CCXXIV. INVESTIGATIONS ON THE NATURE OF HAEMOPOIETIN, THE ANTI-ANAEMIC SUBSTANCE IN HOG'S STOMACH.

II. THE PRODUCTION OF A THERMOSTABLE HAEMO- POIETICALLY ACTIVE SUBSTANCE SIMILAR TO OR IDENTICAL WITH THE ANTI-ANAEMIC PRINCIPLE OF LIVER BY THE ACTION OF THE THERMO- LABILE HAEMOPOIETIN ON BEEF.

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IN Part I of this series [Klein and Wilkinson, 1933] we reported the preparation and properties of concentrates containing haemopoietin, the anti-anaemic substance in hog's stomach, and these experiments seemed to suggest that haemopoietin had different properties from the active anti-anaemic principle present in liver and was probably enzyme-like in its nature. Further work has confirmed our suggestion that at least two different anti-anaemic factors exist; one, a thermolabile substance, haemopoietin, present in hog's stomach and not as yet separable from the proteins; the other, a thermostable protein-free substance found in liver. The relationship between these two substances, which are so different chemically but nevertheless apparently similar in their clinical effects on cases of pernicious anaemia, is of considerable interest.

This question has already been mentioned in a preliminary report published elsewhere [Wilkinson and Klein, 1933]. The experiments there described confirm our views as to the enzymic nature of haemopoietin and demonstrate the formation of a substance very similar to, if not identical with, the active principle of liver *in vitro* without the use of liver by the action of concentrates of haemopoietin on beef. This change has hitherto been believed to take place only *in vivo*. In the present paper we describe further experiments along these lines and have indicated the possible relationship between the two anti-anaemic principles.

EXPERIMENTAL.

METHODS OF TESTING HAEMOPOIETIC ACTIVITY.

As we have previously indicated, no chemical or reliable biological tests are known for assaying the haemopoietic value of preparations used for the treatment of pernicious anaemia, and the only available method involves clinical trial on persons suffering from this disease.

The details of the method have previously been described elsewhere [Wilkinson, 1933, 2; Klein and Wilkinson, 1933] but it appears necessary to emphasise here once again the importance of choosing very carefully and adequately controlling all the clinical material used in these tests.

Since liver preparations can be freed from proteins and blood-pressure depressants without impairing their haemopoietic activity, it is possible to administer them not only orally but also by intramuscular or intravenous injection. The latter parenteral route is a far better method, since the active principle thereby finds its way into the blood-stream very quickly, while there is no loss through lack of absorption or destruction in the alimentary tract; consequently, there is a much more rapid response to the treatment. Stomach preparations containing haemopoietin, on the other hand, cannot be given parenterally but only by mouth, since it has not yet been found possible to remove proteins from them without removing the associated haemopoietin.

EXPERIMENTS WITH HOG'S STOMACH FRACTIONS.

Fractionation of press juice by isoelectric precipitation.

In a typical experiment, 1 litre of press juice obtained from the mucosa of hog's stomach [Klein and Wilkinson, 1933] was brought to p_H 4.2 by the careful addition of $NHCl$ and the mixture filtered through a series of No. 41 Whatman papers. The precipitate, fraction J 1 (mucosa), was washed with absolute alcohol and dried over sulphuric acid in vacuum-desiccators. The yield was about 8-9 g.

The filtrates from fraction J 1 (mucosa) were adjusted to p_H 6.2 and precipitated by stirring into 4 volumes of 90 % alcohol cooled to 0° . The precipitate of fraction J 2 (mucosa) was collected, washed with absolute alcohol and dried over sulphuric acid in vacuum-desiccators. The yield was about 9-10 g.

Properties of fraction J 1 (mucosa). Fraction J 1 (mucosa) was a pale yellowish-brown powder giving all the usual protein reactions. It contained 11.4 % N, 1.3 % P and 4.5 % ash. The ash gave a very strong phosphate reaction and contained also traces of sodium, calcium and sulphate. Fraction J 1 (mucosa) was extremely active peptically (*cf.* Table I) but contained no haemopoietin when tested clinically.

Properties of fraction J 2 (mucosa). Fraction J 2 (mucosa) was a very pale buff powder almost completely soluble in water giving all the usual protein reactions. It contained 12.5 % N, 2.4 % P and 10.0 % ash. The ash contained sodium, potassium, calcium, iron, magnesium and phosphate, and a trace of sulphate. Fraction J 2 (mucosa) had only moderate peptic activity (*cf.* Table I) and was only moderately active in the treatment of pernicious anaemia. Thus, case 390, receiving 5 g. daily, gave the following blood picture: Maximum reticulocyte response, 10.8 % on the eighth day of treatment. First day of treatment: red cells, 1,220,000; haemoglobin, 34 %; colour index, 1.4. Thirteenth day: red cells, 1,750,000; haemoglobin, 54 %; colour index, 1.6.

Properties of various stomach fractions.

In previous communications [Wilkinson and Klein, 1932; Klein and Wilkinson, 1933], the preparation of concentrates of haemopoietin from the press juice of hog's stomach was described. The properties of some of these fractions, in so far as they have any bearing on later experiments in this paper, are summarised in Table I.

Table I. *Properties of stomach fractions.*

Fraction	Method of preparation	Peptic activity	Time taken to digest egg-albumin mins.	Haemo-poietic activity	Daily dose to case of P.A. g.
P 5	Precipitation of press juice from whole hog's stomach with alcohol	+	7	+	5
P 5 (mucosa)	Precipitation of press juice from mucosa of hog's stomach with alcohol	+	4-5	+	5
P 5 (i)	Dissolution of fraction P 5 in N/10 HCl and precipitation of addition by NaOH to p_H 4.2	+	4-5	-	7.5
P 5 (ii)	Filtrate from fraction P 5 (i) brought to p_H 6.2 and precipitated with alcohol	Slight	40	+	5
J 1 (mucosa)	Precipitation of press juice from mucosa of hog's stomach by bringing to p_H 4.2 with HCl	+	5-6	-	5
J 2 (mucosa)	Filtrate from fraction J 1 (mucosa) brought to p_H 6.2 and precipitated with alcohol	Moderate	15	Moderate	5
Commercial scale pepsin	Treatment of hog's stomach by the usual commercial methods	+	2	-	60

Inactivation of haemopoietin by heat.

5 g. of fraction P 5 were ground up with 100 ml. of water, and the mixture was heated to 60-65° for half an hour with constant stirring on the water-bath. The resulting product was found to have lost all its peptic activity, and daily administration to cases of pernicious anaemia indicated that it no longer possessed any haemopoietic activity, since no reticulocyte crisis or improvements in the blood counts were noted.

Incubation of fraction P 5 with water.

5 g. of fraction P 5 were ground up with 150 ml. of water, and the mixture was incubated for 6 hours at 37°. The product was then heated to 60-65° for half an hour to inactivate haemopoietin, cooled and given daily by mouth to each of three cases of pernicious anaemia. A small reticulocyte peak (12.4 %) was observed in one patient and may have been due to a spontaneous remission; two other cases failed to show any responses to this treatment, consequently the haemopoietic activity of the product must have been almost negligible.

INCUBATION OF STOMACH FRACTIONS WITH BEEF.

The general procedure was as follows. The experimental stomach fraction was added to minced lean beef-steak and water and after thorough mixing brought to a temperature of 37° by careful heating on the water-bath and finally incubated at that temperature with occasional stirring. All the incubation mixtures had a slightly acid reaction (p_H 5.7) which remained unaltered during the incubation.

The subsequent procedure was based on the fact that haemopoietin is inactivated by exposure to a temperature of 60-65° for half an hour, whereas the active principle of liver is thermostable under these conditions.

A. Oral experiments.

Incubation of beef with fraction P 5. A mixture of beef (100 g.), fraction P 5 (5 g.) and water (120 ml.) was incubated for 2 hours at 37°, heated to 60–65° for half an hour and cooled. The product given daily by mouth to cases of pernicious anaemia was found to be active. Even better responses were obtained in another experiment using 150 g. beef, 5 g. fraction P 5 and 200 ml. water when the incubation time was increased to 6 hours. In this case, the product was filtered and the clear liquid alone administered orally. For example, the effect on the blood picture of case 357 was as follows: Maximum reticulocyte response, 22.4% on the ninth day of treatment. Initial count: red cells, 1,440,000; haemoglobin, 35 %; colour index, 1.2. Twenty-second day; red cells, 2,440,000; haemoglobin, 64 %; colour index, 1.3.

Incubation of beef with fraction P 5 (ii). Similar haemopoietically active products were obtained using a mixture of 100 g. beef, 2 g. fraction P 5 (ii) and 120 ml. water.

Effect of heating fractions P 5 and P 5 (mucosa) before incubation with beef. A mixture of fraction P 5 (5 g.) and water (200 ml.) was heated to 60–65° for half an hour and then incubated for 6 hours with 150 g. beef. The strained juice given daily by mouth to cases of pernicious anaemia produced no haemopoietic responses.

A similar negative result was obtained using fraction P 5 (mucosa).

These experiments confirm the result obtained in a previous experiment that the haemopoietin present in these stomach fractions is inactivated by heating to 60–65° for half an hour.

Incubation of beef with fraction P 5 (i). The material resulting from the incubation of 100 g. beef, 5 g. fraction P 5 (i) and 120 ml. water for 3 hours was given daily by mouth to each of two cases of pernicious anaemia but produced no haemopoietic response. Since fraction P 5 (i) consists largely of pepsin, this experiment confirms our own previous observations and those of Castle *et al.* [1930] that incubation of beef and pepsin yields a haemopoietically inactive product.

B. Injection experiments.

Since a product similar to, or identical with, the active principle of liver appears to be formed in these experiments, the method adopted for the isolation of an active fraction suitable for injection was similar to that used for preparing parenteral liver fractions [*cf.* Wilkinson and Klein, 1934]. It depends upon (a) the solubility of the active principle of liver in 70 % alcohol and its insolubility in 95 % alcohol [*cf.* Cohn *et al.* 1930], and (b) the solubility of blood-pressure depressants in 95 % alcohol. By utilising these solubility relations, products suitable for intramuscular injection could be easily obtained and they did not possess any marked vasodepressor activity. The products were extremely hygroscopic and were preserved in vacuum-desiccators until required.

Injection product from incubation of fraction P 5 and beef. A mixture of 4 kg. of beef, 80 g. of fraction P 5 and 4 litres of water was incubated at 37° for 6 hours. The product was then heated to 60–65° for half an hour, cooled and filtered at the pump. The clear liquid and washings were treated with sufficient alcohol (about 2½ volumes) to give a concentration of about 70 %, allowed to stand overnight and filtered from the large protein precipitate. The alcoholic filtrates were concentrated *in vacuo* below 50° to about 500 ml., and the last trace of protein was removed by adding absolute alcohol to a concentration of about 70 %, cooling in the refrigerator and filtering. The clear liquid was then

reduced to a thick syrup *in vacuo* below 50° and poured into 10 vols. of absolute alcohol. After stirring until the product was almost solid, the alcohol was replaced by fresh liquid and the stirring continued until the substance became brittle and could be easily broken up and powdered. The pale yellow-brown solid was collected, washed with absolute alcohol and dried and preserved over sulphuric acid in a vacuum-desiccator (yield, 43 g.). This product (S.I. 1) dissolved in the minimum amount of water and sterilised by filtration through a Seitz filter was found to be very active when given by intramuscular injection; *e.g.* case 370 received a total dosage of 43 g. over a period of twelve days. Maximum reticulocyte response, 19.1 % on nineteenth day of treatment. The red cell count rose from 1,160,000 to 2,500,000 and the haemoglobin percentage from 31 % to 74 % without further treatment during 41 days. At the commencement of this treatment the patient was showing a very severe and rapidly relapsing condition—the response was therefore more satisfactory than the figures would appear to suggest.

DISCUSSION.

The results of the experiments described in this paper are summarised in Table II.

Table II. *Responses of cases of pernicious anaemia to various stomach fractions and incubation products.*

Haemopoietically active	Haemopoietically inactive
Fraction P 5 alone	Pepsin alone
Fraction P 5 (mucosa) alone	Fraction P 5 (i) alone
Fraction P 5 (ii) alone	Fraction J 1 (mucosa) alone
Fraction J 2 (mucosa) alone	Beef + water alone
Fraction P 5 or P 5 (mucosa) + beef + water incubated at 37°	Beef + pepsin + water incubated at 37°
Fraction P 5 (ii) + beef + water incubated at 37°	Fraction P 5 + water heated to 60–65° for half an hour
Fraction P 5 + beef + water incubated at 37°, then heated 60–65° for half an hour	Fraction P 5 or P 5 (mucosa) + water heated to 60–65° for half an hour, then incubated with beef at 37°
Fraction P 5 + beef + water incubated at 37°, then heated at 60–65° for half an hour and filtrate worked up for parenteral administration	Fraction P 5 (i) + beef + water incubated at 37°

Inspection of Table II shows that although haemopoietin in the form of fractions P 5, P 5 (mucosa) *etc.*, is active in the treatment of pernicious anaemia, the haemopoietic activity can be destroyed by heating with water to 60–65° for half an hour. Moreover, when the active fractions are heated with water to 60–65° for half an hour they do not yield any active products when subsequently incubated with beef. If, however, any of these unheated concentrates of haemopoietin is incubated with beef and water at 37°, an active product is produced whose activity can no longer be destroyed by heating to 60–65° for half an hour. Thus, during the incubation, the thermolabile haemopoietin produces or is converted into a haemopoietically active product that is now thermostable. Control experiments in which the active fractions were incubated with water alone without beef and subsequently heated to 60–65° gave products of negligible activity. It seems not unreasonable to suppose that the haemopoietin has acted on some constituent in beef and produced another substance similar to or identical

with the relatively thermostable "liver active principle" itself, thus representing a synthesis of the latter from beef and haemopoetin outside the body and without the use of liver. Moreover, the products were worked up by methods similar to those employed for parenteral liver fractions and were found to be active when injected intramuscularly into patients with pernicious anaemia.

The striking resemblance in chemical and other properties between the active principle of liver and the product formed in the above incubation experiments seems to indicate their probable identity and differentiates them sharply from haemopoietin, the active principle of stomach. This is clearly shown in Table III.

Table III. *Comparison of properties of haemopoietin, the active principle of liver and the product formed by the action of haemopoietin on beef.*

Haemopoietin	Active principle of liver	Product formed by incubation of haemopoietin with beef
(1) Precipitated with proteins	Not precipitated with proteins	Not precipitated with proteins
(2) Gives protein reactions	Does not give protein reactions	Does not give protein reactions
(3) Soluble in water	Soluble in water	Soluble in water
(4) Insoluble in 70 % alcohol	Soluble in 70 % alcohol	Soluble in 70 % alcohol
(5) Insoluble in 95 % alcohol	Insoluble in 95 % alcohol	Insoluble in 95 % alcohol
(6) Thermolabile	Thermostable	Thermostable
(7) Gives thermostable product, when incubated with beef	Unchanged when incubated with beef	Unchanged when incubated with beef
(8) Inactivated by acetone etc.	Unaffected by acetone	Unaffected by acetone

It seems justifiable then, to assume, that the relationship of haemopoietin to the active principle of liver is that of an enzyme to an end-product and that the latter is formed by enzymic action on some unknown constituent of beef in accordance with the scheme:

Bearing of these results on the work of other investigators.

Many observations recorded not only by ourselves but also by other workers are in harmony with the results just described.

Castle [1929] and his co-workers [1930; 1931; 1932] showed that normal human gastric juice when incubated *in vitro* with the proteins of beef-muscle produced haemopoietically active material, although neither the gastric juice nor the muscle proteins administered separately were active. They suggested, therefore, that there was an "intrinsic factor" of an "enzyme nature" in normal gastric juice which, reacting with the proteins of beef-muscle (the so-called "extrinsic factor"), gave rise to haemopoietically active material. Their evidence, however, was not quite conclusive since they did not record the properties of the active product formed in their experiments or its effect when purified and administered parenterally; but in the light of our own experiments it appears to us justifiable to assume that during incubation of gastric juice and beef enzymic action had taken place similar to that which occurs in our own incubation experiments. In support of this contention, it is interesting to note that one of us [Wilkinson, 1930] has shown that when normal human gastric juice is given with protein food (*e.g.* beef) to patients suffering from pernicious anaemia, a response is obtained, although as Castle also found, the juice is not active when given alone.

Further experiments on the haemopoietic factor in gastric juice have been reported by Morris *et al.* [1932], who have claimed that large amounts of normal human gastric juice after concentration *in vacuo* and neutralisation produced reticulocyte responses when injected into patients having pernicious anaemia. They described the substance in gastric juice (for which they proposed the name *addisin*) as being "thermolabile, dialysable and exhaustible" and concluded that it was probably a hormone. The clinical responses obtained by these workers are, however, open to question and it appears to us that there is not sufficient evidence from their observations to justify their assumption of the existence of yet another anti-anaemic factor [Wilkinson, 1932; 1933, 1]. In this connection, the recent work of Fouts *et al.* [1934] is of great interest. These authors have shown that some change takes place in normal human gastric juice during the process of vacuum-distillation. Fresh gastric juice was subjected to ultrafiltration, but the portions held back by the ultrafilter as well as the ultrafiltrate were inactive haemopoietically. After concentration *in vacuo*, however, the gastric juice gave a response on injection. Moreover, when gastric juice was concentrated by vacuum-distillation and then subjected to ultrafiltration, the portion passing through the ultrafilter was active. In another experiment, gastric juice was subjected to ultrafiltration, and the material held back by the ultrafilter was, after concentration *in vacuo*, found to be active, whereas the ultrafiltrate after concentration *in vacuo* was not active. It is evident, therefore, that the material that could be made active during the process of concentration *in vacuo* was held back by the ultrafilter and was therefore presumably a substance having a large molecular weight, whereas the product formed was a small molecule capable of passing through the ultrafilter. The authors suggest that the haemopoietically active substance is probably formed by action of an "intrinsic factor" upon an "extrinsic factor" in the gastric juice.

These results and their explanation are substantially in agreement with our own work. The enzyme present in gastric juice is probably identical with our haemopoietin and during the prolonged concentration of the juice *in vacuo* acts upon an unknown substrate present in the juice thereby producing the active principle of liver.

The nature of the substrate present in beef (and also in stomach-muscle and apparently in much lesser amount in normal human gastric juice), upon which haemopoietin acts has been the subject of investigation by Castle and his co-workers [1931; 1932] who termed it the "extrinsic factor" and found that it was present in the washed proteins of beef precipitated at p_H 6.0 but not in washed caseinogen or wheat-gluten. It was therefore assumed to be a protein or closely related substance. Castle and Strauss [1932] later found that incubation of normal human gastric juice with autolysed yeast produced haemopoietically active material and they suggested that the "extrinsic factor" was probably vitamin B_{12} . These conclusions are, however, vitiated by the observations of later workers [Davidson, 1932; Goodall, 1932; Ungley, 1933; Wilkinson and Klein, 1933] that autolysed yeast products (*e.g.* "marmite" or "vegex") taken alone are frequently active when administered to cases of pernicious anaemia. Evidently, the responses obtained by Castle and Strauss might have been due to the autolysed yeast alone. Moreover, the non-identity of the "extrinsic factor" and vitamin B_{12} is consistent with the observation of Wills and Naish [1933] that when an extract of egg-white rich in vitamin B_{12} is incubated with gastric juice, the resulting material is inactive haemopoietically.

The relationship between the haemopoietic factor in marmite and the active principles of stomach and liver has not yet been satisfactorily elucidated. The

marmite factor, in its general properties (*e.g.* heat-stability, solubility in 70–80 % alcohol, *etc.*) resembles the active principle of liver rather than haemopoietin. Judging by the many therapeutic failures following the use of marmite in the treatment of pernicious anaemia, the active haemopoietic factor must be present in relatively low concentration, and marmite cannot therefore be considered to be a satisfactory substitute for stomach and liver products.

Many workers [Riemann, 1931; Walden and Clowes, 1932; Helmer *et al.*, 1933; Fouts and Zerfas, 1933] have reported that the haemopoietic activity of liver or liver extracts can be markedly increased by incubation with hog's stomach or with normal human gastric juice. These observations all find a ready explanation on the basis of our experiments described in this paper and are in harmony with the view that the enzyme haemopoietin finds its necessary substrate in the fresh gastric or liver tissues.

The results we have obtained appear to have an important bearing on the aetiology and pathology of pernicious anaemia and are consistent with the theory that pernicious anaemia is a deficiency disease characterised by the absence from the gastric mucosa and secretion of a specific enzyme, haemopoietin. It is significant in this connection that pernicious anaemia is almost invariably associated with achylia gastrica and moreover complete or partial removal of the stomach in human subjects has been followed in many cases by an anaemia of the Addisonian type [Wilkinson, 1933, 3].

It is evident that since the reaction

cannot occur in cases of pernicious anaemia owing to the absence of haemopoietin, the livers of such cases must be deficient in the anti-anaemic factor that is present in normal livers. We have recently reported elsewhere the results of experiments bearing on this point [Wilkinson and Klein, 1934] and have shown definitely that whilst normal human livers, like the livers of various mammals and fishes, contain an active anti-anaemic principle, the latter is not present in livers from untreated cases of pernicious anaemia in relapse. If, however, a case of pernicious anaemia is treated with suitable active liver or stomach preparations, the active principle is stored in the liver and consequently the livers of such cases of pernicious anaemia in active remission are found to contain the anti-anaemic liver principle.

Finally, some animal experiments reported by Bence [1933] are also of interest in this connection, for he found that if the stomachs of pigs were completely removed and several months later the animals were killed, the livers were no longer active haemopoietically.

SUMMARY.

1. A relationship of fundamental importance has been shown to exist between the anti-anaemic factors in hog's stomach and in liver.
2. It is found that when concentrates containing the thermolabile haemopoietin (the active anti-anaemic factor in hog's stomach) are incubated *in vitro* with beef-muscle, haemopoietically active material is obtained which is now relatively thermostable. The substance formed, although obtained without the use of liver, appears to resemble the "liver active principle" very closely in its properties, and it can be prepared in a form suitable for intramuscular injection by methods similar to those used for obtaining parenteral liver fractions from liver.
3. A fraction from hog's stomach containing haemopoietin but practically free from pepsin gave similar results when incubated with beef.

4. Pepsin itself, or fractions from hog's stomach shown clinically to be free from haemopoietin, gave negative results when incubated with beef.

5. It is considered that the relationship between the anti-anaemic principles in stomach and in liver is that of an enzyme to an end-product; that is to say, the enzyme haemopoietin requires for its effective action some unknown substrate apparently present in beef, and the result of this enzymic action is a haemopoietically active end-product which is ultimately stored as the active principle in liver until it is required by the body for the production of the red blood corpuscles.

6. This enzymic action which we have been able successfully to imitate outside the body, namely

Unknown substrate in beef + the enzyme haemopoietin $\rightarrow$ the "liver active principle"

probably takes place in the stomachs of normal individuals but not in cases of pernicious anaemia.

7. Various observations of our own and of other workers are reviewed and shown to be in harmony with the results we have described and the views we have put forward.

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