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

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Introduction.

The successful treatment of pernicious anaemia depends upon the use of a diet containing fresh liver or liver extracts, or fresh or desiccated hog's stomach [Minot and Murphy, 1926; Wilkinson, 1930].

The nature of the active anti-anaemic principle in liver has been investigated by many workers. West and Howe [1930] and Dakin, West and Howe [1930] reported the isolation from liver extract of an active amorphous substance which they regarded as a dipeptide of *l*-γ-hydroxyproline and β-hydroxyglutamic acid; this claim was later, however, withdrawn [West and Howe, 1931]. Cohn, Minot and their co-workers [1927; 1928; 1929; 1930, 1, 2] obtained an active amorphous solid from liver extract by removing the proteins with either alcohol or basic lead acetate and precipitating with phosphotungstic acid when it was found that the material obtained from the acetone-soluble phosphotungstates was rich in the active principle. Further concentration was achieved by fractionating with various mixtures of alcohol, ether and water, when finally an intensely active amorphous substance was obtained of which the injection of only 0·025 g. daily was sufficient to give a maximum reticulocyte response in a case of pernicious anaemia. This substance has been shown to be neither a protein, lipin, carbohydrate, α-amino-acid nor polypeptide, and Cohn *et al.* [1930, 2] are of the opinion that it is probably an ω-amino- or imino-acid.

The use of preparations of hog's stomach is a direct consequence of the finding of Castle [1929] and his co-workers [1930; 1931; 1932] that normal human gastric juice, after incubation *in vitro* with the proteins of beef muscle, was able to induce remissions in cases of pernicious anaemia. Neither the gastric juice nor the proteins of beef muscle administered separately, however, were active. It was suggested, therefore, that there was an "intrinsic factor" of enzymic nature in normal gastric juice which, reacting with the proteins of beef muscle (the "extrinsic factor"), gave rise to haemopoietically active material. Wilkinson [1930] has, moreover, shown that when normal human gastric juice is given with protein food to patients suffering from pernicious anaemia, a reticulocyte response does occur although the juice is quite inactive when administered alone.

These experiments led one of us [Wilkinson, 1930] to consider the use of stomach tissue itself as a source of the active anti-anaemic principle. It was

found that responses could be obtained with hog's stomach but not with the stomachs of the ox or sheep.

The superiority of the hog's stomach treatment over the older liver therapy is manifested not only by the quickness with which normal health is reached but also by the greater speed of remission of the disease as measured by the increase in the number of red blood cells and in the percentage of haemoglobin [*cf.* Wilkinson, 1930; 1931, 1, 3]. This suggests either that the stomach is a richer source of the active anti-anaemic principle than liver, or else that the active principles in these two organs are not identical, though there must evidently be some fairly close relationship between them. Our experiments on the nature of the active anti-anaemic principle in hog's stomach support the second of these possibilities.

Several factors were found to destroy the stomach active principle and consequently led to the formation of inactive stomach extracts. Among the most striking of these were (*a*) exposure to temperatures greater than 45°, (*b*) autolysis, (*c*) prolonged digestion with pepsin or trypsin. Moreover, it was not found possible to obtain active stomach extracts by methods of extraction analogous to those employed in the case of liver.

It appeared, therefore, that the active principle of stomach, for which we have temporarily suggested the name "haemopoietin" [Wilkinson and Klein, 1932], was much more unstable and had different properties from the active principle in liver. Later experiments showed that an active press juice could be prepared from hog's stomach by the method used by Buchner [1897; 1898] to obtain zymase from yeast, and, by precipitating the juice with alcohol, a protein fraction was obtained in which the whole haemopoietic activity of the juice appeared to be concentrated. The active principle of liver, on the other hand, is always found in the filtrate obtained after the removal of proteins [*cf.* Cohn *et al.*, 1928]. This protein fraction from the stomach press juice is highly active peptically but, by dissolution in $N/10$ hydrochloric acid followed by fractional isoelectric precipitation, we have isolated a product which, while haemopoietically active, contains relatively little pepsin. There is, moreover, abundant experimental evidence that the active principle in hog's stomach is certainly not pepsin or rennin [Castle, 1929; Wilkinson, 1930, 1932, 1; Wilkinson and Brockbank, 1930]. The experiments described in this paper indicate that even the relatively mild methods employed for the preparation of concentrates of haemopoietin cause some loss of activity, and in consequence the possibility of isolating it in a chemically pure state seems at present to be remote.

Experimental.

Clinical technique.

The investigation of the potency of the various fractions obtained from hog's stomach involves feeding experiments carried out on well-authenticated and specially controlled cases of pernicious anaemia and the observation of the effect of such treatment in producing firstly a sharp rise or peak in the number of reticulocytes in the circulating blood, followed subsequently by an increase in the number of red blood cells and in the percentage of haemoglobin. The cases used for this purpose were all carefully chosen according to the rigid criteria that have already been laid down by one of us [Wilkinson, 1932, 1].

As an illustration of the use of this method, the results obtained with one of our active fractions, P 5, are shown in Fig. 1. It will be observed that there was a maximum reticulocyte response on the 13th day of treatment, followed by

a gradual fall to normal values with significant rises in both the red cell count and the haemoglobin percentage.

The clinical method, though necessarily crude and possessing the disadvantages of involving the use of relatively large quantities of each fraction and

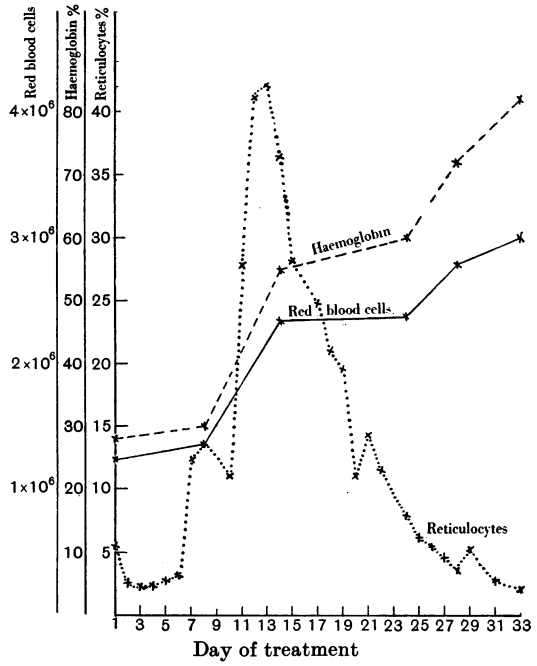


Fig. 1. Effect of fraction P 5 (5 g. per day) on a case of pernicious anaemia.

requiring the use of human test material, is the only one at present available. Although in the course of our work on the treatment of pernicious anaemia with hog's stomach some 400 patients have passed through the hands of one of us (J. F. W.), we have been considerably handicapped by the scarcity of suitable cases for experimental treatment with our various fractions.

Preparation of haemopoietically active desiccated stomach powder.

Active preparations of desiccated hog's stomach are prepared [*cf.* Wilkinson, 1932, 1] by desiccating minced whole hog's stomach, freed from fat by dissection, in thin layers at 40°. The drying must be carried out as quickly as possible and on no account must a temperature of 45° be exceeded.

Desiccated hog's stomach powder is a light, sparingly soluble biscuit-coloured powder, 1 g. of which is equivalent haemopoietically to approximately 4–5 g. of fresh whole hog's stomach. It has strong peptic activity and is active in the treatment of pernicious anaemia, the effective daily dose being 10–30 g. In the course of this work we have frequently had to use much larger amounts of material than we could prepare ourselves and have been kindly supplied with almost unlimited quantities of a very active preparation "Pepsac" made according to our directions by Messrs Boots Pure Drug Co., Ltd., of Nottingham, whom we take this opportunity of thanking.

Peptic activities of preparations from hog's stomach.

Wilkinson [1932, 1] has shown that the pepsin content of desiccated preparations of hog's stomach appears to run parallel with the haemopoietic activity since any conditions which inactivate pepsin during the desiccation also destroy the effectiveness of haemopoietin. Peptic activity determinations have therefore been carried out on all the preparations and fractions described in this paper and the results show that, whilst the above observation holds for preparations of desiccated hog's stomach, peptic activity and haemopoietic activity do not always go hand in hand in the case of other fractions.

Negative attempts to obtain active stomach extracts.

Large scale extractions were tried not only with fresh stomach tissue but also with the more easily handled desiccated stomach powder (p. 602). All the methods of extraction used cannot be described here, but brief details of a few typical experiments are appended by way of illustration.

1. Various methods of extracting both fresh and desiccated stomach were investigated using water, dilute hydrochloric acid at different p_H values, with or without the addition of pepsin or glycerol, but in every case the extracts were inactive whilst the solid or semi-solid residues when separated and desiccated at 40° were still active.

2. The use of organic solvents likewise failed to furnish any active extracts.

Thus, 4500 g. of desiccated hog's stomach were extracted with ether in a Soxhlet apparatus which removed about 10 % of its weight of haemopoietically inactive fat. Subsequent extraction at room temperature with aqueous ethyl alcohol of varying strengths (60–70 %)[1] gave extracts which deposited very small amounts of a wax-like, sparingly soluble lipoid solid (M.P. 175–185°) on cooling in the refrigerator. The extracts and the solid were haemopoietically inactive. The residual stomach powder, however, was still as active as the original powder even after 7 months' continuous extraction with aqueous alcohol. Other organic solvents (*e.g.* glycerol and acetone) similarly failed to yield active extracts.

Preparation of a haemopoietically active press juice from hog's stomach.

In our experiments with stomach tissue a special apparatus was constructed for us by Mr H. W. Baker of the Engineering Department of this University, enabling us to dispense with the use of a cloth bag. Pressure was applied by means of a Tensile and Compressive Testing Machine in the Engineering Department [Wilkinson and Klein, 1932].

The finely minced fresh stomach tissue (1300 g.) was thoroughly mixed with an equal weight of Calais sand and ground in a Bailey's Edge-Runner Mill (about 2–3 minutes) to the correct consistency. The mixture was then packed into the above apparatus with two thicknesses of fine brass gauze and one thickness of stout cloth at the top and bottom, and a pressure of 2–2½ tons per square inch gradually applied until no further quantities of liquid could be obtained. In a series of experiments in which the pressure was 2½ tons per square inch the yield of juice was between 560 and 670 cc. per kg. of fresh tissue. In another series of experiments, using a lower pressure (2 tons per square inch), the yield varied between 525 and 640 cc. per kg.

If the mucosa of hog's stomach was used instead of the whole stomach, a considerably smaller yield of press juice was obtained. In two typical experiments, for example, the yields of juice per kg. of mucous membrane were 305 and 340 cc. (pressure = 2 tons per square inch). This yield was improved somewhat

[1] Alcohol concentrations are given throughout this paper in volume percentages.

by increasing the proportion of sand to mucous membrane from 1 : 1 to $1\frac{1}{2}$: 1, when the yield varied between 390 and 435 cc. per kg.

Properties of press juice from hog's stomach.

The press juice is a reddish, opalescent, rather viscid liquid containing a small amount of suspended matter which can be removed by filtration through cloth. It decomposes rapidly within 24 hours at ordinary temperatures but can be kept for a few days in a refrigerator.

It has p_H about 6·2. It coagulates with heat owing to the presence of proteins (1·6–2·0 g. of protein per 100 cc. of juice, by Esbach's method). The total solids, determined by heating to 100° for 4 hours, amounted to 6·1 % and the ash content was 0·5 %.

The press juice has a good pepsin content but contains no rennin, amylase, lipase, urease or trypsin.

The following protein tests were positive (*cf.* Table II): biuret, Molisch, lead acetate sulphur test, Millon, Folin phenol, Pauly, Adamkiewicz, *p*-dimethylaminobenzaldehyde, ninhydrin, Sakaguchi and xanthoproteic.

Precipitates were obtained with the following protein precipitants: alcohol, acetone, phosphotungstic acid, picric acid, metaphosphoric acid, trichloroacetic acid, tannic acid, hydroferrocyanic acid, sulphosalicylic acid, lead acetate, mercuric chloride, ferric chloride, colloidal ferric hydroxide, uranium acetate, ammonium sulphate and zinc sulphate.

The press juice was found to be very active in the treatment of pernicious anaemia, using 150 cc. daily, representing about 250 g. of fresh stomach tissue.

Fractionation of press juice with alcohol.

The action of various protein precipitants (alcohol, acetone, tannic acid, picric acid and lead acetate) on press juice has been investigated but so far only the use of alcohol has given satisfactory results.

When the filtered press juice was slowly poured with stirring into 5 volumes of 92 % alcohol, there was a copious flocculent precipitate P 5, which was collected, washed with absolute alcohol and with ether and dried in vacuum desiccators over sulphuric acid. The usual yield of fraction P 5 was 15–18 g. per 500 cc. of press juice, 20 g. being thus derived from approximately 1 kg. of fresh whole stomach tissue.

If the press juice from the mucous membrane of hog's stomach was precipitated in the same way with alcohol, a rather higher yield of fraction P 5 (mucosa) was obtained, namely, about 18–22 g. per 500 cc. of juice.

Properties of fraction P 5. Fraction P 5 is a light cream-coloured powder which gave results on analysis corresponding with those one would expect to obtain from a somewhat impure protein (C, 43·1, 43·3; H, 6·8, 6·7; N, 13·5, 13·5; P, 1·2, 1·2 %. Ash, 5·4, 5·7 %). The ash was found to contain calcium, sodium, potassium, iron and phosphate. Traces of copper, magnesium and sulphate were present but chloride was absent.

Fraction P 5 gave positive results with all the protein reagents mentioned above. It was strongly active peptically, but rennin, amylase, urease, lipase and trypsin were absent. It was only partially soluble in water. Enzyme preparations precipitated by means of alcohol are often sparingly soluble in water [Albert and Buchner, 1900]. Other neutral solvents such as 50 % alcohol, 10 % glycerol and 1 % sodium chloride were less satisfactory than water. It was found, however, that almost complete solution could be achieved by trituration with

cold dilute hydrochloric acid, 1 g. of fraction P 5 requiring about 40 cc. of $N/14$ or 25 cc. of $N/10$ hydrochloric acid. These solutions gave precipitates with all the usual protein precipitants.

Fraction P 5 is very active in the treatment of pernicious anaemia, a dose of only 5 g. daily being adequate as compared with 30 g. daily of desiccated stomach powder. It is evident, therefore, that a considerable concentration of haemopoietin has been achieved in the preparation of this fraction, which is the most active stomach preparation hitherto obtained.

The preparation of fraction P 5 in large quantities was rather laborious, and we were fortunate to secure the co-operation of Messrs Boots Pure Drug Co., who very kindly prepared this fraction for us on a large scale by the method described above.

By precipitating the press juice obtained from the mucous membrane of hog's stomach (p. 603) with alcohol a fraction P 5 (mucosa) was obtained, which was similar in its properties to the ordinary fraction P 5, though it appeared to be slightly more active peptically and haemopoietically.

Alcoholic filtrates from fraction P 5. The combined alcoholic filtrates from one series of experiments (56 litres, representing 9·5 litres of press juice) were evaporated *in vacuo* below 35° to a syrup. This, after keeping for several days in a vacuum desiccator over sulphuric acid, gave 164 g. of a yellowish-brown semi-solid mass, B 5.

Fraction B 5 had a strong porcine odour and was almost completely soluble in water, the solution being acid to litmus. On boiling the solution, no coagulation took place. It had no peptic activity, and of the above-mentioned protein reactions, positive results were given only with the Molisch, Folin phenol, Pauly, ninhydrin and xanthoproteic tests; it failed to respond to the biuret test. The usual protein precipitants likewise gave no precipitates. Phosphotungstic acid produced a white precipitate but this reagent precipitates organic bases and amino-acids as well as proteins. The nitrogen content was low (N, 5·8, 5·9 %). The whole of this nitrogen was of a non-protein character, a high proportion being in the amino-form (found by micro-Van Slyke method: NH_2-N 3·2, 3·2 %). Part of the nitrogen was present as ammonium salts since there was a distinct odour of ammonia on shaking with cold caustic soda. The ash content was 13·6 %; it contained calcium, sodium, potassium, a slight trace of iron, and chloride, sulphate and phosphate.

Fraction B 5 (in doses of 5 g. daily, equivalent to about 500 g. of fresh stomach tissue) was found to be inactive in the treatment of pernicious anaemia. It is evident, therefore, that when the press juice from hog's stomach is treated with alcohol, haemopoietin is entirely precipitated with the protein fraction.

Fractional precipitation of press juice with alcohol.

Fractional precipitation with alcohol was used by Sherman and Schlesinger [1913] for the preparation of amylase from malt extract. The principle of the method is to add alcohol to about 40–50 %, filter off the precipitate and then add further quantities of alcohol to about 80–90 %, when a second precipitate appears.

The press juice and alcohol were both cooled to 0° and the alcohol was slowly added to the juice with constant stirring until a concentration of about 40 % was reached. The temperature was not permitted to rise above 20° during the addition of the alcohol. The precipitate (P 5 α) was collected and dried in a vacuum desiccator over sulphuric acid. Filtration at this stage was extremely

slow, consequently the filtrate had to be kept in the refrigerator overnight and the next stage carried out on the following day. The filtrate was treated with further quantities of alcohol until a concentration of about 80 % was attained when a second precipitate (P 5β) was obtained. This was washed with absolute alcohol and ether and dried in a vacuum desiccator over sulphuric acid.

The average yields of these fractions from 500 cc. of press juice were as follows: P 5α, 7–9 g.; P 5β, 4–5 g.

Properties of fraction P 5α. Fraction P 5α is a yellowish-brown powder giving all the usual protein tests. It is only slightly soluble in water and in $N/18$ hydrochloric acid. Its nitrogen content is 13·3 %. It has no peptic activity and is not active in the treatment of pernicious anaemia.

Properties of fraction P 5β. Fraction P 5β is a yellowish powder giving all the usual protein tests. It is moderately soluble in water and almost completely soluble in $N/18$ hydrochloric acid. It contains a higher percentage of nitrogen (13·8 %) than either fraction P 5 or fraction P 5α. It is about as active peptically as fraction P 5 and is also active in the treatment of pernicious anaemia, a case receiving 2–3 g. daily for 13 days showing considerable improvement.

Inactivation of haemopoietin by acetone.

Fraction P 5 is almost completely soluble in $N/10$ hydrochloric acid, but this acid solution no longer gives a precipitate with alcohol, only a turbidity being produced. Acetone, on the other hand, readily gives a copious white precipitate, P 5A, about 10 g. being obtained from 20 g. of fraction P 5.

Fraction P 5A responds to the usual protein tests, contains 13·9 % N, and has a high pepsin content. It was, however, rather surprising to find that it was unable to induce remissions in cases of pernicious anaemia, the acetone used in the course of the preparation having apparently inactivated the haemopoietin.

Fractionation of fraction P 5 by isoelectric precipitation.

It was hoped to achieve a separation of pepsin and haemopoietin in solutions of fraction P 5 by precipitating out most of the pepsin at its isoelectric point. Various values are given in the literature for the isoelectric point of pepsin, ranging from 2·3 to 3·8 [*cf.* Michaelis and Davidsohn, 1910; Pekelharing and Ringer, 1911; Forbes, 1927; Fenger and Andrew, 1927; Northrop, 1930]. The cause of these variations appears to be the degree of purity of the sample of pepsin used, the presence of proteins and inorganic salts in particular having a marked effect on the value of the isoelectric point. Maximum precipitation of the pepsin in fraction P 5 appears to take place at p_H 4·2, which is somewhat higher than the highest value given above.

In a typical experiment, 20 g. of fraction P 5 were dissolved in 500 cc. of $N/10$ hydrochloric acid at 20–25°. The p_H of this solution was 2·3. A solution of N NaOH was then run in drop by drop from a burette with constant stirring. The variations in p_H during this addition were as follows:

After adding 10 cc. of N NaOH, $p_H = 2·4$,
,, 20 ,, ,, $p_H = 3·5$,
,, 25 ,, ,, $p_H = 4·2$,
,, 26 ,, ,, $p_H = 4·2$,
,, 27 ,, ,, $p_H = 4·2$,
,, 28 ,, ,, $p_H = 4·2$.

At this stage, the formation of a slimy gelatinous yellowish precipitate, P 5 (i), appeared to have reached a maximum and the mixture was kept

in the refrigerator for half an hour and filtered through a series of No. 41 Whatman papers. Filtration was rather slow. The filtrate from fraction P 5 (i) was brought up to p_H 6·2 by careful addition of *N* NaOH (9 cc.) and then poured with stirring into about 4 volumes of 92 % alcohol cooled to 0°. After standing half an hour in the refrigerator, the precipitate P 5 (ii) was collected.

Fractions P 5 (i) and P 5 (ii) were both washed with absolute alcohol and dried *in vacuo* over sulphuric acid. 20 g. of fraction P 5 usually gave about 5–6 g. of fraction P 5 (i) and about 3–4 g. of fraction P 5 (ii). The yields of these fractions and also the quantities of caustic soda used in the preparation varied somewhat with the particular batch of fraction P 5 that was being used.

An attempt to achieve a separation of fractions P 5 (i) and P 5 (ii) by a simpler and more rapid method than the above, namely, the extraction of fraction P 5 with a buffer solution at p_H 4·2, did not give sufficiently good yields to warrant further investigation. Thus, 15 g. of fraction P 5 were extracted with 400 cc. of a sodium acetate-acetic acid buffer at p_H 4·2. A peptically active residue of 10 g. was obtained but the extract, after precipitation with alcohol, yielded only a very small fraction weighing less than 1 g.

Properties of fraction P 5 (i). Fraction P 5 (i) was a fawn-coloured powder giving all the usual protein reactions. It was only very sparingly soluble in water (N, 13·7 %, soluble N, 1·9 %). It contained 0·75 % P and 3·2 % ash. The ash gave a very strong iron reaction (thiocyanate test) and contained also traces of sodium, potassium, calcium and sulphate. Phosphate was also present. Fraction P 5 (i) was extremely active peptically but contained no haemopoietin.

Properties of fraction P 5 (ii). Fraction P 5 (ii) was a pale buff powder giving all the usual protein tests. In contrast with fraction P 5 (i) it was almost completely soluble in water (total N, 10·6 %, and total soluble N, 8·6 %). It was remarkable in having a relatively high phosphorus content (3·6 %) and a very high ash content (14·8 %). The ash contained calcium, magnesium, sodium, potassium and phosphate. Chloride and sulphate were absent, and there were only extremely minute traces of iron (thiocyanate test) and copper (sodium diethyldithiocarbamate test). The absence of iron and copper from this fraction, and presumably, therefore, from haemopoietin, is interesting in view of the importance attached to the therapeutic use of these metals in the treatment of secondary anaemias.

Fraction P 5 (ii) was only slightly active peptically, as might be expected since most of the pepsin had been removed by isoelectric precipitation. It gave no blue coloration with hydrogen peroxide and benzidine, thereby indicating absence of enzymes of the peroxidase type. The fraction was active in doses of 5 g. daily in cases of pernicious anaemia.

Although pepsin and insoluble impurities have been eliminated in the preparation of fraction P 5 (ii) the haemopoietic activity does not appear to be any greater than that of fraction P 5, probably owing to inactivation of part of the very sensitive haemopoietin.

Chemical reactions and analyses of preparations from hog's stomach.

The chemical analyses and reactions of the various preparations and fractions from hog's stomach described in this paper are shown in Tables I and II, and a diagrammatic scheme indicating the methods by which these fractions were obtained is shown on p. 609.

Table I. *Analyses of preparations from hog's stomach.*

	Total N % (Kjeldahl)	Soluble N %						Phosphorus %	Ash %
		Total	Peptide	Amino	Non-coagulable	Proteose ($ZnSO_4$ ppt.)	Non-protein		
Stomach press juice	1·2	1·2	0·3	0·1	0·1	0·1	0·3	0·15	0·5
Desiccated stomach	11·5	2·2	1·0	0·5	1·1	0·4	1·1	0·8	3·6
Fraction P 5	13·5	2·6	0·9	0·8	1·6	0·9	0·6	1·5	5·7
Fraction B 5	5·9	5·7	0·1	3·2	5·7	0	5·7	1·4	13·6
Fraction P 5α	13·3	1·8	0·5	0·4	0·9	0·2	0·8	0·9	4·8
Fraction P 5β	13·8	8·2	5·0	1·8	1·3	0·3	1·1	1·2	11·9
Fraction P 5 (i)	13·7	1·9	1·25	0·35	1·9	0·9	0·7	0·75	3·2
Fraction P 5 (ii)	10·6	8·6	5·15	0·95	8·2	0	0·9	3·6	14·8

Table II. *Properties of preparations from hog's stomach.*

Test	Press juice from hog's stomach	Desiccated hog's stomach	Fraction P 5	Fraction B 5	Fraction P 5α	Fraction P 5β	Fraction P 5 (i)	Fraction P 5 (ii)	Fraction P 5 *A*
Biuret	+	+	+	−	+	+	+	+	+
Molisch	+	+	+	+	+	+	+	+	+
Alkaline lead acetate	+	+	+	Very faint	+	+	+	+	+
Folin phenol	+	+	+	+	+	+	+	+	+
Millon	+	+	+	−	+	+	+	+	+
Pauly	+	+	+	+	+	+	+	+	+
Adamkiewicz	+	+	+	Very faint	+	+	+	+	+
p-Dimethylamino-benzaldehyde	+	+	+	−	+	+	+	+	+
Ninhydrin	+	+	+	+	+	+	+	+	+
Metaphosphoric acid	White ppt.	White ppt.	White ppt.	No ppt.	White ppt.	White ppt.	White ppt.	White ppt.	White ppt.
Trichloroacetic acid	,,	,,	,,	,,	,,	,,	,,	,,	,,
Sakaguchi	+	+	+	−	+	+	+	+	+
Xanthoproteic	+	+	+	+	+	+	+	+	+
Murexide	−	−	−	−	−	−	−	−	−
H_2O_2 + benzidine	Blue colour	Blue colour	Blue colour	No colour	Blue colour	Blue colour	Blue colour	No colour	Blue colour
Peptic activity	+	+	+	−	Very slight	+	+	Slight	+
Time taken to digest egg-albumin	28 min.	10–12 min.	7 min.	No digestion	>60 min.	9 min.	4–5 min.	40 min.	9 min.
Effect on cases of pernicious anaemia	Active	Active	Active	Not active	Not active	Active	Not active	Active	Not active

These tables reveal a number of features of interest the most noteworthy being the following.

(*a*) The close parallelism between protein reactions and haemopoietic activity, *i.e.* all the haemopoietically active products gave the protein colour and other tests.

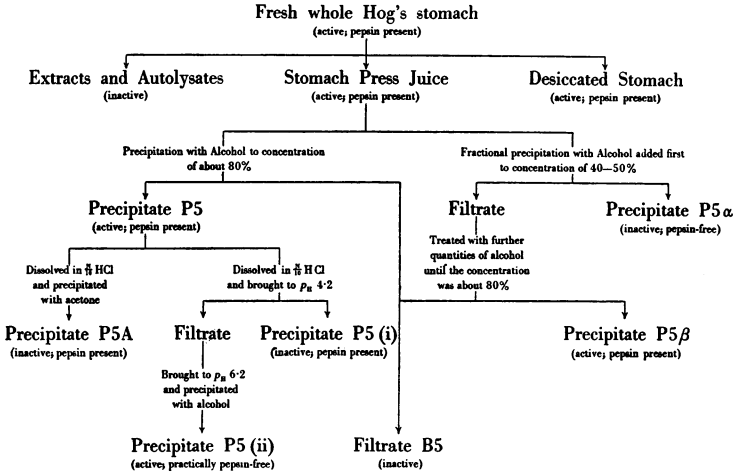
(*b*) Peptic activity and haemopoietic activity are not necessarily related to each other. For example, fractions P 5A and P 5 (i) were very active peptically but inactive haemopoietically, whereas fraction P 5 (ii) had strong haemopoietic activity but contained very little pepsin.

(*c*) The relatively high proportion of nitrogen (13·5 %) in the haemopoietically active fraction P 5 and the low value for non-protein nitrogen (0·6 %).

(*d*) The low nitrogen content (5·9 %) of the inactive fraction B 5, a very

high proportion of which (3·2 %) was in the amino-form and practically the whole of which was of a non-protein character.

(*e*) The relatively high phosphorus content (3·6 %) of the active fraction P 5 (ii).



Scheme of fractionation.

Summary.

1. Experiments are described on the preparation of extracts and fractions from hog's stomach containing haemopoietin, the active substance effective in the treatment of pernicious anaemia.

2. An active extract has only been obtained by the method used by Buchner in the case of zymase. This involves the subjection of a mixture of fresh stomach tissue and sand to high pressure in a specially constructed apparatus.

3. Addition of alcohol to this press juice yields a precipitate in which the whole of the activity of the juice appears to be concentrated, the alcoholic filtrates being inactive.

4. Other active fractions have been prepared by fractional precipitation with alcohol and by fractional isoelectric precipitation.

5. Our experiments show that haemopoietin is more unstable than and has different properties from the active anti-anaemic principle in liver.

6. It is considered that the clinical experiments with gastric juice, the necessity for an extrinsic substrate, the association of pernicious anaemia with deficient enzyme secretion [Wilkinson, 1932, 2], the difficulty of extracting haemopoietin from stomach tissue, its digestion by the prolonged action of the proteolytic enzymes pepsin or trypsin, its association with the protein fraction of stomach press juice, its instability to heat and its sensitiveness to chemical treatment all harmonise with the view that haemopoietin is an organic substance of complex structure, probably a protein and possibly enzyme-like in its nature.

We have to thank the Honorary Physicians of the Manchester Royal Infirmary for placing at our disposal the patients necessary for these investigations and the Assistant Clinical Pathologists of the Manchester Royal Infirmary for their careful determinations of the blood counts. We also gratefully

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