SCIENTIFIC SECTION

THE BIOLOGICAL ASSAY OF THE WATER-SOLUBLE ANTINEURITIC AND ANTIPELLAGRIC VITAMINS.*

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(It is our purpose in this paper to present a concise review of the methods used for the assay of the antineuritic and antipellagric vitamins, with the object in view of establishing some standard method which will combine simplicity and accuracy, and be acceptable to the various commercial laboratories engaged in this work.)

It is only within the last half decade (1) that the existence of two water-soluble factors in the vitamin B complex has been established. Since then the symptoms of the respective vitamin deficiencies have been extensively studied. Previously in testing for the vitamin B complex, a sample was evaluated in terms of its ability to promote growth in a rat on a vitamin B deficient diet. The renewed growth was due to a combination of both factors and no distinction was made between the antineuritic vitamin and the antipellagric vitamin.

Originally, of course, vitamin B was known only as the antineuritic vitamin and was evaluated in terms of its ability to cure polyneuritis in pigeons. The pigeon as a test object has many disadvantages, and since at the present time it is seldom used in vitamin standardization, we shall disregard this method and consider only those methods which make use of the white rat.

As regards the nomenclature of the vitamins, the tendency of American writers has been to employ the designation "Vitamin B" for the antineuritic factor and "Vitamin G" for the antipellagric factor, while the British workers use the designation "B_1" and "B_2" for the same two factors, respectively. However, this differentiation is by no means general and usually both designations are used together, one in parenthesis succeeding the other. Therapeutically, the vitamin B complex, as such, is of great value and it is our opinion that the term "Vitamin B" should be reserved for the complex as was the case originally, and the terms "B_1" and "B_2" used for the antineuritic and antipellagric vitamins, respectively. This idea seems still more reasonable in view of the fact that Reader (2, 3, 4) has shown the existence of a third factor (B_4) in the vitamin B complex. However, in this paper, we shall use the terms antineuritic vitamin and antipellagric vitamin and leave the specific designations to the future.

SYMPTOMS OF DEFICIENCY OF THE ANTINEURITIC VITAMIN.

When the mature white rat, about $3^1/_2$ to 4 weeks old and weighing about 40 to 50 Gm., is placed on a diet devoid of the antineuritic factor, no appreciable change takes place for about 10 days, except for a noticeable loss in appetite. Anywhere from the 18th to the 25th day the characteristic syndrome of antineuritic vitamin deficiency appears rather rapidly and is in complete evidence in practically every case before the 28th day.

In addition to the characteristic loss of appetite, we have noticed in the animal a condition of extreme irritability when touched or disturbed, which is

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considerably in evidence even as early as the 14th day. The animal assumes a hunched attitude and becomes lethargic, displaying no interest in its surroundings. The fur loses its gloss and becomes ragged and ruffled. In the final stages of the decline the animal becomes very pale and extremely emaciated with more or less lack of neuromuscular control. The animal seldom survives more than 4 weeks, death usually taking place from inanition.

SYMPTOMS OF DEFICIENCY OF THE ANTIPELLAGRIC VITAMIN.

In demonstrating deficiency of the antipellagric vitamin, immature white rats about 4 or 5 weeks old and weighing about 50 to 55 Gm. are used. When placed on a diet devoid of the antipellagric vitamin, no change takes place for 3 or 4 weeks, until a slight raggedness of the hair appears, with some irritation and reddening of the tip of the nose. During this period, there is no definite change in weight, either gain or loss, and the appetite is fair with the animal displaying active interest in its surroundings. About the 5th week, there is considerable blood around the margins of the nostrils, with a characteristic loss of hair from the eyelids. During the 5th and 6th weeks the various skin symptoms rapidly develop, with the loss of hair from the head and abdomen. The front paws become stained with blood from the nostrils; also there is in evidence considerable inflammation of the tips of the digits of the paws. The urine is very frequently bloody. It has been reported that the animal undergoes a change in temper, becoming irritable and apt to bite. However, in this laboratory, we have observed just the opposite, the animal being very tame and docile. In fact, this condition seems very characteristic in differentiating between the antineuritic and antipellagric vitamin deficiencies. In every case the extreme irritability is almost a certain symptom in deficiency of the antineuritic vitamin, while it seldom, if ever, appears in deficiency of the antipellagric vitamin. The animal will exist in this condition of antipellagric vitamin deficiency for some time before death intervenes—at least three to four months.

In standardizing a method for the assay of the separate factors in the vitamin B complex, there should be no trouble in differentiating between the syndromes of antineuritic vitamin deficiency and antipellagric vitamin deficiency.

THE QUANTITATIVE DETERMINATION OF ANTINEURITIC ACTIVITY.

The method of assay is essentially the same as that of all the vitamin assays. The immature rat is placed on a diet adequate in all respects except for the antineuritic vitamin. When the symptoms of the vitamin deficiency are pronounced (about 3 weeks) the material to be tested is fed along with the basal diet and the minimum amount which will give a definite increase in weight over a definite period of time can be designated as a unit. Several methods for assay of the antineuritic vitamin content have been proposed, particularly those of Chick and Roscoe (5), Sherman and Spohn (6) and the injection methods used by the United States Public Health Service (7, 8). The only difference in these various methods, aside from the mode of administration of the material to be tested, is in the preparation of the proper basal diet, and the normal growth rate to be accepted as the basis of the unit. The basal diet consists of casein, starch, butter fat, salt mixture and cod liver oil and is supplemented with an adequate supply of the anitpellagric factor. In both the Sherman method and the Chick and Roscoe method, autoclaved yeast

was originally used as the source of the antipellagric factor, but recently Chick and Roscoe (9) have discovered that egg white is an excellent source of the antipellagric factor, practically free from the antineuritic factor. Their objection to autoclaved yeast was its tendency to cause diarrhea, but in this laboratory we have used both autoclaved yeast and egg white in our diets without any noticeable difference in results.

In the choice of rats, there is close agreement in the use of animals about $3^1/_2$ to 4 weeks old, the feeding experiments being conducted in parallel between groups matched as to sex and weight after the depletion period. As regards the length of the test period, Chick and Roscoe advocate a period of 8 weeks, while Sherman recommends 4 weeks, due to the fact that on the basal diet other vitamins, not yet well defined, may begin to be interfering factors. Sherman suggests that the unit be based on a growth rate of 3 Gm. per week while Chick and Roscoe advocate a unit growth rate of 11 to 14 Gm. per week. Both units are purely arbitrary, but the 3-Gm. per week unit seems to follow out the idea used in determining the normal growth rate for the vitamin A assay.

The actual housing and care of the animals are carried out the same as in any vitamin assay. Individual metal cages are provided with raised screen floors to prevent coprophagy, the basal diet and distilled water being given *ad libitum*.

BASAL DIET.

Casein	18%
Corn-starch	53%
Salt mixture	4%
Butter fat	8%
Cod liver oil	2%
Autoclaved yeast	15%
	100%

Casein.—We have found that if a good grade of casein is used, two alcoholic extractions are sufficient to remove all traces of the antineuritic vitamin. In the first extraction, 5 pounds of casein are extracted 24 hours at room temperature with 3 gallons of 60% alcohol. In the second extraction, 3 gallons of 60% alcohol are used at the boiling point for about 2 to 3 hours. The casein is sucked dry on a Buchner funnel and washed with 60% alcohol after each extraction.

Corn-starch.—Any good grade is satisfactory.

Salt Mixture.—McCollum Salt Mixture No. 185.

Butter Fat.—All excess water must be removed, and the fat carefully strained through fine cheese cloth.

Cod Liver Oil.—Sometimes it is desirable to give the cod liver oil along with the sample to be tested. This improves the flavor and facilitates the feeding to the rat.

Autoclaved Yeast.—In the preparation of the autoclaved yeast, a dried yeast should be selected which is active in 150 mg. dose in causing normal growth in the rat which has been on a basal diet completely deprived of the vitamin B complex. We have autoclaved the yeast in the form of a thin powder with satisfactory results, but for routine work it is advisable to make a paste with 0.1 *N* sodium hydroxide and autoclave at 15-pounds pressure for 5 or 6 hours. It may be necessary to neutralize slightly after autoclaving. The product is then dried and powdered.

THE QUANTITATIVE DETERMINATION OF ANTIPELLAGRIC ACTIVITY.

In the assay for antipellagric activity, the method is essentially the same as the previous method described for testing antineuritic vitamin activity, except that a potent source of the antineuritic vitamin is incorporated into the diet in place of autoclaved yeast. A slightly older animal, about 4 to 5 weeks of age, is usually used for this test. The housing and care of the animal during the depletion period are exactly the same as in the assay for antineuritic activity—except that a longer depletion period is necessary (about $4^1/_2$ to 5 weeks). The test period as recommended by Chick and Roscoe is 8 weeks, while Sherman suggests a 4-week period from the time the minimum body weight is obtained. Chick and Roscoe suggest 10–14 Gm. per week as normal growth rate, while Sherman suggests 2–5 Gm. per week as being the limits at which the growth rate is proportional to the allowance of the antipellagric vitamin.

As a source of antineuritic vitamin free from the antipellagric factor, several materials have been tried. Goldberger and his co-workers (1) used an 85% alcoholic extract of corn meal; Sherman and Sandels (10) used a 75% alcoholic extract of whole wheat; Chick and Roscoe (11) used Peter's antineuritic concentrate; and Evans and Burr (12) used tikitiki, a dilute alcoholic extract of rice polishings. In this laboratory we have found that an alcoholic extract of whole wheat properly dried on corn-starch is quite satisfactory as a source of antineuritic vitamin and is effective in developing symptoms of antipellagric vitamin deficiency.

During the depletion period of about 5 weeks, and continuing through the actual test period, the animals are housed in individual cages under exactly the same conditions as described in the method for antineuritic vitamin assay. Some investigators prefer to keep their animals in one large cage during the depletion period, but it has been our experience that more consistent results can be obtained by keeping the rat in a separate cage during the depletion period, as well as during the actual test period.

BASAL DIET.

The basal diet and specifications are exactly the same as the basal diet for the assay of the antineuritic vitamin except that an alcoholic extract of whole wheat is substituted for the autoclaved yeast. It is also advisable to use a slightly acid and stronger alcoholic solution in the casein purification.

Casein	18%
Corn-starch	68% (containing extract of whole wheat)
Salt mixture	4%
Butter fat	8%
Cod liver oil	2%
	100%

Casein.—Five pounds of casein are extracted with 3 gallons 75% alcohol, containing 10 cc. of glacial acetic acid, at room temperature over night. A second extraction at the boiling point is carried out for 2 hours. The casein is washed and sucked dry in a Buchner funnel as in the previous method.

Antineuritic Concentrate.—Five pounds freshly ground whole wheat are given three extractions with one-gallon portions of 75% alcohol for 2 hours at room

temperature, with frequent shaking. The combined extracts are concentrated *in vacuo* and the extract dried *in vacuo* on corn-starch. The equivalent of 5 pounds whole wheat is added to each 10 pounds of finished diet.

The specifications for the corn-starch, butter fat and cod liver oil are the same as given previously.

SUMMARY.

The following is a brief summary of the methods which have given successful results in this laboratory.

ANTINEURITIC VITAMIN ASSAY.

Animals.—Immature white rats $3^1/_2$ to 4 weeks old and weighing about 40 to 50 Gm.

Depletion Period.—Twenty-four animals are placed on the special basal diet and after the first week are weighed every other day and the weight curve plotted. The condition and weight curve of each animal is separately studied. When the animals demonstrate a definite decline in the weight curve and show the primary symptoms of the antineuritic vitamin deficiency—loss of appetite, ruffled hair and hunched attitude, the series is divided into groups of four each. One group is kept as control and allowed to develop the full symptoms of the vitamin deficiency, finally dying. Each of the other groups of four are used for separate doses of the material to be tested.

Test Period.—The material to be tested is usually weighed out into the feeding cup, slightly moistened with water or cod liver oil, and given to the rat before the regular food. In practically every case, the animal consumes the test sample, while under observation. After a test period of 4 weeks, the average growth rate of each group is computed, and the unit based on an increase in weight of three Gm. per week.

ANTIPELLAGRIC VITAMIN ASSAY.

Animals.—Immature white rats 4 to 5 weeks old and weighing about 50 to 55 Gm.

Depletion Period.—Twenty-four animals are placed on the special basal diet and after the first two weeks are weighed every other day and the weight curve plotted. When the weight curve shows a definite decline and the animal demonstrates the symptoms of antipellagric vitamin deficiency, ragged hair, reddening of the tip of the nose, and loss of hair around the eyelids and nostrils, the series is divided into groups of four each. One group is kept as control and each of the remaining groups receive a separate dose of the material under test.

Test Period.—The manner of feeding is the same as described. After a test period of 4 weeks, the average growth rate is computed and the unit based on an increase in weight of 3 Gm. per week. (In this laboratory, we prefer a unit based on 3 Gm. rather than 11–14 Gm. per week, since in conjunction with the unit of antineuritic activity, it gives a more correct comparison of the relative antineuritic and antipellagric activity of the product under test.)

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THE DETERMINATION OF HALOGENS IN PHARMACOPŒIAL ORGANIC COMPOUNDS.

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The United States Pharmacopœia, X, recognizes a large number of organic compounds containing halogens. For some of these a purity rubric based on halogen content is required; in the case of the others a similar requirement might be desirable.

Until comparatively recently the only accurate methods available for the determination of halogens were those of Carius[1] and the Lemp and Broderson[2] modification of the Parr[3] and Pringsheim[4] methods.

The Carius method is not desirable for general use because of several disadvantages which it presents. Among these are the use of a sealed tube, a bomb furnace in which to heat it, and the chance of fragments of glass falling into the tube upon opening it. This procedure requires an excessive amount of time, and it is not easy to determine when the material is completely oxidized.

When the Lemp and Broderson method is used there is always the possibility of incomplete fusion of the material, the danger of too rapid oxidation when the sample is mixed with sodium peroxide, and the difficulty in obtaining accurate results with volatile compounds.

With the exceptions of chloramine and dichloramine, the pharmacopœial assay methods for the determination of halogens are restricted to compounds containing iodine. In the determination of iodine in calcium iodobehenate, thymol iodide and thyroxin, the principle involved depends upon carbonizing the material which has previously been mixed with potassium carbonate, extracting the residue with water, and oxidizing the iodide thus formed to iodate by means of potassium permanganate. After filtering and collecting an aliquot portion of the filtrate, potassium iodide is added, the solution is acidified, and the iodine liberated is determined by titrating with sodium thiosulphate solution using starch as indicator.

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* Parke, Davis and Company Fellow, 1930–1931.

[1] Carius, *Ann.*, 136 (1865), 129.

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