XXXIV. NOTE ON THE NON-IDENTITY OF LACTOFLAVIN AND THE "EXTRINSIC FACTOR" IN PERNICIOUS ANAEMIA.

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THE maintenance of a normal red cell count in human blood is apparently dependent on the presence in the gastric secretion of an "intrinsic factor"—haemopoietin—which is of the nature of an enzyme [Castle *et al.*, 1929; 1930; Klein and Wilkinson, 1933; 1934; Wilkinson and Klein, 1932; 1933].

A deficiency of this factor in the gastric secretion is closely associated with the development of the megalocytic anaemia—pernicious anaemia. Under the influence of this haemopoietin some as yet unknown constituent of the diet—the so-called "extrinsic factor"—is converted into the substance, probably identical with the anti-anaemic liver principle, which is essential for normal erythropoiesis in the bone-marrow. The nature of this "extrinsic factor" has naturally been a source of much speculation. Strauss and Castle [1932], noting the similarity of distribution of the vitamin B₂ complex and the extrinsic factor, carried out experiments using both autolysed yeast and an 80 % alcohol extract of autolysed yeast as substrates and put forward the suggestion that "the extrinsic factor may now be defined as a substance closely related to vitamin B₂, if not vitamin B₂ itself".

Since then a considerable amount of research has been directed to the examination of this point; it may be said at once that practically all the results reported fail to support this suggestion, and hence the literature requires only brief review. Wills and Naish [1933], using an egg-white extract rich in vitamin B₂ as substrate, were unable to obtain a remission when this was fed after incubation with normal gastric juice to a patient with pernicious anaemia. Lassen and Lassen [1934], using various yeast extracts, rich in vitamin B₂ and sometimes also in vitamin B₁, concluded that the extrinsic factor was not identical with vitamin B₁ or B₂. Diehl and Kühnau [1933], using a purified source of vitamin B₂, concluded that the two factors were distinct. On the other hand Miller and Rhoads [1934] obtained contrary results to Wills and Naish when using egg-white containing vitamin B₂ and also when using rice polishings concentrate as substrate. Their results, however, are somewhat equivocal as to the identity of vitamin B₂ with the extrinsic factor, since the responses obtained with the egg albumin with a high vitamin B₂ content (20 rat units per day) were poor whilst the responses with the rice polishings containing less than half that amount of vitamin B₂ were very good. They concluded that "if vitamin B₂ is the extrinsic factor it must be effective in exceedingly low concentration", and again—"conclusive proof of the identity of the dietary anti-anaemic factor and vitamin B₂ must be deferred until the isolation of the vitamin in a pure form has been effected". The reason for re-opening the question here is that the researches of György *et al.* [1934] and György [1935, 1, 2] have provided very strong evidence

of the identity of one growth-promoting constituent of vitamin B₂ with lactoflavin.¹ This substance has been placed at our disposal in quantity through the kindness of Messrs Bayers, Ltd., and this has made it possible for us to reinvestigate the point. The results though negative are considered of sufficient importance to be reported here.²

EXPERIMENTAL.

The clinical technique employed in the following experiments to detect haemopoietic activity has been fully described in previous communications. It is only necessary to emphasise here the necessity firstly for careful choice and adequate control of the test cases of pernicious anaemia used [Wilkinson and Klein, 1934], and secondly for a correct interpretation of the haematological response.

The products have been administered parenterally, since it is well established that active liver extracts are much more effective when administered intramuscularly than when given orally. Klein and Wilkinson [1934] showed that, following incubation of beef and fraction P 5 (*i.e.* the active fraction prepared by alcoholic precipitation of the press juice from hog's stomach [Klein and Wilkinson, 1933]), a protein-free substance can be prepared which is therapeutically active in pernicious anaemia and is suitable for administration by intramuscular injection. A similar technique has been employed in the experiments to be reported here.

The lactoflavin used was supplied by Messrs Bayer Products, Ltd., in ampoules each containing 2 ml. of an aqueous solution of 1 mg. lactoflavin.

The gastric juice was obtained from normal human subjects after histamine injection, only samples containing adequate amounts of free hydrochloric acid and pepsin being used.

Exp. I. Effect of lactoflavin alone. Lactoflavin solution (32 ml. = 16 mg.) was injected intramuscularly into case P.A./538 over a period of several days. There was no haematological response even after 9 days but the patient subsequently responded normally to a known active parenteral liver preparation (Table I). It will be seen indeed that the patient's condition was steadily relapsing during the injection of lactoflavin.

Exp. II. The effect of incubating lactoflavin with fraction P 5. Fraction P 5 (10 g.) was dissolved in 100 ml. of cold *N*/10 HCl, diluted to 400 ml. with distilled water and brought to p_H 4.3 by addition of *N* NaOH. Lactoflavin solution (32 ml. = 16 mg.) was then added and the mixture warmed to 37° and incubated at that temperature for 5 hours. The product was adjusted to p_H 6.0 and 2 volumes of 90 % alcohol were added. After standing for 3 days, the mixture was filtered and the alcoholic filtrate concentrated *in vacuo* to one-tenth of the volume. Two volumes of 90 % alcohol were then added, and after standing overnight the precipitate was removed and the filtrate again concentrated to small volume. After standing in the ice-chest overnight the precipitate which settled was filtered off and the filtrate reduced to a syrup *in vacuo*. Ten volumes of absolute alcohol were then added and the precipitate so obtained after standing was collected, washed with absolute alcohol and dried in a vacuum desiccator. The yield of the product (S.I. 22) was 1.5 g. Parenteral administration of this fraction

¹ György [1935, 1] regards "vitamin B₂" as a complex containing lactoflavin and vitamin B₆ [see also Chick *et al.*, 1935].

² Since carrying out our experiments, the important results of Groen [1935] have come to our notice. He obtained negative results when 5 mg. lactoflavin, incubated with 150 ml. normal gastric juice, were administered each day orally for 10 days. His previous experiments using different sources of vitamin B₂ pointed in the same direction.

Table I. *Showing lack of response in case P.A./538 to injection of lactoflavin and subsequent response to injection of an active liver extract.*

Day	Reticulocytes %	Red blood cells per μ l.	Haemo- globin %	Treatment
1	1.9	1,500,000	45	Control period. No treatment
2	—	—	—	
3	1.6	—	—	
4	1.7	—	—	
5	—	—	—	
6	1.7	1,552,000	45	2 ml. }
7	1.8	—	—	6 ml. }
8	1.7	—	—	6 ml. }
9	1.5	—	—	6 ml. }
10	1.6	—	—	6 ml. }
11	1.0	—	—	6 ml. }
12	—	—	—	Nil
13	1.4	1,176,000	36	2 ml. }
14	1.3	—	—	4 ml. }
15	1.6	—	—	4 ml. }
16	4.1	—	—	6 ml. }
17	10.6	—	—	Nil
18	23.0	—	—	Nil
19	30.8	—	—	Nil
20	37.5	1,520,000	48	Nil
21	26.2	—	—	Nil
29	5.1	2,960,000	70	Nil
34	1.6	3,200,000	74	Nil
41	0.8	4,064,000	90	Nil

Table II. *Showing lack of response in case P.A./553 to injection of fraction S.I. 22 and subsequent response to injection of an active liver extract.*

Day	Reticulocytes %	Red blood cells per μ l.	Haemo- globin %	Treatment
1	1.5	1,340,000	36	Control period. No treatment
2	1.8	—	—	
3	2.2	—	—	
4	2.3	—	—	
5	1.7	1,250,000	34	
6	1.1	—	—	0.2 g. }
7	2.8	—	—	0.4 g. }
8	1.2	—	—	0.8 g. }
9	2.1	1,110,000	32	S.I. 22
10	2.0	—	—	Nil
11	1.3	—	—	Nil
12	1.1	890,000	26	4 ml. }
13	1.3	840,000	26	4 ml. }
14	—	—	—	4 ml. }
15	2.6	830,000	24	Intramuscular
16	6.1	—	—	liver extract
17	24.4	880,000	—	S.P. 202
18	16.9	—	—	Nil
19	—	—	—	Nil
20	21.7	1,120,000	27	Nil
21	17.6	—	—	Nil
22	—	—	—	Nil
23	16.1	1,320,000	34	Nil
24	13.6	—	—	Nil
25	9.8	—	—	Nil
26	7.2	—	—	Nil
27	5.1	—	—	Nil
28	—	—	—	Nil
29	3.6	1,740,000	40	Nil

to case P.A./553 during 3 days produced no clinical response after 8 days, although normal responses were obtained subsequently using a known active preparation (Table II).

The negative result obtained might have been due to an insufficient amount of substrate since the fraction P 5 used had been clinically tested and found to be potent. A further experiment using larger quantities of lactoflavin and normal human gastric juice instead of fraction P 5 (Exp. III) confirms the findings of Exp. II.

Exp. III. The effect of lactoflavin after incubation with normal gastric juice.

A mixture of 78 ml. (= 39 mg.) of lactoflavin solution and 1940 ml. of normal fresh gastric juice was adjusted to p_H 3.0 by addition of *N* NaOH and incubated for 3 hours at 37°. The p_H was adjusted to about 6.0 and after heating at 60–65° for half an hour to inactivate the enzymes, 2 volumes of 90 % alcohol were added. After standing for 3 days, the mixture was filtered and the alcoholic filtrate worked up as described in Exp. II. The yield of this fraction (G.I. 2) was 10 g.

The administration of this fraction intramuscularly failed to produce any haemopoietic response. Thus in case P.A./365 when 10 g. were given over a period of 4 days no clinical response was noted after 23 days, although a normal response was subsequently obtained following the administration of a known active preparation (Table III).

Table III. *Showing lack of response of case P.A./365 to fraction G.I. 2 and subsequent response to injection of an active liver extract.*

Day	Reticulocytes %	Red blood cells per μ l.	Haemo- globin %	Treatment
1	0.4	1,890,000	54	{ Control period. No treatment. Reticu- locytes below 2.5 %
7	1.3	1,775,000	52	
14	2.3	—	—	
15	1.9	1,680,000	50	
16	2.1	—	—	{ 1 g. 2 g. 3 g. 4 g. } Fraction G.I. 2
17	1.9	—	—	
18	0.9	1,440,000	43	
19	1.7	—	—	
21	0.9	—	—	Nil
23	0.6	—	—	"
25	0.7	1,200,000	36	"
26	0.7	—	—	"
28	1.3	—	—	"
37	1.1	1,260,000	38	{ 4 ml. 4 ml. 4 ml. } Intramuscular liver extract
38	1.0	—	—	
39	1.9	—	—	
40	1.2	—	—	
41	4.2	—	—	Nil
42	18.3	—	—	"
43	17.7	—	—	"
44	19.6	—	—	"
45	7.9	2,056,000	52	"
46	5.1	—	—	"
47	2.1	—	—	"
52	1.8	2,820,000	64	"
59	1.0	3,320,000	82	"

DISCUSSION.

The illustrative experiments reported here make it clear that lactoflavin is neither the anti-anaemic liver principle nor the "extrinsic factor" of Castle. In connection with the latter finding it should be noted, however, that Castle

himself (quoted by Lassen and Lassen [1934] and Davidson [1933]) is "prepared to relinquish the suggestion that vitamin B₂ and the extrinsic factor are identical".

The exact nature of the "extrinsic factor" or factors is as yet unknown, and its distribution among dietary foodstuffs has led to conflicting results among different workers. Yeast and yeast products have been much investigated from this point of view but there is still no satisfactory evidence as to whether the active principle in certain autolysed yeast products is solely of the nature of "extrinsic factor" or a substance similar to the anti-anaemic liver principle or even a mixture of the two produced by autolysis. The literature on the subject is full of contradictions and quite uncritical experimentation. It is clear that the methods of testing haemopoietic activity and the interpretation of the findings are still not fully understood by some workers and, indeed, cases other than those of typical uncomplicated pernicious anaemia have sometimes been employed for test purposes. In fact the whole problem of the nature of the extrinsic factor, and of the nature of the active principle in autolysed yeast preparations, needs careful and critical reinvestigation.

It appears certain on the one hand that autolysis of yeast yields a substance capable of curing certain tropical megalocytic anaemias where there is little or no gastric impairment [Wills, 1931]. This autolysate or its extracts can apparently be digested with haemopoietin, or "intrinsic factor", and the digest employed to initiate remissions in pernicious anaemia [Groen, 1935; Ungley, 1933]. Yeast preparations without preliminary autolysis are apparently inactive. It is less clear, on the other hand, whether autolysed yeast products can really be used satisfactorily to treat typical cases of pernicious anaemia. Occasional success has been described [Davidson, 1931; 1933; Goodall, 1932; Russell, 1934; Ungley, 1933; Ungley and James, 1934] but it is evident that in the published series of cases, very few have responded to this treatment, and Davidson [1933] has concluded that marmite is not in any way an efficient or reliable substitute for stomach or liver preparations.

It will suffice therefore to remark that the occurrence of the extrinsic factor in beef muscle protein has been amply demonstrated by Castle *et al.* [1929; 1930] and by Klein and Wilkinson [1934] and this material furnishes a very suitable source for testing digestion methods in conjunction with normal gastric juice or hog's stomach fractions. The possibility of the extrinsic factor being related to the glycoproteins is suggested by the recent work of Dakin and West [1935], and is also being investigated in this laboratory. It will be recalled that Reimann [1931; Reimann and Fritsch, 1934] has shown that the potency of fresh liver is much increased by incubation with normal gastric juice, presumably owing to a high content of extrinsic factor in fresh liver, and more particularly, in the liver proteins. On the other hand, the report of Walden and Clowes [1931] and of Helmer *et al.* [1932], that the potency of liver extract (Lilly No. 343) is increased by incubation with normal gastric juice is difficult to explain and obviously requires reinvestigation. (The theory that vitamin B₂ was the extrinsic factor afforded an obvious and easy explanation of this observation since liver extract is a rich source of the vitamin.) Of a different nature is the claim of Herron and McEllroy [1933] that simple autolysis for 10 days enhances the haemopoietic potency of liver. This claim is not justified by their published results and has since been disproved by Castle and Strauss [1935].

One further point requires clarification. According to György [1935, 1, 2] the lactoflavin of the vitamin B₂ complex is concerned largely with its growth-promoting properties and has no well-marked "antipellagra" or "antidermatitis"

properties for rats. The nature of the antipellagra factor is at present unknown. It is therefore of importance in this connection that Spies *et al.* [1934] have concluded that the "extrinsic factor" and the "antipellagra" factor for human subjects are distinct, although both may be contained in yeast. [See also Spies, 1935.] On the other hand several observers, *e.g.* Ramsdell and Magness [1933] and Spies [1933], have found parenteral liver extract very efficacious in the treatment of human pellagra.

SUMMARY.

The growth-promoting constituent of vitamin B₂ in the form of pure lactoflavin has been shown to be neither the liver anti-pernicious anaemia principle nor the "extrinsic factor" concerned in haemopoiesis.

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