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EXPERIMENTS WITH TWO METHODS FOR THE STUDY OF VITAMIN B

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The further progress of our knowledge of the vitamine must depend largely upon the development of methods for the experimental study of these substances which shall be both practicable and quantitative. When such methods are sufficiently developed they can be applied to the quantitative measurement of the concentration of the vitamin at successive steps in attempts to isolate the substance from natural sources and bring it to a condition of maximum purity for the determination of its chemical nature. Moreover, the importance of vitamin B as a factor in food values makes it urgently desirable that quantitative methods of known precision be applied in all studies of the vitamin contents of different foods or of the same food before and after heating or other treatment to which foods may be subjected.

As has been fully explained elsewhere[1,2] such methods imply feeding to a suitable organism, in or with an otherwise adequate diet, known amounts or proportions of the material to be tested as the sole source of the vitamin in question.

In this and the two following papers dealing with vitamin B, we use this term to designate the growth-promoting, water-soluble B vitamin without regard to the question as to whether this is or is not identical with the antineuritic substance. Hence, we have taken the growth of young rats rather than the prevention or cure of polyneuritis in pigeons as our principal criterion. Several investigators have worked actively in the belief[3] that experiments based on acceleration of yeast growth could serve the same purpose as those based on rat growth and in a quicker, more economical and more readily controllable way. Different methods of ascertaining the stimulating or growth-promoting effect of the vitamin upon the yeast were proposed: (a) counting the cells produced; (b) measuring the carbon dioxide produced; (c) measuring the increase in volume of yeast cells; (d) weighing to determine the increase in yeast gravimetrically.

The counting of yeast cells, even as reported by those who have appeared to have most confidence in this method, has yielded data showing such wide variations in duplicate tests as to preclude its employment in quantitative investigations.

[1] Chick and Hume, *J. Biol. Chem.*, **39**, 203 (1919).

[2] Sherman, LaMer and Campbell, THIS JOURNAL, **44**, 165 (1922).

[3] For further discussion, see Sherman and Smith, "The Vitamins," Chemical Catalogue Company, New York, **1922**, pp. 72–75.

In the estimation of yeast growth (or activity) by measurement of the carbon dioxide produced, there is the difficulty that the difference in carbon dioxide pressure at the two surfaces of the liquid in the fermentation tube permits a loss of carbon dioxide by passage through the solution. Obviously also even small changes in temperature may result in further error. This method therefore does not seem promising from a quantitative point of view.

In measuring the volume of the yeast, the fact that yeast-substance is largely protein, having an iso-electric point on either side of which it will show differences in amount of swelling, depending on the hydrogen-ion concentration of the medium, must be taken into account and plainly detracts much from the ease and precision which might otherwise be expected of this method.

The gravimetric method seemed, therefore, the most promising from the standpoint of quantitative accuracy of results.

Yeast was made up for seeding from a Fleischmann yeast cake according to the method of Williams,[4] and was grown for 18 hours at 30° in a constant-temperature oven, in one of the two media described below.

As source of vitamin B we selected dried skimmed milk, as a material readily available and easily kept uniform throughout the investigation, easy and accurate of manipulation, and usable either in the dry state or reconstituted as fluid skimmed milk by mixing with nine times its weight of water. It was desired to employ a typical and natural form of vitamin B free from any danger of having been altered or fractioned by chemical or physical treatment. It was also thought best, in view of the experience of this and other laboratories and of Osborne's suggestion that vitamin B may occur naturally in chemical combination with the proteins of some foods, to avoid the use of any method which should involve the necessity of employing extracts of the food to be tested.

Experiments with the Growth of Yeast

Using the Medium of Williams.—This medium contains asparagine, 1.5 g.; calcium chloride, 0.25 g.; magnesium sulfate, 0.25 g.; monopotassium orthophosphate, 2.0 g.; ammonium sulfate, 3.0 g.; and sugar 20.0 g. per 100 cc. It was made up in lots of one liter divided into aliquot portions of 100 cc. each, placed in Erlenmeyer flasks stoppered with cotton and sterilized in an autoclave at 10 pounds' pressure for 15 minutes. To each portion, when cooled to 30°, was added 1 cc. of yeast suspension containing 0.3 mg. of yeast prepared according to the method of Williams.[4] These portions were then incubated at 30° for 18 hours, at the end of which time 1 cc. of U.S.P. formaldehyde was added to each flask to prevent further growth of the yeast, and the contents were subsequently filtered through

[4] Williams, *J. Biol. Chem.*, **42**, 259 (1920).

a weighed Gooch crucible prepared according to Williams, washed, and dried at 102° to constant weight. Parallel tests were made with the addition of various amounts of the skimmed milk used as source of vitamin B, sometimes as purchased, sometimes after heating for 6, 12, 24, or 48 hours at 100°. The control involved two tests (1) of medium and yeast without added source of vitamin, (2) of medium plus the amount of milk used as source of vitamin in each experiment.

As this medium itself contained no insoluble material, the sum of the weights obtained in the two control tests just mentioned was taken as the correction to be subtracted from the gross weighing obtained in each test, the remainder showing the amount of yeast growth due to the material added as source of vitamin.

The concentrations of hydrogen ion in (a) the control with yeast only, (b) the medium plus yeast plus 0.4 g. of unheated skim milk powder, (c) the medium plus yeast plus 0.4 g. of skim milk powder heated for 48 hours at 100°, were determined before and after incubation and found to be (a) before incubation, P_H 4.4; after incubation, P_H 5.5; (b) before, P_H 5.7; after, P_H 7.1; (c) before, P_H 5.5; after, P_H 7.0.

While the media seem to become slightly less acid during the process of incubation, the change is not such as to interfere with the correction of results for precipitated milk protein as described above.

In a series of determinations in which 0.4 g. of the milk powder was tested, the average results were as follows.

	G.
Wt. of insoluble matter from medium + yeast	0.0030
Wt. of insoluble matter from medium + milk	.1088
Total wt. to be subtracted	.1118
Average gross wt. in tests	.1572
Accelerated growth due to the milk	.0454

The average results of about 200 quantitative tests made by this method with or without the introduction of various amounts of the milk powder as source of vitamin B are shown in Table I.

It will be seen that the results are such as amply to justify quantitative discussion. The averages of the total, and of the accelerated growths observed show probable errors which in all cases are less than 2%, and in most cases are less than 1% of the quantities concerned. The accelerated growth of the yeast shows progressive increase with the quantity of milk powder added up to 0.4 g. This amount was therefore used in testing for the effect of heat upon the vitamin B content of the milk powder as indicated by this method. The effects attributable to heating at 100° for from 12 to 48 hours are in our judgment too small to permit of definite conclusions as to whether a measurable amount of vitamin B was thus destroyed.

Thus our experiments show that the growth of yeast in the Williams medium is a process capable of acceleration by the addition of vitamin-containing material and that this acceleration of yeast growth can be determined gravimetrically with a satisfactory degree of precision. As yet, however, there is not sufficient evidence that the Williams medium

TABLE I

SUMMARY OF RESULTS BY THE GRAVIMETRIC YEAST METHOD USING WILLIAMS' MEDIUM

Milk powder added G.	Heating at 100° Hours	No. of tests	Av. growth G.	Probable error[a]	Accelerated growth G.	Probable error[b]
None		60	0.0031	±0.00004	...	.....
0.1	unheated	17	.0174	± .00027	0.014	±0.0003
.2	unheated	14	.0285	± .00026	.026	± .0003
.4	unheated	39	.0482	± .00025	.045	± .0003
.6	unheated	16	.0430	± .00027	.040	± .0003
.4	12	19	.0390	± .00040	.036	± .0004
.4	24	13	.0390	± .00036	.036	± .0004
.4	48	20	.0377	± .00056	.035	± .0006

[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 by 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.

contains such favorable proportions of all other essential nutrients as to justify the assumption that acceleration of growth upon addition of food material to this medium is necessarily attributable solely to the introduction of vitamin B, and the experiments of Fulmer and his co-workers[5,6] published while our own work was in progress tend strongly to the opposite view. Experiments with the medium of Fulmer, Nelson and Sherwood were therefore undertaken.

Using the Medium of Fulmer, Nelson and Sherwood.—For the reason above given, the method of counting cells did not seem suitable for our work and we have, therefore, tried to adapt the gravimetric method to the use of this medium, which contains undissolved calcium carbonate and precipitated phosphate. These insoluble buffers apparently yield variable amounts of residue at the end of the incubation period, for neither by drying and weighing nor by ignition of the dry residue and subtraction of the mineral matter, were we able to obtain satisfactory gravimetric measurements of yeast growth when this medium was used.

Experiments with Growth of Rats

The rat was preferred as experimental animal because it has been best standardized by previous work. Several rats of the same litter and approximately the same size were, when 28 to 30 days old, divided into groups as closely matched as possible in size and in distribution of the

[5] Fulmer, Nelson and Sherwood, THIS JOURNAL, **43**, 191 (1921).

[6] Nelson, Fulmer and Cessna, *J. Biol. Chem.*, **46**, 77 (1921).

sexes, some groups receiving the skimmed milk powder unheated and others receiving heated milk powder as stated beyond. This skimmed milk powder was analyzed with the following results: moisture, 3.0; fat, 0.3; protein, 33.3; ash, 7.2; and carbohydrate by difference, 56.2%. It was fed mixed with the other food materials in a diet composed of starch, 50; skimmed milk powder, 40; purified butter fat, 9; and sodium chloride, 1%. Except for a different distribution of calories as between starch and fat, this diet corresponds fairly closely to the whole-milk-starch-fat diet which Osborne and Mendel have shown to be adequate.

Furthermore, unpublished experiments in this Laboratory have shown that 40% of the food mixture is about the minimum proportion of skimmed milk powder for optimum growth when this constitutes the sole source of protein and water-soluble vitamin and that when growth is retarded by further dilution of milk powder with starch, vitamin B becomes the first limiting factor in the growth of young rats. Hence, the use of 40% of skimmed milk powder in the food mixture for the experiments on growth of rats was considered analogous to the addition of 0.4 g. of this powder per 100 cc. of Williams' medium in the experiments upon the growth of yeast.

The experiments with rats were carried out in three series, (1) in the autumn of 1920, (2) in the summer of 1921, (3) in the autumn of 1921. In all cases the animals were weighed weekly and the feeding experiments continued for eight weeks. Using the diet described above, the gains in weight during the eight weeks, averaged for all the rats in each of the three series separately, with the probable errors of these averages, were as shown in Table II.

TABLE II

GAINS IN WEIGHT OF RATS DURING EIGHT WEEKS ON EXPERIMENTAL DIET CONTAINING UNHEATED MILK POWDER

	No. of rats	Av. gain G.	Probable error
First series	11	125	±4.0
Second series	39	92	±2.2
Third series	14	99	±2.9

Here it will be seen that averages of 11 to 39 animals in a series are subject to probable errors of about 2 to 3% of their numerical values. The differences in average gains from season to season are sufficient to emphasize the importance of making quantitative comparisons directly with control groups fed simultaneously rather than with pre-established normal rates of growth alone. The latter, however, are valuable as a check upon all work of this character.

The differences in general average gains between all animals receiving milk powder unheated, and all those receiving the same after heating are shown in Table III.

TABLE III

GAINS IN WEIGHT OF RATS DURING EIGHT WEEKS ON DIETS WITH UNHEATED AS COMPARED WITH HEATED MILK POWDER

Time of heating milk powder at 100° Hours	No. of rats	Av. gain G.	Av. gain Probable error	Difference from those on unheated food G.	Difference from those on unheated food Probable error	Food eaten G.
Unheated	64	100	±2.0	..	...	537
6	28	86	±2.7	14	±3.4	513
12	23	77	±1.8	23	±2.7	501
24	21	59	±2.5	41	±3.4	439
48	22	40	±3.3	60	±3.9	406

Here it is plain that the differences in growth are too large to be accidental; but it is not plain whether the slower growth on the heated food is due to heat destruction of vitamin or to other causes. The last column of Table III shows that with increasing heat treatment of the food there was a progressive decrease in the amount of food consumed. The decreased food consumption would probably be sufficient to account for the decreased gain in weight whether heat destruction of vitamin had occurred or not. Since vitamin B promotes appetite it might be argued that decreased food intake is due to decreased vitamin content of the food through its heating; but the heating might make the food less appetizing in other ways, as through changing its flavor, and there is some reason to believe that this was actually a factor in these experiments because at the time they were made we had not entirely eliminated uneven heating, with consequent occasional local over-heating of the food. The thorough mixing of the food would result in distributing through the whole food mixture any particles which might have acquired a cooked flavor through local over-heating. In subsequent experiments described in the paper which follows, the heat treatment was more perfectly controlled, the food consumption was more uniform and the differences in gains of weight attributable to dry heating of the food at 100° practically disappear.

When 10 animals on each variation of the heat treatment were compared with 10 litter-controls receiving unheated food, the average results were as shown in Table IV.

TABLE IV

COMPARISONS OF GAINS AND PROBABLE ERRORS ON AVERAGES OF GROUPS OF TEN ANIMALS EACH

Time of heating milk powder at 100° Hours	Av. gain G.	Av. gain Probable error	Decreased gain on heated food G.	Decreased gain on heated food Probable error
Unheated	99	±3.6	..	...
6	97	±3.6	2	±5.1
Unheated	97	±3.6	..	...
12	86	±2.4	11	±4.3
Unheated	100	±2.2	..	...
24	56	±3.6	44	±4.2
Unheated	116	±2.2	..	...
48	51	±4.9	65	±5.4

As explained above, these experiments do not answer the question as to whether the heat treatment here employed destroyed a measurable proportion of the vitamin. They do eliminate seasonal influences and illustrate well the degree of precision to be expected from this method when dealing with averages of groups of ten. The average gains in groups of 10 rats on unheated food are subject to probable errors of 2 to 4% while in those groups in which heating of food has decreased the average rate of gain the results are more variable and the probable errors are relatively larger.

Summary and Conclusions

The purpose of this work was to examine the availability of the gravimetric yeast-growth method and of the rat-growth method for quantitative study of vitamin B.

The gravimetric yeast-growth method using the Williams medium and procedure yielded consistent results. The coefficients of variation and the probable errors of the averages obtained in the different series were within limits quite satisfactory for work in this field, the probable errors being of the order of 1% of the observed numerical values. This method is, however, open to the objection that the increased growth of yeast which it measures may be due to the introduction of other substances favorable to yeast growth and is therefore not necessarily a measure of relative amounts of vitamin B. The medium devised by Fulmer, Nelson and Sherwood to overcome this objection did not, in our hands, prove adaptable to use with the gravimetric method of determining the growth of the yeast.

The rat-growth method also yielded quantitative results. It was found desirable to use rats four weeks of age for these experiments and to continue them upon the experimental diets for eight weeks. So far as is practicable each experimental animal should have a closely comparable control, taken at the same time from the same litter and of approximately the same initial size so that a series of comparisons can be made between animals confidently regarded as directly and accurately comparable. The conclusions reached through such comparisons should then be verified by comparison of the general averages of groups so large as to insure that individual peculiarities cannot appreciably affect the final result. In the work here recorded averages were drawn in each of these ways, and the probable errors of these averages were found to be of the order of 2 to 4% of their numerical values.

While the rat-growth method involves somewhat larger probable errors than the gravimetric yeast-growth method as here used, the results can be interpreted in terms of vitamin B with much greater certainty and we therefore consider the use of the growing rat to be the preferable method.

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