Section of Experimental Medicine and Therapeutics

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RECENT WORK ON VITAMIN B_{12}

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Isolation and Chemistry of Vitamin B_{12}

In 1946, that is to say twenty years after the initial discovery by Minot and Murphy of the efficacy of liver in pernicious anæmia, my colleagues Emery and Parker (1946) announced the preparation of a concentrate fully effective in a single dose of 1 mg. This achievement seemed so remarkable at the time that the claim was received with some scepticism. Yet in another two years we had the preparation one hundred times more potent, viz. the crystalline anti-pernicious factor itself, now generally known as vitamin B_{12}.

The secrets of this greatly accelerated progress were, first: the use of new fractionation techniques, especially chromatography and, secondly, improved assay facilities. The U.S. team invented a new microbiological assay method, while we were fortunate in enlisting the assistance of Dr. C. C. Ungley with a large number of suitable clinical cases. The American team, directed by Dr. Karl Folkers of Merck & Co. Inc., have published some of the chemical and physical properties of vitamin B_{12} isolated both from liver and from *Streptomyces griseus* fermentation liquors (Rickes, Brink, Koniuszy, Wood and Folkers, 1948*a*, 1948*b*; Brink, Wolf, Kaczka, Rickes, Koniuszy, Wood and Folkers, 1949). They have, however, released no details of methods of isolation except in a recently published patent specification which indicates that, like ourselves, they found chromatography to be the key to the problem. The isolation of crystalline vitamin B_{12} in Glaxo Laboratories was announced only a few weeks later, and was later followed by information about our isolation techniques (Lester Smith, 1948; Lester Smith and Parker, 1948; Fantes, Page, Parker and Lester Smith, 1949). In our hands the relatively new technique of partition chromatography, invented by Martin and Synge (1941), proved invaluable. Our product was later shown to be identical with that isolated by the Merck workers; vitamin B_{12} has since been isolated in other industrial laboratories in Britain, America and Holland.

Beginning with a commercial liver extract we usually applied at least six chromatographic procedures in succession, interspersed with other steps of purification, such as salting out with ammonium sulphate, proteolysis, precipitation with phosphotungstic acid and partition between aqueous solutions and solvent mixtures. The chromatographic steps included adsorption on alumina, silica and charcoal, elution being effected usually with aqueous alcohol, as well as partition chromatography on moist silica using butanol as flowing solvent. Some of these steps had to be repeated. This complicated succession of steps was necessitated by the approximately millionfold concentration involved in passing from liver to pure vitamin B_{12}. Unfortunately, the substance had no chemical properties that could be usefully employed to separate it from impurities. We had therefore to rely almost entirely on physical methods. Naturally the losses *en route* were considerable and we considered ourselves fortunate to finish with 20 mg. from a ton of liver. We have been able to obtain larger amounts, though with not very much less difficulty, from *S. griseus* fermentation liquors.

Vitamin B_{12} occurs as dark red needles. The anhydrous substance has a molecular weight of about 1,300. It is remarkable in containing not only the usual elements, carbon, hydrogen, oxygen and nitrogen but also phosphorus and, most surprising of all, cobalt. It has a rather characteristic absorption spectrum, the main bands being one in the ultraviolet at 360 mμ and one in the visible at 550 mμ. A large number of its physical constants have been determined but they have not so far been of much value in helping to elucidate the structure of this somewhat complex molecule. Vitamin B_{12} appears to be stable in the solid state and also in solution at neutral or slightly acid pH values. It is not completely destroyed, for example, during several days at room temperature at extreme pH values of 2 or 12. Hydrolysis by boiling with strong acid, however, disrupts the molecule, liberating the following substances: phosphoric acid, ammonia, dimethylbenzimidazole, an unidentified compound giving a weak ninhydrin reaction and a red cobalt-containing acidic substance, which still comprises the greater part of the original molecule (Ellis, Petrow and Snook, 1949; Beaven, Holiday, Johnson, Ellis, Mamalis, Petrow and Sturgeon, 1949; Cooley, Ellis and Petrow, 1950). Little more is known except that on fusion with alkali it yields pyrrole-like substances. Its physiological mode of action is equally a mystery. We had hoped to determine at any rate its site of action by using vitamin B_{12} labelled with radio-active cobalt or phosphorus but so far our attempts to prepare the vitamin "tagged" in these ways have not been successful.

There are three or more forms of vitamin B_{12}, all active both microbiologically and clinically. My colleagues and I were the first to produce chromatographic evidence for a second and possibly a third form. One of these, called vitamin B_{12b}, was, however, first obtained crystalline by a team at the Lederle Laboratories (Pierce, Page, Stokstad and Jukes, 1949). In the meantime vitamin B_{12a} had been prepared by catalytic hydrogenation of vitamin B_{12} by the Merck workers. It now appears to be identical with B_{12b} (Kaczka, Wolf and Folkers, 1949; Brockman, Pierce, Stokstad, Broquist and Jukes, 1950). My colleagues and I at Glaxo Laboratories have lately isolated another crystalline factor, for which the name vitamin B_{12c} is now proposed (Buchanan, Johnson, Mills and Todd, 1950).[1] Its clinical activity will be mentioned in succeeding papers.

This, however, does not end the complications; evidence is accumulating for various forms of bound vitamin B_{12}, probably linked with peptides or proteins. Such bound forms appear to occur, for example, in liver, in fermentation liquors and possibly in milk. Ternberg and Eakin (1949) have shown that vitamin B_{12} appears to combine with a constituent of gastric juice, presumably Castle's intrinsic factor, to produce a non-dialysable microbiologically inactive complex. On autoclaving, however, free vitamin B_{12} is released again. Further, although it appears certain that vitamin B_{12} will do anything, clinically speaking, that a liver extract can do, it is perhaps premature to dismiss entirely the earlier "multiple factor theory" put forward by Jacobsen and others. In other words it is still possible that other factors may exert a "sparing action" on vitamin B_{12}. Certainly evidence is beginning to accumulate that folic acid as well as vitamin B_{12} may be needed for the control of some anæmias. It is now well established that vitamin B_{12} is needed by chicks, turkeys and pigs on diets low in animal protein. In this field again there is some evidence that one or more of some additional unidentified factors may be essential for optimal growth.

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[1] At the meeting vitamin B_{12c} was referred to as "the unnamed factor" or as "vitamin B_{12x}"—a temporary designation.

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Vitamin B_{12} and other Dietary Factors in Megaloblastic Anæmias and in Subacute Combined Degeneration of the Cord

Two groups of megaloblastic anæmia will be considered: pernicious anæmia, in which gastric atrophy leads to permanent loss of Castle's intrinsic factor and deficient absorption of vitamin B_{12}; and non-Addisonian megaloblastic anæmias associated with pregnancy and intestinal disorders where a different mechanism is at work.

The effects of parenteral administration of vitamin B_{12} in pernicious anæmia have already been described (Ungley, 1949*b*). Single doses of 10 μg. or more produced, on average, a maximal reticulocyte response. A better yardstick, however, is the increase of red blood cells in fifteen days. Ten μg. produced a response up to the "average" standard of Della Vida and Dyke (1942), but doubling the dose anywhere in the range from 5 to 80 or even 160 μg. produced a constant increase in response. It was thus possible to forecast the expected response to any dose within this range. The response in single cases might deviate from the expected response by 300,000 cells per c.mm. but for groups of 10 cases the error was not usually more than 100,000 cells.

About 80% of the total increase of red blood cells in 15 days occurred in the first ten days (Ungley, 1949*b*).

These findings form a useful basis for comparison, for example, in assessing the efficacy of vitamin B_{12} in other types of megaloblastic anæmia and for comparing vitamin B_{12} from liver with a similar red crystalline compound obtained from *Streptomyces griseus*.

Vitamin B_{12} in subacute combined degeneration of the cord.—Maintenance doses of 10 μg. a fortnight have prevented the development or exacerbation of neurological disorders in pernicious anæmia although larger doses are desirable for routine use.

More direct evidence of the efficacy of vitamin B_{12} against the neurological manifestations of pernicious anæmia has been obtained by the intensive treatment of 9 fully-established cases of subacute combined degeneration of the cord (Ungley, 1949*c*). Neurological findings were assessed on a quantitative basis and scored. During a control period anæmia was counteracted in some cases by transfusion without improvement in the neurological condition. Subsequently the weekly injection of 40 microgrammes of vitamin B_{12} led to a gradual reduction in the total score for neurological defect. Improvement continued for about six months leaving, as usual, a residuum of irreversible damage in the nervous system. The degree of improvement was related to the duration of the disease as measured by the duration of difficulty in walking. Comparison with 44 cases treated before the war showed that vitamin B_{12} was as effective as parenteral liver extracts, whether crude or refined.

Cobalt-containing red pigments related to vitamin B_{12}.—(1) *Free forms*: Vitamin B_{12a} is an artificial product obtained chemically by hydrogenation of vitamin B_{12}.

Vitamin B_{12b} is a constituent of liver extract and appears in the slow-moving red component of the chromatogram.

An allied substance, now named vitamin B_{12c}, has been isolated by Lester Smith from *Streptomyces griseus*.

All these factors are clinically active when administered by injection.

Comparisons between crystalline vitamin B_{12c} from *Streptomyces griseus* and vitamin B_{12} from liver have been made firstly on the basis of the increase of red blood cells in fifteen days following a single dose. 17 patients with pernicious anæmia in relapse have received single doses of 10, 20, 40 and 80 μg. On the average, the responses are equal to those which would be expected from similar doses of vitamin B_{12} obtained from liver.

Secondly, improvement has been observed in 3 patients with subacute combined degeneration of the cord, but it is too early to say whether there is any quantitative difference between the effect of this substance and that of crystalline vitamin B_{12} obtained from liver.

Thirdly, requirements for maintenance are being assessed.

Fourthly, comparisons are being made by the double reticulocyte response method.

(2) *Bound forms*: "Animal protein factor" is apparently the same as vitamin B_{12} either free or bound. Information about bound forms of vitamin B_{12} is scanty. They are probably unavailable to bacteria and inactive by injection. When given by mouth the linkage to protein is presumably split by gastric or pancreatic digestion.

Interrelationship between vitamin B_{12} and folic acid and other nutrients.—In bacteria needs for vitamin B_{12} can be partly met by thymidine and certain other deoxy-ribosides, or even under some conditions by ascorbic acid or methionine.

In a case of pernicious anæmia the injection of 48 mg. of thymidine produced a negligible response (Ungley, 1949*a*). Hausmann (1949) claims positive results with doses of 1 to 2 grammes. This is approaching the enormous doses required for thymine.

We know little about the interrelationship between folic acid and vitamin B_{12}, but three questions may be considered.

Firstly, do patients with pernicious anæmia ever need folic acid in addition to vitamin B_{12}?

Patients with pernicious anæmia with or without subacute combined degeneration of the cord can be successfully treated for indefinite periods with refined liver extracts or vitamin B_{12}. The following is an exceptional case in which vitamin B_{12} failed until needs for folic acid had been met. The patient was a man aged 55 years. Treatment for syphilis had been successfully completed some months before, otherwise the case was typical of pernicious anæmia with complete achlorhydria and megaloblastic marrow. His diet had been reasonably good. The first dose of 10 μg. of vitamin B_{12} produced a very poor response. Thereafter small amounts of folic acid, 15 mg. in all, led to a satisfactory reticulocyte response and increase in red blood cells. When the hæmatopoietic effects had worn off and counts were falling a similar dose of the same batch of vitamin B_{12} caused a satisfactory increase in red blood cells. This case recalls the pigs described by Heinle, Welch and Pritchard (1948) which were so completely depleted of hæmatopoietic factors that folic acid failed unless liver extract was supplied; and liver extract failed unless small amounts of folic acid were supplied.

I should like to stress here once again, that this case is exceptional and that ordinary patients with pernicious anæmia and subacute combined degeneration can be treated for years without requiring the administration of anything more than refined liver extract or vitamin B_{12}. Does this mean that vitamin B_{12} aids in the synthesis of folic acid in man as it seems to do in chicks (Dietrich, Nichol, Monson and Elvehjem, 1949), or that there was no deficiency of folic acid in the first place?

Patients with pernicious anæmia are said to have difficulty in deriving free folic acid from conjugated forms in natural foodstuffs, many of which contain conjugase inhibitors. Even some of the responses to pure folic acid conjugates (pteroyltri- and di-glutamic acids (Wilkinson and Israëls, 1949)) were not very good considering the large doses administered.

Nevertheless patients with pernicious anæmia seem able to excrete in the urine as large a percentage of a loading dose of 20 mg. of folic acid as do normal persons. This does not suggest any deficiency of folic acid but tests must be repeated using smaller—"more physiological"—loading doses.

The second question is, why do some patients with pernicious anæmia treated with folic acid alone respond first but later become progressively worse even if the dose is increased? Why should folic acid work at all if there is no deficiency of this substance? Is it that an excess of folic acid improves the function of traces of vitamin B_{12} still remaining in the tissues? On this hypothesis the increased tendency to involvement of the nervous system would be explained by an accelerated utilization and ultimate exhaustion of these traces of vitamin B_{12}.

Even in megaloblastic anæmia associated with the sprue syndrome prolonged administration of folic acid alone may lead to neurological disorders. This occurred in 2 patients described by Davidson and Girdwood (1948), and in one of my own who developed a psychosis which responded promptly to a source of vitamin B_{12}.

The third question is, can folic acid potentiate the action of small doses of vitamin B_{12}? In a patient with pernicious anæmia the daily injection of 0·5 μg. of vitamin B_{12} led to a reticulocytosis. Thereafter the same daily dose of vitamin B_{12} supplemented by 100 μg. of folic acid caused a second reticulocyte response. This, however, may have been merely a summation of effects and not a catalytic effect. In another patient the dose of vitamin B_{12} in the second period was reduced to 0·4 μg. and there was no secondary reticulocytosis. Much more work is necessary before conclusions can be drawn.

In chickens, feeding folic acid increased the stores of vitamin B_{12} in the liver and giving vitamin B_{12} increased the stores of folic acid (Dietrich, Nichol, Monson and Elvehjem, 1949). We do not yet know whether the same is true in man.

Absorption of vitamin B_{12} from the alimentary tract.—When vitamin B_{12c} from *Streptomyces griseus* and later vitamin B_{12} itself were given by mouth in the enormous dosage of 80 μg. a day the responses were poor. After a total of 1,920 μg. in twenty-four days the increase of red blood cells was less than half the expected increase in fifteen days from a single injection of 5 μg: it was in fact rather less than the mean increase in fifteen days observed in 6 patients who received a single injection of 2·5 μg. (Ungley, 1949*b*, Table V). The subsequent daily administration of only 1 μg. by injection was followed by a satisfactory rise of red blood cells (equivalent to the expected rise from a single dose of 10 μg.). In this case the amount of orally administered material necessary to produce a given response was several hundred times that which would have been required by injection.

The daily administration of 5 μg. of vitamin B_{12} by mouth was ineffective whereas the same quantity given daily with 50 ml. of normal unfiltered gastric juice produced a satisfactory response. A total of 75 μg. in fifteen days produced an increase of erythrocytes equivalent to the response expected from a single dose of 10 μg. by injection.

In another case similar amounts of material for ten days produced a response equivalent to that expected from 20 μg. by injection.

Incidentally filtration of the gastric juice through a Seitz filter led to loss of most of the intrinsic factor activity.

In one case the administration of 40 μg. of vitamin B_{12} given with only 150 ml. of gastric juice as a single dose was inadequate.

In another patient a single dose of 50 μg. and 500 ml. of gastric juice given by stomach tube produced a good response. The increase in red blood cells in fifteen days more than equalled the expected response from a single dose of 40 μg. by injection.

Further work is necessary but in the 3 cases mentioned the amounts of vitamin B_{12} which had to be given orally with gastric juice were approximately 1·25, 2·5 and 8 times as great as would have had to be given by injection to produce the same effect (*see* Ungley, 1949*b*). Possibly the results might be more consistent with larger amounts of gastric juice.

Does intrinsic factor directly facilitate the absorption of vitamin B_{12} or merely prevent its destruction in the gastro-intestinal tract?

The daily application of 5 μg. of vitamin B_{12} to the mucous membrane of the floor of the mouth produced no hæmatopoietic response, whereas the same quantity given by mouth with 50 ml. of gastric juice produced a good response, equivalent to the response expected from a single injection of 20 μg.

We next tried to determine whether vitamin B_{12} would be absorbed from the intestine without gastric juice if we prevented contact with intestinal contents which might destroy it or render it unavailable.

A segment of small intestine was isolated between two balloons on a Miller-Abbott tube. This segment was washed clear of intestinal contents and 40 μg. of vitamin B_{12} was instilled. A small sample withdrawn after one hour still showed a high vitamin B_{12} content. There was no hæmatopoietic response. Later the same amount of vitamin B_{12} given orally with 150 ml. of normal gastric juice produced a reticulocyte response. The response was submaximal probably because the volume of gastric juice was too small. An increase of erythrocytes almost equal to the predicted response followed the injection of 40 μg.

Contents aspirated from various levels of the small intestine have been assayed microbiologically by Dr. W. F. J. Cuthbertson. The subjects were 2 untreated cases of pernicious anæmia. Some samples contained thymidine or minute amounts of vitamin B_{12}. No greater amounts were released after heat or digestion with papain. The intestinal contents did not contain anything which inhibited the growth of the lactobacilli.

The remarkably high excretion of vitamin B_{12} in the stools of patients with pernicious anæmia reported by Callender, Mallet, Spray and Shaw (1949) is not necessarily due to deficient absorption—it might equally well be due to biosynthesis of vitamin B_{12} in the colon.

Is there any interaction of vitamin B_{12} and intrinsic factor?

In microbiological assays carried out last year Cuthbertson and I were surprised to find that what little vitamin B_{12} activity there was in a beef digest disappeared after incubation with normal gastric juice, whereas pernicious anæmia gastric juice had little or no effect.

Recent work by Ternberg and Eakin (1949) seems to show that something in the gastric juice (probably Castle's intrinsic factor) combines with vitamin B_{12} *in vitro* and renders it unavailable to bacteria. The combination seems to be quite loose. Heating the compound breaks the linkage and leaves the vitamin B_{12} once again available to bacteria.

We still do not know whether this combination with a gastric factor facilitates absorption of the vitamin or merely protects it from destruction in the gastro-intestinal tract.

Vitamin B_{12} in megaloblastic anæmias of pregnancy.—In 6 cases of megaloblastic anæmia associated with pregnancy or the puerperium, the injection of vitamin B_{12} in doses of 65 to 80 μg. was completely ineffective except for a slight reticulocytosis in one case. The patients subsequently responded to folic acid, often in small doses—2·5 μg. daily (Ungley and Thompson, 1950).

Vitamin B_{12} in megaloblastic anæmias associated with intestinal disorders.—Here the results are variable.

In a case associated with intestinal stenosis the injection of 80 μg. produced quite a good response. But even allowing for an initial fall in the first three days the increase of red blood cells by the fifteenth day was equivalent only to the response expected from 20 μg. in Addisonian pernicious anæmia.

In a patient with idiopathic steatorrhœa the response to 80 μg. of vitamin B_{12} was about equal to the response expected from 5 μg. in Addisonian pernicious anæmia.

A patient with steatorrhœa associated with thyrotoxicosis failed to respond to vitamin B_{12} whereas folic acid was effective.

Two further cases associated with steatorrhœa are now under treatment. One showed no response to vitamin B_{12} but a good response to folic acid. The other is responding to vitamin B_{12}.

Toxic and hæmolytic aspects.—Not all the facts can be explained on a simple nutritional basis. Both in true pernicious anæmia and in non-Addisonian megaloblastic anæmias toxic and hæmolytic factors may play a part. Since methæmalbumin may be present in the plasma some of the hæmolysis must be intravascular. Destruction of poorly formed red cells is not sufficient explanation. In collaboration with Dr. W. Walker we have followed the survival of transfused cells from normal donors. In most patients with pernicious anæmia such cells are eliminated at a normal rate, surviving about 120 days. In three cases, however, the transfused cells were rapidly destroyed. A change to a normal rate of elimination occurred after vitamin B_{12} in 2 cases and spontaneously in 1 case. In three patients with non-Addisonian megaloblastic anæmia associated with pregnancy or intestinal disorders excessive hæmolysis changed to a normal rate of elimination about two weeks after giving folic acid. The dramatic change in the rate of destruction after treatment suggests that vitamin B_{12} and folic acid may be concerned in detoxicating or preventing the production of a hæmolytic agent.

My colleague, Dr. R. B. Thompson (1950), confirms the finding of Rusznyák, Löwinger and Lajtha (1947) that the maturation of megaloblasts in marrow culture is accelerated by the addition of normal plasma but inhibited by "pernicious anæmia" plasma. The greater the concentration of "pernicious anæmia" plasma the less the megaloblasts mature. This suggests active inhibition rather than mere absence of a maturation factor. Low concentrations of folic acid (1 μg. / ml.) added to an inert medium caused rapid maturation of megaloblasts, but "pernicious anæmia" plasma antagonizes this effect. The maturing effect of small amounts of normal plasma is also antagonized by the addition of "pernicious anæmia" plasma. Larger amounts of folic acid or of normal plasma overcome this antagonism. Cerebrospinal fluid from patients with pernicious anæmia has an effect similar to their plasma so that the inhibiting factor is probably ultrafiltrable.

The action of vitamin B_{12} on maturation of megaloblasts *in vivo* is presumably indirect, for unlike folic acid it fails to accelerate maturation *in vitro*.

Other relevant facts follow:

Early lesions in the spinal cord in pernicious anæmia are spotty in distribution and often related to vessels. They suggest the action of a substance destructive to myelin rather than a simple nutritional deficiency.

The urinary excretion of certain phenolic compounds is excessive in relapse and becomes normal after treatment with vitamin B_{12} (Abbott and James, 1950).

Liver slices from rats deficient in folic acid failed to metabolize tyrosine completely until folic acid was added (Rodney, Swendseid and Swanson, 1949). Intermediary products of tyrosine metabolism include phenolic substances.

Another potentially toxic substance is indol, a product of the metabolism of tryptophane. Indol fed to pigs on a diet deficient in vitamin B complex produces hæmolysis and macrocytic anæmia, a result not observed in normal pigs (Rhoads, Barker and Miller, 1938).

For the production of macrocytic anæmia following intestinal stenosis, loops or blind sacs, stagnation and bacterial infection of intestinal contents seem to be essential. In the rats of Watson, Cameron and Witts (1948) many weeks elapsed before the animals became suddenly ill and anæmic. My tentative interpretation is that a toxic and hæmolytic factor was produced in the infected contents of the blind sac. During the latent period detoxication occurred through enzymes using folic acid and possibly vitamin B_{12}, stores of which were gradually depleted in the process. When these stores were exhausted detoxication failed, resulting in sudden illness and anæmia. Folic acid restored the power of detoxication and relieved the anæmia.

Something of the same kind may occur in pernicious anæmia where bacteria flourish in the small intestine in a medium rendered abnormal by lack of gastric acid and enzymes.

A tentative hypothesis based on these findings, some of which require confirmation, is that in megaloblastic anæmias toxic as well as nutritional factors play a part. These are responsible for megaloblastic erythropoiesis, for some of the hæmolysis and possibly for the lesions in the spinal cord. Potentially toxic material, for example indol or a phenolic compound, arises either from bacterial action on protein metabolites in the small intestine or from a defect in intermediary metabolism of some substance such as tyrosine or tryptophane. Detoxication or a return to normal metabolism in which production of toxic material ceases occurs through the action of enzymes using folic acid and vitamin B_{12}.

Yeast.—Wills' factor: Is there a hæmatopoietic factor other than vitamin B_{12} or folic acid present in whole liver and in yeast? Why should yeast extracts which appear to contain no vitamin B_{12} when tested microbiologically or in animals be effective as a source of extrinsic factor? In pernicious anæmia yeast extracts such as marmite have to be given in large doses (e.g. marmite 120 grammes) to produce even a moderate effect. When given with a source of intrinsic factor even small doses (e.g. marmite 12 to 24 grammes) are effective.

In megaloblastic anæmias associated with pregnancy and sprue, yeast extracts are sometimes effective by mouth in relatively small doses. Yet a patient with non-tropical sprue failed to respond to doses by injection, one-tenth of those which were successful when given by mouth. If vitamin B_{12} had been the effective agent injections should have been effective in doses 60 to 100 times less than the oral dose.

Can the effect be due to folic acid or folic acid conjugates?

In a patient with megaloblastic anæmia of pregnancy the daily dose of yeast extract which produced a good reticulocyte response contained less than 40 μg. of folic acid tested both microbiologically and in animals for conjugates. Moreover, the daily excretion of folic acid in the urine during the period of administration of the yeast was extremely low, only 1 to 5 μg. per day. During the next period 2·5 mg. folic acid daily produced no secondary reticulocyte response such as one might have expected if the initial response to yeast had been due to traces of folic acid. The mean excretion of folic acid now rose to 700 μg. per day. It is true that conjugase inhibitors in yeast make it difficult to assay folic acid conjugates microbiologically, but this difficulty does not apply to rat assays which were used as a check in this instance. This leads me to think that there may be a Wills' factor after all, despite current tendencies to explain the hæmatopoietic effect of yeast in terms of folic acid conjugates.

In conclusion I thank my colleagues, notably Dr. R. B. Thompson who has been responsible for marrow cultures, Dr. W. Walker who followed the survival of transfused erythrocytes and Dr. L. W. Carstairs for intubation of the small intestine. I am grateful to the medical, nursing and lay staff of the hospital and medical school and to many general practitioners for their co-operation. Dr. E. Lester Smith supplied the whole of the vitamin B_{12} used in this investigation and Dr. W. F. J. Cuthbertson was responsible for microbiological assays. I wish to thank both these members of the Research and Development Division of Glaxo Laboratories for their co-operation and Glaxo Laboratories for a research grant made to King's College, University of Durham, which provided for the Research Fellowship now held by Dr. R. B. Thompson.

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Observations on the Relationship between the Red Cell and Reticulocyte Responses and Changes in the Bone-marrow of Patients Suffering from Pernicious Anæmia Treated with Injections of Liver Extracts or Vitamin B_{12}.

This work has been carried out on a series of patients suffering from uncomplicated Addisonian pernicious anæmia. We have been particularly interested in the changes which take place in the red cell and reticulocyte counts of patients treated with single injections of liver extract or with vitamin B_{12} and the correlation of these changes with alterations in the cellularity and morphology of the bone-marrow which take place at the same time.

Several well-known brands of liver extract have been employed, both of British and American origin, all of known B_{12} content as judged by microbiological assay[1]. We have also had available to us, by courtesy of Glaxo Laboratories, a B_{12} concentrate prepared from *Streptomyces griseus* cultures and, in addition, preparations of B_{12c} and of crystalline B_{12} derived from liver.

Although about 30 patients have been studied, the observations that are now recorded are based on an intensive study of a relatively small group of typical cases. It is thought, nevertheless, that these observations have a general application.

Methods.—Venous blood has been used throughout. We have taken great pains with the red cell counts and have counted a minimum of 2,000 cells in each count; under these circumstances our results are believed to be accurate within ± 5%, a range embracing ± twice the coefficient of variation. Translated into figures, this means ± 100,000 cells per c.mm. in a two million count or ± 50,000 cells in a count of 1 million per c.mm.

The *reticulocytes* have been counted in dry, uncounterstained films made from the deposit, suspended in plasma, obtained after staining for 15 min. at 37° C. and then centrifuging a weak suspension of red cells in 0·4% cresyl blue in saline. As with all counts concerned with a varying proportion of abnormal cells, it is difficult to generalize as to the errors involved in these reticulocyte counts; with 10% of reticulocytes, the error is thought to have been about ± 20% of this percentage; with higher proportions of reticulocytes, the error is likely to have been less, and with lower proportions correspondingly greater.

The *bone-marrow* has been studied in smears made from material aspirated by sternal puncture; and in most patients a series of observations has been made.

Results.—The first point to be considered is concerned with the length of time a bone-marrow will remain normoblastic in type following a single injection of liver extract or vitamin B_{12}, given to a case of pernicious anæmia. With doses of liver extracts or of B_{12} of sufficient potency to produce a so-called "average satisfactory" rise in the red cells according to the criteria of Della Vida and Dyke (1942), the marrow is likely to have reverted to a partial megaloblastic state several days before the end of the fifteen-day period. Usually quite well-marked megaloblastic change is present by the eleventh day, and there may be early indications by the eighth day. This tendency to reversion after single injections are given is reflected in a falling off in the response in the peripheral blood. With smaller dosages the marrow may never become normoblastic at all, although "intermediate" types of megaloblasts may appear (*see* Dacie and White, 1949). In these cases, nevertheless, there may be considerable (but usually unsatisfactory) rises in the red cell counts. With very small dosages, the megaloblastic marrow may hardly alter at all; in these circumstances the peripheral response may be a mere flicker, sufficient perhaps only to halt a previous downward trend for a few days. With large single doses of the order of 30–40 μg. of crystalline B_{12}, 40–50 μg. of B_{12} concentrate or of a liver extract assayed at 30–40 μg., the marrow will probably remain normoblastic for the whole of the fifteen-day period or even longer; and in these patients the red cell responses in the peripheral blood will usually be greater than the expected response as calculated from the Della Vida and Dyke formula. These points are illustrated in Fig. 1.

In cases responding maximally with a normoblastic marrow throughout, the red cell response curve for the first fifteen days seems to follow a definite biphasic form; first there is a sharp rise starting on the second or third day and lasting three to four days, then there is a pause for two to three days and finally a second sharp rise starting on about the ninth day which gradually tails off (Fig. 2).

Marrow studies indicate that the first sharp rise is the sequel to a tremendous development of ripe (pyknotic) normoblasts. These cells all ripen within a short period of time and are clearly derived from the large accumulation of frustrated primitive cells of approximately the same age typically present in the severe case before treatment. By the sixth to seventh day, however, the marrow is relatively hypocellular and "empty". Ripe normoblasts are much less conspicuous, and the rise in the peripheral count is in consequence slowed down or almost halted. This deficiency of ripe cells in the marrow does not, however, last long, and the formation of a second generation of normoblasts results in a further rapid outpouring of cells and the second phase of increase in the peripheral blood.

When doses of liver or B_{12} are given sufficient to satisfy the requirements of an "average satisfactory" response only, it is likely that the first rise will be the only sharp one that is observed, and that the pause from the sixth to ninth day will merge into a less distinct and much less steep secondary rise. The response curve under these circumstances is typically sigmoid in shape (Fig. 3).

[1]The microbiological assay of these extracts has been kindly carried out by Dr. W. F. J. Cuthbertson of Glaxo Laboratories using *L. lactis Dorner* as the test organism, and in certain cases by Lederle Laboratories using *Lactobacillus leichmannii* 313.

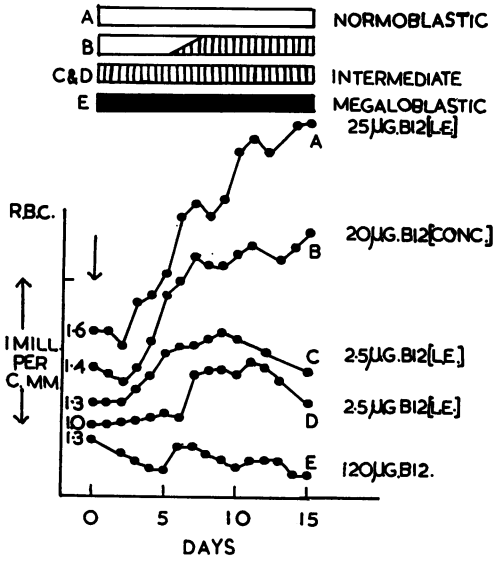

FIG. 1.

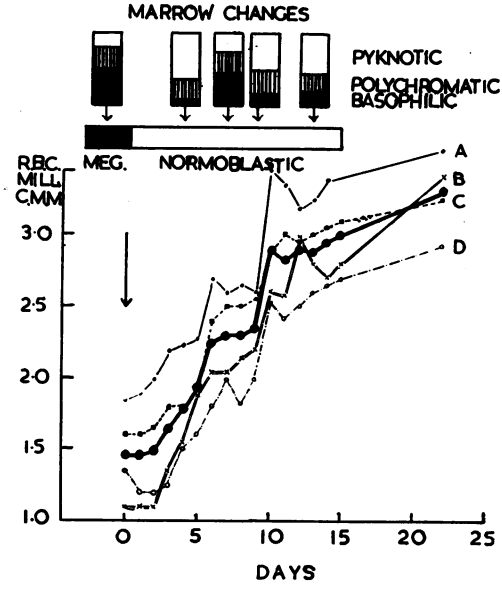

FIG. 2.

FIG. 1.—The bone-marrow changes and the initial red cell responses in 4 patients with pernicious anæmia (A–D), and in 1 patient with refractory megaloblastic anæmia (E), who were treated with single injections of crystalline vitamin B_{12}, vitamin B_{12} concentrate or with liver extracts of known vitamin B_{12} content. The bone-marrow changes are indicated in the rectangles at the top of the figure. The black shading indicates a megaloblastic bone-marrow, the "hatched" shading indicates an intermediate marrow, and the absence of shading a normoblastic marrow.

FIG. 2.—The initial red cell responses of 4 patients with pernicious anæmia in whom erythropoiesis remained normal for at least fifteen days after the start of treatment. The proportions of basophilic, polychromatic and pyknotic erythroblasts, as revealed by serial marrow punctures, are also given. The basophilic component includes hæmocytoblasts, pronormoblasts (or promegaloblasts) and early basophilic normoblasts. *Patients A and C* received single injections of liver extract; the vitamin B_{12} content of the doses given was assayed as 125 μg. and 30 μg. respectively. *Patient B* received 20 mg. folic acid daily. *Patient D* received a single injection of 30 μg. crystalline vitamin B_{12}. The dark continuous line is the average of the four responses.

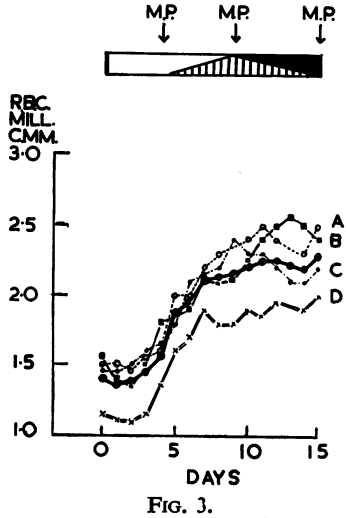

FIG. 3.

FIG. 3.—The initial red cell responses of 4 patients with pernicious anæmia in whom normoblastic erythropoiesis was not maintained for the whole of the fifteen-day period. *Patient A* received a single injection of 20 μg. vitamin B_{12} c. *Patients B and D* received single injections of 20 μg. vitamin B_{12} concentrate. *Patient C* received a single injection of liver extract; the vitamin B_{12} content was assayed as 2·5 μg. The dark continuous line is the average of the four responses.

The marrow changes are indicated above. M.P. denotes a marrow puncture. The black shading indicates a megaloblastic marrow, the "hatched" shading an "intermediate" marrow, and the absence of shading a normoblastic marrow.

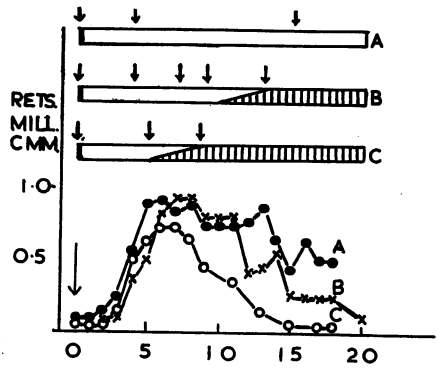

FIG. 4.

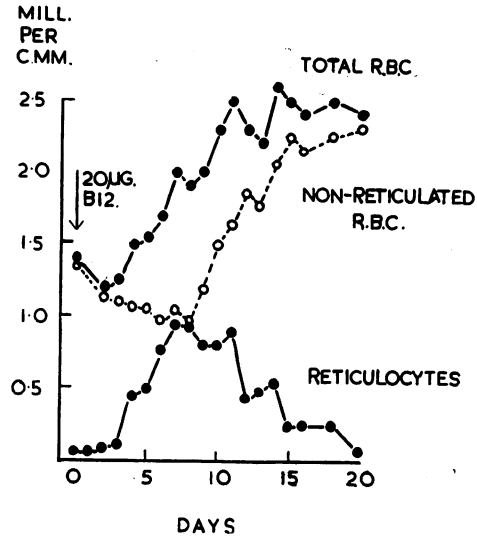

FIG. 5.

FIG. 4.—The form, height and duration of the reticulocyte responses in relation to the bone-marrow changes in 3 patients (A, B, C) with pernicious anæmia treated with folic acid, crystalline vitamin B_{12} or vitamin B_{12} concentrate respectively. The reticulocyte counts are expressed in absolute numbers. *Patient A* was treated with 20 mg. folic acid daily. Erythropoiesis remained normoblastic throughout the period of observation. *Patient B* received a single injection of 20 µg. crystalline vitamin B_{12}. Reversion to megaloblastic erythropoiesis was present by the thirteenth day. *Patient C* was treated with 20 µg. vitamin B_{12} concentrate. Reversion to megaloblastic erythropoiesis was present by the eighth to ninth day. The arrows indicate the dates of sternal punctures. The "hatched" shading denotes an "intermediate" marrow, and the absence of shading a normoblastic marrow.

FIG. 5.—The reticulocyte response, initial red cell response and non-reticulated red cell count in a patient with pernicious anæmia treated with crystalline vitamin B_{12}.

The reticulocyte count and the red cell count paralleled one another until the seventh day. The counts diverged after this date as the reticulocytes became transformed into mature red cells. The non-reticulated count started to rise after the seventh day.

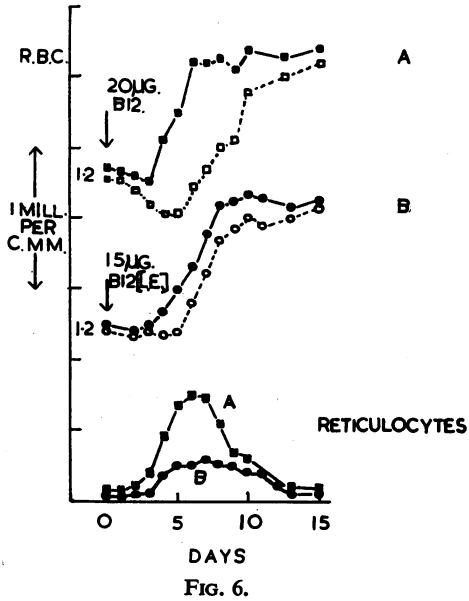

FIG. 6.

FIG. 6.—The reticulocyte responses, initial red cell responses and non-reticulated red cell counts of two patients (A, B) with pernicious anæmia, treated with single injections of vitamin B_{12} and liver extract respectively. Continuous lines: Total red cell counts. Interrupted lines: Non-reticulated red cell counts.

The sharper response of patient A was associated with a higher reticulocyte curve and a more prolonged reticulocyte life, compared with the changes observed in the case of patient B.

The reticulocyte response to liver therapy has been extensively studied for many years. Riddle's paper (1930) is particularly noteworthy. Working with large single doses of liver extract, he studied the absolute numbers of reticulocytes in the circulation and came to the conclusion that all the new cells appearing in a patient's blood during the initial response to treatment with liver were, in fact, reticulocytes. If this view is correct, and we believe that it is, almost as much information should be obtainable by studying the reticulocyte response in the first few days of therapy as by studying the rate of rise of the red cells themselves.

Nevertheless, most workers using reticulocyte percentages as criteria have come to the conclusion that there is no reliable correlation between the percentage reticulocyte response and the rise in red cells. One reason for this is clearly the assessment of reticulocyte responses as *percentages*. This method of assessment is convenient and will certainly demonstrate small differences, but it has its drawbacks. For instance, a reticulocyte count of 50% with the total red cell count at 1 million per c.mm. and a 25% count at the 2 million level represent the same *total* number of reticulocytes; in both cases, 500,000 per c.mm. In the above example there is a temptation to believe that the 25% response indicates a less intense marrow effort than does the 50% response, which is simply not the case. Another objection to plotting the reticulocyte response as a percentage is that the scale of references is altering all the time; at the end of a fifteen-day period the red cell count may have been almost doubled, so that 5% of reticulocytes at the end of the period corresponds with 10% in the early days of the response.

A number of factors probably contribute to the form and duration of the reticulocyte response. Firstly, the primitiveness of the marrow—in the most severe cases of pernicious anæmia many basophilic primitive promegaloblasts are present and the marrow as a whole is usually tremendously hyperplastic. When a marrow of this type is permitted to develop by the administration of a sufficient quantity of B_{12}, there is a tremendous surge of activity and many normoblasts mature within a day or so, and a tremendous outpouring of reticulocytes results. The first factor which controls the reticulocyte response is thus the marrow—how hyperplastic it is, and how homogeneous. The next factor is, of course, the potency of the material administered in relation to the patient's requirements. This will determine the response of the marrow which in turn will be reflected in the number of reticulocytes that are formed and the speed with which they appear in the peripheral circulation.

Then, of fundamental importance in controlling the form of the reticulocyte response curve, particularly its height and width, is the speed with which the reticulocytes ripen in the peripheral circulation. This depends, at least in part, on their maturity as they are delivered. It seems that the more vigorous the response the more immature are the reticulocytes which appear in the blood stream and in consequence the more prolonged is their average life-span. Ripening may also depend on humoral ripening factors, in which category perhaps B_{12} and liver extracts themselves may be included. This is, however, rather a matter for speculation at the moment.

The typical reticulocyte response curve is asymmetrical, the initial rise being steeper than the subsequent fall. The better and more sustained the marrow response (the more potent the preparation given) the more asymmetrical are the curves. A rise followed by a relatively quick fall to relatively low levels means that the marrow is soon ceasing to form many new cells, and the reticulocyte count will fall as soon as those cells initially delivered ripen (Fig. 4).

It is instructive to calculate the non-reticulated red cell count by subtracting from the total count the reticulocyte count in absolute numbers, and to plot the totals separately. If this is done, it will be seen, firstly, that the total red cell count and the reticulocyte count rise in a strikingly parallel fashion, strongly suggesting that the total cell rise, for at least the first seven days, is wholly due to increments of reticulocytes. Secondly, it becomes clear that the non-reticulated count does not rise until several days after the start of the reticulocyte rise (Fig. 5).

The ripening time of reticulocytes as illustrated in Fig. 5 seems to be as long as five days. In most cases it appears to be three to four days. It may, however, appear to be even shorter than this. In these cases the total reticulocyte count may be disappointingly low, although sustained, and yet the total red cell rise for the whole fifteen-day period may be quite good (Fig. 6). These differences can be partially explained on the basis of different degrees of immaturity of the cells as they are delivered from the marrow, depending upon the intensity of the response. It would be premature to speculate as to whether there was any difference in the behaviour of patients treated with liver extracts and those treated with B_{12} preparations in this respect.

Conclusions

(1) It is necessary to give large doses of vitamin B_{12} or liver extract to patients with pernicious anæmia, if it is desired to maintain a normoblastic marrow for fifteen days following a single injection. Doses of the order of 30–40 μg. of crystalline B_{12} or 40–50 μg. of B_{12} concentrate are required, or an injection of a liver extract assayed to contain 30–40 μg. B_{12}. In patients thus treated the red cell responses tend to occur in two phases, separated by a relative pause at about the sixth to ninth day.

(2) So-called "average satisfactory" responses can be obtained in the majority of patients with half the quantities of B_{12} or less than are mentioned above. In these cases, however, the marrow will have become megaloblastic again well before the end of the fifteen-day period, and the second phase of red cell response will be diminished. The potency of an extract can in fact be quite reliably assessed clinically by studying the changes it produces in the bone-marrow.

(3) It seems that all the red cells produced as a response to the administration of an adequate dose of liver extract or B_{12} probably enter the blood stream as reticulocytes, for the rises in reticulocyte numbers and red cell numbers closely parallel each other in the early stages of the response.

(4) The form of the reticulocyte response curve is determined by several factors; the hyperplasia in the marrow, the potency of the material administered in relation to the patient's requirements, the degree of immaturity of the reticulocytes as delivered into the blood stream and the rate at which they ripen therein. It is this rather complicated relationship which limits the value of the reticulocyte response as a means of assessing clinically the potency of liver extracts and vitamin B_{12}.

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