan contemplated experiments upon the heating of the milk in both the dry and the fluid condition, it seemed best for the sake of uniformity of conditions as affecting the experimental animals, to restore the dry milk to fluid form in all cases before feeding. This was done by mixing the weighed milk powder with distilled water in such proportions as to make 10 cc. of ("reconstructed") fluid milk from each gram of the skimmed milk powder. The daily dose of the fluid milk thus prepared was measured by means of a graduated pipet into a small feeding cup and placed in the cage at a uniform hour each day. Usually the rat consumed the daily allowance of milk promptly upon receiving it. When necessary in order to induce this, the water cup was removed until the milk had been consumed. This was necessary in only a few cases and then only for a few feedings. In general there was no difficulty in securing prompt and complete consumption of the daily allowance of milk.

In several series of experiments the dry milk was fed mixed with the rest of the diet, replacing a smaller or larger part of the starch of Diet 94. The results are summarized graphically in Fig. 2, Part 1. The greatest



Part 1

Part 2

Fig. 2.—Part 1. Curve 1, Average-gain curve of rats on Diet 101 (30% milk); Curve 2, 100 (25% milk); Curve 3, 99 (20% milk); Curve 4, 95 (15% milk); Curve 5, 96 (10% milk); Curve 6, 97 (5% milk); Curve 7, 98 (2.5% milk); Curve 8, 94 (no milk).

Fig. 2.—Part 2. Curve 1, Average-gain curve of rats on Diet 100 (milk unheated); Curve 2, 100 (milk heated 6 hrs. at 100°); Curve 3, 100 (milk heated 24 hrs. at 100°); Curve 4, 100 (milk heated 48 hrs. at 100°). Heating was in dry air in these cases.

K = killed D = died

The last point on the curve is the average age and weight at death.

Curves 4, 5 and 6 in Part I could not be completed, as some of the rats died before the end of the experimental period of eight weeks.

difference in rate of growth for a given increment of milk in the diet was found between the diet containing 20% and that containing 25% of the skimmed milk powder in the dry food mixture. Hence on the same principle that 8 cc. was selected as the daily feeding in the experiments described above, we here selected 25% as the proportion of milk powder to be included in the food mixture in the cases in which the effect of heating was to be tested by feeding the milk in admixture with the rest of the food. Table

IV shows the average numerical results obtained from 11 comparisons of the three diets, 99, 100 and 101, which contained 20, 25 and 30%, respectively, of the dry skimmed milk in place of equal weights of the starch contained in Diet 94.

Table IV

Comparison of Growth on Diets with Different Percentages of Skimmed Milk Powder as Source of Vitamin B

Percentage of milk powder in diet	Number of rats	Average gain G.	Probable error[a]	Increase over preceding diet G.	Probable error[b]	Coefficient of variation in gains[c]	Food eaten G.
20	11	54	±5.7	..	...	52	348
25	11	106	±6.6	52	±8.7	31	441
30	11	133	±5.4	27	±8.5	20	511

[a] See Note [a] of Table III.

[b] See Note [b] of Table III.

[c] "Coefficient of variation" $= \frac{\text{Standard Deviation}}{\text{Mean}} \times 100.$

The data summarized in Table IV illustrate well certain relationships usually found in vitamin experiments of this general type. Actual magnitude of the probable error of the average gain remains about the same as the gain increases with increased proportion of vitamin in the diet. Also the difference in gain in passing from diet to diet is considerably larger between 20 and 25, than between 25 and 30% of milk powder in the diet, yet the actual magnitude of the probable error of the two differences is about the same. The coefficient of variation is greatest for the diet containing (of those here compared) the smallest amount of vitamin and becomes less as the vitamin intake approaches the optimum. This is in accordance with the general experience of this Laboratory, that on diets permitting some growth but at a distinctly subnormal rate, individual variations are apt to be larger than on normal diet; and as the partially adequate diet is brought toward the optimum the rate of growth becomes both more rapid and more uniform. Certain observations in this Laboratory indicate further that as the diet is enriched in growth-promoting substances beyond the quantity or concentration required for optimum results individual variations again tend to increase, but we are not yet ready to generalize on this latter point.

The natural increase in total food intake with increasing rate of growth adds to the difficulty of interpreting growth rates quantitatively in terms of vitamin intake alone.

Effect of Heating at 100° upon Vitamin B in Milk in the Dry and Fluid States.—Dry heating was carried out by spreading the milk powder in a layer about 1 cm. deep in a flat-bottomed dish and heating in a constant-temperature oven, the temperature taken by means of a thermometer extending into the dry milk. When milk powder which had been heated for

6, 24 or 48 hours at 100° in the dry state, with free access of air, was substituted for the unheated dry milk, no effect of the heating could be detected, from the results as shown in Table V and in Fig. 2, Part 2.

Table V

Comparison of Diets Containing 25 per cent. of Skimmed Milk Powder, Unheated or Heated at 100°

Experiments with 15 rats

Time of heating of milk powder Hours	Average gain in weight in eight weeks G.	Probable error[a]	Difference from those receiving unheated milk G.	Probable error[b]	Coefficient of variation in gains[c]	Average food intake G.
Unheated	84	±5.0	.	...	34	520
6	88	±5.0	4	±7.1	33	511
24	87	±3.0	3	±5.8	20	506
48	82	±4.2	2	±6.5	29	455

For Notes *a*, *b* and *c*, see Tables III and IV.

Examination of the data in Table V shows plainly that the small differences in average gains on the heated and unheated milk powder are not significant; nor do the coefficients of variation or the data of food intake reveal any differences attributable to the heating of the milk. In view of the fact that the milk was the sole source of vitamin B and was fed at a level at which decrease of the vitamin B intake results in decrease of gain in weight, the results constitute strong evidence that the dry heating at 100° as here described caused no appreciable destruction of vitamin B, even when continued for 24 to 48 hours.

In another series of experiments the heated milk was fed separately from the basal diet, and in this series was also included the study of milk heated for 6 hours at 100° in fluid form, that is, in the presence of 9 to 10 parts by weight of water to 1 part of the skimmed milk solids. The results of the tests of the milk thus heated together with parallel tests on the same milk unheated and heated for 6 and 48 hours in the dry state are shown in Table VI. (Tests with milk heated dry for 24 hours were rejected because of accidental irregularities.)

Table VI

Comparison of Tests with Milk as Source of Vitamin B, Unheated and Heated in Dry or in Fluid Form

Experiments with 10 rats. Basal Diet 107.8 cc. of milk daily

Heat treatment of milk Hours	at °C.	Average gain in weight in 8 weeks G.	Probable error[a]	Difference from that with unheated milk G.	Probable error[b]	Coefficient of variation of gains[c]	Average food intake G.
Unheated		42	±3.0	..	...	33	283
6	100 (dry)	42	±2.6	0	±4.0	29	276
48	100 (dry)	37	±1.8	5	±3.5	22	270
6	100 (wet)	6	±1.6	36	±3.4	126	199

For Notes *a*, *b* and *c*, see Tables III and IV.

Here there was plainly no appreciable destruction of vitamin B in heating the milk powder dry for 6 hours at 100°, and probably no significant result when the same heating was continued for 48 hours. The animals receiving, as their sole source of vitamin B, the milk which had been heated 6 hours at 100°, wet, made distinctly lower average gains but showed much greater variability and a much lower average food intake. The average-weight curve for the rats receiving 8 cc. of milk thus heated approximates that of those receiving 6 cc. of unheated milk, so that if the difference due to heating the milk can be attributed wholly to destruction of vitamin B, the results would indicate a heat destruction of about $^1/_4$ of the vitamin B, as the result of heating for 6 hours in water at 100°. (The heating was carried out in thin-walled flasks immersed in briskly boiling water. A thermometer standing directly in the milk registered 99.5° to 99.8°.)

Thus there appears to have been an appreciable destruction in 6 hours' heating at 100° in water while there was no appreciable destruction in heating at 100° even up to 48 hours when the milk was heated in the dry state. Any seasonal differences in the growth of rats, and possible seasonal variations in the vitamin B content of milk, are eliminated here because all comparative tests were carried out simultaneously using milk powder from the same lot, and as previously explained the rats were all of exactly known age and relationship and were so distributed in the different tests that comparisons were nearly always with litter-controls.

Summary and Conclusions

A quantitative method for the determination of relative amounts of vitamin B by means of rat-growth experiments is described, and the known factors affecting the accuracy of the results of such experiments are discussed.

The data presented are representative of the work as a whole which comprised 11 series of experiments with a total of 38 variations of the experimental diet and included over 600 quantitative records of the food and growth of young rats.

The experiments were made upon rats of known age and relationship, the experimental period covering the fifth to the twelfth weeks of the rat's age, inclusive. It was found that the diet of the mothers from which the young rats are taken for experiment is a factor which must be kept uniform if results are to be as strictly quantitative as is possible.

Basal diets are described which are believed to be free from vitamin B and which are shown by numerous and rigorous tests to furnish practically optimum amounts of all other nutrients required by rats, the complete salt mixture of Osborne and Mendel being here considered as a unit.

The quantities of milk selected as most advantageous to feed in order to detect possible reduction in vitamin B content on heating were 0.8

g. of dry or 8 cc. of fluid skimmed milk per rat per day when fed separately from the basal ration, or 25% of the total food mixture when the skimmed milk powder was mixed with the basal diet.

By feeding the diets described to experimental animals of suitable age and size and sufficiently matched as to nutritional history and litter controls, it is believed to be possible, when dealing with averages of ten or more rats on each diet, to detect a diminution certainly of 25% and probably of 15% in the vitamin B content of the food tested.

There was no evidence of any measurable diminution of vitamin B in milk powder heated dry with free access of air at 100° even when this heating was continued for 48 hours. When the milk was heated in the fluid state for 6 hours at 100° there was an apparent diminution in its vitamin B content; probably about $^1/_4$ of the vitamin was thus destroyed. Vitamin B in the form in which it exists in milk is, therefore, comparatively stable to heating at 100° in the dry state, but less stable when heated at the same temperature in water solution.

New York, N. Y.

[Contribution from the Department of Chemistry, Columbia University, No. 425]

A QUANTITATIVE STUDY OF THE DESTRUCTION OF VITAMIN B BY HEAT

By H. C. Sherman and M. R. Grose

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In the study of vitamins the property of thermostability bears important relations both to the behavior of the vitamin in attempts to isolate it and to the conservation of the vitamin values of foods. Hence, statements regarding this property are frequently met, but most of the work which has been done to determine the effect of heating upon vitamins A and B has been qualitative, or little more than qualitative, in nature.

Building upon the work described in the two preceding papers[1,2] we have attempted in the experiments here recorded to establish a quantitative relation between temperature and the rate of destruction by heat of vitamin B, somewhat as had been done in the previous work of this Laboratory with vitamin C.[3,4]

Vitamin B is more stable than vitamin C both toward heat and toward oxidation but its destruction by heat can be studied on the same general principle by the use of higher temperatures. Using tests of the amounts of food required to cure polyneuritis in fowls or pigeons as the measure of

[1] Sherman and Edgeworth, This Journal, **45**, 2712 (1923).

[2] Sherman and Spohn, *ibid.*, **45**, 2719 (1923).

[3] Sherman, LaMer and Campbell, *Proc. Nat. Acad. of Sci.*, **7**, 279 (1921).

[4] LaMer, Campbell and Sherman, This Journal, **44**, 172 (1922).