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# Effect of drugs on vitamin $B_{12}$ levels obtained using the *Lactobacillus leichmanii* method

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SYNOPSIS In 750 consecutive assays of serum vitamin $B_{12}$ levels using *Lactobacillus leichmanii*, 25 (3·3%) showed inhibition. A satisfactory history of drug therapy was obtained in 23 cases. Seventeen of these were receiving ampicillin at the time. Two patients showed inhibition while receiving phenoxmethyl penicillin or phenethicillin. A prospective study of 11 patients detected inhibition in seven at varying intervals during ampicillin therapy. It was shown that the vitamin $B_{12}$ as measured by the radioisotope and *Euglena gracilis* methods was not affected. Inhibition of *L. leichmanii* was reproduced *in vitro* with concentrations of ampicillin corresponding to those obtained in therapy. Benzylpenicillin, streptomycin, and chloramphenicol had no such effect.

A prospective study of patients receiving chlorpromazine yielded negative results.

Inhibition may occasionally be seen in the absence of any known intake of drugs. Measurement by other methods may be required to distinguish this from vitamin $B_{12}$ depletion.

Where serum $B_{12}$ is estimated using *Lactobacillus leichmanii*, the phenomenon of inhibition is encountered not infrequently. Boczarow (1961) reported on the effect of penicillin on the test organism and found that penicillin therapy invalidated the test. However, Watts (1967) claimed that the dilution and heating involved in the assay procedure resulted in inactivation of the antibiotic, except where novobiocin was given. In view of this discrepancy we decided to measure the incidence of the phenomenon of inhibition and its possible relationship to antibiotic and drug therapy.

## MATERIAL AND METHOD

Serum vitamin $B_{12}$ was estimated using *Lactobacillus leichmanii* (ATCC 7830) and DANO-$B_{12}$ assay medium (Dano Chemicals, Copenhagen). The method (Thompson, Dietrich, and Elvehjem, 1950; Spray, 1955) was basically as described by Dano Chemicals, except that the serum dilution was adjusted so that the vitamin $B_{12}$ activity was measured using both 0·1 and 0·25 ml of serum (corresponding to dilutions of 1:100 and 1:40 respectively). The tests were done in duplicate. All the assays were carried out by one of us (C.D.) who has been using the method for five years. It was first established that there was insufficient diurnal or daily variation to invalidate the study and that the normal levels were between 120 and 600 $\mu\mu$g/ml.

The *Euglena gracilis* and $^{57}$Co methods were performed according to the descriptions of Ross (1952) and Lau, Gottlieb, Wasserman, and Herbert (1965).

The incidence of inhibition was determined by assessing the results of the last 750 consecutive vitamin $B_{12}$ assays, 480 from cases in a psychiatric hospital and 270 from general medical cases. All the accepted cases showed a marked reduction (usually more than twofold) of the vitamin $B_{12}$ level with the larger volume of serum (1:40 dilution). Drug ingestion was then investigated.

When it was realized that ampicillin was implicated, tests *in vivo* and *in vitro* were carried out with this antibiotic. Eleven patients who required therapy were studied. Blood was taken to establish a basal serum vitamin $B_{12}$ level before ampicillin was given in a dose of 250 mg intramuscularly every eight hours. Subsequent samples were collected half an hour after the morning injection on the third and fifth treatment days. A final sample was collected between five and seven days after the cessation of therapy. A similar procedure was adopted in 10 patients receiving chlorpromazine hydrochloride 50 mg twice daily.

A further five patients were studied during ampicillin therapy in order that parallel vitamin $B_{12}$ levels by the radioisotope and/or *E. gracilis* methods could be determined in the inhibitory phase.

Tests *in vitro* with ampicillin and other antibiotics were carried out by adding the antibiotics to the serum before the assay. The quantities added were adjusted to give levels which might be encountered *in vivo*.

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## RESULTS

INCIDENCE OF INHIBITION Twenty-five instances of probable inhibition were found among the 750 consecutive vitamin $B_{12}$ assays (incidence 3·3%). Only three instances were in the psychiatric group, while 22 occurred in the much smaller general group. The patients' ages ranged from 44 to 92 years. A reliable drug history could not be obtained in two patients. No drugs had been prescribed in five patients. One patient was receiving phenethicillin. The remaining 17 were all on ampicillin (Table I). Patients 4 (a case of pernicious anaemia) and 15 had been given cytamen; patient 6 was also on chlorpromazine, imipramine, and promazine hydrochloride; and patient 11 was on streptomycin. The corresponding figures for the five patients for whom no drugs had been prescribed are also shown.

TABLE I

VITAMIN $B_{12}$ LEVELS IN 22 PATIENTS[1]

| *Patient* | *0·1 ml Serum (1:100 dilution)* | *0·25 ml Serum (1:40 dilution)* |
|---|---|---|
| *Patients receiving ampicillin* | | |
| 1 | 40 | 0 |
| 2 | 120 | 75 |
| 3 | 350 | 30 |
| 4 | 1,300 | 140 |
| 5 | 50 | 0 |
| 6 | 580 | 340 |
| 7 | 55 | 0 |
| 8 | 1,200 | 600 |
| 9 | 50 | 10 |
| 10 | 110 | 20 |
| 11 | 45 | 0 |
| 12 | 300 | 95 |
| 13 | 130 | 80 |
| 14 | 0 | 0 |
| 15 | 1,700 | 860 |
| 16 | 135 | 90 |
| 17 | 20 | 0 |
| *Patients receiving no drugs* | | |
| 18 | 75 | 30 |
| 19 | 420 | 250 |
| 20 | 50 | 20 |
| 21 | 50 | 20 |
| 22 | 830 | 530 |

[1]Results are expressed in $\mu\mu$g/ml.

Many other drugs, including tetracycline, frusemide, digoxin, imipramine hydrochloride, methyldopa, and barbiturates, were being administered to the other patients in this survey at the time of sampling. None of these were associated with inhibition.

PROSPECTIVE STUDY OF AMPICILLIN THERAPY The effect of ampicillin was studied prospectively in 11 patients (Table II). Seven showed clear evidence of inhibition on the third and/or fifth days of therapy. Case 23 showed almost complete inhibition on both days; four of the others (cases 24, 28, 29, and 31)

TABLE II

EFFECT OF AMPICILLIN ON VITAMIN $B_{12}$ LEVELS[1]

| *Case No.* | *Basal Level* | | *Days after Ampicillin* | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | *Third Day* | | *Fifth Day* | | *Five to Seven* | |
| | *0·1 ml* | *0·25 ml* | *0·1 ml* | *0·25 ml* | *0·1 ml* | *0·25 ml* | *0·1 ml* | *0·25 m* |
| 23 | 320 | 300 | 0 | 0 | 20 | 0 | 280 | 260 |
| 24 | 170 | 190 | 0 | 0 | 160 | 0 | 150 | 160 |
| 25 | 120 | 120 | 110 | 110 | — | — | 80 | 110 |
| 26 | 370 | 380 | 380 | 380 | 350 | 0 | 410 | 380 |
| 27 | 210 | 230 | 190 | 200 | 350 | 350 | 220 | 280 |
| 28 | 650 | 500 | 240 | 0 | 550 | 470 | 770 | 660 |
| 29 | 430 | 410 | 0 | 0 | 390 | 360 | 250 | 300 |
| 30 | 230 | 230 | 280 | 280 | 0 | 0 | Died | |
| 31 | 620 | 510 | 370 | 10 | 440 | 350 | 770 | 660 |
| 32 | 80 | 90 | 60 | 70 | 50 | 10 | 110 | 130 |
| 33 | 220 | 220 | 210 | 190 | 270 | 290 | 220 | 250 |

[1]Results are expressed in $\mu\mu$g/ml.

showed marked inhibition on the third day, but only one of these (case 24) continued to show clear evidence of inhibition on the fifth day. On the other hand two patients (cases 26 and 30) showed inhibition on the fifth day only. Sometimes the degree of inhibition was complete with both 0·1 and 0·25 ml of serum. In others the presence of an inhibitor was indicated by the higher $B_{12}$ levels in the more dilute sample. This difference may be marked, for example, in case 26 on the fifth day from 350 $\mu\mu$g/ml to zero. On the other hand, the difference may be much smaller and more difficult to interpret, for example, in case 32 from 50 to 10 $\mu\mu$g/ml, or in case 31, in which both pre- and post-antibiotic levels were above 600 $\mu\mu$g/ml, from 440 to 350 $\mu\mu$g/ml on the fifth day.

A further five patients were studied to check the effect of ampicillin therapy on vitamin $B_{12}$ levels, as measured by the radioisotope and *E. gracilis* methods. Because of insufficient serum it was not possible to perform all three methods on all samples. The first samples were collected before ampicillin therapy was begun, and the second on the third day of treatment (Table III). Three of the five (M.T., H.H., and M.E.H.) showed typical inhibition with *L. leichmanii*, but when measurements were performed by the other two methods levels of vitamin $B_{12}$ were within the normal range. The ampicillin levels in two of the sera that showed inhibition (M.T. and H.H.) were 0·4 $\mu$g and 3·1 $\mu$g per ml respectively.

Patient F.G. is of interest in that the low serum vitamin $B_{12}$ levels obtained with *L. leichmanii* were not related to ampicillin therapy, as shown by a similar level after treatment. This patient, aged 88 years, was on thyroid therapy for myxoedema, but was not anaemic. Assay with *E. gracilis* gave the same reading as with *L. leichmanii* and a true vitamin $B_{12}$ deficiency was confirmed by a Schilling radioisotope absorption test which showed only 2·6% excretion in the first 24 hours.

TABLE III

COMPARISON OF ASSAY METHODS FOR SERUM $B_{12}$ IN PATIENTS GIVEN AMPICILLIN

| Patient | Sample | L. leichmanii | | $^{57}Co$ | E. gracilis |
|---|---|---|---|---|---|
| | | 0·1 ml Serum | 0·25 ml Serum | | |
| M.T. | 1 | 250 | 250 | — | — |
| | 2 | 110 | 0 | 270 | — |
| H.H. | 1 | 290 | 300 | — | — |
| | 2 | 250 | 80 | 594 | — |
| E.H. | 1 | 200 | 220 | — | — |
| | 2 | 240 | 240 | 530 | 327 |
| M.E.H. | 1 | 360 | 330 | — | — |
| | 2 | 340 | 40 | — | 373 |
| F.G. | 1 | 30 | 0 | — | — |
| | 2 | 50 | 30 | — | — |
| | Thereafter | 30 | 30 | — | 30 |

Sample 1 refers to that taken before the start of ampicillin, and sample 2 on the third day of therapy. Results are expressed in $\mu\mu$g/ml.

EFFECT OF ADDING AMPICILLIN IN VITRO The effect of adding ampicillin *in vitro* to normal serum before vitamin $B_{12}$ assay is shown in Table IV. The $B_{12}$ value before the addition of ampicillin was 210 $\mu\mu$g/ml. Ampicillin was added to give levels of 1·0 to 10·0 $\mu$g per ml and it was shown that 4·0 $\mu$g and above produced inhibition.

The specificity of the inhibition was confirmed, as shown in Table IV, where 2 and 8 $\mu$g of ampicillin were added to the pre-antibiotic serum of case 26 (Table II). Slight inhibition was seen in the 0·25 ml sample at 2 $\mu$g, and complete inhibition in 0·1 ml and 0·25 ml at 8 g. This patient subsequently showed inhibition on the fifth day of ampicillin therapy.

*Ampicillin sensivity of L. leichmanii* The organism was tested using the standard assay medium in the presence of ampicillin. The organism was found to be highly sensitive, giving a minimum inhibitory concentration of 0·04 $\mu$g per ml.

TABLE IV

EFFECT OF ADDING AMPICILLIN TO CONTROL SERUM *IN VITRO* BEFORE VITAMIN $B_{12}$ ASSAY

| Ampicillin ($\mu$g) | Vitamin $B_{12}$ | |
|---|---|---|
| | 0·1 ml Serum | 0·25 ml Serum |
| 1·0 | 190 | 180 |
| 2·0 | 170 | 70 |
| 4·0 | 100 | 0 |
| 6·0 | 0 | 0 |
| 8·0 | 0 | 0 |
| 10·0 | 0 | 0 |
| No ampicillin | 210 | 210 |

TABLE IVA

EFFECT ON AMPICILLIN *IN VITRO* IN CASE 26 (TABLE II)

| | 0·1 ml Serum | 0·25 ml Serum |
|---|---|---|
| Basal sample | 340 | 310 |
| 2 $\mu$g ampicillin added | 340 | 170 |
| 8 $\mu$g ampicillin added | 0 | 0 |

INHIBITION WITH PHENOXYMETHYL PENICILLIN One patient, investigated after completion of the survey, showed complete inhibition of *L. leichmanii* at all serum dilutions. This patient had been given phenoxymethyl penicillin, 250 mg four times daily, and complete inhibition was observed on the third and sixth days of treatment. The levels obtained by *E. gracilis* and radioisotope methods showed no inhibition. A level of $B_{12}$ of 230 $\mu\mu$g per ml was found two weeks after cessation of treatment.

When more than 5 $\mu$g per ml of phenoxymethyl penicillin was added *in vitro* complete inhibition was found at both serum dilutions.

EFFECT OF ADDING BENZYLPENICILLIN IN VITRO The vitamin $B_{12}$ levels obtained when sodium benzylpenicillin (Crystapen, Glaxo) was added before assay showed no evidence of inhibition (Table V).

TABLE V

EFFECT OF ADDING BENZYLPENICILLIN *IN VITRO* BEFORE VITAMIN $B_{12}$ ASSAY

| Benzylpenicillin (units) | Vitamin $B_{12}$ | |
|---|---|---|
| | 0·1 ml Serum | 0·25 ml Serum |
| 0 | 280 | 270 |
| 0·5 | 270 | 270 |
| 1·0 | 290 | 270 |
| 2·0 | 290 | 280 |
| 3·0 | 290 | 270 |
| 5·0 | 290 | 270 |
| 10·0 | 290 | 240 |
| 15·0 | 290 | 270 |
| 20·0 | 270 | 300 |

OTHER ANTIBIOTICS Chloramphenicol and streptomycin (both in strengths of up to 50 $\mu$g per ml) were without any inhibitory effect when tested *in vitro*.

EFFECT OF AMPICILLIN ON HIGH VITAMIN $B_{12}$ LEVELS Two additional patients who showed high vitamin $B_{12}$ levels were studied. The first had received vitamin $B_{12}$ therapy until three weeks before admission. Ampicillin was prescribed and the vitamin $B_{12}$ results obtained on the second day of therapy were 1,300 $\mu\mu$g per ml with 0·1 ml serum and 160 $\mu\mu$g per ml with 0·25 ml serum. The second patient presented with diabetes mellitus and features consistent with liver damage. She had received neither vitamin $B_{12}$ nor antibiotic therapy, but $B_{12}$ levels were 1,100 $\mu\mu$g per ml with 0·1 ml serum and 640 $\mu\mu$g per ml with 0·25 ml serum.

PROSPECTIVE STUDY OF CHLORPROMAZINE THERAPY The $B_{12}$ levels were obtained before, during, and after chlorpromazine therapy but only the mean value for the dilutions of 0·1 ml and 0·25 ml are

TABLE VI

VITAMIN $B_{12}$ LEVELS DURING CHLORPROMAZINE THERAPY

| *Basal $B_{12}$ Level* | *Chlorpromazine Therapy* | | |
|---|---|---|---|
| | *Third Day* | *Fifth Day* | *Five to Seven Days Later* |
| 250 | 240 | 240 | 230 |
| 120 | 120 | 110 | 100 |
| 140 | 140 | 140 | 150 |
| 210 | 430 | 500 | 310 |
| 280 | 290 | 240 | 400 |
| 620 | 650 | 500 | 540 |
| 340 | 290 | — | 360 |
| 140 | 260 | 290 | 300 |
| 240 | — | 220 | — |
| 270 | 240 | 220 | 230 |
| 190 | 110 | 190 | 120 |

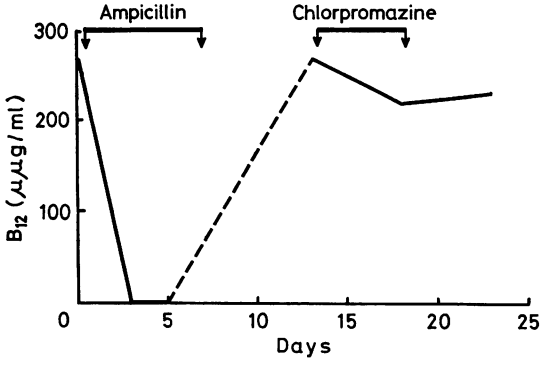


FIG. 1. *Vitamin $B_{12}$ levels during courses of ampicillin and chlorpromazine (C.P.Z.) therapy in one patient*

given in Table VI because no significant differences were seen.

One patient who received ampicillin followed by chlorpromazine therapy showed complete inhibition on ampicillin but no inhibition while receiving chlorpromazine (Fig. 1).

## DISCUSSION

In this survey it has been shown that when *L. leichmanii* is used for the assay of serum vitamin $B_{12}$ and the serum tested at dilutions of 1:40 and 1:100, approximately 3·3% of samples showed inhibition of the test organism. A large number of inhibitory samples were from patients receiving ampicillin. Patients in a psychiatric hospital, although being treated with a variety of drugs, showed a much lower incidence of inhibition.

A prospective study has confirmed that the *L. leichmanii* used in vitamin $B_{12}$ assay is often inhibited in the course of ampicillin therapy. In this investigation the phenomenon was observed in approximately 60% of the patients receiving ampicillin. The stage at which inhibition was observed was also variable. In some it was present within three days, but in others it was not manifest until the fifth day. Alternatively inhibition may appear within three days, only to disappear by the fifth. The degree of inhibition also varies. It may be complete in both dilutions as tested, or may only become apparent as the concentration of serum is increased. The volumes used in this method, namely 0·1 and 0·25 ml, appear to be appropriate, otherwise partial inhibition at one level could be misleadingly interpreted as a low value. Even when two volumes are used partial inhibition may still be difficult to assess, especially over the lower range of vitamin $B_{12}$ levels. We have, in fact, noticed that the low levels obtained in proven examples of pernicious anaemia showed higher readings in the more diluted serum sample. This may be related to the observation that traces of serum usually depress the growth of *E. gracilis* (Anderson, 1964). The additional cases reported showed that inhibition can occur even with the high vitamin $B_{12}$ levels induced by cytamen therapy. Occasional instances occur where no history of drug therapy can be obtained. If the $B_{12}$ levels are below the normal range, then other steps must be taken to exclude true deficiency. Thus the results of *L. leichmanii* assays are not reliable as a solitary index of vitamin $B_{12}$ deficiency in large surveys.

The range of blood ampicillin levels that might be expected within 30 minutes of intramuscular injection (as supplied by Beecham Limited) determined the levels used in our tests *in vitro*. These confirmed that ampicillin-induced inhibition occurs at the expected therapeutic blood levels 2 to 6 $\mu$g per ml. Sera from patients, free from inhibition before ampicillin therapy, showed a degree of inhibition similar to that seen *in vitro* on the addition of comparable concentrations of ampicillin. It is of interest to speculate on the mechanisms that might account for inconstancy of inhibition *in vivo*. Presumably this is not a simple penicillin versus organism equation. Although among the penicillins ampicillin shows the least degree of protein binding, a variable degree of binding may be one possible explanation.

Boczarow (1961) found that *L. leichmanii* showed a sensitivity to penicillin similar to that of the Oxford staphylococcus. He examined five patients being treated with penicillin, and, although he did not specify the type of penicillin, or the time of sampling, he found complete inhibition with serum dilutions of 1:20 and 1:50. Boczarow further stated that even low concentrations of penicillin invalidated the $B_{12}$ result in practically every case. We have confirmed the occurrence of inhibition with ampicillin but have noted inexplicable exceptions.

Watts (1967) did not specify the heating procedure adopted, although in the method supplied by the Danocheme Company the diluted serum is steamed for 30 minutes. This is the procedure we adopted. Our results are in conflict with those of Watts, who confirmed the sensitivity *in vitro* of *L. leichmanii* to penicillin (and many other antibiotics), but claimed that dilution, heat, and precipitation of protein removed the antibiotic.

Ross (1952) showed that several antibiotics, including penicillin, had no effect on *E. gracilis* when added *in vitro* in concentrations comparable to those found during treatment. We have confirmed that this also applies *in vivo*. The radioisotope method is also unaffected.

Herbert, Gottlieb, and Altschule (1965) reported chlorpromazine inhibition of *E. gracilis* in vitamin $B_{12}$ assays. In the correspondence that followed, Forshaw and Harwood (1966) showed that this did not occur when *L. leichmanii* was used as the test organism. Davis and Nicol (1966) thought that there might be differences in the effect of Largactil and Thorazine, the latter being the trade name under which chlorpromazine is sold in the United States. However, they failed to show inhibition with either, although it is not clear which test organism was used. Our results confirm those of Forshaw and Harwood with the *L. leichmanii* assay and suggest that this is a valuable routine method in a psychiatric hospital (of 2,000 beds) where chlorpromazine and other psychotropic drugs are used extensively.

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