Extraction of serum vitamin B₁₂ for radio-isotopic and *Lactobacillus leichmannii* assay

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SYNOPSIS The protein precipitates discarded during the extraction process of the *Lactobacillus leichmannii* vitamin B₁₂ assay have been shown to contain significant amounts of vitamin B₁₂. This loss of vitamin B₁₂ provides a satisfactory explanation for many of the discrepancies between the serum vitamin B₁₂ values obtained by the *L. leichmannii* method and the radio-isotopic method of Raven *et al* (1969). It is possible to produce lower results by the method of Raven *et al* (1969) by incorporating into that method the *L. leichmannii* extraction process; it is also possible to produce higher results by the *L. leichmannii* method using a papain extraction process.

Over the last nine years, a radio-isotopic assay of vitamin B₁₂ in serum has been found to give higher results for many sera than the *Lactobacillus leichmannii* microbiological assay. For some sera from post-gastrectomy patients and from patients with folate deficiency of many different causes the discrepancies in results have been especially marked and often of clinical importance (Raven *et al*, 1969; Raven *et al*, 1972). This paper gives the results of investigations into the reasons for the differences between the results obtained by the radio-isotopic and microbiological assays.

Material and Methods

RADIO-ISOTOPIC STUDIES

The radio-isotopic assay was that of Lau *et al* (1965) modified by Raven *et al* (1969). Individual backgrounds were used for each serum sample, and the normal range for this method is 200-1000 pg/ml. In this method, serum vitamin B₁₂ is extracted from its binding proteins by the heating of serum in the presence of cyanide and N/4 HCl. A turbid solution is obtained and no precipitation of protein occurs (fig 1).

For the assay of the serum extracts and protein precipitates obtained from the extraction process used in the *L. leichmannii* assay, the radio-isotopic assay was modified as follows:

Fig 1 Serum extracts for the assay of vitamin B₁₂. From left to right: water; radio-isotopic method of Raven *et al* (1969); *L. leichmannii* method; papain modification of *L. leichmannii* method. Each extract was prepared from 2 ml of the same serum, and the bottles have not been centrifuged.

L. leichmannii Serum Extracts

Into a 20 ml screw-capped glass universal container are placed 1 ml serum, 8 ml water, 0.5 ml 0.1% NaCN solution, and 0.25 ml 0.4M acetate buffer (pH 4.5). With the screw cap in place, the container is heated at 115°C for 10 minutes and, after cooling, 0.25 ml phosphate buffer (M/5 K₂HPO₄) is added. The container is then centrifuged and 8 ml of the clear supernatant (equivalent to 0.8 ml serum) is transferred to another universal container together with 2 ml N/4 HCl. This acid-serum extract mixture is then treated as in the radio-isotopic assay above.

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Received for publication 14 January 1975.

An appropriate intrinsic factor control is prepared similarly from an *L. leichmannii* serum extract of either a serum containing less than 10 pg/ml of vitamin B₁₂ (by radio-isotopic assay) or a 1 in 20 dilution of vitamin B₁₂-deficient serum. The vitamin B₁₂ value derived by calculation is multiplied by 10/8 to give the serum vitamin B₁₂ level.

L. leichmannii Protein Deposits

The protein deposit obtained from 1 ml serum by means of the *L. leichmannii* extraction process is placed in a 20 ml universal container and washed thoroughly with three changes of distilled water. After the last wash, the supernatant fluid is removed, and the volume of the deposit is made up to 10 ml with 2 ml N/4 HCl and the required volume of 0.1% (w/v) NaCN solution. With the screw cap in position, the container is then autoclaved at 115°C for 15 minutes and allowed to cool. The extract is then treated as in the radio-isotopic assay above. An appropriate intrinsic factor control is prepared in the same way from the protein deposit obtained from a serum shown by radio-isotopic assay to have a B₁₂ content of less than 10 pg/ml.

L. leichmannii STUDIES

The *L. leichmannii* assay was that of Rosenthal and Sarett (1952) modified by Spray (1955) and Chanarin (1969). It is the method used in the previous studies of Raven *et al* (1969; 1972) and is described in detail by Chanarin (1969). Its normal range is 150-850 pg/ml, although occasionally sera from patients with untreated pernicious anaemia have vitamin B₁₂ values of between 150 and 200 pg/ml (Ardeman *et al*, 1966; Raven *et al*, 1972). In this method, serum vitamin B₁₂ is extracted from its binding proteins by a deproteinization technique involving the heating of serum in the presence of cyanide and acetate buffer (pH 4.5). Only the clear supernatant obtained is used in the *L. leichmannii* assay (fig 1). The protein precipitate is discarded before assay. For some studies the *L. leichmannii* method was modified to incorporate a papain extraction process as follows:

Preparation of Papain Solution

Ten grams of papain powder (Merck) is ground in a mortar with 100 ml Sorensen's phosphate buffer (0.066M, pH 5.4), and the mixture is removed and centrifuged for 10 minutes. Ten ml of 0.5M cysteine HCl solution is added to the supernatant to activate the papain, and the mixture is made up to 200 ml with phosphate buffer and incubated at 37°C for one hour. The 5% papain solution thus obtained is dispensed into 25 ml aliquots and stored for up to six months at -20°C.

Papain—*L. leichmannii* extraction process

Into a 20 ml universal container are placed 1 ml serum, 7 ml water, 0.5 ml 0.1% NaCN solution, and 1 ml 5% papain solution. The container is then heated in a 60°C water bath for one hour and, after the addition of 0.25 ml 0.4M acetate buffer (pH 4.5), autoclaved at 115°C for 10 minutes. After cooling, 0.25 ml phosphate buffer (M/5 K₂HPO₄) is added and the universal container is placed in a 4°C refrigerator overnight to allow the fine protein particles to precipitate, leaving a clear supernatant (fig 1). (The protein particles are so fine that centrifugation often produces a turbid supernatant.) For the *L. leichmannii* assay, this supernatant is then treated in exactly the same way as the ordinary *L. leichmannii* serum extract. The standard curve for the papain-*L. leichmannii* assay is set up in the same way as for the standard *L. leichmannii* assay.

Results

RADIO-ISOTOPIC STUDIES

Assay of *L. leichmannii* Serum Extracts

The vitamin B₁₂ levels of 331 miscellaneous sera were determined by the radio-isotopic and *L. leichmannii* methods and by the radio-isotopic method modified to incorporate the *L. leichmannii* extraction process. The radio-isotopic method produced generally higher values than the *L. leichmannii* method (fig 2); there was closer agreement between the *L. leichmannii* method and the radio-isotopic method using the *L. leichmannii* extraction process

Fig 2 Comparison of vitamin B₁₂ values obtained for 331 sera by the *L. leichmannii* method and the radio-isotopic method of Raven *et al* (1969). In this and subsequent figures, the line drawn through the ordinate represents the line of identity for the two results on the same serum.

Fig 3 Comparison of vitamin B₁₂ values obtained for 331 sera by the *L. leichmannii* method and the radio-isotopic method of Raven et al (1969), modified to incorporate the *L. leichmannii* extraction process.

(fig 3). Similar conclusions may be reached about the results obtained by the three different assays for 110 sera from post-gastrectomy patients, 45 folate deficient sera, and 40 sera from patients with vitamin B₁₂ deficiency due to untreated pernicious anaemia (figs 4 to 6).

Fig 4 Vitamin B₁₂ values obtained for 110 sera from post-gastrectomy patients by the radio-isotopic method, the radio-isotopic method modified to incorporate the *L. leichmannii* extraction process, and the *L. leichmannii* method. In this and subsequent figures, the horizontal lines represent mean values.

Assay of *L. leichmannii* protein deposits

The amount of vitamin B₁₂ detected in the protein deposits obtained from 331 miscellaneous sera by means of the *L. leichmannii* extraction process

Fig 5 Vitamin B₁₂ values obtained for 45 sera from patients with folate deficiency by the radio-isotopic method, the radio-isotopic method modified to incorporate the *L. leichmannii* extraction process, and the *L. leichmannii* method.

Fig 6 Vitamin B₁₂ values obtained for 40 sera from patients with vitamin B₁₂ deficiency due to pernicious anaemia by the radio-isotopic method, the radio-isotopic method modified to incorporate the *L. leichmannii* extraction process, and the *L. leichmannii* method.

ranged from 0 to 365 pg per ml of whole serum. The mean value was 65 pg (fig 7). The values for 48 vitamin B₁₂ deficient sera, 48 folate deficient sera, 138 sera from post-gastrectomy patients, and 70 normal sera are shown in fig 8, the highest values being given by the folate deficient and post-gastrectomy groups of sera.

The agreement between radio-isotopic and *L. leichmannii* methods (fig 2) is improved when to the *L. leichmannii* value is added the amount of vitamin B₁₂ detected in the *L. leichmannii* protein deposit (fig 9).

Fig 7 Distribution of amounts of vitamin B_{12} detected by the radio-isotopic method of Raven et al (1969) in the protein deposits obtained from 331 miscellaneous sera. The mean value is 65 pg/ml of whole serum.

Fig 8 Amounts of vitamin B_{12} detected by the radio-isotopic method of Raven et al (1969) in the protein deposits (from 1 ml of whole serum) obtained from 48 B_{12} deficient sera, 48 folate deficient sera, 138 post-gastrectomy sera, and 70 normal sera.

L. leichmannii STUDIES

Tests for the Presence of *L. leichmannii* Inhibitor

(a) Twenty sera shown to have considerably higher serum vitamin B_{12} values by the radio-isotopic assay than the *L. leichmannii* assay were mixed with equal parts of a serum whose vitamin B_{12} value was found to be 500 pg/ml by both radio-isotopic and *L. leichmannii* assays. The serum mixtures were then assayed by the *L. leichmannii* method. For each mixture the vitamin B_{12} values obtained were the

Fig 9 Comparison of vitamin B_{12} values obtained for 331 sera by the radio-isotopic method of Raven et al (1969) and by the *L. leichmannii* method, when to each *L. leichmannii* value has been added the amount of vitamin B_{12} detected in the *L. leichmannii* protein deposit by the radio-isotopic method.

same as those expected by calculation, thus excluding the presence of some *L. leichmannii* inhibitor in the sera tested.

(b) Replicate vitamin B_{12} assays were carried out by the *L. leichmannii* method for 397 sera, first using the usual volume of serum extract and, secondly, twice this volume (with the results divided by two to obtain the serum vitamin B_{12} value). The results obtained (fig 10) show the double-volume values to be a little lower than the single-volume values. However, the differences are of little importance and

Fig 10 Comparison of vitamin B_{12} values obtained for 397 sera by the *L. leichmannii* method in replicate assays, one assay using a single volume of serum extract and the other a double volume.

can be explained easily by the reading of results from an insensitive part of the standard curve. They provide no real evidence of a serum *L. leichmannii* inhibitor.

Recovery Experiments for Normal and Post-gastrectomy Sera

To each of 27 normal sera and 24 post-gastrectomy sera was added 250 pg of ⁵⁷CoB₁₂, and the ⁵⁷CoB₁₂-serum mixtures were then treated by means of the *L. leichmannii* extraction process. The post-gastrectomy sera had been selected from those shown to have considerably higher vitamin B₁₂ values by the radio-isotopic method than the *L. leichmannii* method. For the normal sera, the recovery of added ⁵⁷CoB₁₂ ranged from 91 to 100% (mean 98%) and for the post-gastrectomy sera from 88 to 100% (mean 98%).

Papain Extraction Process

Replicate vitamin B₁₂ assays were carried out for 308 sera by the *L. leichmannii* method, first using the standard extraction process and, secondly, a papain modification of the extraction process. The papain method produced higher vitamin B₁₂ values than the standard method (fig 11). The possibility of falsely high values due to non-specific stimulation of *L. leichmannii* growth by the papain extracts was excluded by the preparation on three occasions of two different standard curves. Each standard curve was formed from mixtures of standard

vitamin B₁₂ solutions and vitamin B₁₂ deficient serum, the mixtures for one curve being treated by means of the standard *L. leichmannii* extraction process and for the other by the papain modification. The two curves thus obtained were similar on each occasion.

Discussion

Quite naturally, the early radio-isotopic assays of vitamin B₁₂ in serum were expected to produce results as close as possible to the conventional microbiological assays (*L. leichmannii* and *Euglena gracilis*). The method of Lau *et al* (1965) was reported to give a good correlation with the *E. gracilis* assay but it soon became apparent that there was a defect in this radio-isotopic method, tending to produce falsely low serum vitamin B₁₂ values or even negative values for vitamin B₁₂ deficient sera (Raven *et al*, 1966, 1968, 1969; Hillman *et al*, 1969); many modifications have been suggested to overcome this defect which was due to the absence of serum proteins from the standards (intrinsic factor controls) (Raven, 1970).

Experience since 1966 with a modification of the method of Lau *et al* (1965) has shown that in general the serum vitamin B₁₂ values obtained by the radio-isotopic assay are higher than the *L. leichmannii* microbiological method (Raven *et al*, 1969; 1972). The discrepancies have been particularly marked for some folate deficient sera and post-gastrectomy sera. Even the unmodified method of Lau *et al* (1965) has been reported to give higher vitamin B₁₂ values than the *L. leichmannii* method for some sera from pregnant women (Lawrence and Klipstein, 1967).

The purpose of the investigations in this paper was to find the reason for differences in the results obtained by the radio-isotopic and *L. leichmannii* assays. The possible explanations included:

TECHNICAL DEFECT IN THE RADIO-ISOTOPIC METHOD

To anyone with long experience with a microbiological vitamin B₁₂ assay and a belief in the accuracy of its results this would seem the likely explanation. The radio-isotopic results would then be considered falsely high. In the radio-isotopic method of Raven *et al* (1969), some defect in the acid extraction process, the binding of vitamin B₁₂ by intrinsic factor, the separation of free and bound B₁₂ by albumen-coated charcoal or in the calculation of results by equation could lead to consistently high serum vitamin B₁₂ values but it would be difficult to explain why some folate deficient or post-gastrectomy sera should give especially discrepant

Fig 11 Comparison of vitamin B₁₂ values obtained for 308 sera by the *L. leichmannii* method and by the *L. leichmannii* method modified to incorporate a papain extraction process.

results and why accurate values should be given for vitamin B₁₂ solutions and dilutions of sera. It has already been shown that the calculation of results by equation gives similar results in the 0-1000 pg/ml range to those obtained from a standard curve (Raven *et al*, 1968) and, in addition, other quite different radio-isotopic assays have produced higher serum vitamin B₁₂ values than the *L. leichmannii* assay (Raven and Robson, 1974; van de Wiel *et al*, 1974). The acid extraction process, used in the method of Raven *et al* (1969) and suggested by Rothenberg (1968) as a cause of falsely high serum vitamin B₁₂ values, will be discussed later.

DETECTION OF DIFFERENT FORMS OF VITAMIN B₁₂

Both the radio-isotopic method of Raven *et al* (1969) and the *L. leichmannii* method use cyanide in their extraction processes and so both assays are detecting cyanocobalamin. There remains the possibility that some analogue of vitamin B₁₂ which does not support the growth of *L. leichmannii* is also being detected by the radio-isotopic assay. It is difficult to exclude this possibility but the experience of Raven and his colleagues over the last nine years is that when the radio-isotopic assay gives a normal value and the *L. leichmannii* assay a subnormal value on the same serum, a full clinical and haematological study fails to detect any evidence of vitamin B₁₂ deficiency. Also the sera from all patients with haematological evidence of vitamin B₁₂ deficiency have given subnormal vitamin B₁₂ values by the radio-isotopic method, whether the vitamin B₁₂ deficiency was due to pernicious anaemia or followed gastric surgery. Any hypothetical vitamin B₁₂ analogue cannot therefore be distorting the serum vitamin B₁₂ values so that false clinical information is being given by the radio-isotopic assay. These clinical findings, the results of extraction and papain studies described in this paper, and a comparison of *L. leichmannii* and *E. gracilis* assays (Raven *et al*, 1972), all make it unlikely that some vitamin B₁₂ analogue is responsible for the discrepancy between radio-isotopic and *L. leichmannii* results.

SERUM *L. leichmannii* INHIBITOR

Such an inhibitor causing falsely low *L. leichmannii* values has been postulated by Lawrence and Klipstein (1967) but no good evidence, including the experiments described in this paper and those of Green *et al* (1974), has ever been found except, of course, such obvious examples as sera containing antibiotics.

TECHNICAL DEFECT IN THE *L. leichmannii* METHOD

The variability of results that one might expect from a microbiological assay can be seen both from studies of the reproducibility of the *L. leichmannii* method (Spray, 1955; Matthews, 1962; Raven *et al*, 1966; Wagstaff and Broughton, 1971) and from its comparison with the *E. gracilis* assay (Raven *et al*, 1972) but cannot be an explanation for any systematic difference in radio-isotopic and *L. leichmannii* results. One possible explanation, however, is the difference in extraction processes used by the radio-isotopic and *L. leichmannii* assays to separate vitamin B₁₂ from its binding proteins. The radio-isotopic assay uses a whole serum method in which serum is heated in the presence of cyanide and N/4 HCl. A turbid solution is obtained and, because of the low pH (approximately 2.0), no protein precipitation occurs. The *L. leichmannii* method uses a deproteinization technique in which serum is heated in the presence of cyanide and an acetate buffer (pH 4.5). A clear supernatant is used for the vitamin B₁₂ assay and a flocculent white protein precipitate is discarded.

Ever since the introduction of cyanide into the *L. leichmannii* extraction process it has been believed that no vitamin B₁₂ remains in the protein precipitate and that all the serum vitamin B₁₂ is available for assay (Matthews, 1962). Only occasionally has it been suggested that the extraction of vitamin B₁₂ by a deproteinization method may lead to falsely low serum vitamin B₁₂ values (Barakat and Ekins, 1963; Carmel and Coltman, 1969; Raven *et al*, 1969). The studies described in this paper show that often significant amounts of vitamin B₁₂ are left behind in the protein precipitates and so are discarded before *L. leichmannii* assay. This loss of vitamin B₁₂ provides an explanation for the *L. leichmannii* results being generally lower than those of the radio-isotopic assay. The especially discrepant results for some folate deficient or post-gastrectomy sera can be explained by the increased loss of vitamin B₁₂ in the protein precipitates formed from these groups of sera (fig 8).

Although these findings supported the belief that the difference in extraction processes was at least one of the factors responsible for the difference in radio-isotopic and *L. leichmannii* results, it was felt that the evidence would be stronger if it were possible to modify the microbiological method so that it could produce higher serum vitamin B₁₂ values. It proved possible to convert the acidic serum extract used in the radio-isotopic assay into a form suitable for *L. leichmannii* assay by heating it in the presence of Difco brand B₁₂ assay medium, USP. Complete protein precipitation occurred and

a clear extract of appropriate pH was obtained. Unfortunately, the vitamin B₁₂ results were similar to those obtained by standard *L. leichmannii* assay. It did prove possible to produce higher *L. leichmannii* values by adding another step to the serum extraction process; before centrifugation to remove precipitated proteins, N/4 HCl was added and the extract was heated a second time. However, for some sera the turbidity of their extracts was too great, and individual serum blanks were necessary. It was also necessary to modify the standard curve so that the pH of the standard curve and serum tubes was similar. Because of these difficulties it was decided to try a completely different form of extraction process using some proteolytic enzyme.

Enzyme modifications of microbiological assays had been used previously in the assay of vitamin B₁₂ in serum, milk, bile, and tissue. Gregory (1954) had shown that prior digestion with cyanide-activated papain was necessary before the vitamin B₁₂ content of human and sow's milk could be measured by the *L. leichmannii* assay, and both Shenoy *et al* (1957) and Hall and Allen (1964) had shown that higher serum vitamin B₁₂ values could be obtained by the *E. gracilis* method if a papain extraction process were used. The papain-*L. leichmannii* method described in this paper proved simple to use and produced higher serum vitamin B₁₂ values than the standard *L. leichmannii* method.

The extraction process in the *L. leichmannii* assay has now been used without modification for almost 20 years. Undoubtedly the reason for the continued belief that deproteination in the presence of cyanide produces complete extraction of vitamin B₁₂ has been the use of the recovery experiment. Recovery by the *L. leichmannii* assay of vitamin B₁₂ added to serum has been excellent ever since cyanide was introduced into the extraction process, and the recovery results shown in this paper are typical of others' experience. The usual assumption has been that if any vitamin B₁₂ assay, microbiological or radio-isotopic, can recover 100% of (exogenous) vitamin B₁₂ added to a serum, then the extraction of the endogenous vitamin B₁₂ in the serum is also complete (Jacobs and Zondag, 1969; Frenkel *et al*, 1970). It is most unlikely that this assumption is correct. Endogenous vitamin B₁₂ in serum is tightly bound largely to transcobalamin 1 (Benson *et al*, 1972); vitamin B₁₂ added to serum is bound in a labile fashion largely to transcobalamin 2 from which vitamin B₁₂ can be separated by simple measures such as heating at 56°C for 30 minutes (Gullberg, 1970). Accordingly, a more severe extraction process is required for the assay of endogenous vitamin B₁₂ than for the recovery of vitamin B₁₂ added to serum, and an extraction

process which is suitable for the recovery of added vitamin B₁₂ is not necessarily efficient for the extraction of endogenous vitamin B₁₂.

In general, those radio-isotopic assays which use an acid extraction process, either hydrochloric or glutamic acid, produce higher serum vitamin B₁₂ values than the *L. leichmannii* method (Raven *et al*, 1969; van de Wiel *et al*, 1974; Raven and Robson, 1974). Those radio-isotopic assays which use a deproteination extraction process produce values in agreement with the *L. leichmannii* method (Matthews *et al*, 1967; Britt *et al*, 1969; Wagstaff and Broughton, 1971). The only exception is the radio-isotopic method of Green *et al* (1974), which uses a deproteination extraction process yet has the highest normal range of all the radio-isotopic assays described so far (400-1100 pg/ml).

In two other radio-isotopic assays (Rothenberg, 1968; Jacobs and Zondag, 1969) an acid extraction process has been found to produce higher serum vitamin B₁₂ values than a deproteination method. Rothenberg suggested that the higher values obtained for pernicious anaemia sera with an acid extraction process were falsely high due to interference by denatured proteins with the vitamin B₁₂ binder (transcobalamin 1). He thought that the lower values obtained with the deproteination extraction process could not be due to less efficient extraction since recovery of added ⁵⁷CoB₁₂ by the deproteination method was of the order of 95%. Apart from the confusion of extraction and recovery, mentioned previously, this cannot provide an explanation for Rothenberg's results or for the radio-isotopic method of Raven *et al* (1969) producing higher serum vitamin B₁₂ values than the *L. leichmannii* method. It is true that denatured serum proteins affect the binding of vitamin B₁₂ by intrinsic factor and so play an important part in radio-isotopic assays which use intrinsic factor as the vitamin B₁₂ binder (Rothenberg, 1963; Raven *et al*, 1968; Hillman *et al*, 1969; Raven, 1970; Mortensen, 1972). Similar findings have been reported for a radio-isotopic method using an acid extraction process and serum as the vitamin B₁₂ binder (van de Wiel *et al*, 1974) but the effect of the denatured proteins is to increase the binding of vitamin B₁₂ by intrinsic factor or the serum protein binder—Mortensen (1972) used the term 'negative interference'. In a radio-isotopic assay such as that of Rothenberg (1968) in which there are no serum proteins in the standards, the presence of the denatured proteins produced by an acid extraction process would lead to falsely low serum vitamin B₁₂ values if it were not outweighed by improved extraction of vitamin B₁₂ from the serum proteins. Rothenberg's argument, however, is an

important one since it is likely to be repeated as it becomes apparent that an acid extraction process is associated with increased serum vitamin B₁₂ values. It would be simple to explain the high values obtained by the method of Raven *et al* (1969) as being due to the presence of some interfering protein degradation product. One would then expect to obtain lower values when a deproteinated serum extract was used and also to detect the same hypothetical degradation protein in the protein precipitates formed during the *L. leichmannii* extraction process. This argument was the main stimulus for the attempts, described in this paper, to modify the *L. leichmannii* method so that higher serum vitamin B₁₂ values could be obtained. Since it did prove possible by both acid and papain modifications to obtain higher results by the *L. leichmannii* method, it is unnecessary to postulate the presence of some serum degradation product causing artefactually high serum vitamin B₁₂ values by the method of Raven *et al* (1969).

All the studies in this paper refer to the extraction process used by the *L. leichmannii* method of Chanarin (1969). Is it possible to apply the results to the other varieties of *L. leichmannii* assay in common use? The extraction process of Chanarin (1969) is almost the same as that of Dacie and Lewis (1968) but it might be argued that the extraction processes used by the *L. leichmannii* methods of Spray (1955) and Matthews (1962) are more efficient because they both use twice as much acetate buffer per millilitre of serum as the methods of Chanarin (1969) and Dacie and Lewis (1968). However, this does not appear to be so since the reported lower limits of the normal ranges for these two methods are no higher than that of the method of Chanarin (1969) (Spray, 1955: 150 pg/ml; Spray and Witts, 1958: 150 pg/ml; Batata *et al*, 1967: 140 pg/ml; Matthews, 1962: 120 and 115 pg/ml). The likelihood remains that the radioisotopic method of Raven (1969) is producing generally higher values than different varieties of the *L. leichmannii* method because it is using a more efficient extraction process to separate serum vitamin B₁₂ from its binding proteins.

We should like to thank Mr J. Holliday, FAIMT, for carrying out the *L. leichmannii* assays shown in figure 10.

References

- Ardeman, S., Chanarin, I., Krafchik, B., and Singer, W. (1966). Addisonian pernicious anaemia and intrinsic factor antibodies in thyroid disorders. *Quart. J. Med.*, **35**, 421-431.
- Barakat, R. M. and Ekins, R. P. (1963). An isotopic method for the determination of vitamin B₁₂ levels in blood. *Blood*, **21**, 70-79.
- Batata, M., Spray, G. H., Bolton, F. G., Higgins, G., and Wollner, L. (1967). Blood and bone marrow changes in elderly patients, with special reference to folic acid, vitamin B₁₂, iron, and ascorbic acid. *Brit. med. J.*, **2**, 667-669.
- Benson, R. E., Rappazzo, M. E., and Hall, C. A. (1972). Late transport of vitamin B₁₂ by transcobalamin II. *J. Lab. clin. Med.*, **80**, 488-495.
- Britt, R. P., Bolton, F. G., Cull, A. C., and Spray, G. H. (1969). Experience with a simplified method of radioisotopic assay of serum vitamin B₁₂. *Brit. J. Haematol.*, **16**, 457-464.
- Carmel, R. and Coltman, C. A., Jr. (1969). Radioassay for serum vitamin B₁₂ with the use of saliva as the vitamin B₁₂ binder. *J. Lab. clin. Med.*, **74**, 967-975.
- Chanarin, I. (1969). *The Megaloblastic Anaemias*, pp. 198-204. Blackwell Scientific Publications, Oxford.
- Dacie, J. V. and Lewis, S. M. (1968). *Practical Haematology*, 4th edition, pp. 398-400. Churchill, London.
- Frenkel, E. P., McCall, M. S., and White, J. D. (1970). Recognition and resolution of errors in the radioisotopic assay of serum vitamin B₁₂. *Amer. J. clin. Path.*, **53**, 891-903.
- Green, R., Newmark, P. A., Musso, A. M., and Mollin, D. L. (1974). The use of chicken serum for measurement of serum vitamin B₁₂ concentration by radioisotopic dilution: description of method and comparison with microbiological assay results. *Brit. J. Haematol.*, **27**, 507-526.
- Gregory, M. E. (1954). The microbiological assay of 'vitamin B₁₂' in the milk of different animal species. *Brit. J. Nutr.*, **8**, 340-347.
- Gullberg, R. (1970). Effect of heat on the binding of vitamin B₁₂ to serum transcobalamins. *Clin. chim. Acta*, **29**, 97-100.
- Hall, C. A. and Allen, E. S. (1964). Improved method for the extraction of vitamin B₁₂ from serum. *J. Lab. clin. Med.*, **63**, 517-523.
- Hillman, R. S., Oakes, M., and Finhold, C. (1969). Hemoglobin-coated charcoal radioassay for serum vitamin B₁₂. A simple modification to improve intrinsic factor reliability. *Blood*, **34**, 385-390.
- Jacobs, W. L. W. and Zondag, H. A. (1969). Radioisotope assay of vitamin B₁₂ in human blood serum. *Clin. chim. Acta*, **24**, 93-103.
- Lau, K. S., Gottlieb, C., Wasserman, L. R., and Herbert, V. (1965). Measurement of serum vitamin B₁₂ level using radioisotope dilution and coated charcoal. *Blood*, **26**, 202-214.
- Lawrence, C. and Klipstein, F. A. (1967). Megaloblastic anemia of pregnancy in New York City. *Ann intern. Med.*, **66**, 25-34.
- Matthews, D. M. (1962). Observations on the estimation of serum vitamin B₁₂ using *Lactobacillus leichmannii*. *Clin. Sci.*, **22**, 101-111.
- Matthews, D. M., Gunasegaram, R., and Linnell, J. C. (1967). Results with radioisotopic assay of serum B₁₂ using serum binding agent. *J. clin. Path.*, **20**, 683-686.
- Mortensen, E. (1972). Negative interference by residual proteins in the supernatant fluid used in radioisotopic assay of serum vitamin B₁₂. *Clin. Chem.*, **18**, 895-900.
- Raven, J. L. (1970). Radioisotopic assay of vitamin B₁₂. *Blood*, **35**, 288-289.
- Raven, J. L. and Robson, M. B. (1974). Experience with a commercial kit for the radio-isotopic assay of vitamin B₁₂ in serum: the Phadebas B₁₂ Test. *J. clin. Path.*, **27**, 59-65.
- Raven, J. L., Robson, M. B., Morgan, J. O., and Hoffbrand, A. V. (1972). Comparison of three methods for measuring

- vitamin B₁₂ in serum: radioisotopic, *Euglena gracilis* and *Lactobacillus leichmannii*. *Brit. J. Haemat.*, **22**, 21-31.
- Raven, J. L., Robson, M. B., Walker, P. L., and Barkhan, P. (1968). The effect of cyanide, serum and other factors on the assay of vitamin B₁₂ by a radio-isotope method using ⁵⁷CoB₁₂, intrinsic factor and coated charcoal. *Guy's Hosp. Rep.*, **117**, 89-109.
- Raven, J. L., Robson, M. B., Walker, P. L., and Barkhan, P. (1969). Improved method for measuring vitamin B₁₂ in serum using intrinsic factor, ⁵⁷CoB₁₂ and coated charcoal. *J. clin. Path.*, **22**, 205-211.
- Raven, J. L., Walker, P. L., and Barkhan, P. (1966). Comparison of the radioisotope dilution-coated charcoal method and a microbiological method (*L. leichmannii*) for measuring vitamin B₁₂ in serum. *J. clin. Path.*, **19**, 610-613.
- Rosenthal, H. L. and Sarett, H. P. (1952). The determination of vitamin B₁₂ activity in human serum. *J. biol. Chem.*, **199**, 433-442.
- Rothenberg, S. P. (1963). Radioassay of serum vitamin B₁₂ by quantitating the competition between Co⁵⁷B₁₂ and unlabeled B₁₂ for the binding sites of intrinsic factor. *J. clin. Invest.*, **42**, 1391-1398.
- Rothenberg, S. P. (1968). A radioassay for serum B₁₂ using unsaturated transcobalamin I as the B₁₂ binding protein. *Blood*, **31**, 44-54.
- Shenoy, K. G., Narayanan, M. S., and Ramasarma, G. B. (1957). An improved *Euglena gracilis* method for assay of serum vitamin B₁₂ activity. *Indian J. med. Sci.*, **11**, 155-162.
- Spray, G. H. (1955). An improved method for the rapid estimation of vitamin B₁₂ in serum. *Clin. Sci.*, **14**, 661-667.
- Spray, G. H. and Witts, L. J. (1958). Results of three years' experience with microbiological assay of vitamin B₁₂ in serum. *Brit. med. J.*, **1**, 295-298.
- Wagstaff, M. and Broughton, A. (1971). A simple routine radio-isotopic method for the estimation of serum vitamin B₁₂ using DEAE cellulose and human serum binding agent. *Brit. J. Haemat.*, **21**, 581-593.
- van de Wiel, D. F. M., Koster-Otte, L. J., Goedemans, W. T., and Woldring, M. G. (1974). Competitive protein binding analysis of vitamin B₁₂ using vitamin B₁₂-free serum as a standard diluent. *Clin. chim. Acta*, **56**, 131-142.