CCLX. A NEW FACTOR IN THE PRODUCTION AND CURE OF MACROCYTIC ANAEMIAS AND ITS RELATION TO OTHER HAEMOPOIETIC PRINCIPLES CURATIVE IN PERNICIOUS ANAEMIA[1,2]

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Previous work has shown that a macrocytic anaemia can be produced in Rhesus monkeys by feeding a deficient diet based on one in common use among sufferers from tropical macrocytic anaemia in Bombay. Reasons were given for considering this anaemia the animal counterpart of the tropical condition [Wills & Stewart, 1935]. After the publication of this work our attention was drawn to papers by Miller & Rhoads [1933; 1935] reporting the production of a similar anaemia in dogs, which however failed to respond to parenterally administered liver extract. Our animals were known to react to treatment with marmite and liver extract by mouth but had not been treated by the parenteral route. On account of these findings, preliminary experiments (2(*b*) below) were carried out which showed that the nutritional macrocytic anaemia of the monkey does respond to parenteral liver treatment and in this respect also corresponds to the tropical condition in man. A suitable animal therefore being available the work reported below was planned to investigate the nature of the haemopoietic factor present in yeast and other substances, a factor curative in the nutritional macrocytic anaemias of man and monkeys, and the relation of this factor to the haemopoietic principle curative in pernicious anaemia.

1. *Experimental methods*

The methods of feeding the animals and the technique used for the blood examinations have been described in a previous paper [Wills & Stewart, 1935]. In the present work the diet was slightly modified: its composition is shown in Table I. Vitamin B_1 was at first given weekly as 1 g. acid clay adsorbate from rice polishings, but this dose was inadequate and was later increased to 2 g. In addition the animals received 2 g. daily of iron and ammonium citrate as certain of them showed signs of an iron deficiency and this corrected it. The diet was considered adequate in vitamins A, D and C and in salts but was poor in protein and in the vitamin B_2 complex. All the animals on this diet ultimately became

[1] A preliminary account of some of the results recorded in this paper has appeared in the *Lancet*, 6 February 1937.

[2] The expenses of this work were met by a grant to one of us (L. W.) from the Lady Tata Memorial Trust.

Table I. *Diet*

Daily ration	Parts	Preparation
Polished rice	40	Cooked together with water till rice soft
Margarine	12	
White bread	70	Added after cooking
Salt mixture	5 g. per animal	
Cod liver oil	3 ml. per animal	
Apple, orange or tomato	20–30 g. given separately	—

anaemic; no animal, however, became anaemic under 3 months and a few took over a year before showing symptoms. For economy of space the preliminary periods are not shown on the charts.

For test purposes the oral doses were given by stomach tube with the exception of marmite and yeast, which were mixed with the food. The parenteral doses were given into the buttock or the shoulder muscles. A preparation was only considered active when it induced, in an animal known to be going downhill or to be resistant to other preparations, a good reticulocyte response followed by an increase in the red cell count and haemoglobin percentage in the blood. Sufficient animals have not been studied to determine the maximum reticulocyte response for different initial red cell levels, but Table II gives the figures found

Table II. *Average reticulocyte response to treatment*

Initial r.b.c. in millions per μl.	No. of observations	Maximum reticulocytes % after treatment: Range	Maximum reticulocytes % after treatment: Average
0·5	1	—	49
1·0	—	—	—
1·5	1	—	28·1
2·0	2	18·5–18·6	18·55
2·5	6	18·2– 9·3	12·4
3·0	5	10·6– 3·8	7·7
3·5	7	9·4– 2·2	7·9
4·0	16	6·2– 2·2	4·2
4·5	5	5·3– 2·1	4·5

in animals showing good response to treatment and therefore the average values to be expected. It should be noted that, however given, the curative dose per unit of body weight of either liver or yeast preparations is large in comparison with the dose required in the similar condition in man. This need for relatively large doses is not limited to the substances under consideration, but applies to other drugs.

2. *Preliminary experiments confirming the suitability of the experimental animal*

(*a*) *Activity of liver extract given by mouth.* Previous work [Wills & Bilimoria, 1932] had shown that liver extract by the mouth was curative in this macrocytic anaemia of monkeys but, to confirm this in the present series, an animal was given 30 ml. of a fluid extract equivalent to 227 g. fresh liver daily for 6 days. Chart I of Fig. 1 shows the excellent response. The animal was very ill when treatment was commenced and the clinical improvement after 2 days was very remarkable and paralleled that seen in man. In view of the earlier work it was not considered necessary to repeat this experiment.

(*b*) *Activity of liver extract given parenterally.* It was next sought to ascertain whether this monkey anaemia could be cured by liver extract given parenterally.

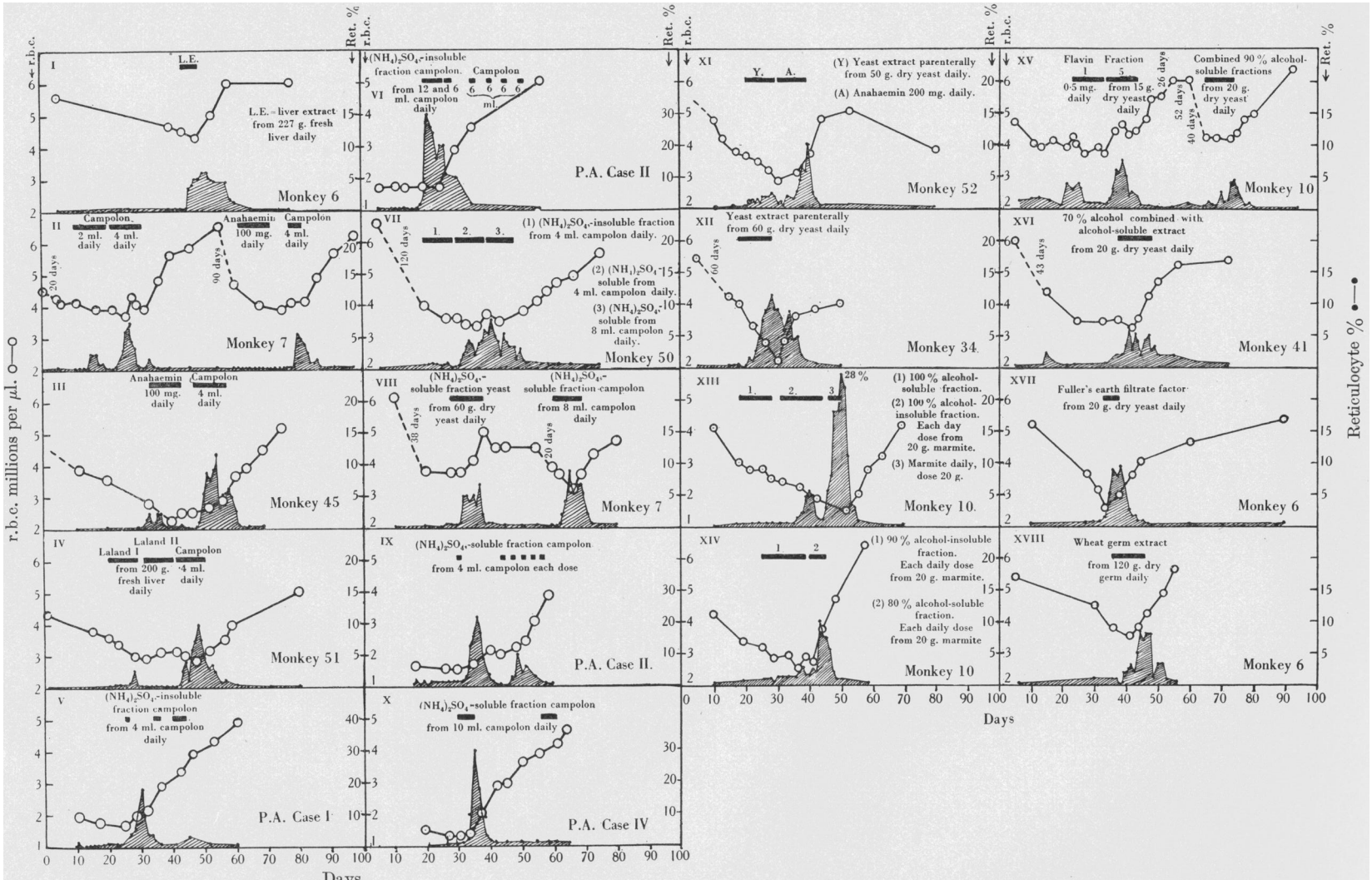

Fig. 1. Note different scale for reticulocyte graphs in human subjects and monkeys.

Campolon had been used for human cases in India so it was selected for the present experiment. Twelve trials were made of this preparation and in all positive results were obtained. One animal had, for a monkey, the amazing count of 600,000 r.b.c. per ml., yet with campolon and a transfusion of about 16 ml. blood it made a splendid recovery. Chart II of Fig. 1 is of especial interest as it shows the response of the same monkey to the same treatment in two different relapses. The first response was delayed and only occurred after large doses, 60 ml. in all, had been given. It illustrates the inhibitory effect of severe sepsis, in this case marked pyorrhoea and gingivitis, which evidently has the same unfavourable effect on haemopoiesis in monkeys as in man. Later the same animal was again treated with campolon: this time there was no sepsis as the mouth had cleared under the previous treatment and 20 ml. campolon were enough to induce a rapid and sustained rise in the red cell count. The activity of liver products both by the oral and the parenteral routes having been established, work was begun on the nature of the haemopoietic factor present in yeast and liver, and curative in the macrocytic nutritional anaemia of monkeys, referred to in this paper as the monkey factor.

3. *Preparation of fractions*

Fractions active either in true pernicious anaemia or in the monkey anaemia or in both have been prepared from liver, yeast, marmite and wheat germ extracts. In this work, whenever a concentration of alcohol is expressed as a percentage, it is intended to convey that the water content of the extract in question has been determined and a weight percentage of alcohol added. Similarly with $(NH_4)_2SO_4$, the water content was determined and the solution saturated by stirring mechanically with a very slight calculated excess of this salt.

(*a*) *Preparation of fractions of the liver extract "campolon".* (i) *Fractionation with Reinecke's salt.* The method used was the same as that of Dakin & West [1935].

(ii) *Fractionation with* $(NH_4)_2SO_4$. A known volume of campolon was saturated as above with $(NH_4)_2SO_4$ and the solution kept in the cold room overnight. The precipitate and slight excess of sulphate were filtered off, dissolved in a small volume of water, 3 vol. of alcohol added to precipitate most of the $(NH_4)_2SO_4$, and the last traces of sulphate removed as $BaSO_4$ with rhodizonic acid as external indicator. This solution was concentrated to the volume of the original campolon. The filtrate from the original precipitation was similarly treated with alcohol and barium to remove all the sulphate and also concentrated to the volume of original campolon. As these fractions were required for injection, the alcoholic filtrates, after complete removal of sulphate, were filtered through a sterile Seitz filter, concentrated with removal of alcohol in sterilized apparatus, and transferred to vaccine bottles.

(*b*) *Preparation of fractions from yeast.* (i) *Fraction 5.* All yeast fractions were made from a dilute acetic acid extract of brewer's yeast, referred to as fraction 5, prepared as described by Chick & Roscoe [1930]. This extract was autoclaved at *p*H 5 at 120° for 5 hr. to destroy vitamin B_1 and the volume so adjusted that 1 ml. was equivalent to 0·5 g. dry yeast. Simple alcoholic extracts were made from this extract.

(ii) *Yeast reineckate fractions.* These fractions were obtained by taking the original yeast extract, precipitating by calcium acetate and 75% alcohol and applying the methods of Dakin & West [1935].

(iii) *Alcohol-soluble and insoluble fractions from the* $(NH_4)_2SO_4$*-soluble portion of an 80% alcohol-soluble extract of fraction 5.* In another series of experiments,

alcohol was added up to 80 % to yeast fraction 5 derived from 3000 g. of pressed yeast and containing 130 g. of total solids. The filtrate, after removal of alcohol, contained 60 g. of total solid. It was then saturated with $(NH_4)_2SO_4$, kept, filtered and the sulphate removed as before; a filtrate containing 30 g. of total solid remained which retained practically the whole of the activity. This material was finally subdivided into portions soluble in 95 % alcohol (total solids 21 g.) and insoluble in 95 % alcohol (total solids 9 g.).

(iv) *Yeast fuller's earth filtrate.* An extract containing the filtrate from fuller's earth adsorption of autoclaved fraction 5 was prepared according to the method of Edgar *et al.* [1937]. The original fraction 5 from 3000 g. wet yeast contained 130 g. of total solids: the filtrate from the fuller's earth adsorption of this amount of fraction 5 contained 76 g. of total solids. The water content of this final filtrate was determined and alcohol added up to 80 % by weight. After filtering, removing the alcohol and concentrating by distilling *in vacuo* at 45° the alcohol-soluble material was made up to 100 ml. and contained 28 g. of total solids.

(c) *Preparation of fractions from marmite.* Marmite (300 g. containing 25 % H_2O) was dissolved in warm water (522 ml.) and alcohol (3000 ml.) added in a thin stream with shaking. Taking the water content of the marmite into account, the alcohol concentration amounted to 80 % by weight. After standing overnight in the cold room, the contents were filtered and the filtrate evaporated *in vacuo* to a small volume (bath temperature 40–45°). The total solids then amounted to 140 g. and dissolved with difficulty in sufficient water to make the volume up to 150 ml. The material was further subdivided into fractions soluble in 90 % alcohol (weight 75 g.) and insoluble in 90 % alcohol (weight 65 g.), and the 90 % alcohol-soluble fraction was divided still further into fractions soluble in 100 % alcohol (26 g.) and insoluble in 100 % alcohol (49 g.).

(d) *Preparation of an alcoholic extract of wheat germ.* This extract was prepared by the method of Edgar *et al.* [1937] and the extracted material from 600 g. (dry weight 525 g.) dissolved in 200 ml. water.

4. *Relation of the monkey haemopoietic factor to the liver principle and Castle's extrinsic factor*

(a) *Activity of purified liver extracts. Anahaemin.* Three animals were treated with the enormous dose of 100 mg. daily, two for 10 and one for 11 days. All three animals failed to show any response at all, though all responded well later to campolon, 4 ml. daily for 9, 5 and 7 days respectively (Charts II and III, Fig. 1). These results were very surprising in view of the activity of this preparation in pernicious anaemia [Ungley *et al.* 1936] and the fact that till this extract was tried all extracts curative in pernicious anaemia, either alone or after incubation with normal gastric juice, had been active curatively in this monkey anaemia. The question therefore arose as to the potency of the particular sample of anahaemin used. Dr C. C. Ungley very kindly made a trial of the remainder of our batch. Two cases of pernicious anaemia in relapse with initial counts of 1,500,000 responded well with reticulocyte crises of 28 and 29 % respectively and a rise in each case of 1,000,000 in 10 days. One dose of 400 mg. only was given to each patient. There was no question of the activity of the material in true pernicious anaemia.

The three monkeys used above had all been on the diet for a considerable time and were therefore suffering from a relative deficiency in the B_2-vitamins and protein. It was thought possible that this might be a limiting factor so another monkey was treated with anahaemin. This animal had only been on the

experimental diet for 3 months, and was anaemic for the first time and except for its anaemia was in good condition. It too received 1 g. anahaemin in all. Actually there was a slight rise in the red cell count but no reticulocyte crisis.

Laland's extract. A trial was also made of Dr Laland's[1] [Laland & Klem, 1936] highly purified liver extract. This extract is potent in pernicious anaemia but like anahaemin was completely inactive in the monkey anaemia. Two animals were treated and received the enormous dose of the equivalent of 200 g. liver daily for 10 days (Chart IV, Fig. 1); both went downhill but responded to campolon parenterally later. It is therefore obvious that preparations that will induce responses in cases of pernicious anaemia in man may be entirely inactive in the macrocytic anaemia of monkeys: this question is dealt with more fully in the discussion.

(*b*) *Activity of fractions prepared from campolon and yeast extracts by the use of Reinecke's salt* (3 (*a*, i) and (*b*, ii)). In an attempt to elucidate the problem further Dakin & West's [1935] method for the preparation of anahaemin was used, but in our hands Reinecke's reagent inactivated the liver preparations and the same inactivation occurred when the method was applied to yeast extracts, with one exception, out of seven trials, when the Reinecke-soluble fraction of an alcoholic extract of fraction 5 induced an excellent remission in an anaemic animal. Similar variable results have been obtained by other workers using this method and as no definite conclusions can be deduced from them the method was abandoned. Fractionation with phosphotungstic acid also led to complete inactivation of our material.

(*c*) *Activity of fractions prepared from campolon and yeast extracts by the use of saturated* $(NH_4)_2SO_4$ (3 (*a*, ii) and (*b*, iii)). This method also separates any anahaemin-like substances, which are precipitated and therefore present in the insoluble fraction, and in this way two fractions were obtained from both campolon and yeast fraction 5. The fractions from campolon were tested on both man and monkeys, the yeast fractions on monkeys only. All extracts from campolon were given parenterally; the insoluble fraction which would contain the bulk of any anahaemin present induced an immediate remission in two untreated, controlled cases of pernicious anaemia (Charts V and VI, Fig. 1). In one case (Chart V) a single dose, derived from 4 ml. of original campolon, i.e. from about 20 g. liver, induced a good reticulocyte response associated with an immediate rise of red blood cells at the rate of 100,000 a day for 10 days. The extract was obviously highly potent in the treatment of pernicious anaemia. This same insoluble fraction was completely inactive in monkeys, who, however, responded to the administration of the fraction soluble in $(NH_4)_2SO_4$ (Chart VII, Fig. 1). The first animal received a daily dose of the soluble fraction derived from 4 ml. of campolon: this induced a good reticulocyte response but the rise in the red cell level was slow till the dose was doubled. A second anaemic animal was therefore given 10 daily doses, each derived from 8 ml. campolon: here the rise in the red cell count was much more rapid (Chart VIII, Fig. 1). The $(NH_4)_2SO_4$-soluble fraction from campolon was then tested in four untreated, uncomplicated controlled cases of pernicious anaemia and was found to be extremely potent (Charts IX and I). These results were somewhat surprising, as the insoluble fraction from the $(NH_4)_2SO_4$ fractionation had already been shown to be extremely active curatively: there was however, as judged by the few cases treated, very little difference in activity between the two preparations (compare Charts V and IX and VI and X, Fig. 1). Yeast fraction 5 similarly treated yielded two fractions, soluble

[1] Kindly supplied by Dr Laland through the courtesy of Messrs Glaxo Laboratories, Ltd.

and insoluble, which were tested on anaemic monkeys—the doses being given by the oral route. The insoluble fraction which should contain any anahaemin-like bodies present in yeast was completely inactive in two anaemic monkeys, who, however, responded to treatment with the soluble fraction (Chart VIII). On the assumption, which as the result of its clinical trials [Ungley *et al.* 1936] appears justified, that anahaemin contains Castle's liver principle, it is clear that the factor active in the monkey anaemia cannot be this liver principle alone, and if the liver principle, the final product in the body of the interaction of the extrinsic and intrinsic factors, is inactive, the deficiency in the animals must, it seems, be other than a lack of the extrinsic factor. To test this further a trial was made of a yeast extract by the parenteral route. If the yeast factor and the extrinsic factor are identical, a yeast extract thus given would probably be inactive. The extract was made by concentration from the $(NH_4)_2SO_4$-soluble fraction of an 80 % alcohol extract of yeast fraction 5. This extract was given to three animals in daily doses equivalent to 50 g. in one case and 60 g. in the other two for 10-day periods. The first case was negative but gave a striking positive result (Chart XI, Fig. 1) after 0·1 g. anahaemin daily for a further 10 days. The other two cases responded to treatment but both the reticulocyte response and the rise in the red cell count were somewhat delayed (Chart XII, Fig. 1). These results suggest that generally in the anaemic monkey there is a deficiency only in the $(NH_4)_2SO_4$-soluble fraction of yeast or liver, but that in exceptional animals there may be a deficiency in the anahaemin principle as well: the significance of these results will be discussed later.

5. *Relation of the monkey haemopoietic factor to the vitamin B_2 complex*

Vitamin B_1 was not considered in the present work as it had been excluded both in tropical macrocytic anaemia and in the experimental condition. Actually several animals were given this vitamin in the form of acid clay, 1 g. daily for 10 days, in an attempt to improve their general condition but without any effect on their blood count and little, if any, on their general condition.

As a source of the vitamin B_2 complex marmite was used at first as it was known to be active in similar nutritional anaemias in man as well as in the monkey condition. Later a non-autolysed yeast extract was substituted and liver extracts, wheat germ extract and flavin were also tested.

(*a*) *Activity of marmite and its simple alcoholic extracts* (3 (*c*)). 5 g. of marmite daily prevented the development of this anaemia and kept the experimental animals in good health for periods up to 18 months, in which time all the other experimental animals had become anaemic or died of intercurrent disease. Curatively 5 g. daily for 10 days induced a satisfactory remission, but when the dose was reduced to 3 g. there was only a submaximum response. The effect of 5 daily doses of 20 g. each is shown on Chart XIII of Fig. 1. The rise of 1,100,000 in the erythrocyte count in 7 days is, by comparison with figures from human cases, taken as approximately maximum. To allow for loss in extraction and to ensure a maximum response, the fractions tested were given in daily doses equivalent to 20 g. or more of the original marmite or yeast for periods of 10 days.

Five monkeys were treated with an 80 % alcohol-soluble fraction of marmite and all responded well: Chart XIV (Fig. 1) shows its dramatic effect in a severe case. This extract appeared to contain the bulk of the active substance in marmite since a dose equivalent to 10 g. of the parent substance induced a good response after 10 days' treatment. A little, however, is probably carried down with the insoluble fraction, as one monkey treated with this fraction for 10 days in doses equivalent to 20 g. of the parent substance showed a slight reticulocyte

response, unaccompanied by any increase in the erythrocyte count. A similar 80 % alcohol-soluble extract of unsalted marmite was also highly active.

A trial of both soluble and insoluble fractions from 90 and 100 % alcohol extracts, alone and combined, indicated that the haemopoietic factor is partially or wholly destroyed by such fractionation, as certain anaemic animals failed to respond at all and others gave only a doubtful, submaximum response (Charts XIII and XIV, Fig. 1).

These results with marmite and its simple alcoholic extracts are compatible with the view that the haemopoietic factor is closely related to some part of the vitamin B_2 complex, for though certain authors [Guha, 1931] report that vitamin B_2 is not inactivated by 90 % alcohol, others [Chick & Roscoe, 1930] find that even 70 % alcohol will inactivate certain solutions of vitamin B_2. It is possible that inactivation depends on the source and method of preparation of the extracts.

(*b*) *Activity of dried brewers' yeast powder* (*Harris*). This yeast powder has been biologically tested and is known to be a rich source of the vitamin B_2 complex. A monkey treated with 10 daily doses of 20 g. responded with a submaximum reticulocyte response and a small temporary increase in the red cell count. A possible explanation of the relative inactivity of yeast is the fact that it is not digested in the stomach: in this connexion a trial was made both in a monkey and in a case of pernicious anaemia of zymin. A case of pernicious anaemia failed to respond to 10 days' treatment with daily doses of 15 g. zymin incubated with 200 ml. gastric juice and a monkey treated with daily doses of 4 g. zymin showed only a slight rise in the red cell count that was not sustained. In view of these negative and doubtful results no further trials of dried yeast were made.

(*c*) *Activity of 0·1 % acetic acid extract of fresh brewer's yeast* (*fraction 5*) *and its simple alcoholic extracts* (3 (*b*, i)). An excellent remission was induced, in an anaemic monkey, by 10 daily doses of fraction 5, each dose derived from 15 g. dried yeast (Chart XV). An extract made from the combined soluble and insoluble fractions from a 90 % alcohol extract of fraction 5 also induced a good remission in the same monkey, though the daily dose in this case was derived from 20 g. of the original dry yeast: there may, however, have been some slight loss of potency as the reticulocyte crisis did not occur till the 9th day (Chart XV, Fig. 1). These findings were somewhat surprising, first, because yeast extracts similarly prepared gave uniformly negative results [Wills, 1934] in the treatment of cases of tropical macrocytic anaemia and also because 90 % alcohol inactivates marmite. It is possible that the extracts used in India were inactivated by storing, heat and, as no precautions were taken against it, by exposure to light. Similar differences in the effect of alcohol on vitamin B_2 extracts from different sources have already been discussed. However, as yeast extracts were found to be active under the present experimental conditions, it was decided to use them rather than extracts of marmite, as the former are less complex and can be more easily standardized.

(*d*) *Activity of* $(NH_4)_2SO_4$*-soluble fractions of various alcoholic extracts of fraction 5* (3 (*b*, iii)). These extracts were active but there was some loss of potency. Fractionation of the $(NH_4)_2SO_4$-soluble portion of an 80 % alcohol-soluble extract of fraction 5 with 90 and 95 % alcohol did not lead to further separation of the active principle, since though all the soluble extracts were active, the insoluble gave variable results, sometimes being active and at other times inactive. There was also, as judged by the size of the doses necessary for maximum responses, some loss of potency, so the method was abandoned.

(*e*) *Activity of fraction prepared by treatment of fraction 5 with fuller's earth* (3 (*b*, iv)). The first sample of this fraction which contains the yeast fuller's earth filtrate factor of the vitamin B_2 complex was very kindly prepared for us by Miss Edgar at the Lister Institute and was tested biologically for this factor and shown to be active. The extract was free from vitamin B_1 and flavin, as the original fraction 5 was autoclaved before treatment with fuller's earth, which process destroys vitamin B_1, and the flavin was removed on the fuller's earth. This extract and an 80% alcoholic extract of it were both very active haemopoietically when tested on four anaemic monkeys (Chart XVII, Fig. 1). These experiments demonstrate the activity in the cure of the monkey anaemia of extracts free from vitamin B_1 and the flavin fraction of the vitamin B_2 complex but containing the yeast fuller's earth filtrate fraction of Edgar & Macrae [1937].

(*f*) *Activity of hepatoflavin.* The preceding experiments, by demonstrating the activity of fractions from which the flavin had been removed by adsorption on fuller's earth, had excluded this substance as the haemopoietic factor, but to confirm this three anaemic animals were treated with pure hepatoflavin. Hepatoflavin was used, as it has the same properties as lactoflavin and a supply was available through the generosity of Dr Chick. The daily dose was 0·5 mg.: this dose was used as it was forty times the rat dose and therefore equivalent in terms of this factor to the curative dose of marmite used in the original Indian work [Wills, 1931]. In no animal was there a response (Chart XV): in all three the dose appeared to be slightly toxic, as the animals were more ill than would have been expected from their blood counts and in one (Chart XVII, Fig. 1) there was a slight increase in the reticulocyte level, with no associated rise in the red cell count. Hepatoflavin can therefore be excluded as the haemopoietic factor.

(*g*) *Response to wheat germ extracts* (3 (*d*)). Wheat germ extracts have been used as sources of Castle's extrinsic factor [Groen, 1935]. Tested on the anaemic monkeys they were found to contain the haemopoietic factor, but very large doses were necessary to obtain good responses. Four animals were treated in doses derived from 15 to 120 g. of dry wheat germ: with the smaller doses submaximum responses only were obtained, whereas with a dose derived from 120 g. there was excellent response (Chart XVIII, Fig. 1). Wheat germ was not used further, on account of the size of the dose and the time needed to extract the material, but its activity was tested as it was a known source of Castle's extrinsic factor and of the vitamin B_2 fraction necessary to supplement lactoflavin to ensure optimum growth in the rat.

Discussion

The nutritional macrocytic anaemia of monkeys, by virtue of its method of production and its response to orally and parenterally administered marmite, yeast and liver, is considered the animal counterpart of tropical macrocytic anaemia. It has been suggested that the tropical condition is a deficiency disease [Wills, 1934] due to a lack in the diet of Castle's extrinsic factor; in other words, the final deficiency in the body is the same as that in pernicious anaemia [Castle *et al.* 1935]. There are, however, certain difficulties in accepting this suggestion if all the pathological and clinical findings of pernicious anaemia are due only to the absence of the liver principle. For example, an increased bilirubin content of the blood is a constant finding in untreated pernicious anaemia, whereas in uncomplicated tropical cases and in the experimental animals the bilirubin content is normal or subnormal. Similarly there is no increase of urobilin output in the tropical condition, though this is a constant finding in true pernicious

anaemia. It is also a remarkable fact that no description of nervous lesions in the tropical condition has yet appeared, although the majority of pernicious anaemic patients show some signs of subacute combined degeneration.

This difference between idiopathic pernicious anaemia and the experimental condition is further emphasized by the findings reported in this paper. The experimental evidence indicates that at least two factors are concerned in the cure of these two conditions. Crude extracts of liver, yeast, marmite and wheat germ are curative in both conditions, some only after mixture with normal gastric juice, but the more highly purified extracts of the liver principle are inactive in the monkey anaemia although still retaining good activity in true pernicious anaemia. An extensive trial was made of the purified liver extracts anahaemin (Dakin & West) and examen (Laland & Klem), but these alone were inactive in the monkey anaemia, though the actual samples tested were known to be fully potent in cases of true pernicious anaemia. The liver extract campolon, and yeast extracts were fractionated by full saturation with ammonium sulphate into insoluble and soluble fractions. The insoluble fractions should contain, according to Dakin & West [1935], practically the whole of the anahaemin or anahaemin-like substances present in the liver and yeast extracts respectively. The insoluble extract from campolon was extremely potent haemopoietically in untreated cases of pernicious anaemia, but in the monkey condition it was completely inactive even in large doses. These results were similar to those obtained with the commercial preparation anahaemin. A therapeutic trial of the soluble fraction from campolon in cases of pernicious anaemia and in the monkey anaemia showed, surprisingly, that this extract was highly active in both the human and animal conditions, a single dose of 4 ml. producing in one human case a response almost identical with that made by the patient treated with a similar amount of the insoluble fraction.

Two possible explanations immediately present themselves. First, it is suggested that the material active in man is not completely precipitated, as claimed by Dakin & West [1935], by saturation with ammonium sulphate and that therefore some passes into the soluble fraction along with an entirely distinct factor active in the monkey anaemia. As a matter of fact it is generally agreed by other workers in this field that anahaemin is not completely precipitated but that the amount in the soluble fraction is only a small percentage of that originally present. It would therefore be necessary to assume that very small amounts of anahaemin are necessary to induce a maximum response, at least in the presence of the second factor. Secondly, it is possible that the liver principle, inactive in the monkey anaemia but active in pernicious anaemia, and precipitated in its anahaemin form by ammonium sulphate, is also present in the soluble fraction but is present in some other form, probably more complex, which is not precipitable by this reagent. The principle active in the monkey anaemia may be either this more complex substance or again an entirely distinct factor.

Subbarow *et al.* [1935; 1936] have produced evidence that at least three distinct factors, one of which is tyrosine, are necessary for maximum haemopoietic activity in the cure of pernicious anaemia. Tyrosine in enormous doses was inactive in the monkey anaemia but the relation of the other two factors to those described in the work mentioned above has not yet been investigated.

It is not suggested that monkeys do not require the liver principle contained in anahaemin, but only that the anaemia under discussion is not produced by a simple lack of this factor, and cannot therefore be due solely to absence of Castle's extrinsic factor from the diet as originally suggested. This monkey

anaemia appears to be due, in part at least, to the lack of some other factor at present unidentified. It seems possible, however, that the two factors are concerned both in the monkey anaemia and in pernicious anaemia, and there is some evidence that this view is correct. For example, the more sustained response in the monkey to the crude extracts in comparison with the response to the more highly purified fractions, even when these are given in doses derived from far larger amounts of the parent substance (yeast or liver) than those used in the preparation of the cruder extracts, points in this direction. The fact that one monkey responded well to very large doses of anahaemin when given immediately after the parenteral injection of the monkey fraction from yeast, which in other animals had been shown to be active without anahaemin, also suggests that this principle is necessary for normal haemopoiesis in the monkey. The crude extracts probably contain both factors; campolon certainly does. If, then, there is in the monkey suffering from macrocytic nutritional anaemia a deficiency of both factors, the administration of this latter may activate the small supply of the liver principle still available in the body. The liver principle thus activated will cause an initial rise in the red cell count, which however will not be sustained when the available store of the liver principle is exhausted. Accepting this explanation of the action of the new factor as correct, then there may be exceptional cases of pernicious anaemia, in which there is a deficiency, not only of the liver principle contained in anahaemin but also of the new haemopoietic factor. Such cases would either not respond to anahaemin or make a poor temporary response, but would respond to the cruder preparations such as campolon. Such a case has recently been treated by one of us. Further, the extremely good response made by cases of pernicious anaemia to the ammonium sulphate-soluble fraction of campolon would also support this view. Such a preparation must contain only minimum amounts of the anahaemin principle, but is a rich source of the monkey factor.

The nature of the new factor active in the monkey condition is at present unknown. Its distribution in natural products, yeast, marmite, wheat germ, liver, and the fact that it is water-soluble, suggest a relationship to the vitamin B complex. Vitamins B_1 and B_4 and the flavin component of the vitamin B_2 complex have been excluded as sources of this factor.

More recent work on the vitamin B_2 complex [Edgar & Macrae, 1937] has shown that autoclaved acidulated yeast extract can be fractionated into two fractions other than lactoflavin, termed the yeast fuller's earth filtrate factor and the yeast fuller's earth eluate factor, both of which are required to supplement lactoflavin. Rats receiving as the source of the vitamin B complex these two fractions supplemented by lactoflavin and crystalline vitamin B_1 hydrochloride increased in weight as rapidly as rats on a diet containing untreated yeast extract or a good mixed diet. The yeast fuller's earth filtrate factor is probably not identical with György's vitamin B_6 [1935, 1, 2]. That this filtrate factor may possibly be identified with the monkey haemopoietic factor is suggested by the fact that both are present in the more soluble fractions of yeast, marmite, liver and wheat germ extracts, both are extracted from yeast with dilute acetic acid, both resist autoclaving at *p*H 5 for 5 hr. at 120°, neither is precipitated by 80% alcohol or by saturated ammonium sulphate, and both resist adsorption by fuller's earth. Still further fractionation is in hand, and though haemopoietic activity in the monkey has in the earlier stages followed the presence of the yeast fuller's earth filtrate factor of the vitamin B_2 complex, evidence is now accumulating which suggests that the haemopoietic factor is probably not identical with the filtrate factor.

It will be noted that the active factor of marmite is inactivated by 90% but not by 80% alcohol, whereas that of acetic acid extracts of brewer's yeast and of wheat germ can be extracted with 90% alcohol. The explanation of this difference may lie in the fact that protracted autolysis during the preparation of marmite has resulted either in the partial degradation of the active substance or in the loss of some stabilizing agent rather than in the presence of two entirely distinct factors.

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