asthma began after the age of 15 there were 82% who were clear and not disabled from asthma.

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ORAL TREATMENT OF PERNICIOUS ANAEMIA: FURTHER STUDIES

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During the past two and a half years the results have been published of 11 cases of pernicious anaemia treated with the oral vitamin-B₁₂-peptide complex, H.P.P./1, which was derived from a fermentation of a streptomyces mutant (Heathcote and Mooney, 1958a, 1958b; Mooney and Heathcote, 1960a). As a result of these findings we formulated a new hypothesis concerning the aetiology of pernicious anaemia—namely, that the disease is due to failure of gastric proteolysis and that intrinsic factor, as currently understood, does not exist. Table I summarizes the present state of 10 of these same patients. Case 39 (Mooney and Heathcote, 1960a) has been withdrawn from the series because he received, inadvertently, an injection of crystalline vitamin B₁₂. Nine of the remaining cases are still maintained on a daily dose of 10 µg. of vitamin B₁₂ (78 µg. H.P.P./1) or less. Case 30, however (Mooney and Heathcote, 1960a), was barely maintained on a daily dose of 10 µg., and this has been increased to 20 µg. with satisfactory results (Table I). Haematological remission remains complete

in all cases. No neurological complications have developed and there is no refractoriness. The duration of treatment now ranges from 476 to 1,409 days.

The main purpose of the present paper, however, is to describe the clinical results obtained in cases of pernicious anaemia, using a new peptide complex derived from fermentations of propionibacterium and to discuss these results and the others obtained from the streptomyces preparation in the light of present knowledge.

Properties of the Complex.—Six batches of a vitamin-B₁₂-peptide complex derived from fermentations of propionibacterium have been used in the clinical studies. These have been designated H.P.P./4, H.P.P./7, H.P.P./9, H.P.P./12, H.P.P./16, and H.P.P./17. The spectrophotometric curves of these preparations were similar to one another and to that of H.P.P./1 (Heathcote and Mooney, 1958a), having maxima at 277, 361, and 550 mµ. Amino-acid analysis of the preparations was also similar to that for H.P.P./1. The vitamin-B₁₂-peptide ratio of the batches varied, on a weight basis, from 1:3.7 to 1:7.1, thus showing that the preparation was not a chemical compound in the strict sense but rather a loose combination of peptide and vitamin which can occur in different proportions. Ultracentrifuge studies, carried out at the Microbiological Research Station, Porton, with one of these batches (H.P.P./7) have shown that it produces a single peak corresponding to a molecular weight of about 1,200–1,400—that is, of the same order as vitamin B₁₂. These and further ultracentrifuge studies at the Biochemical Laboratory, Cambridge, have clearly shown that there is no protein in the complex. Furthermore, the batches are dialysable.

Maturation of Megaloblasts.—It has previously been shown (Heathcote and Mooney, 1958a) that the vitamin-B₁₂-peptide complex H.P.P./1 matured megaloblasts *in vitro* in 18-hour cultures as rapidly as folic acid. This property is not shared by crystalline vitamin B₁₂ alone, nor by gastric juice alone (Callender and Lajtha, 1951). Of the present vitamin-B₁₂-peptide batches only H.P.P./4 was tested in this way, but it also proved to be approximately as effective as folic acid in bringing about the maturation of megaloblasts.

Clinical Studies

Fourteen newly diagnosed uncomplicated cases of pernicious anaemia were treated with the propionibacterium complex. Three patients were males and 11 were females. The ages range from 36 to 80 years. Case 6A was used as a control, and Cases 11 and 14 in maximum response experiments. As all three of these received

TABLE I.—Uncomplicated Cases of Pernicious Anaemia Treated Exclusively with Streptomyces Complex (H.P.P./1)

Case No.*	Age	Sex	Duration Treatment (Days)	Vitamin-B ₁₂ -Peptide Complex (as µg. Vitamin B ₁₂)			Initial Blood Picture			Current Blood Picture			
				Total Dose	Mean Daily Dose	Current Maintenance Dose	Hb %	P.C.V. %	M.C.H.C. %	Hb %	P.C.V. %	M.C.H.C. %	Serum Vitamin B ₁₂ µg./ml.
1	48	M	1,409	15,370	10.8	10	66	22	44	100	48	32.5	48
2	75	F	1,354	15,450	11.4	10	48	19	37	93	40	34	62
3	67	M	1,316	13,270	10.1	10	39	15	38	98	43	34	66
4	66	M	1,262	14,020	11.1	10	76	27	42	112	46	36.0	90
5	44	M	1,156	12,040	10.4	10	53	19	42	114	53	32	49
25	58	F	912	4,560	5.0	5	66	30	32	101	51	29.5	46
26	55	M	825	2,890	3.5	3.5	50	25	30	100	49	31.5	—
27	61	M	821	3,046	3.7	3.5	56	26	32	100	43	35.5	13
30	66	M	740	10,870	14.9	20.0	56	22	37	110	46.5	35.0	140
38	70	F	476	2,200	4.6	4.0	29	14	31	81	39.0	30.5	23
Average	61		1,026		8.6	9.0	54	22	36.5	101	44	34	60

* The numbers here refer to those same cases reported in the papers by Heathcote and Mooney (1958a) and Mooney and Heathcote (1960a).

crystalline vitamin B₁₂, either orally or parenterally, they are not included in Table II, but are described separately (see below).

Methods

The diagnosis of pernicious anaemia was confirmed by full examination of the peripheral blood, histamine alcohol test meal, and biopsy of the sternal marrow. Progress was assessed by packed cell volume (P.C.V.) readings (3,000 r.p.m. for 30 minutes) and by haemoglobin estimations (Hb 100% = 14.8 g./100 ml.).

The method of assay of serum vitamin B₁₂ was essentially that described by Ross (1952), but using the "Z" strain of *Euglena gracilis* (Hutner *et al.*, 1956). The results obtained from 16 samples from normal controls ranged from 100 to 340 µg./ml. of vitamin B₁₂, and the mean reading was 170 µg./ml.

Dosage

For ease of administration of the minute doses of vitamin-B₁₂-peptide complex the material has always been mixed with mannitol. At the time of publication of our previous work it was being suggested in the literature (Chow *et al.*, 1956, 1958) that sorbitol in relatively large amounts (3–12 g.) aided the absorption of crystalline vitamin B₁₂. Although the amount of mannitol which we were giving per dose was extremely small (about 0.1 g.), it was decided to exclude the possibility of any improved absorptive effect which might be due to this. Accordingly, a portion of H.P.P./7 was admixed with lactose, and this preparation was coded H.P.P./7 L.B. It has since been shown, however, that sorbitol has no effect in aiding the absorption of crystalline vitamin B₁₂ in pernicious anaemia (Herbert, 1958) or, indeed, even in normal subjects (Chalmers and Shinton, 1959). For all practical purposes, therefore, H.P.P./7 L.B. is the same as H.P.P./7 and, as will be seen from Case 16 (Table II), the response to the lactose-base preparation is equally good. No P.C.V. readings are available in this case, however, owing to the difficulty of obtaining blood by venepuncture.

For the sake of simplicity and consistency the doses of the different batches of the peptide complex are given in terms of the vitamin B₁₂ content alone. Where crystalline vitamin B₁₂ has been used it is mentioned specifically in the text.

In most cases a relatively high initial dose of H.P.P. was given for a short time. In this way the patients were exposed to minimum risk at a time when the blood picture was at its lowest. This initial dose was then followed by a low daily maintenance dose—usually the

equivalent of 10 µg. of vitamin B₁₂—which was increased if necessary. No attempt was made to reduce the maintenance dose below 10 µg. daily in any of the cases.

Results

Control Case 6A.—A man aged 72 was given crystalline vitamin B₁₂ (60 µg. a day) for 10 days. At the end of this period the blood count was unchanged, the haemoglobin remaining at 30% and the P.C.V. at 13% (Fig. 1). There was a trivial reticulocytosis which gave a maximum reading of 3.6% on the eighth day. On the 11th day 60 µg. of H.P.P./7 daily was given instead of the crystalline vitamin. A brisk reticulocytosis occurred which was maximum on the 17th day with a reading of 20.8%. By the 28th day—that is, when he had been on H.P.P./7 for 17 days—the haemoglobin had risen from 30% to 58%, and the P.C.V. from 13% to 28%.

Test Cases

The results obtained in the 11 test cases are summarized in Table II. At the time of writing four of these (Cases 8, 13, 16, and 22) were all satisfactorily maintained on daily doses of 10 µg., and the duration

FIG. 1.—Control Case 6A.

of treatment of these cases ranged from 900 to 1,077 days. Cases 31 and 45 were receiving 20 µg. a day. One patient (Case 17) died from cerebral haemorrhage on the 670th day, but up to the time of death she also was stabilized satisfactorily on a daily dose of 20 µg. Cases 10, 15, and 28 were being maintained on 30 µg. a day, and Case 12 was maintained on 40 µg. a day. Case 15 (Fig. 2) showed the typical behaviour of the blood picture during the period of stabilization. This patient was a woman aged 63. Initially she received 100 µg. of H.P.P./4 a day for eight days, which was followed by a daily maintenance dose of 15 µg. The blood picture began to deteriorate about the 62nd day (the average daily dose at this time was 26 µg.), and on the 146th day the daily dose was increased to 30 µg. This produced such a good response that on the 245th day the dose was reduced to 20 µg. a day. By the 300th day, however, it was apparent that this dosage was barely adequate for

TABLE II.—Uncomplicated Cases of Pernicious Anaemia Treated Exclusively With the Propionibacterium Complex

Case No.	Age	Sex	H.P.P. Batch	Duration Treatment (Days)	Vitamin-B ₁₂ -Peptide Complex (as µg. Vitamin B ₁₂)			Initial Blood Picture			Current Blood Picture			
					Total Dose	Mean Daily Dose	Current Maintenance Dose	Hb %	P.C.V. %	M.C.H.C. %	Hb %	P.C.V. %	M.C.H.C. %	Serum Vitamin B ₁₂ µg./ml.
8	61	F	4	1,077	11,770	10.9	10	39	19	31	104	50	31	226
10	72	F	7	1,044	24,070	23.0	30	45	19	35	102	44	36	71
12	70	F	7	971	41,630	42.8	40	47	20	35	94	48	29	153
13	76	F	7	1,018	12,210	11.9	10	37	16	34	102	44	34	—
15	63	F	4	939	23,270	24.7	30	50	19	39	96	45	31	141
16	36	F	7	933	10,020	10.7	10	63	—	—	100	—	—	—
17	76	F	7	652	12,780	19.8	20	22	14	24.5	76	39	29	72
22	80	F	9	900	9,190	10.2	10	38	16	35	91	40	34	38
28	66	M	12	779	19,930	27.4	30	45	19	35	88	40	33	50
31	68	M	17	740	10,750	14.5	20	69	31	33	105	42	37	10
45	37	F	16	293	5,980	20.4	20	57	26	32.5	106	46	34	23
Average	64	—	—	849	—	19.7	21.0	46	20	34	95	44	31.5	76

satisfactory maintenance, and it was then restored to 30 μ g. The blood picture began to improve once more on this higher dosage, and has remained satisfactory after 939 days.

FIG. 2.—Case 15. Blood picture during period of stabilization.

This relationship between dosage and response (Fig. 2) is typical also of all the other cases in which it has been necessary to increase the dosage above the original maintenance level.

Maximum Response Experiments

In Cases 11 and 14 an attempt was made to discover the dosage required to produce a maximum response.

Case 11.—A woman aged 56 had a haemoglobin of 18% on admission. She was given a dose of 300 μ g. of H.P.P./7 daily for nine days followed by 100 μ g. a day for a further week, and the response was very good (Fig. 3). From the 17th to the 26th day inclusive the dose was reduced to 10 μ g. daily. On the 27th day 500 μ g. of parenteral vitamin B₁₂ was administered, but no secondary reticulocytosis was observed. She has since been satisfactorily maintained on 10 μ g. of H.P.P./7 a day for 1,031 days, and her haemoglobin at the time of writing was 91% and the P.C.V. 46%.

FIG. 3.—Blood picture of Case 11.

Case 14.—A woman aged 80 received 100 μ g. of H.P.P./7 a day for 14 days. A slow response occurred. From the 15th to the 22nd day 10 μ g. was given daily, and then 300 μ g. daily from the 22nd to the 32nd day. A slight secondary reticulocyte response to the higher dosage then occurred, but a further 500 μ g. of parenteral vitamin B₁₂ given on the 39th day failed to produce any further reticulo-

cytosis (Fig. 4). In spite of the apparently sluggish nature of the initial response to H.P.P./7, it would appear to have been maximal in her particular case. This patient was also satisfactorily maintained on 10 μ g. a day, the haemoglobin being 95% and the P.C.V. 49% on the 1,104th day.

From the above results it would seem that a daily dose of 300 μ g. of H.P.P./7 has been sufficient in each case to produce a maximum response.

FIG. 4.—Blood picture of Case 14.

Discussion

Comparison with Crystalline Vitamin B₁₂.—The effect of oral crystalline vitamin B₁₂ in pernicious anaemia has been studied extensively, and most of the work on this subject has been reviewed by Ungley (1951), by Brody *et al.* (1959), or by ourselves (Heathcote and Mooney, 1958a, 1958b). The differing schemes of dosage employed in the studies, however, make it difficult to summarize the findings in precise terms. There appears to be fairly general agreement, nevertheless, that daily doses of at least 100 μ g. are required for maintenance in most cases (Hall, 1950; Chalmers and Shinton, 1958; Hemsted and Mills, 1958; Brody *et al.*, 1959), and some patients may require more. As the body requirements are about 1 μ g. a day (Bethell *et al.*, 1948; Vilter, 1955; Pollycove and Apt, 1956) it can be inferred that the absorption of oral crystalline vitamin B₁₂ into the haemopoietic system is probably on average no greater than 1% of a 100- μ g. dose. This inference is supported by the work of Ungley (1951), who, from his series of 27 cases, concluded that absorption of the crystalline vitamin was rarely greater than 1%, and by Chalmers and Hall (1954), who found that an oral dose of 2,000 μ g. was needed to produce the same effect as a parenteral dose of 20 μ g.

In our experiments, maintenance over long periods has been possible in 10 cases on an average daily dose of 8.6 μ g. of the streptomycin complex H.P.P./1. Similarly, 11 cases have been maintained on the propionibacterium complex on an average daily dose of 19.7 μ g. These results, supported by the evidence from the control experiment described above and others reported elsewhere (Mooney and Heathcote, 1960a), demonstrate that both preparations are much more effective in the treatment of pernicious anaemia than crystalline vitamin B₁₂ alone.

Serum Vitamin B₁₂ Estimations.—Although we recognize that the serum vitamin B₁₂ assay has a limited value in the study of pernicious anaemia, present knowledge hardly justifies placing its importance on a par with the actual clinical and haematological responses in assessing the efficacy of a particular preparation. Apart from the inherent limitations of microbiological assay—which are particularly great in the case of vitamin B₁₂—the actual interpretation of the results during H.P.P. therapy still remains largely a matter of conjecture (Mooney and Heathcote, 1960b). Nevertheless, it can be asserted with confidence, even now, that relatively low serum vitamin B₁₂ levels on vitamin-B₁₂-peptide therapy are quite consistent with good clinical remission and absence of neurological complications and refractoriness (see below).

Refractoriness.—This has been constantly observed in cases of pernicious anaemia treated with all so-called purified intrinsic-factor preparations, and it may appear at any time between 3 and 48 months after the commencement of therapy (Blackburn *et al.*, 1955; Meulengracht, 1953; Nørregaard, 1955; Bastrup-Madsen, 1957; Schwartz *et al.*, 1957, 1958). Schwartz *et al.* (1958) reported that 11 out of 16 cases developed refractoriness after an average period of 18 months. In our series of 21 patients (Tables I and II) who have been treated exclusively with the H.P.P. preparations for an average period of over two years no case of refractoriness has occurred as yet.

Aetiology of Pernicious Anaemia

The premature conclusion, still open to dispute, that crystalline vitamin B₁₂ is the extrinsic factor, and possibly the haemopoietic factor itself (Castle, 1953), has obstructed critical work on the aetiology of pernicious anaemia for several years, and has led to another possible error—namely, the belief that the missing gastric factor merely facilitates absorption of the crystalline vitamin. It is perhaps unfortunate, too, that the Schilling (1953) test—which has become the popular method for measuring the activity of the gastric factor—depends on these and certain other assumptions which have not yet been proved. We believe that the final solution to this problem will be reached, not by studying the behaviour of the vitamin in its unnatural crystalline form, but only by returning to the investigation of the behaviour of the naturally occurring—that is, protein-bound—form, under the influence of various gastric enzymes. This we have already discussed in our previous publications (Heathcote and Mooney, 1958a, 1958b).

Unpublished *in vitro* experiments which we have carried out with normal gastric-juice digests of both beef muscle and cheese have indicated that the vitamin is present after digestion in a dialysable form but still firmly bound to polypeptide. The vitamin-B₁₂-peptide complexes which we have described in this paper are more loosely complexed than those of the gastric-juice digests and probably simulate more closely the intestinal forms. This association between vitamin and peptide may be due to hydrogen-bonding, Van der Waal's forces, or some similar type of attachment common to polypeptide association phenomena; but, whatever the nature of the association between the vitamin and the peptide, there is no doubt that both solubility and clinical effectiveness are greatly increased when compared with the crystalline vitamin.

The mounting evidence that vitamin-B₁₂-peptide complexes are so highly efficacious in the oral treatment of

pernicious anaemia enhances the view we expressed previously, that the disease is caused essentially by a failure of gastric proteolysis and that "intrinsic factor," as hitherto understood, does not exist. Furthermore, we believe that the evidence to date has failed to establish that crystalline vitamin B₁₂ is either the extrinsic or the haemopoietic factor.

Summary

Our previous clinical studies in which we used a vitamin-B₁₂-peptide complex derived from *Streptomyces griseus* (H.P.P./1) have been extended to include cases treated with batches of a vitamin-B₁₂-peptide derived from fermentations of a species of *Propionibacterium*.

Ten patients receiving H.P.P./1 have now been maintained for an average period of 1,026 days on an average daily dose equivalent to 8.6 µg. of vitamin B₁₂.

Eleven patients receiving the propionibacterium peptide complex have been maintained for an average period of 849 days on an average daily dose equivalent to 19.7 µg. of vitamin B₁₂.

The superiority of the propionibacterium complex over crystalline vitamin B₁₂ in the oral treatment of pernicious anaemia has been demonstrated in a controlled experiment, and daily doses of 300 µg. of vitamin B₁₂ equivalent have given a maximum response in two other patients.

No refractoriness or neurological complications have developed and all patients remain in complete clinical remission.

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