Estimation of serum *L. casei* activity

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SYNOPSIS Measurement of the serum *Lactobacillus casei* ('folic-acid') activity is widely used as an index of folic-acid deficiency. Present methods of assay result in recovery of about half the active material in normal serum. A modified method of assay is described which gives higher *L. casei* values and a clearer distinction between the sera of normal subjects and of patients with folic-acid deficiency.

The assessment of folic-acid deficiency by the microbiological assay of serum with *Lactobacillus casei* was introduced by Baker, Herbert, Frank, Pasher, Hutner, Wasserman, and Sobotka (1959). Present methods of assay measure about half the *L. casei* activity in normal serum. As the technique is widely used in the investigation of patients with megaloblastic anaemia, it was thought desirable to publish details of a modified assay procedure which overcomes this problem.

MATERIALS AND METHODS

PREPARATION OF SERUM An 0.1 M phosphate buffer containing 150 mg. ascorbic acid per 100 ml. at pH 6.1 was prepared before each assay. Serum was added to the buffer to give dilutions varying from 1 in 10 to 1 in 150. The diluted serum was heated at 10 pounds pressure per square inch for 10 minutes and coagulated protein removed by filtration through no. 1 Whatman paper.

With the higher dilutions the serum extract after filtration was sometimes cloudy. In such cases the turbidity of the extract was allowed for by including an uninoculated tube containing serum extract and culture medium in the assay. The turbidity of this tube was subtracted from the mean value of similar inoculated tubes. More recently the pH of the ascorbate buffer was reduced to 5.7 and this resulted in clear serum extracts. Variations in pH from 5.7 to 7.2 did not influence the *L. casei* activity of serum extracts.

CULTURE MEDIUM The culture medium was that described by the United States Department of Agriculture (Toepfer, Zook, Orr, and Richardson, 1951).

ORGANISM *Lactobacillus casei*, N.C.I.B. 8010, was used. In order to obtain minimum growth in tubes with only

water and culture medium a broth was inoculated in the morning of the assay and removed from the incubator as soon as some turbidity was visible, *i.e.*, within about six hours of inoculation. The organism was washed three times in a single-strength culture medium before inoculation of the assay.

OTHER DETAILS Other details of the assay have been described by Herbert (1961) and by Waters and Mollin (1961). Provided the ingredients used in making the culture medium were initially free of substances supporting the growth of *L. casei*, the elaborate treatment of glassware recommended was found to be unnecessary. We have found an occasional batch of casein to be contaminated with 'folate-like' compounds and a single batch of glucose was similarly contaminated (Dr. T. Vanier, personal communication).

RESULTS

EFFECT OF DILUTION IN PREPARATION OF THE SERUM EXTRACT It had been the practice to dilute serum 1 in 10 in preparing serum extracts although further dilution is usually necessary at the time of the assay. The effect of varying the dilution from 1 in 10 to 1 in 150 is shown in Figure 1. Higher *L. casei* values were always obtained when higher dilutions were used, reaching a plateau after a 1 in 70 dilution.

When more ascorbate-phosphate buffer was added to the coagulated proteins removed in preparing the serum extract and the suspension re-autoclaved, considerably more *L. casei* activity was recovered. The highest values were obtained by reheating the deposit from the lower dilutions, *i.e.*, far more was obtained from the 1 in 10 deposit than from the 1 in 50 deposit. However, the rise in the *L. casei* activity was only partly accounted for by the recovery of *L. casei*-active material from the precipitate and it must be

FIG. 1. The effect of varying the dilution of serum in the preparation of a serum extract for microbiological assay with *L. casei*. The results with sera from four normal subjects are shown.

assumed that some of the material was destroyed by heating.

Serum *L. casei* activity assayed at the 1 in 10 and the 1 in 100 dilution in various groups of patients is shown in Figure 2. The effect of dilution is striking in normal subjects, the mean *L. casei* level being 8.9 μ mg. per ml. at 1 in 10 dilution and 15.8 μ mg. per ml. at 1 in 100 dilution. The range at the 1 in 100 dilution was 8.0 to 31.4 μ mg. per ml.

On the other hand dilution had little effect on the very low values with sera from patients with megaloblastic anaemia requiring folic-acid therapy. The results in patients with vitamin B12 deficiency are also shown.

Effect of ascorbate in preserving L. casei activity of sera Waters and Mollin (1961) found that the decline of serum *L. casei* activity could be prevented by the addition of ascorbic acid to the serum before freezing. Herbert (personal communication), however, found that the *L. casei* activity of serum was stable in the frozen state. The addition of ascorbate to sera has the disadvantage of rendering these

FIG. 2. A comparison of the *L. casei* activity of serum, when a 1 in 10 and a 1 in 100 dilution of serum is used in the preparation of the serum extracts. The sera were obtained from 28 normal subjects, nine patients with megaloblastic anaemia due to B12 deficiency, and 14 patients with megaloblastic anaemia requiring therapy with folic acid.

FIG. 3. The effect of storage of sera at -20°C . on their *L. casei* activity. Blood was taken from 30 normal subjects. One portion of blood was added directly to dry ascorbate and serum separated (blood + C). This procedure caused some haemolysis and this probably produced slightly raised *L. casei* values. Serum from the rest of the sample was divided into two and ascorbate added to one portion (serum + C), the remaining serum being stored without preservative (serum alone). The *L. casei* activity was assayed periodically during the next six months. Serum extracts were prepared at a 1 in 10 dilution and the mean values of the 30 samples are shown.

specimens unsuitable for vitamin B12 assay, since the ascorbate is said to destroy B12 present as hydroxycobalamin. The effect of ascorbate (5 to 10 mg.) on the preservation of *L. casei* activity was investigated by bleeding 30 normal subjects. The blood sample was divided into three portions. One aliquot was added to a tube with 5 to 10 mg. of dry ascorbate and the serum retained. Ascorbate, 5 to 10 mg., was added to the serum from the second aliquot. Serum from the third portion was retained without any addition. All the sera were stored at -20°C . and *L. casei* activity assayed periodically over the next six

months. The results (Fig. 3) confirm the observations of Waters and Mollin (1961) that ascorbate largely prevented the decline of *L. casei* activity in storage.

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