ASSAY FOR VITAMIN B COMPLEX IN THE PRESENCE OF INTERFERING SUBSTANCES.*

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In the usual method of assay for the vitamin B complex young rats are starved for this complex until a sharp decline of weight is observed. The minimum dose of the material under investigation, necessary to restore a normal rate of growth, is then determined.

In applying this method the following factors may well be taken into account: (1) The accuracy of the method must obviously depend somewhat upon the nicety of judgment as to whether the normal rate of growth is restored or not. (2) The interpretation of results is facilitated by the use of a physical standard of reference, a material of well-known vitamin B complex potency, to which all assays for this vitamin complex may be referred. (3) The method is not quite generally applicable because of the fact that some food and medicinal products contain salts, drugs, laxatives, poisons or odd flavoring materials, which experimental animals either refuse to eat or from which complicated abnormal or ill effects follow. In other specimens, the vitamin is in such dilution that a concentration must be made.

1. TEST FOR THE RESTORATION OF NORMAL RATE OF GROWTH.

It is frequently observed that litter mates of the same sex do not grow at the same rate even when kept under identical conditions of housing and diet. When animals which have declined in weight due to starvation for the vitamin B complex are fed on several different doses of a given test material, animals on different doses may all gain in weight but it is frequently difficult to be certain that any one animal has been restored to its individual normal growth rate. This element of uncertainty may be eliminated if at the end of the test period all test doses are discontinued and each animal is then given a daily dose of dried brewers' yeast which is known to be several times the minimum amount required for normal growth. The growth curves of the animals are then followed for a week or two. If the curve breaks upward with the feeding of the brewers' yeast, the previous dose of the test material was inadequate. If the growth curve continues as when the previous dose was given, the previous dose was adequate for normal growth.

2. PHYSICAL STANDARDS OF REFERENCE.

In order to overcome the known variation in the response of animals to vitamin feeding this laboratory has adopted the procedure of making assays for the vitamin B complex comparative. Various doses of a material to be tested for the vitamin B complex are fed in comparison with fresh bakers' yeast cake (Fleischmann) and dried brewers' yeast (Harris). Only a few animals need to be placed on doses of the yeast cake or of dried brewers' yeast, because it has been learned from several years of experience that 1500 mg. of the fresh yeast cake is approximately equivalent to 100 mg. of the dried brewers' yeast and that the vitamin B complex potency of these doses is just about adequate for normal growth of young rats. It is also the experience of this laboratory that the dried brewers' yeast, when kept in closed con-

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tainers, shows no detectable variations in potency over a period of about three years. This material has, therefore, been adopted as a standard of reference for this laboratory. The general adoption of some such standard would greatly facilitate interpretation of the results obtained by different laboratories.

3. ASSAY OF A FOOD MATERIAL WHICH ANIMALS DO NOT EAT READILY.

An attempt was made to assay the vitamin B complex in bouillon cubes[1] prepared from an extract of brewers' yeast and presumably containing vitamin B. On account of the salts and flavoring of these cubes, experimental animals refused, after the first few days of the test, to eat the portions of bouillon cube supplied to them, either in the form of small (weighed) pieces of the cube or as similar amounts of the cube material mixed with some inert substance such as starch or dextrin. For purposes of assay it was therefore necessary to devise some method for the extraction of the vitamin-bearing constituents of the bouillon cubes.

The approximate composition of the bouillon cubes is indicated in the following table (Table I).

TABLE I.

Moisture	5.84%
Nitrogen	3.54
Protein ($N \times 6.25$)	22.13
Ash	61.35
NaCl	55.02

I. EXTRACTION EXPERIMENTS OF BOUILLON CUBES.

A survey of the literature dealing with the properties of the vitamin B complex lead to a consideration of the three following possible methods for the extraction of the vitamin B bearing material from bouillon cubes.

1. Extraction with dilute alcohol (approximately 70%).
2. Adsorption of the vitamin on fuller's earth
3. Extraction of the vitamin-bearing material with glacial acetic acid.

Method No. 1 was tried and discarded because it was found that a complete extraction by this method required repeated extractions and chilling of the extract in the refrigerator over night. Such procedure results in so much exposure of the extract to the influence of atmospheric oxygen as to very largely destroy the vitamin B present in the original cube material.

Method No. 2 was tried and discarded because it was found that while it was very easy to adsorb a portion of the vitamin-bearing material on fuller's earth, there appeared to be no satisfactory way in which we might be assured that the vitamin-bearing material was quantitatively adsorbed. The probability of a selective adsorption of the vitamin B factors was also taken into consideration.

Method No. 3—extraction with acetic acid—was finally adopted for use in this series of studies. The method, as first tried, gave rather irregular results because of apparent differences in the amount of exposure to the air which occurred in the preparation of different batches of the extract. A standardized procedure finally

[1] Yeast Bouillon Cubes—Harris, prepared by the Harris Laboratories, Tuckahoe, New York.

was selected, after repeated trials, which yields rather uniform results. This method of extraction is briefly described below.

Ten bouillon cubes, weighing on the average 45 Gm., are crushed in a small mortar and washed into a small beaker with 135 cc. of glacial acetic acid. The cubes and acid are heated as rapidly as possible, with constant stirring, to boiling. As soon as the mixture is boiling vigorously, the beaker is removed from the burner and the acetic acid-insoluble material is allowed to settle for a few moments. The clear, supernatant liquor is then decanted through a funnel in which a filter mass of absorbent cotton has been previously moistened with glacial acetic acid.

As soon as a small amount of the filtrate (acetic acid extract) is obtained, it is drawn, little by little, by vacuum, into a 150-cc. weighed distillation flask. The acetic acid extract is drawn into the distilling flask through a glass tube drawn out to a capillary so that the acetic acid solution falls into the flask a few drops at a time and evaporates almost as rapidly as it is delivered to the flask. The flask is immersed in a water-bath kept at a temperature of 70° C. The flask is connected by means of a Liebig condenser with a vacuum pump so that the acetic acid is distilled off under a high vacuum.

The residue remaining in the beaker after the first extraction is again extracted with 100 cc. glacial acetic acid and the acetic acid solution is decanted through the same filter into the same receiving flask. The residue from this extraction is again similarly extracted twice with 50-cc. portions of glacial acetic acid and then once extracted with 25 cc. of glacial acetic acid. The final portion of acetic acid (25 cc.) is practically colorless and serves the purpose of removing all but the last traces of the acetic acid-soluble material which remains on the cotton filter through which each extract is poured.

The vacuum distillation is continued during the time that the several extractions are being made, so that at the time the final extraction is completed, only a small amount of the acetic acid solution remains to be distilled. As soon as all of the acetic acid solution has been transferred to the distilling flask, the inlet tube is connected to a reservoir of nitrogen gas and the distillation is continued under a stream of nitrogen until the final volume of the material in the flask has been reduced to approximately 60 cc.

At this point in the procedure the flask is removed from the hot water-bath and the flask is cooled with either tap water or ice water. The flow of nitrogen gas is continued during the cooling of the flask. When the flask is cool, starch is added to the semi-solid residue and the flask is placed in a vacuum desiccator over sodium hydroxide sticks. The desiccator is evacuated under the highest possible vacuum and the mixture of starch and extract of the cubes is allowed to remain in the flask in the desiccator for two days. At the end of two days' time the vacuum is released with dry nitrogen gas and the flask with its contents is removed and accurately weighed. The weight of the flask alone was taken before the experiment was begun. Additional starch is now added in an amount which will bring the contents of the flask to three times the weight of the ten cubes taken for the extraction.

It is obvious that three Gm. of the mixture of starch and cube extract now contains approximately the same amount of acetic acid-soluble material as was contained in one Gm. of the original cube. This is the material that is fed to the experimental animals in testing the vitamin B (complex) potency of the bouillon

cubes. If, then, we assume that all of the vitamin B complex is soluble in acetic acid and if we further assume that no losses have occurred during the laboratory manipulations to which the material has been subjected, we may then assume that the vitamin B complex potency of the starch mixture represents the vitamin B complex potency of the original bouillon cube in the proportion of three to one. There is apparently no simple way in which the validity of the two essential assumptions upon which the third assumption is based, may be definitely verified. It is almost certain that the second assumption is not entirely correct, because we find that if any delay in the extraction and distillation procedure occurs, a less potent vitamin extract is obtained. A more correct statement of this assumption might then well be that, by standardizing as nearly as possible our procedure and making our extractions as rapidly as possible and with as little exposure to air as possible, we are able to make the extraction with a minimum loss of vitamin B complex potency.

TABLE II.—GROWTH OF ANIMALS ON TEST DOSES—EFFECT OF SURPLUS OF BREWERS' YEAST.

Animal.	Material Fed.	Daily Dose in Mg.	Days on Given Dose.	Total Weight Change.	Daily Weight Change.	Dose of B. Y. in Mg.	Days on B. Y.	Total Weight Change.	Daily Weight Change.
7 F	B. Y.	25	54	27	0.5	1000	26	37	1.423
8 M	B. Y.	50	54	56	1.037	1000	26	54	2.076
2 F	B. Y.	50	53	44	0.8301	1000	26	35	1.346
1 M	B. Y.	100	53	90	1.698	1000	26	36	1.384
19 M	B. Y.	100	53	95	1.792	1000	26	41	1.576
4 F	B. Y.	150	55	62	1.127	1000	13	11	0.8461
23 M	B. Y.	150	52	81	1.553	1000	27	48	1.777
6 M	B. Y.	200	50	90	1.800	1000	12	11	0.9166
17 M	B. Y.	200	50	90	1.800	1000	12	22	1.833
27 M	Y. B. C.	100	54	11	0.2037	1000	26	87	3.346
10 F	Y. B. C.	100	54	− 3	−0.055	1000	26	56	2.153
12 F	Y. B. C.	100	54	11	0.2037	1000	26	53	1.203
15 F	Y. B. C.	200	54	33	0.6111	1000	26	56	2.153
13 M	Y. B. C.	200	54	31	0.5740	1000	26	77	2.961
29 M	Y. B. C.	300	56	60	1.071	1000	26	50	1.923
22 F	Y. B. C.	300	54	68	1.259	1000	26	21	0.8076
28 F	Y. B. C.	400	56	56	1.000	1000	26	23	0.8846
3 F	Y. B. C.	400	56	55	0.9821	1000	26	19	0.7307
20 F	F. Y. C.	500	54	23	0.4259	1000	27	45	1.666
9 M	F. Y. C.	500	34	12	0.3527	Died at end of 34 days			
30 M	F. Y. C.	1000	54	55	1.018	1000	27	74	2.740
11 M	F. Y. C.	1000	54	28	0.5185	1000	27	28	1.036
21 M	F. Y. C.	1000	54	58	1.036	1000	27	60	2.222
24 M	F. Y. C.	1500	54	61	1.129	1000	27	31	1.148
26 F	F. Y. C.	2000	50	43	0.8600	1000	26	14	0.5384
25 F	F. Y. C.	2000	54	56	1.036	1000	27	24	0.8888

NOTE: B. Y. = Brewers' Yeast, Y. B. C. = Yeast Bouillon Cube, F. Y. C. = Fleischmann's Yeast Cake.

From the above it appears to us that the minimum vitamin B complex dose for normal growth of the young, white rat is as follows:

Brewers' yeast.....	100 mg.
Bouillon cubes.....	300 mg.
Yeast cake.....	1500 mg.

II. ANIMAL EXPERIMENTS.

Some seventy-five animals were used in establishing the relative vitamin B complex potency of dried brewers' yeast (Harris), fresh yeast cake (Fleischmann) and yeast bouillon cube (Harris). It is not necessary to present the complete data obtained from all of these animals because the complete data are in strict conformity with the data of one typical test, using twenty-six animals, and employing the technic described above. The data of this test are presented in Table II.

It will be noted from the data of Table II that those animals that received daily doses of less than 100 mg. of brewers' yeast; less than 300 mg. of yeast bouillon cube and less than 1500 mg. of fresh yeast cake, when changed from the test doses to 1000 mg. of brewers' yeast showed a daily gain of weight greater than the daily gain of weight on the test doses. Those animals receiving daily doses of at least 100 mg. of brewers' yeast; 300 mg. of yeast bouillon cube and 1500 mg. of yeast cake do not show a marked increased rate of gain when transferred to 1000 mg. of brewers' yeast.

From these observations it is possible to draw the conclusions that 100 mg. of the dry brewers' yeast, 300 mg. of the material of the yeast bouillon cubes, and 1500 mg. of the fresh yeast cakes are each adequate for the normal growth of rats for the period of this test.

The vitamin B complex potency of yeast cakes and the yeast bouillon cubes used in these studies may be expressed in terms of the potency of the physical standard[1] employed in this laboratory as follows:

$$\frac{\text{Minimum adequate dose of the standard}}{\text{Minimum adequate dose of bouillon cube}} = \frac{100}{300} = \frac{1}{3}$$

The bouillon cube then has a vitamin B complex potency of 33% that of the standard.

$$\frac{\text{Minimum adequate dose of the standard}}{\text{Minimum adequate dose of yeast cake}} = \frac{100}{1500} = \frac{1}{15}$$

The yeast cake then has a vitamin B complex potency of 6.6% that of the standard.

If one wishes to reduce the figures obtained to the basis of the dry materials contained in each product, it is only necessary to take into account the fact that the brewers' yeast contains approximately 1% moisture and that the bouillon cubes and the yeast cakes contain approximately 5.8% and 66% moisture, respectively. Thus 99 mg., 286 mg. and 495 mg. of the dry material of brewers' yeast, of bouillon cube and of yeast cake, respectively, may each be said to contain one adequate dose of the vitamin B complex which might well be called one unit of the vitamin B complex.

It might be well to point out that one bouillon cube weighs approximately 4.5 Gm. (4500 mg.) and that one yeast cake weighs approximately 12.5 Gm. (12,500 mg.). One bouillon cube will then contain:

$$\frac{4500}{300} = 15 \text{ units of the vitamin B complex}$$

[1]Dried brewers' yeast—Harris.

One yeast cake will then contain:

$$\frac{12{,}500}{1500} = 8.33 \text{ units of the vitamin B complex.}$$

We have found that a simple and convenient method for the graphic representation of the growth of animals which have been used in a test employing the technic described above is to lay off on cross section paper a line representing the weight during the test period, *i. e.*, a line joining the graphic points representing the weights at the beginning and the end of the test period. A second line representing the weight of the animal during the period in which the excessive dose of brewers' yeast is fed is similarly laid off on the same paper. The angle at which

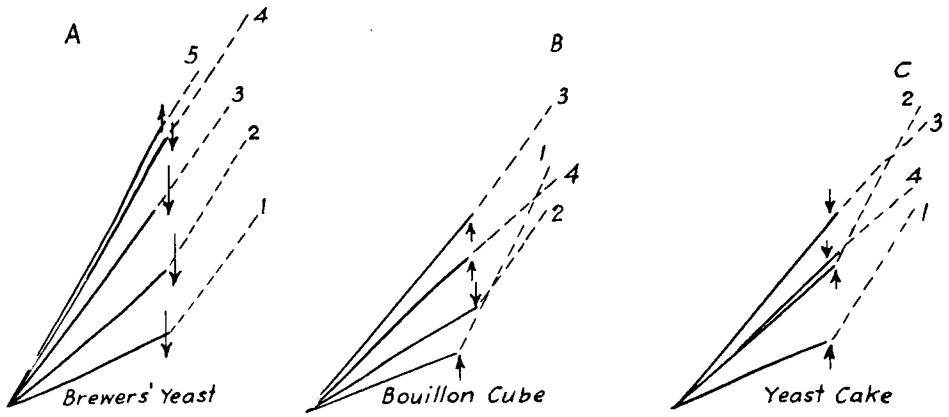

Fig. 1.

Curves No. 1, 2, 3, 4 and 5, division A, of Fig. 1 are constructed from the composite weight figures of animals fed on 25, 50, 100, 150 and 200 mg. of brewers' yeast, respectively. Arrows indicate the feeding of 1000 mg. of brewers' yeast. Curves 1, 2, 3 and 4, division B, represent animals on 100, 200, 300 and 400 mg. of bouillion cube. Curves 1, 2, 3 and 4, division C, represent animals fed on 500, 1000, 1500 and 2000 mg. of fresh yeast cake. The upward breaks in curves 1 and 2 of each series indicate:

25 mg. brewers' yeast is inadequate for normal growth.
50 mg. brewers' yeast is inadequate for normal growth.
100 mg. bouillon cubes is inadequate for normal growth.
200 mg. bouillon cubes is inadequate for normal growth.
500 mg. yeast cake is inadequate for normal growth.
1000 mg. yeast cake is inadequate for normal growth.

these lines intersect indicates any change in the rate of growth during the two periods. In cases in which several animals are on each dose, the composite weight figures for the several animals may conveniently be used in the construction of such figures.

The composite weight figures for the animals employed in this test were used for the construction of Fig. 1.

SUMMARY.

1. A simple method is described for the extraction of the vitamin B complex bearing material from such food products as bouillon cubes prepared from an extract of yeast. This extraction is by means of glacial acetic acid. The acetic acid is removed by distillation in vacuum and in a stream of inert gas such as nitrogen.

The paste remaining in the distillation flask is mixed with starch and aliquot parts of the starch mixture are fed as known doses of the material of the bouillon cube.

It is obvious that this method will be inapplicable for the separation of the vitamin B complex from interfering substances which are soluble in glacial acetic acid.

2. A simple method is described which employs each test animal used in an assay for the vitamin B complex as its own control. Experimental animals, depleted of the vitamin B complex, are fed on given doses of the material to be tested. At the end of the test period a daily dose of a material (dried brewers' yeast) known to contain several times the minimum adequate dose of the vitamin B complex required for normal growth is substituted for the daily dose of the test material under investigation. If the growth curve breaks upward with the feeding of the large dose of brewers' yeast, the given dose of the test material was inadequate for normal growth.

3. Dried brewers' yeast known to be stable for a period of at least three years is suggested as a physical standard of reference of assays for the vitamin B complex.

4. It has been found that 100 mg. of brewers' yeast, 300 mg. of bouillon cube and 1500 mg. of yeast cake are equivalent in vitamin B complex potency.

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THE COLORIMETRIC AND ELECTROMETRIC p_H DETERMINATIONS OF SOLUTIONS OF CERTAIN ALKALOIDAL SALTS.*[1]

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A review of the literature reveals several reports of investigations on the determination of the hydrogen-ion concentration of solutions of alkaloidal salts. Evers (1) determined the colorimetric p_H values of solutions of the pure hydrochlorides of morphine, quinine and atropine and suggested the use of certain indicators which would give more accurate results in the titration of the corresponding free alkaloids. McGill (2), and later Wagener and McGill (3), using electrometric methods of measurement, obtained values for pure quinine, morphine, strychnine and atropine salts. Krantz (4), using a hydrogen electrode, determined the p_H values of pure quinine hydrochloride and also obtained data on the p_H values of quinine, strychnine and atropine dissolved in varying quantities of tenth-normal hydrochloric acid in excess.

Masucci and Moffat (5) reported electrometric values for many commercial samples of morphine, codeine, quinine, strychnine, atropine and caffeine salts. Wales (6) determined titration curves for various alkaloids and from these he obtained p_H values for pure salts. Based on the latter, he recommended the use of certain indicators to minimize titration errors. Finding great variations in commercial samples of alkaloidal salts, Eder (7) recommended the adoption of definite p_H limits for alkaloidal salt solutions. More recently, Mellon and Tigelaar (8)

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