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RESULTS OF THREE YEARS' EXPERIENCE WITH MICROBIOLOGICAL ASSAY OF VITAMIN B_{12} IN SERUM

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An improved method for the microbiological assay of vitamin B_{12} in serum using *Lactobacillus leichmannii* as test organism was developed in this department (Spray, 1955) and has been in regular use for the past three years. Although no new facts have been discovered, results have been obtained from patients with a variety of conditions. It is felt that a summary of these results may be of interest, illustrating the value of the method in diagnosis and confirming and extending observations already published.

Methods

Blood is collected in carefully cleaned all-glass syringes with stainless-steel needles, and is allowed to clot in acid-cleaned bottles. The clot is broken up with a glass rod, the blood is centrifuged, and the serum is removed and stored in a deep-freeze until required for assay. Details of the extraction process and the technique of the assays have been described elsewhere (Spray, 1955). In accordance with the accepted convention, results are expressed as micromicrogrammes ($\mu\mu$g. or 10^{-12} g.) of vitamin B_{12} activity per ml. of serum.

Results

The accompanying Chart illustrates the results obtained in the main groups of patients with macrocytic anaemia and related conditions.

Normal Subjects.—Results have been obtained from 123 normal subjects. The values ranged from 150 to 1,000 $\mu\mu$g. per ml., with a mean of 450 $\mu\mu$g. per ml.

Untreated Pernicious Anaemia.—Results have been obtained from 94 patients with pernicious anaemia in relapse. Of these, 85 gave values of 100 $\mu\mu$g. per ml. or less. In 7 the values were between 100 and 170 $\mu\mu$g. per ml., but only one of these (170 $\mu\mu$g. per ml.) was above the observed lower limit of the normal range. The mean of the results from these 92 patients is 64 $\mu\mu$g. per ml. The figures for the remaining two patients, who appeared to satisfy all the usual criteria for untreated pernicious anaemia, were 380 and 500 $\mu\mu$g. per ml. No explanation for these anomalous results was found, but contamination of the specimens from syringes or containers, or the possibility that the blood may have been taken after vitamin B_{12} had been given to the patients, cannot be excluded.

Diseases of the Central Nervous System.—In a few patients the first evidence of a deficiency of vitamin B_{12} is subacute combined degeneration of the spinal cord rather than anaemia. Determination of vitamin B_{12} in the serum is a simple way to see whether a patient believed to be suffering from subacute combined degeneration is deficient in vitamin B_{12}, or whether some other cause should be sought for the symptoms. The estimation has been carried out on sera from 27 patients in whom a diagnosis of subacute combined degeneration was suspected. In eight of these patients the clinical and laboratory findings were suggestive of vitamin-B_{12} deficiency, but there was usually some feature, such as a relatively normal blood picture or a normoblastic bone marrow, which raised doubts about the diagnosis. All these patients showed abnormally low levels, seven being below 100 $\mu\mu$g. per ml. and the other being 110 $\mu\mu$g. per ml. Thus these results confirmed the clinical diagnosis, and seven of the patients showed improvement after treatment with vitamin B_{12}. In four patients the response was very good, and it was good in another two. One patient showed only slight improvement, and one had not improved up to six months after starting treatment. Despite this lack of response the neurologist who was asked to assess the case felt that the evidence for deficiency of vitamin B_{12} was quite good.

The remaining 19 patients in whom deficiency of vitamin B_{12} was thought possible on clinical grounds included four on whom the final diagnosis was cervical spondylosis, three with disseminated sclerosis, three with unexplained paraesthesiae, and six with miscellaneous diagnoses such as pre-senile dementia, unexplained peripheral neuropathy, arteriosclerotic parkinsonism, and dizziness. All of these patients had normal concentrations of vitamin B_{12} in the serum, ranging from 160 to 780 $\mu\mu$g. per ml. Only two of the results (160 and 170 $\mu\mu$g. per ml.) were so low as to suggest the possibility of deficiency of vitamin B_{12}, but both of these figures were much higher than the highest figure found in patients with proved subacute combined degeneration. In most instances vitamin B_{12} was not given to the patients, but in those patients in whom it was tried there was little or no suggestion of a response.

Pernicious Anaemia of Pregnancy and the Puerperium.—Sera from 22 patients with this condition have been examined. The results ranged from 60 to 600 $\mu\mu$g. per ml., with a mean of 237 $\mu\mu$g. per ml. Only four of the values were below the normal range, but the mean for this group of patients is significantly lower than the normal mean ($n=143$, $t=5.65$, $p<<0.001$).

Partial Gastrectomy.—Badenoch, Evans, Richards, and Witts (1955) showed that megaloblastic anaemia can occur after partial gastrectomy, and that the patients may be unable to absorb vitamin B_{12} from the gut. It was therefore

of interest to measure vitamin B_{12} in the serum of patients who developed macrocytic anaemia after partial gastrectomy. Serum has been obtained from 14 such patients. The bone marrow was examined in most of these patients and showed megaloblastic degeneration. The results ranged from 18 to 480 $\mu\mu$g. per ml., with a mean of 164 $\mu\mu$g. per ml. Repeat observations were obtained on four of these patients at times between two weeks and six months after the date of the first serum. The later figures were all in accord with the first, in one instance confirming a deficiency of vitamin B_{12}, and in another suggesting an aggravation of the deficiency. The supplementary figures are not included in the quoted range or mean. Of the 19 observations on these 14 patients, 11 are below the normal range and 3 more are towards the lower limit of normal.

These results confirm the earlier observations (Badenoch *et al.*, 1955; Mollin and Ross, 1957) that deficiency of vitamin B_{12} may develop some years after partial gastrectomy. Nevertheless the mean value is higher than that in pernicious anaemia, and this would suggest that lack of vitamin B_{12} is not the only factor in the development of megaloblastic anaemia after partial gastrectomy. In an attempt to obtain further information on the frequency of this sequel to the operation, the level of vitamin B_{12} was estimated in the serum of 21 randomly selected patients who had had a partial gastrectomy five years or more previously. The results ranged from 99 to 750 $\mu\mu$g. per ml., with a mean of 270 $\mu\mu$g. per ml. Two of the patients gave results below the observed lower limit of the normal range, and the mean of all these values was significantly lower than the normal mean ($n=142$, $t=4.53$, $p<0.001$). However, the mean was significantly higher than the mean for the 14 patients who showed signs of faulty blood formation ($n=33$, $t=9.31$, $p<<0.001$).

Total Gastrectomy.—It has been possible to study only one patient with megaloblastic anaemia following total gastrectomy. He gave the very low value of 17 $\mu\mu$g. per ml. as the mean of the results of three separate assays.

Diverticulosis of Small Intestine and Blind Loop Syndrome.—Because of the occurrence of megaloblastic anaemia in some patients with diverticulosis of the small intestine (Badenoch, Bedford, and Evans, 1955), or with blind loops left after surgery (Cameron, Watson, and Witts, 1949), estimations of vitamin B_{12} have been made on the serum of three patients with diverticulosis and two with a blind loop. One patient with diverticulosis had a megaloblastic marrow, but the marrow was not examined in the other two cases; both patients with blind loops had megaloblastic marrows. All five of these patients had abnormally low levels of vitamin B_{12} in the serum.

Malabsorption Syndrome.—Some patients with steatorrhoea develop megaloblastic anaemia, so that it was of interest to determine the concentration of vitamin B_{12} in the serum of such patients. Serum was obtained from 11 patients in whom malabsorption was confirmed by fat balance or similar tests, and who showed changes in the bone marrow or peripheral blood suggestive of deficiencies of vitamin B_{12} or folic acid. The results ranged from 10 to 320 $\mu\mu$g. per ml., with a mean of 176 $\mu\mu$g. per ml. Four of the results were below the normal range, and two more were only just above the lower limit of normal, so that in a considerable proportion of these patients there was probably a deficiency of vitamin B_{12}.

Nutritional Megaloblastic Anaemia.—Estimations have been made on sera from two patients with megaloblastic anaemia due to dietary deficiencies. The first patient, who had taken a strict vegetarian ("vegan") diet for 20 years, had a level of 46 $\mu\mu$g. per ml. The second patient, whose diet had been deficient in meat and meat products, milk, and fish for at least six years, gave the slightly subnormal level of 120 $\mu\mu$g. per ml.

Megaloblastic Anaemia Due to Anticonvulsants.—Only two patients with this condition have been available for study. One gave the normal result of 260 $\mu\mu$g. per ml., and in the other the abnormally low level of 73 $\mu\mu$g. per ml. was obtained.

Leukaemia, Polycythaemia, and Related Conditions.—The results obtained in leukaemia, polycythaemia, and related conditions are summarized in the Table. No children were studied, which explains the absence of cases of acute lymphatic leukaemia. The serum was examined before any treatment was given. All the patients with chronic myeloid leukaemia gave abnormally high results, while patients with chronic lymphatic leukaemia gave normal values. In

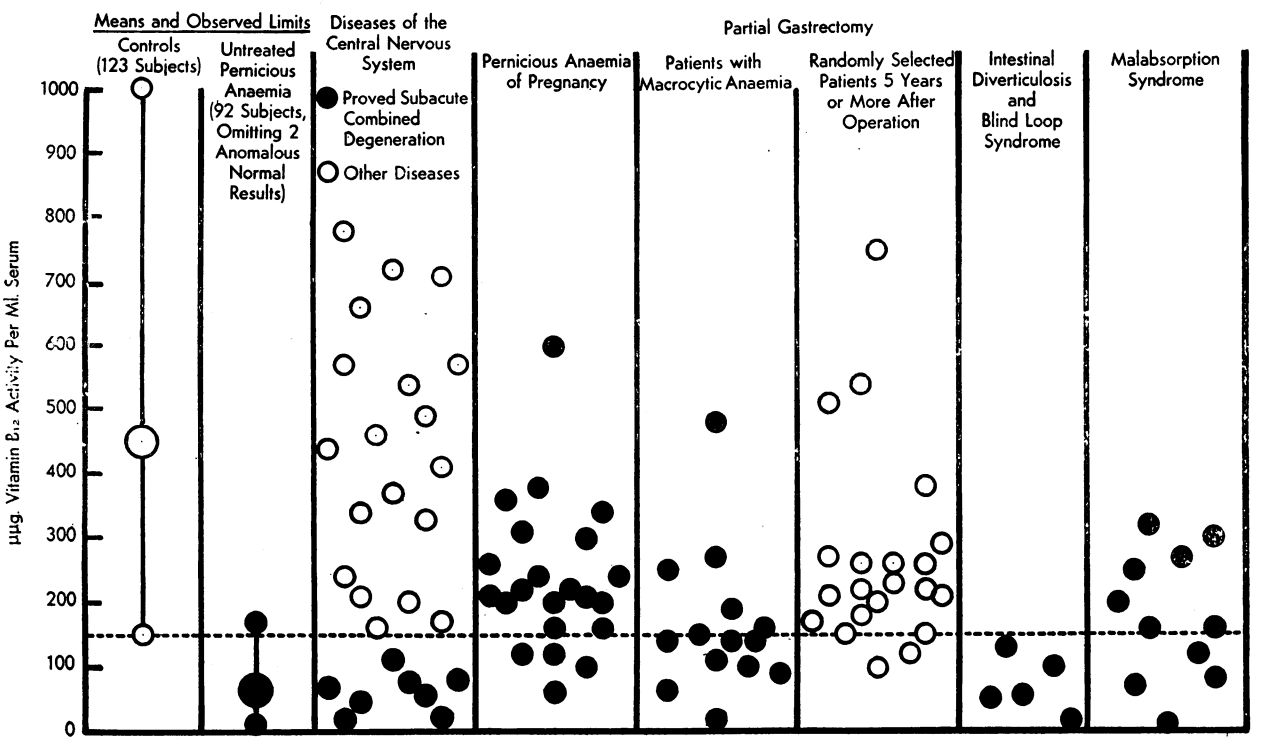

Chart showing concentration of vitamin B_{12} in the serum of control subjects and of patients with macrocytic anaemia and related conditions. The broken line shows the lower limit of normal range.

monocytic leukaemia, the acute leukaemias, and polycythaemia vera both normal and high values were obtained, but the highest figures found in polycythaemia were much lower than in the leukaemias. One patient with polycythaemia showed an abnormally low level, for which no explanation was found. Of two patients with erythroleukaemia, one gave a normal value and in the other the level was slightly raised. The only patient with myelosclerosis gave a slightly raised result, while two patients with multiple myeloma both showed levels a little below the lower limit of the normal range. In general these findings are in agreement with published results (Beard *et al.*, 1954; Erdmann-Oehlecker and Heinrich, 1956; Mollin and Ross, 1955).

Concentration of Vitamin B_{12} in Serum of Patients with Leukaemia, Polycythaemia, and Related Conditions

Condition	No. of Cases	μμg. Vitamin B_{12} Activity per ml. Serum		No. of Patients Showing Abnormally High Values
		Range	Mean	
Chronic myeloid leukaemia ..	5	1,600–6,400	4,580	5
,, lymphatic leukaemia ..	2	460– 510	485	Nil
Monocytic leukaemia	9	300–3,000	1,050	4
Acute myeloblastic leukaemia ..	5	400–5,800	1,800	2
,, undifferentiated leukaemia	3	540–6,600	3,880	2
Polycythaemia vera	10	100–2,200	831	2
Erythroleukaemia	2	510–1,800	1,160	1
Myelosclerosis	1	1,300	—	1
Multiple myeloma	2	110– 120	115	Nil

Haemochromatosis.—Assays have been carried out on serum from two patients with haemochromatosis. The results were 1,400 and 1,800 μμg. per ml. The reason for these abnormally high results in this condition is not clear. Perhaps the excessive deposits of haemosiderin in the liver and elsewhere immobilize the sites where vitamin B_{12} is normally stored, so that the vitamin cannot be taken up in the usual way. This could result in the presence of abnormally large amounts of the vitamin in the serum. Abnormally high levels have been found in patients with cirrhosis of the liver (Rachmilewitz *et al.*, 1956), and it was suggested that in these patients stored vitamin B_{12} might be released from damaged liver cells.

Discussion

With a method of the sort used in this investigation it is impossible to make a strict comparison of the results with those of other workers, because different methods yield rather widely different figures in different types of subject. Hence abnormal figures can be compared only with the normal range established by the same method, and comparison with the results of other methods must be limited to general trends.

The results make it possible to draw certain inferences about the usefulness of serum vitamin B_{12} estimations, both in diagnosis and for making decisions about treatment. The value of the test in untreated pernicious anaemia is well established, and the present results provide further evidence that, although occasional anomalous results are obtained, the test gives clear evidence of vitamin B_{12} deficiency in the vast majority of patients with this condition. In neurological disorders this comparatively simple test makes it possible in most instances to distinguish clearly between patients whose disease is due to deficiency of vitamin B_{12} and those in whom there is some other cause for the symptoms.

The test shows that the megaloblastic anaemias which occasionally develop after partial gastrectomy, or in patients with massive diverticulosis of the small intestine or with blind loops left after surgery, are usually associated with deficiency of vitamin B_{12}, though this may not be the only deficiency present. It is also apparent that in patients who have had a partial gastrectomy for five years or more the level of vitamin B_{12} in the serum tends to be lower than in health.

The results obtained in pernicious anaemia of pregnancy and the puerperium indicate that these patients often have a mild degree of vitamin B_{12} deficiency, which may explain why they respond to large doses of vitamin B_{12} (Moore *et al.*, 1955), but depression below the lower limit of the normal range is infrequent. Heinrich (1954) has shown that the level of vitamin B_{12} in the serum is low in 20% of pregnant women who are otherwise normal, so that there is probably no difference in the vitamin B_{12} status of pregnant women with megaloblastic anaemia and those with uncomplicated pregnancy.

Low levels of vitamin B_{12} were found in the serum of a considerable proportion of patients with the malabsorption syndrome, where folic acid is the treatment of choice. In such patients the test may be of value because some patients need vitamin B_{12} before they respond to folic acid (Nieweg *et al.*, 1952), and the finding of a low serum vitamin B_{12} would indicate that vitamin B_{12} as well as folic acid should be given. For instance, one of the patients included in this group had a serum vitamin B_{12} of 70 μμg. per ml., and was thought to have pernicious anaemia. He failed to show any response to vitamin B_{12}, but had a typical response as soon as folic acid was given. Fat-balance studies later showed that he had steatorrhoea.

The low results in the two patients with nutritional megaloblastic anaemia provide further evidence that patients may become depleted of vitamin B_{12} after taking a diet which is deficient in animal products for a long period. The result in one of the two patients with megaloblastic anaemia after anticonvulsant therapy indicates that this type of anaemia may sometimes be associated with deficiency of vitamin B_{12}. This is in keeping with the observation that a few of these patients respond to vitamin B_{12}, although the majority appear to require folic acid.

In leukaemia and allied conditions abnormally high levels of vitamin B_{12}, rather than low ones, are often found, and this observation may provide the basis for a useful aid to diagnosis. High figures seem to be typical of chronic myeloid leukaemia, whilst normal values are found in chronic lymphatic leukaemia. In other types of leukaemia and in polycythaemia vera the result may be either normal or high, but the test may still be of value. For instance, in a patient suspected to be suffering from polycythaemia vera a high result would confirm the diagnosis, but a normal one, although not excluding this diagnosis, would suggest the possibility that there may be another cause for the clinical findings.

Summary

The results obtained with the microbiological assay of vitamin B_{12} in the serum of various types of subject are presented.

The range of values found in 123 normal subjects was from 150 to 1,000 μμg. per ml., with a mean of 450 μμg. per ml. Of 94 patients with pernicious anaemia in relapse, 85 gave values of 100 μμg. per ml. or less, seven gave values between 100 and 170 μμg. per ml., and two anomalous normal results were obtained.

In subacute combined degeneration of the spinal cord the vitamin B_{12} in the serum is reduced to the same levels as in pernicious anaemia, and this has proved useful in differentiating the condition from other diseases of the nervous system.

There is a moderate reduction in the vitamin B_{12} in the serum in pernicious anaemia of pregnancy, but not usually to the same degree as in Addisonian pernicious anaemia.

A considerable proportion of patients who develop megaloblastic anaemia in association with partial or total gastrectomy, diverticulosis of the small intestine, blind loops left after surgery, the malabsorption syndrome, dietary deficiency and anticonvulsant therapy are shown to be deficient in vitamin B_{12}.

Abnormally high results were obtained in all the patients with chronic myeloid leukaemia, but in chronic

lymphatic leukaemia the results were normal. In the acute leukaemias and in polycythaemia vera both normal and high results were found.

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PORPHYRIA

A SOUTH AFRICAN SCREENING EXPERIMENT

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Garrod (1923) credits Gunther with the first recognition that porphyria was an "inborn error of metabolism." If the term "porphyria" is confined to inborn errors of porphyrin metabolism, it excludes purely acquired disturbances whether of a temporary or permanent nature. An increase of porphyrin production and excretion without a genetic basis could be termed "hyperporphyrinism."

It is essential to distinguish between the genetic and purely acquired disturbances of porphyrin metabolism, because an attack of "acute porphyria" with the possible death of the patient occurs only if the gene for porphyria has been inherited. Diagnosis of porphyria in the latent phase is most valuable because in this disorder acute attacks are usually precipitated by the inadvertent use of certain commonly prescribed drugs, particularly barbiturates.

The classification of the porphyrias has in the past presented many difficulties, and the classification was symptomatic. In 1937 Jan Waldenström made a brilliant clinical survey of 103 cases of acute porphyria in Sweden; among these cases light sensitivity did not occur. He also reviewed the published cases where light sensitivity had only occurred in adult life, and these cases he termed "porphyria cutanea tarda." A third very rare type of porphyria has been described. In this type light sensitivity is present from birth, the urine is dark red because of the high excretion of porphyrin, and anaemia and splenomegaly are present. This very uncommon type of porphyria has been called "congenital porphyria" (Gunther, 1922). Schmid, Schwartz, and Watson (1954) classified the porphyrias on the basis of the porphyrin content of the liver and bone marrow of 31 cases. In two cases of congenital porphyria they found porphyrins concentrated in the bone marrow, and this rare group they named "porphyria erythropoietica." In the remaining 29 cases porphyrins were mainly found in the liver and they were therefore classified as porphyria hepatica. This group included the type described by Waldenström, a mixed type in which photosensitivity and acute symptoms may occur in the same patient, and possibly a third group of purely cutaneous type unassociated with acute attacks.

Genetic Classification

A plea is now made that the term "porphyria" should be reserved for the genetic disease only, for inborn errors of porphyrin metabolism, and that the porphyrias should be classified according to their genetic basis, as this would appear to be a fundamental method of classification.

There are three clearly defined genetic types of porphyria; there may be more.

1. *The Congenital Type, or Porphyria Erythropoietica.*—This is exceedingly rare and should be easily diagnosed by the marked light sensitivity from birth, the dark-coloured urine, anaemia, splenomegaly, and pink staining of the teeth. It is thought to be inherited as a Mendelian recessive characteristic (Cockayne, 1933).

2. *The Swedish Type of Porphyria Hepatica.*—This is the type described by Jan Waldenström, who has now collected over 320 patients with this disorder (Waldenström, 1957). He has shown that it is inherited as a Mendelian dominant characteristic and many of his families trace back to an original forebear from Northern Sweden. This type corresponds to the acute intermittent group of the classification of Schmid *et al.* Most cases of acute porphyria in Europe and in the United States belong to this group.

It has the following characteristics: (1) It is inherited as a Mendelian dominant gene and is not sex-linked. (2) Acute attacks occur most commonly in females between the ages of 16 and 50. (3) Acute attacks are often precipitated by drugs, especially barbiturates. (4) Light sensitivity does not occur. (5) There is a high excretion of porphobilinogen G. and amino-laevulinic acid in the urine during the acute attack and for a long time afterwards. If the disorder is causing symptoms, the Erhlich reaction (Schmid *et al.*) is usually positive. (6) There is either no increase or very little increase in urinary and faecal porphyrin excretion except during an acute attack. (7) In a porphyric family it is difficult to detect all the latent cases. However, the majority of latent cases can be suspected if not proved by using quantitative methods of analysis for porphobilinogen and amino-laevulinic acid. If there has been an acute attack in the previous few years the increase in porphobilinogen is usually sufficient to give a positive Ehrlich reaction.

3. *The South African Type.*—A number of papers (Barnes, 1945, 1951; Murray, 1949; Barnes and Marshall, 1952; Dean, 1953, 1956, 1957; Dean and Barnes, 1955) have shown that porphyria is a common disorder among the European population of South Africa. It is genetically different from the Swedish, or Waldenström, type, and has the following characteristics: (1) It is inherited as a Mendelian dominant gene and is not sex-linked (Barnes, 1945). (2) Acute attacks occur most commonly in females between the ages of 16 and 50. (3) Acute attacks are in our experience always precipitated by drugs, especially barbiturates. The acute attack resembles the acute attack in the Swedish type and in the past was usually fatal. (4) Light sensitivity will usually be found in some of the affected male members of the family, but it may be very slight or absent in the females. (5) There is a high excretion of porpho-