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The influence of penicillin on *Lactobacillus leichmannii* serum B_{12} assay

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SYNOPSIS The influence of penicillin on vitamin B_{12} assay using *L. leichmannii* as the test organism was investigated, and it was found that penicillin even in a low serum concentration invalidated the test.

The assay of vitamin B_{12} in serum is now accepted as a routine laboratory test. In some laboratories the method using *Lactobacillus leichmannii* as the test organism is used in preference to the more time-consuming *Euglena gracilis* method. It gives satisfactory results and does not require special equipment but the fact that *L. leichmannii* used in the test is sensitive to antibiotics is sometimes ignored and specimens for the test are collected during antibiotic treatment. The purpose of this investigation is to draw attention to possible errors in B_{12} assay by using the *L. leichmannii* method on sera collected while patients were receiving penicillin.

METHODS

B_{12} ASSAY IN SERUM A modification of methods described by Hawkins and Meynell (1958) and Cooper (1959) was used.

Medium Double strength Difco B_{12} assay broth was prepared according to the makers' instructions.

Preparation of serum extracts To 1 vol. of serum one fifth volume of 0·1% sodium cyanide and 1 vol. of acetate buffer, *p*H 4·6, were added. The mixture was left at room temperature for about 30 minutes and then distilled water was added to make a total of 10 vol. (If the water is added immediately after the buffer some extracts may be opalescent or milky; all extracts are water clear when the proper interval is observed.) The mixtures were placed in a boiling water bath for 30 minutes, cooled, and centrifuged. The clear supernatant extracts were used for the assay.

Preparation of inoculum A fresh, 24-36-hour-old culture of *L. leichmannii* in Difco inoculum broth was centrifuged and the deposit washed twice with sterile saline and then re-suspended in saline. The suspension was further diluted with saline till it was just slightly opalescent.

Procedure In the standard test two extract strengths are used, neat and diluted 1:2·5. In this investigation extracts were used, neat and diluted 1:2, 1:5, 1:10, 1:20, 1:50. Sterile vitamin B_{12} aqueous solutions containing 0 to 50 $\mu\mu$g. per ml. were prepared for the standard B_{12} curve. To 5 ml. aliquots of neat and diluted extracts and of B_{12} standard solutions in sterile tubes an equal amount of double-strength Difco B_{12} assay broth and 0·1 ml. of *L. leichmannii* inoculum were added. The tubes were incubated anaerobically at 37°C. for 36 hours. After incubation the cultures were diluted with an equal volume of 3% HCl in saline and the opacity measured in a Unicam SP 500 spectrophotometer (wavelength 590 millimicrons, large tube with light path of 24 mm.). Dilution with acid saline has two advantages: it confines all opacity readings in the test to the more sensitive part of the logarithmic scale and it produces a uniform yellow colour in all culture tubes, which, after incubation, are of different shades from reddish-brown to yellow.

DETERMINATION OF L. LEICHMANNII SENSITIVITY TO PENICILLIN A series of twofold dilutions of penicillin in sterile distilled water was prepared. The solutions contained between 0·0038 and 0·48 units of penicillin per ml. To 5 ml. aliquots of each solution an equal amount of double-strength Difco B_{12} assay broth containing 50 $\mu\mu$g of B_{12} per ml. and 0·1 ml. of *L. leichmannii* inoculum were added. The tubes were incubated anaerobically at 37°C. for 36 hours and the presence or absence of growth was read macroscopically.

PENICILLIN ASSAY IN SERUM AND SERUM EXTRACTS The method used was that described by Mackie and McCartney (1953). From both neat serum (or extract) and from its 1:100 dilution in sterile broth a series of twofold dilutions in sterile broth was prepared (from 1:1 to 1:64). At the same time a series of twofold penicillin dilutions in sterile broth containing between 0·0019 and 0·24 units per ml. was also prepared. Then 0·3 ml. aliquots of all serum (or extract) and of penicillin dilutions were inoculated

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with a standard loopful of 1:300 dilution in broth of a 24-hour-old broth culture of the Oxford staphylococcus. The tubes were incubated overnight and the presence or absence of growth observed macroscopically.

The lowest penicillin concentration that inhibited growth of the Oxford staphylococcus was invariably 0·03 units per ml. The same penicillin concentration was assumed to be present in the highest serum or extract dilution that inhibited growth of the Oxford staphylococcus.

RESULTS

The lowest level of penicillin in the serum to be assayed which might, by inhibiting the growth of the test organism, interfere with the assay depends on (1) the sensitivity of *L. leichmannii* to penicillin, (2) the dilution of the serum to be assayed, and (3) the loss of penicillin activity during preparation of extract.

L. LEICHMANNII SENSITIVITY TO PENICILLIN *L. leichmannii* was found to be resistant to 0·015 units per ml. and sensitive to 0·03 units per ml. penicillin. These limits are the same as for the Oxford staphylococcus and this fact was found useful in this investigation.

LOSS OF PENICILLIN ACTIVITY IN SERUM EXTRACTS To evaluate the deterioration of penicillin activity during the preparation and storage of extracts penicillin levels in 12 sera and their extracts were determined and compared. The results are given in Table I.

TABLE I

PENICILLIN CONCENTRATIONS IN SERA AND THEIR EXTRACTS

Sample	*Penicillin Content in 1 ml. Serum (units)*	*Penicillin Content in 10 ml. Extract (units)*	*Time between Collection of Serum and Preparation of Extract*	*Residual Penicillin Activity in Extract (%)*
1	4·8	2·4	Prepared from fresh serum	50
2	19·2	9·6	Prepared from fresh serum	50
3	3·0	1·2	Prepared from fresh serum	40
4	3·0	2·4	Prepared from fresh serum	80
5	3·0	1·2	5 days	40
6	24·0	9·6	5 days	40
7	6·0	4·8	5 days	80
8	6·0	2·4	5 days	40
9	24·0	9·6	10 days	40
10	2·0	1·2	10 days	60
11	2·0	0·6	10 days	30
12	48·0	19·2	10 days	40

Serum penicillin concentrations were estimated on fresh sera and again after they had been stored frozen at −40°C. for 10 days. There was no deterioration in penicillin activity after storage. On the other hand, some penicillin activity was lost during preparation of extracts. The residual activity was between 30% and 80% of that originally present in the serum. The double dilution method used for penicillin assay is not very accurate and, therefore, the figures in Table I give only approximate values.

L. LEICHMANNII ASSAYS ON SERA FROM PATIENTS RECEIVING PENICILLIN The inhibitory action of penicillin on *L. leichmannii* explains findings in routine B_{12} assay work when on some occasions the presence of penicillin in tested sera invalidated the test. The results obtained in five sera submitted for routine B_{12} assay from patients who were being treated with penicillin are shown in Fig. 1. A striking finding in these five tests was the complete inhibition of growth of *L. leichmannii* in tubes containing large concentrations of serum extracts. These are the concentrations used in routine B_{12} assay

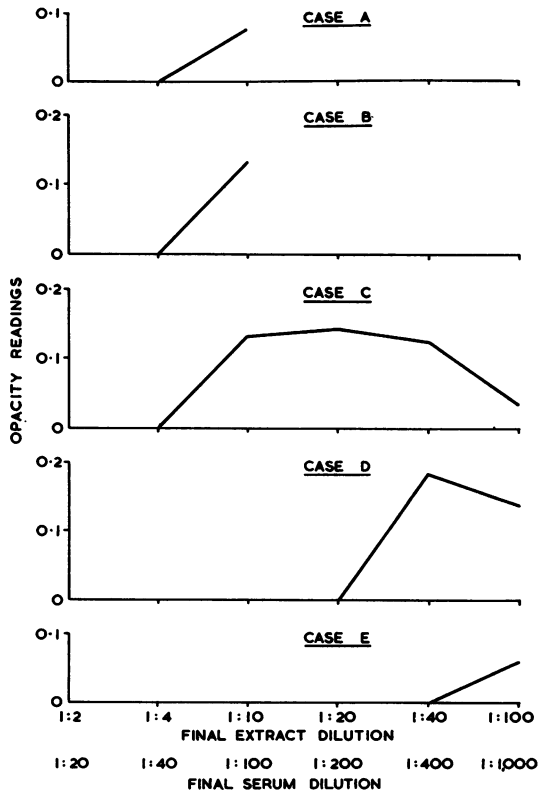

FIG. 1. *Opacity readings in B_{12} assay on five sera collected during penicillin treatment. Penicillin content in extracts: Case A, 0·06 units/ml.; Case B, 0·12 units/ml.; Case C, 0·24 units/ml.; Case D, 1·0 unit/ml.; Case E, 1·9 unit/ml. Penicillin levels in sera: Case A, 2·0 unit/ml.; Case E, 48·0 unit/ml.; there was insufficient serum in the remaining three cases.*

(extracts diluted 1:2 and 1:5 corresponding to serum dilutions of 1:20 and 1:50). The inhibition corresponded roughly to the pencillin level in the extract. In Cases A, B, and C, in which extract penicillin levels were less than 0·25 units per ml., the growth of *L. leichmannii* was completely inhibited in the first two tubes only. In Case D, in which extract penicillin level was 1 unit per ml., there was no growth in the first four tubes, and in Case E with an extract penicillin level of 1·9 units per ml. in the first five tubes. Such complete lack of growth in culture tubes inoculated with *L. leichmannii* is never found when sera are free from penicillin. Even with sera from patients with pernicious anaemia in those tubes containing the least amount of extract there is always a slight turbidity visible to the naked eye.

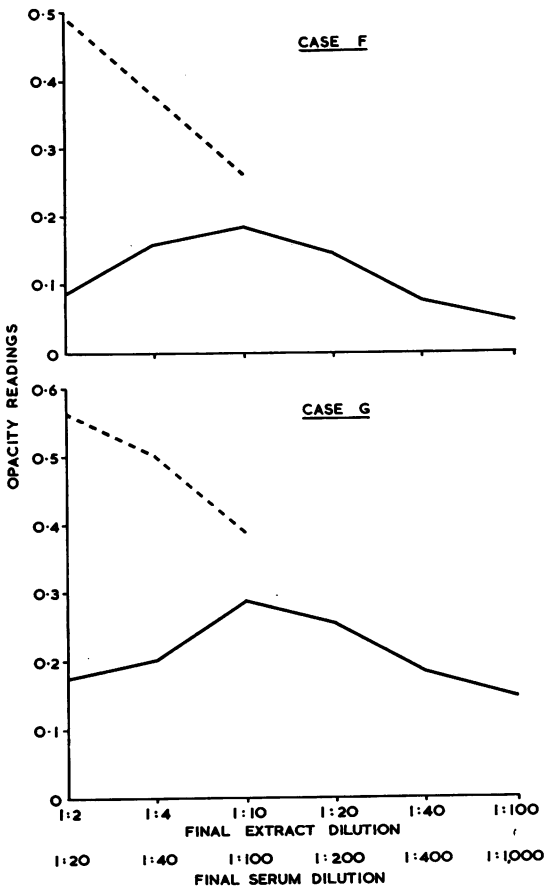

FIG. 2. *Opacity readings in serum B_{12} assays before and during penicillin treatment (interrupted line — — — before, continuous line——— during penicillin treatment). Penicillin doses, serum penicillin levels, and calculated serum B_{12} values are given in Table II.*

While penicillin interference should not be missed in cases where growth of *L. leichmannii* is completely inhibited, it is less easily recognized in tests where extract penicillin levels are so low that growth of *L. leichmannii* is only partly inhibited. Two such cases are shown in Fig. 2. These two patients were treated with fairly low doses of penicillin (Case F, Broxil tablets, 500 mg. daily, and Case G, penicillin injections, 500,000 units daily). Two blood specimens for B_{12} assay were collected from each patient, one before and another on the second day of penicillin treatment. It was assumed that there would be no significant difference in B_{12} levels between two specimens collected at such short intervals. There was a marked difference in the appearance of opacity curves between the first and the second specimens. The specimens collected before penicillin treatment showed a more or less regular slope, while the specimens collected during treatment produced a curve of unusual shape. The growth of *L. leichmannii* was scanty in the first tubes containing undiluted extract. It increased with each dilution till the peak was reached and then diminished with further dilutions. The first, rising part of the curve was obviously related to penicillin and not to B_{12} concentration. The peak values in both cases were less than in corresponding dilutions of penicillin-free extracts and could not be accepted as representing true B_{12} concentrations. The serum B_{12} levels calculated from each dilution are shown in Table II.

TABLE II

SERUM B_{12} VALUES CALCULATED FROM OPACITY READINGS SHOWN IN FIG. 2

Case	*Sample*	*Penicillin Dose*	*Serum Penicillin Level (units/ml.)*	*Extract Dilutions*[1] ½ (1/20)	¼ (1/40)	1/10 (1/100)
F	1	—	—	510	480	500
	2	Oral Broxil, 500 mg. daily	0·06	10	80	250
G	1	—	—	[2]	1000	1100
	2	Penicillin, 500 u. i.m. daily	0·12	32	700	550

[1]Corresponding serum dilutions are shown in brackets.
[2]Opacity too high, outside standard curve range.

Dilutions in the descending part of the curve were outside the useful range. It was also possible that the growth of *L. leichmannii* in these tubes was still influenced by traces of penicillin.

DISCUSSION

The examples described above show that the low concentrations of penicillin in serum, which can be

expected in practically every case treated with penicillin, will invalidate B_{12} assay by the *L. leichmannii* method. When the penicillin level is high enough to cause complete inhibition of growth the fact is easily noticed. A partial inhibition of growth by smaller concentrations of penicillin in serum may not attract attention and consequently false low values may be erroneously reported.

When the serum B_{12} level has to be estimated during penicillin treatment it should be done by the *Euglena gracilis* method. Ross (1952) found that 'sulphathiazole, penicillin, streptomycin, aureomycin, chloramphenicol, and para-aminosalicylic acid when added in concentration likely to be found in human serum during treatment with these drugs were without significant effect on growth of *Euglena gracilis*'. Lear, Harris, Castle, and Fleming (1954), on the other hand, reported that 'sulphonamide derivatives may be present in serum in concentrations which can inhibit *Euglena* growth'. Hutner, Bach, and Ross (1956) showed that the addition of para-aminobenzoic acid to the basal medium abolished the inhibitory effect of sulphonamides but did not interfere with the growth of *Euglena gracilis* in serum B_{12} assay.

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